Thermotolerant Purple Nonsulfur Bacteria from New Zealand Geothermal Areas

A thesis

submitted in partial fulfilment

of the requirements for

the Degree of

Doctor of Philosophy

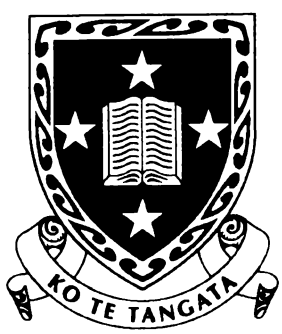
in Biological Sciences

at

The University of Waikato

by

Peter John Charlton



July 2002

Abstract

Purple nonsulfur bacteria (PNSB) are a metabolically and nutritionally diverse group of photosynthetic organisms that have been isolated from many environments. Three thermotolerant or mildly thermophilic strains are known from thermal areas in the United States and Japan. Only mesophilic isolates have been obtained from Russian hot springs.

Enrichment cultures selective for PNSB were set up on samples taken from a range of New Zealand geothermal areas. Photosynthetic purple bacteria could readily be isolated from Waimangu and Orakei Korako samples, but could not be isolated from Tokaanu mats. In total, 35 isolates belonging to the genera *Rhodopseudomonas*, *Rhodoplanes*, *Blastochloris*, *Rubrivivax*, *Rhodomicrobium*, *Rhodospirillum* and *Phaeospirillum* were obtained from 18 positive sample sites. The majority of isolates grew optimally around 40°C and had temperature maxima ranging from 43 to 47°C. Other isolates had lower optima and were thermotolerant. Many *Rhodoplanes* and *Blastochloris* isolates represented novel genospecies, though some could not be unambiguously distinguished by their phenotypic properties. The novel species *Rhodoplanes venustus* sp. nov., *Rhodoplanes segnis* sp. nov., and *Phaeospirillum tepidarium* sp. nov. are proposed.

Molecular methods were used to specifically detect photosynthetic purple bacteria. A clone library of photosynthetic reaction centre *pufL* genes amplified from an Orakei Korako mat sample was constructed and screened. No clone sequences were identical to those of organisms isolated previously or in the current study. The clone library was dominated by sequences attributable to aerobic anoxygenic phototrophic bacteria. Four novel *pufL* sequences attributable to purple nonsulfur bacteria were obtained.

Rhodoplanes strains possessed the ability to use nicotinic acid as sole source of carbon and nitrogen, via a pathway that includes 6-hydroxynicotinate and glutarate as intermediates, as are in the *Azorhizobium caulinodans* pathway. Unlike other nicotinic-acid degrading organisms, growth of *Rhodoplanes* on nicotinate was not inhibited by addition of tungstate.

Acknowledgements

I thank Dr Chris Harfoot for his advice and support as my primary supervisor. I thank Colin Monk (Thermophile Research Unit) for his assistance with HPLC, Wendy Jackson (Department of Chemistry) for assistance with ESMS, David Wild and Alf Harris (MIRINZ) for assistance with SEM and TEM, Dr Barry O'Brien (Department of Biological Sciences) and Ross Clayton (Waikato Photographic Unit) for some photography, and Dr Jackie Aislabie (Landcare-Manaaki Whenua, Hamilton) for providing *Pseudomonas* strains.

I thank Dr Akira Hiraishi of Toyohashi Technical University, Japan for his generosity in carrying out quinone analysis and DNA-DNA hybridization studies. I thank Dr Hiraishi, Prof. Carl Bauer of Indiana University, USA, and Prof. Michael Madigan of Southern Illinois University, USA, for their kind provision of various bacterial cultures.

I thank the Department of Conservation, the staff of Waimangu Volcanic Valley and Orakei Korako Geyserland, Mr Taku Hohepa of Ngati Pikiao, and representatives of Ngati Rangiteaorere for granting permission to take samples.

I thank the University of Waikato Scholarships Committee for provision of a University of Waikato Doctoral Scholarship and the Wilf and Ruth Malcolm Postgraduate Scholarship.

I acknowledge the friendship of other graduate students and lab colleagues, including Marcel Dinger, Bridget Douglas, Dorothee Götz, Gregory Jacobson and Jason McGrath. I thank my family for their support throughout my studies.

List of Contents

List of Figures

List of Tables

List of Abbreviations

PNSB	purple nonsulfur bacteria		
Blc.	*Blastochloris*	*Rov.*	*Rhodovulum*
Phs.	*Phaeospirillum*	*Rpi.*	*Rhodopila*
Rba.	*Rhodobacter*	*Rpl.*	*Rhodoplanes*
Rbc.	*Rhodobaca*	*Rps.*	*Rhodopseudomonas*
Rbi.	*Rhodobium*	*Rsi.*	*Roseospira*
Rbl.	*Rhodoblastus*	*Rsp.*	*Rhodospirillum*
Rci.	*Rhodocista*	*Rss.*	*Roseospirillum*
Rcy.	*Rhodocyclus*	*Rth.*	*Rhodothalassium*
Rfx.	*Rhodoferax*	*Rvb.*	*Rhodovibrio*
Rhs.	*Rhodospira*	*Rvi.*	*Rubrivivax*
Rmi.	*Rhodomicrobium*		

ATCC	American Type Culture Collection
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
IAM	Institute of Applied Microbiology, University of Tokyo, Japan
IFAM	Institut für Allgemeine Mikrobiologie, Universität Kiel, Germany
IFO	Institute For Fermentation, Osaka, Japan
JCM	Japan Collection of Microorganisms
NCIMB	National Collections of Industrial and Marine Bacteria, Aberdeen, UK
NCPPB	National Collection of Plant Pathogenic Bacteria, York, UK
IJSEM	International Journal of Systematic and Evolutionary Microbiology
16S rRNA	16S ribosomal RNA
bp	base pair
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
LB	Luria-Bertani
dNTPs	deoxynucleotide triphosphates
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
SEM	scanning electron micrograph
w/v	weight to volume

Chapter 1
Introduction

The purple nonsulfur bacteria (PNSB) are a physiological group of photosynthetic organisms noted for their metabolic and nutritional diversity. They have been isolated from a wide range of environments, including freshwater lakes, industrial wastewater, marine and hypersaline water bodies, moist soils, and Antarctic lakes (Herbert 1976; Hiraishi and Kitamura 1984; Imhoff and Trüper 1992; Madigan *et al.* 2000). Three thermotolerant or mildly thermophilic PNSB that grow optimally around 40°C were isolated from geothermal areas in 1989 and 1990, and were designated *Rhodospirillum centenum* (Favinger *et al.* 1989), "*Rhodopseudomonas cryptolactis*" (Stadtwald-Demchick *et al.* 1990b) and *Rhodopseudomonas* [*Blastochloris*] strain GI (Resnick and Madigan 1989). Several PNSB species were observed in Russian hot spring systems, but all isolates were mesophilic. New Zealand contains a number of extensive geothermal areas which could provide good habitats for thermotolerant or thermophilic PNSB but which have not been examined for these organisms.

In the present work, the diversity of thermotolerant PNSB in New Zealand geothermal environments was examined. The work was carried out as follows:

1. Enrichment cultures selective for PNSB were set up on a range of samples from geothermal areas, as described in Chapter 4.

2. The resulting isolates were characterised and classified, as described in Chapter 5. In addition, the response of mesophilic PNSB to selection at high temperature was briefly explored.

3. Molecular techniques were used to detect uncultured photosynthetic organisms. This study is described in Chapter 6.

4. Finally, degradation of nicotinic acid by strains of the genus *Rhodoplanes*, including several thermotolerant isolates, was examined, as described in Chapter 7.

Chapter 2

Literature Review

2.1 Introduction

This review of the literature is comprised of four sections: first, a general discussion of the photosynthetic process, defining terms; second, an overview of the diversity of photosynthetic organisms; third, a more detailed examination of the diversity of purple nonsulfur bacteria, arranged in a phylogenetic perspective; and finally a discussion of photosynthetic organisms in geothermal environments. Reviews of specific portions of the literature can be found also in Chapters 6 and 7.

2.2 Photosynthesis

Photosynthesis is the term used to describe the process in which an organism converts light energy into chemical energy which is used for biosynthesis. In a more narrow sense, it refers to the process in which light energy is harnessed to the fixation of carbon dioxide. A phototrophic organism is one which obtains a substantial proportion of its energy from light-driven processes. Photoautotrophic organisms obtain their carbon by fixation of carbon dioxide. Photoheterotrophic organisms obtain much of their carbon by the assimilation of organic compounds. Many organisms are capable of growth photoautotrophically or photoheterotrophically under appropriate conditions.

Photosynthetic processes share as a common feature the involvement of membrane protein-pigment complexes that mediate the conversion of light energy into chemical energy. The archaeal halobacteria produce bacteriorhodopsin, a purple complex which acts as a light-dependent proton pump to enable production of ATP. Recently discovered γ-proteobacteria possess a similar molecule termed proteorhodopsin (Beja *et al.* 2000). As halobacteria do not grow at the sole expense of light energy, they are not usually considered with the other phototrophs.

All "true" phototrophs mediate the capture of light energy through the use of various types of chlorophyll molecule in association with carotenoids or other pigments and membrane proteins. Chlorophyll molecules are arranged in an antenna system that

collects light energy and transfers it to a reaction centre complex consisting of chlorophyll in a specific protein environment. This chlorophyll is promoted to an excited state and transfers an electron to a series of acceptor molecules. The electron flow is coupled to proton translocation across a membrane. The resulting proton gradient can be used for generation of ATP or for other cellular processes. The electron may be transferred back to the oxidised chlorophyll in the process known as cyclic photophosphorylation. In non-cyclic flow, $NADP^+$ is reduced to NADPH, and reduced chlorophyll is regenerated by an external electron donor. In cyanobacteria and chloroplasts, this donor is water, and oxygen is released as a waste product, in the process known as oxygenic photosynthesis. Anoxygenic photosynthesis is carried out by a diverse range of bacteria. Water cannot be used as electron donor; instead reduced inorganic sulfur compounds, molecular hydrogen, simple organic compounds or other less common molecules are used.

2.3 The photosynthetic bacteria

Photosynthetic organisms are found in five divisions of the domain Bacteria. These divisions are the cyanobacteria, green nonsulfur bacteria (filamentous green bacteria), green sulfur bacteria, Gram positive bacteria, and proteobacteria (purple bacteria) (Figure 2.1).

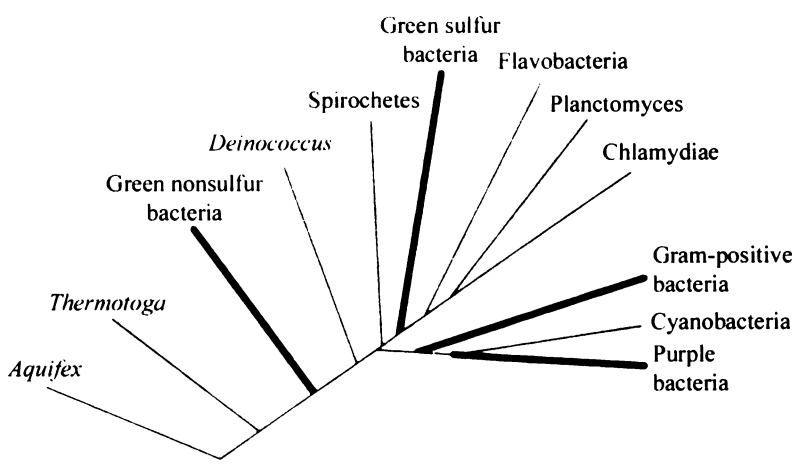


Figure 2.1 Phylogenetic tree of the Bacteria based on 16S rDNA sequences. Lineages containing anoxygenic phototrophic bacteria are shown by bold lines; based on a figure in Madigan (2001)

All known members of the cyanobacteria and green sulfur bacteria groups are photosynthetic. The other divisions contain a mixture of photosynthetic and non-

photosynthetic organisms. Brief descriptions of the photosynthetic members of each group are given in the following sections. Cyanobacteria carry out oxygenic photosynthesis and possess two photosystems. Characteristic pigments are chlorophyll *a* and phycobilins. The group possesses remarkable morphological diversity. Phylogenetically, the cyanobacteria are closely related to the chloroplasts of eukaryotes. Geological evidence suggests that oxygen-producing cyanobacteria as a group are at least 2.7 billion years old (Brocks *et al.* 1999). They will not be considered further.

Anoxygenic phototrophic bacteria, with the exception of "quasi-photosynthetic" (Gest 1993) aerobic organisms discussed in Section 2.3.6, require anaerobic conditions for photosynthetic growth. Synthesis of their photopigments is repressed in the presence of oxygen. Thus, they are predominantly found in anoxic sediments and water bodies exposed to light, where they play important roles in the cycling of carbon compounds as photoautotrophic primary producers and photoheterotrophic consumers.

2.3.1 Green nonsulfur bacteria

The green nonsulfur bacteria consist of filamentous organisms containing bacteriochlorophyll *a* and *c*, or only the former. Organisms containing both bacteriochlorophylls include *Chloroflexus aurantiacus* (Pierson and Castenholz 1974) and *C. aggregans* (Hanada *et* al. 1995), *Chloronema giganteum* and *Oscillochloris trichoides* (Keppen *et al.* 1994). *Heliothrix oregonensis* (Pierson *et al.* 1985) contains only bacteriochlorophyll *a*, as do red filaments observed in Yellowstone microbial mats (Boomer *et al.* 2000). Only a small number of green nonsulfur bacteria have been successfully cultivated, including the two named *Chloroflexus* species, *Heliothrix*, which is maintained in a stable mixed culture, and *Roseiflexus castenholzii* (Hanada *et al.* 2002). These organisms are all thermophilic. Mesophilic *Oscillochloris trichoides* strains have recently been cultivated (Keppen *et al.* 2000). Growth of green nonsulfur bacteria may be photoheterotrophic or chemotrophic under aerobic conditions.

C. aurantiacus has been observed in hot spring environments worldwide, including New Zealand, where mass accumulations together with cyanobacteria occur. *Heliothrix* forms orange mats in a few areas of the western United States (Pierson *et al.* 1985). *Chloroflexus*-like organisms have also been observed in marine and

hypersaline environments (Pierson *et al.* 1994; Nübel *et al.* 2001) and non-thermal freshwater habitats, but few strains have been successfully isolated. Diverse 16S rDNA sequence fragments affiliated with the green nonsulfur bacteria have been obtained from freshwater lakes (Gich *et al.* 2001), and from Yellowstone hot springs (Boomer *et al.* 2002), indicating that the breadth of the group has not yet been fully explored. Several non-photosynthetic organisms are members of the GNS division, and it is unclear whether all environmental 16S rDNA sequences derive from phototrophs.

The green nonsulfur bacteria are phylogenetically deep branching organisms (Madigan 2001), apparently the most ancient of the photosynthetic lineages. In fact, some of their genes involved in photosynthesis appear to be related distantly to those of the purple bacteria and green sulfur bacteria, and the genes of purple bacteria have the most basal position (Xiong *et al.* 2000).

2.3.2 Green sulfur bacteria

The green sulfur bacteria are obligate phototrophs, containing bacteriochlorophylls *c*, *d*, or *e*. They primarily assimilate carbon dioxide using sulfide or thiosulfate as electron donor, oxidising these compounds to elemental sulfur which is deposited outside the cell, and may later be oxidised to sulfate. In the presence of carbon dioxide and an electron donor, they may also assimilate small quantities of organic compounds, particularly acetate. Green sulfur bacteria are able to grow at light intensities which are too low to support growth of other phototrophs. Sulfide tolerance is high. Chemotrophic growth has never been observed. In the dark, green sulfur bacteria ferment storage compounds.

Genera include *Chlorobium*, *Prosthecochloris*, *Clathrochloris*, *Pelodictyon*, *Ancalochloris* and *Chloroherpeton*. Cells of *Chlorobium* are generally rod-shaped. Members of the other genera may be appendaged, filamentous or form multicellular net structures. All known species are non-motile. Some contain gas vacuoles. Green sulfur bacteria are known that form stable close consortia with heterotrophs.

2.3.3 Heliobacteria

Heliobacteria form a separate line of descent in the Gram positive bacteria, showing close relatedness to the clostridia and to *Desulfotomaculum* (Madigan 2001; Redburn

and Patel 1993). All known heliobacteria contain bacteriochlorophyll *g*, a unique form that absorbs maximally around 788 nm (Brockman and Lipinski 1983). They are a distinctive emerald green colour, or brownish-green. Heliobacteria are obligately anaerobic and extremely sensitive to oxygen. They are able to fix nitrogen (Kimble and Madigan 1992). Photoautotrophic growth has never been observed. Growth in darkness under anoxic conditions proceeds slowly by fermentation of fructose, pyruvate or other substrates (Kimble *et al.* 1994; Ormerod *et al.* 1996). No oxic or microaerobic growth has been observed. Heliobacteria are thus close to being obligate photoheterotrophs. Many, if not all, are able to produce endospores, although there is a tendency for strains in cultivation to lose this ability (Ormerod *et al.* 1996).

Rod-shaped species such as *Heliobacterium chlorum* (Gest and Favinger 1983) and *Heliobacillus mobilis* (Beer-Romero and Gest 1987) are known, as well as spirilla such as *Heliobacterium gestii* (Ormerod *et al.* 1996), and organisms of intriguing morphology such as the bundle-forming *Heliophilum fasciatum* (Ormerod *et al.* 1996) and the rod-to-coil shaped *Heliorestis daurensis*. These heliobacteria grow to a maximum temperature of around 44°C. A thermophilic species, *Heliobacterium modesticaldum*, capable of growth to 56°C, and growing optimally at 50-52°C is known (Kimble *et al.* 1995) and appears to be widespread around the world, although samples from Indonesia, Malaysia and Hawaii were negative (Stevenson *et al.* 1997). Samples have not been obtained from New Zealand. In general, only paddy soils and hot springs have proven to be reliable sources of heliobacteria. However, *Heliorestis daurensis* and *H. baculata* were isolated from soda lakes and are alkaliphilic species growing optimally at pH 9 (Bryantseva *et al.* 1999; Bryantseva *et al.* 2000).

2.3.4 Purple sulfur bacteria

The purple sulfur bacteria are members of the γ-subdivision of the proteobacteria that grow photosynthetically primarily autotrophically with sulfide as electron donor, although particular species are more capable of photoheterotrophic growth. They have historically been divided into two families, the Chromatiaceae and the Ectothiorhodospiraceae, and this classification has been confirmed by study of their phylogenetic relationships. Species containing bacteriochlorophyll *a* and *b* are known in both families. The Ectothiorhodospiraceae contain the genera *Ectothiorhodospira* and *Halorhodospira* (Imhoff and Süling 1996). All known species are

haloalkaliphilic. Sulfur is deposited outside the cells. In contrast, members of the Chromatiaceae deposit sulfur inside their cells. Sixteen genera are currently recognised. Many ovoid to rounded rod-shaped species were formerly classified in the genus *Chromatium*, but reclassification was undertaken recently so that the taxonomic arrangement of the group would conform better to phylogenetic information (Imhoff *et al.* 1998b). *Chromatium*-like species include large cell types that are predominantly photoautotrophic, and small cell types that are able to assimilate a range of organic compounds. Among the small cell Chromatiaceae, *Allochromatium vinosum* is notable for its relative metabolic diversity. *Thermochromatium tepidum* is the only purple photosynthetic bacterium capable of growth above 50°C (Madigan 1984; Madigan 1986). Many of the more recently discovered organisms are from salt marsh or marine environments, such as *Thiorhodovibrio winogradskyi* (Overmann *et al.* 1992), *Rhabdochromatium marinum* (Dilling *et al.* 1995), *Thiorhodococcus minus* (Guyoneaud *et al.* 1997), *Thiocapsa litoralis* (Puchkova *et al.* 2000), *Thioalkalicoccus limnaeus* (Bryantseva *et al.* 2000), and *Thioflavicoccus mobilis* (Imhoff and Pfennig 2001). A small number of non-photosynthetic organisms are known that phylogenetically lie within the purple sulfur bacterial radiation (Adkins *et al.* 1993; Head *et al.* 1996; Rijkenberg *et al.* 2001).

2.3.5 Purple nonsulfur bacteria

The purple nonsulfur bacteria (PNSB) are probably the most metabolically versatile organisms on the planet. They are also a phylogenetically disparate group unified only by their common physiology. PNSB include representatives of many different morphologies, including rods, budding rods, spirilla, vibrioids, and half-circles. Representatives are found in the α- and β-subdivisions of the proteobacteria, intermingled with non-photosynthetic organisms. Species may contain either bacteriochlorophyll *a* or *b*. The various genera and species of PNSB are covered in Section 2.4. In this section, general aspects of their biology are discussed.

Photoheterotrophic growth is the preferential mode of growth of all PNSB. In the light, organic carbon sources are efficiently assimilated into cell carbon. In the presence of an inorganic electron donor, organic compounds may be wholly photoassimilated (Imhoff and Trüper 1992). A large number of organic compounds are known to support growth. These include tricarboxylic acid intermediates, fatty

acids (Janssen and Harfoot 1997), alcohols (Lee 1992), amino acids, diols (Charlton 1997; Pantazopoulous and Madigan 2001), simple carbohydrates, some one-carbon compounds, purines and pyrimidines (Yuan 2000) and compounds containing sulfur atoms. *Rhodovulum sulfidophilum* was first reported to use cysteine as carbon source (Heising *et al.* 1996); other purple nonsulfur bacteria use a wide range of organic sulfur compounds (McGrath 1995). A more limited number of PNSB are capable of metabolism of aromatic compounds (Harwood and Gibson 1988; Shoreit and Shabeb 1994). Degradation of unusual organic compounds has been reviewed by Sasikala and Ramana (1997).

In the absence of a fixed carbon source, PNSB can carry out photoautotrophic growth using various compounds as electron donors for generation of NADH required for the fixation of carbon dioxide. Sulfide is used as an electron donor by many PNSB, provided it is at a low enough concentration to avoid toxicity. It is oxidised to sulfate or elemental sulfur, depending on the species (Imhoff and Trüper 1989). Thiosulfate also may serve as electron donor, and many species are capable of using hydrogen. Use of elemental sulfur is a rare property. Strains resembling *Rhodopseudomonas palustris* and *Rhodomicrobium vannielii* and several species of *Rhodovulum* have been shown to be capable of photoautotrophic growth using Fe^{2+} as electron donor (Ehrenreich and Widdel 1994; Heising and Schink 1998; Widdel *et al.* 1993; Straub *et al.* 1999). Mn^{2+} ions are however inhibitory, and lead to repression of pigment synthesis (Horne *et al.* 1998).

In darkness, PNSB are capable of growth using oxygen as terminal electron acceptor, although some species require microaerophilic conditions. Most of the organic compounds used under phototrophic conditions are also used aerobically. Chemolithotrophic growth of *Rba. capsulatus* is possible under aerobic conditions using sulfide as electron donor (Kompantseva 1981). Lithotrophic growth may also occur aerobically using H_2 as electron donor (Madigan and Gest 1979; Siefert and Pfennig 1979).

Growth of some species may also be supported under dark conditions by various forms of anaerobic respiration using nitrate, nitrous oxide, dimethylsulfoxide or trimethylamine oxide as electron acceptors (Ferguson *et al.* 1987). Denitrification occurs in *Rhodobacter azotoformans* (Hiraishi *et al.* 1996), *Rhodoplanes roseus* and

Rpl. elegans (Hiraishi and Ueda 1994), *Rhodobium orientis* (Hiraishi *et al.* 1995c), *Rhodopseudomonas rhenobacensis* (Hougardy *et al.* 2000), and several strains of *Rps. palustris* (Klemme *et al.* 1980), *Rba. sphaeroides* (Satoh *et al.* 1976) and *Rhodoferax fermentans* (Hougardy and Klemme 1995). Dissimilatory reduction of Fe(III) has been found in *Rba. capsulatus* (Dobbin *et al.* 1996), and some *Rhodobacter* strains are capable of chlorate respiration (Roldan *et al.* 1994).

In the absence of light and of external electron acceptors, some PNSB resort to fermentative metabolism, which was first demonstrated in *Rhodospirillum rubrum* using pyruvate (Uffen and Wolfe 1970). *Rfx. fermentans* shows relatively rapid fermentative growth (Hiraishi *et al.* 1991). Poor dark anaerobic growth may occur at the expense of cell storage products such as polyhydroxybutyrate or metabolic intermediates such as fumarate.

PNSB have been isolated from a wide range of environments, including the aquatic environments that generally favour phototrophs.

2.3.6 Aerobic anoxygenic phototrophic bacteria

Aerobic anoxygenic phototrophic bacteria are a physiological group of organisms found intermingled with purple non-sulphur bacteria and non-photosynthetic bacteria, predominantly in the α-subdivision of the proteobacteria, where representatives are found in all of the subdivisions. There is a single β-subdivision member (Suyama *et al.* 1999), and no γ-subdivision species are yet known. These organisms contain bacteriochlorophyll *a* and carotenoid pigments, but are incapable of anaerobic photosynthetic growth, despite production of a complete photosynthetic apparatus. In most cases, light does not appear to provide a significant proportion of energy for growth. Metabolism is predominantly aerobic and heterotrophic. Light may provide a supplementary source of energy, particularly under starvation conditions. The synthesis of the photosynthetic apparatus in *Roseateles depolymerans* is induced by carbon limitation (Suyama *et al.* 2002).

It has been suggested that the term "photosynthetic" is ill-applied to these organisms and that they would be better described as "quasi-photosynthetic" (Gest 1993); while this viewpoint has some validity, the term "aerobic anoxygenic" is now widely used and generally understood.

Many aerobic anoxygenic phototrophic bacteria are marine organisms, classified in the genera *Erythrobacter* (Shiba and Simidu 1982; Yurkov *et al.* 1994) and *Roseobacter* (Shiba 1991). Other marine representatives include *Citromicrobium bathymarinum* which was isolated from a deep sea hydrothermal vent (Yurkov *et al.* 1999), *Rubrimonas cliftonensis* (Suzuki *et al.* 1999) and species of *Roseibium* (Suzuki *et al.* 2000). *Roseovivax halodurans*, *Roseovarius tolerans* and *Roseinatronobacter thiooxidans* inhabit hypersaline environments (Labrenz *et al.* 1999; Sorokin *et al.* 2000; Suzuki *et al.* 1999). Freshwater representatives include members of the genera *Blastomonas* (Hiraishi *et al.* 2000b; Sly and Cahill 1997), *Erythromonas*, *Erythromicrobium* and *Sandaracinobacter* (Yurkov *et al.* 1994; Yurkov *et al.* 1997), *Porphyrobacter* (Fuerst *et al.* 1993; Hanada *et al.* 1997; Hiraishi *et al.* 2002), *Roseateles* (Suyama *et al.* 1999) and *Methylobacterium* (Hiraishi *et al.* 1995a).

Members of the genus *Acidiphilium* (Wakao *et al.* 1993) and *Acidisphaera rubrifaciens* (Hiraishi *et al.* 2000a) grow optimally at low pH, and are unusual in their possession of zinc-containing bacteriochlorophyll (Wakao *et al.* 1996; Hiraishi and Shimada 2001). The sequences of their photosynthetic reaction centre proteins contain unusual features that may be related to this (Nagashima *et al.* 1997b).

Aerobic phototrophic bacteria have also been found as nodulating symbionts and endophytes in association with plants such as the legume *Aeschynomene* and *Oryza* species (Chaintreuil *et al.* 2000; Fleischman and Kramer 1998; Wong *et al.* 1994).

Aerobic anoxygenic phototrophic bacteria can account for 2-5% of the photosynthetic electron transport in the upper ocean (Kolber *et al.* 2000).

2.3.7 Proteorhodopsin phototrophy

Recently, evidence has been found for a new form of phototrophy in marine γ-proteobacteria. Sequencing of large genomic fragments revealed the presence of a gene encoding a form of rhodopsin which produced a functional light-mediated proton pump when expressed in *E. coli* (Beja *et al.* 2000). Further investigation revealed many variant proteorhodopsins that absorbed light at slightly different wavelengths (Beja *et al.* 2001). It is as yet unknown whether light is a major source of energy for these proteobacteria.

2.4 The purple nonsulfur bacteria and their non-photosynthetic relatives

Figure 2.2 shows a phylogenetic tree constructed by the neighbour joining method using Clustal X (Section 3.9.6) based on the sequences of the 16S rRNA genes of validated purple nonsulfur bacterial species of the α-subdivision of the Proteobacteria. A number of species (including *Rhodovibrio sodomensis*, *Rhodovulum iodosum*, *Roseospira mediosalina* and *Roseospirillum parvum*) are omitted from this figure, as available 16S rRNA sequences are substantially less complete than those of other species. Representatives are found in each of the four subdivisions of the α-Proteobacteria.

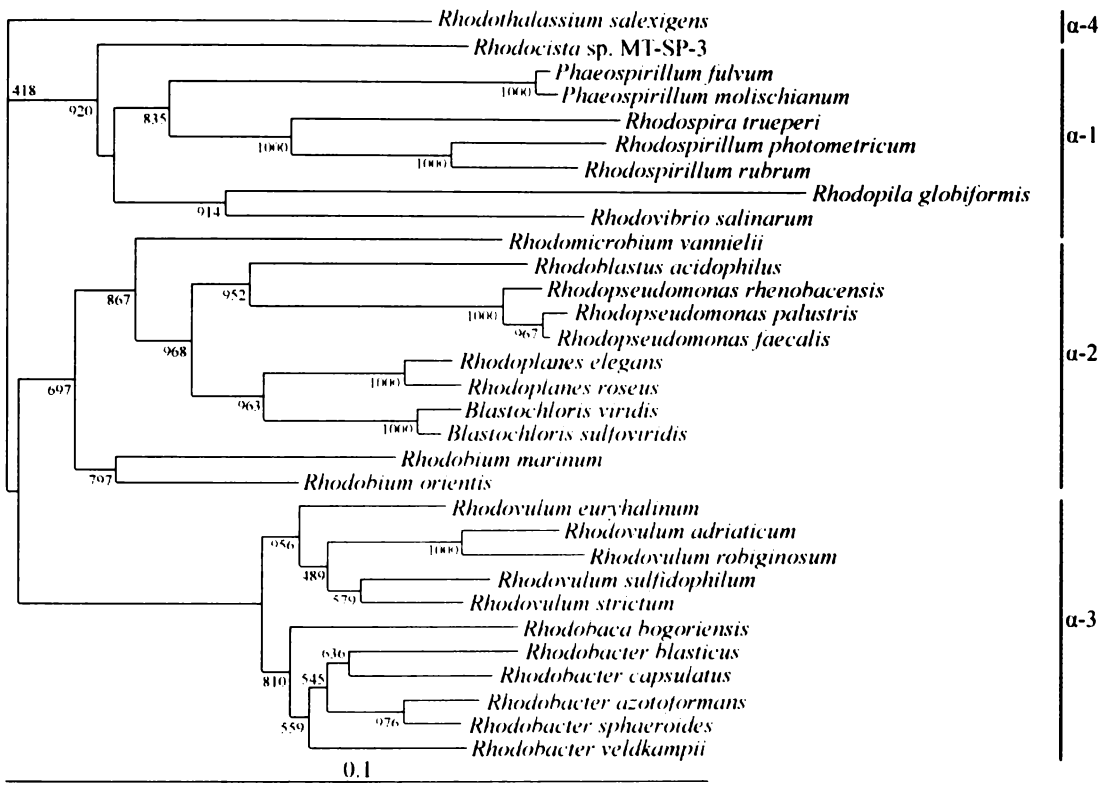


Figure 2.2 Unrooted neighbour-joining tree showing phylogenetic relationships between the purple nonsulfur bacteria of the α subdivision, based on an alignment of 1316 nucleotides of the 16S rRNA gene

A smaller number of PNSB are affiliated with the β-subdivision, and these are shown in Figure 2.3.

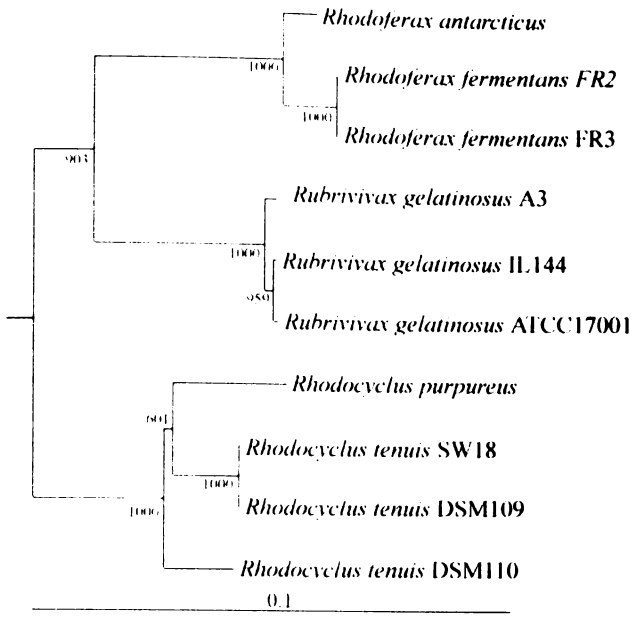


Figure 2.3 Neighbour-joining tree showing phylogenetic relationships between the purple nonsulfur bacteria of the β-subdivision, based on an alignment of 1455 nucleotides of the 16S rRNA gene, and rooted using *Rhodopseudomonas palustris* as outgroup.

2.4.1 The α-1 and α-4 subdivisions

The PNSB genera *Phaeospirillum*, *Rhodocista*, *Rhodospira*, *Rhodospirillum*, *Rhodovibrio* and *Rhodopila* are located in the α-1 and α-4 subdivisions (Figure 2.4). The species of the α-1 subdivision form two well-defined clusters. One consists of organisms of predominantly spiral morphology and includes the majority of α-1 subdivision PNSB. Within this cluster, *Phaeospirillum* species are related to magnetotactic spirilla. The second cluster contains organisms that show a tendency to thrive at low pH (Sievers *et al.* 1994), and includes the single species *Rhodopila globiformis* together with a number of aerobic photosynthetic bacteria, and acetic acid bacteria. The PNSB *Rhodothalassium salexigens* is one of the few species of the α-4 subdivision, and is distantly related to a number of aerobic anoxygenic phototrophic bacteria including *Porphyrobacter* and *Blastomonas*.

Rhodospirillum

Many species classified in the genus *Rhodospirillum* were recently transferred to new genera according to their phylogenetic position in a long overdue rearrangement carried out by Imhoff *et al.* (1998a).

The remaining species of the genus *Rhodospirillum* are vibrioid to spiral-shaped cells that divide by binary fission, are motile by polar flagella, and contain bacteriochlorophyll *a*. *Rsp. rubrum* was the first purple nonsulfur bacterium to be isolated, from the remains of a dead mouse (Esmarch 1887), and is one of the most studied anoxygenic phototrophs. Despite the long time since its isolation, new features remain to be uncovered, exemplified by the recent discovery of melatonin in *Rsp. rubrum* cells (Manchester *et al.* 1995).

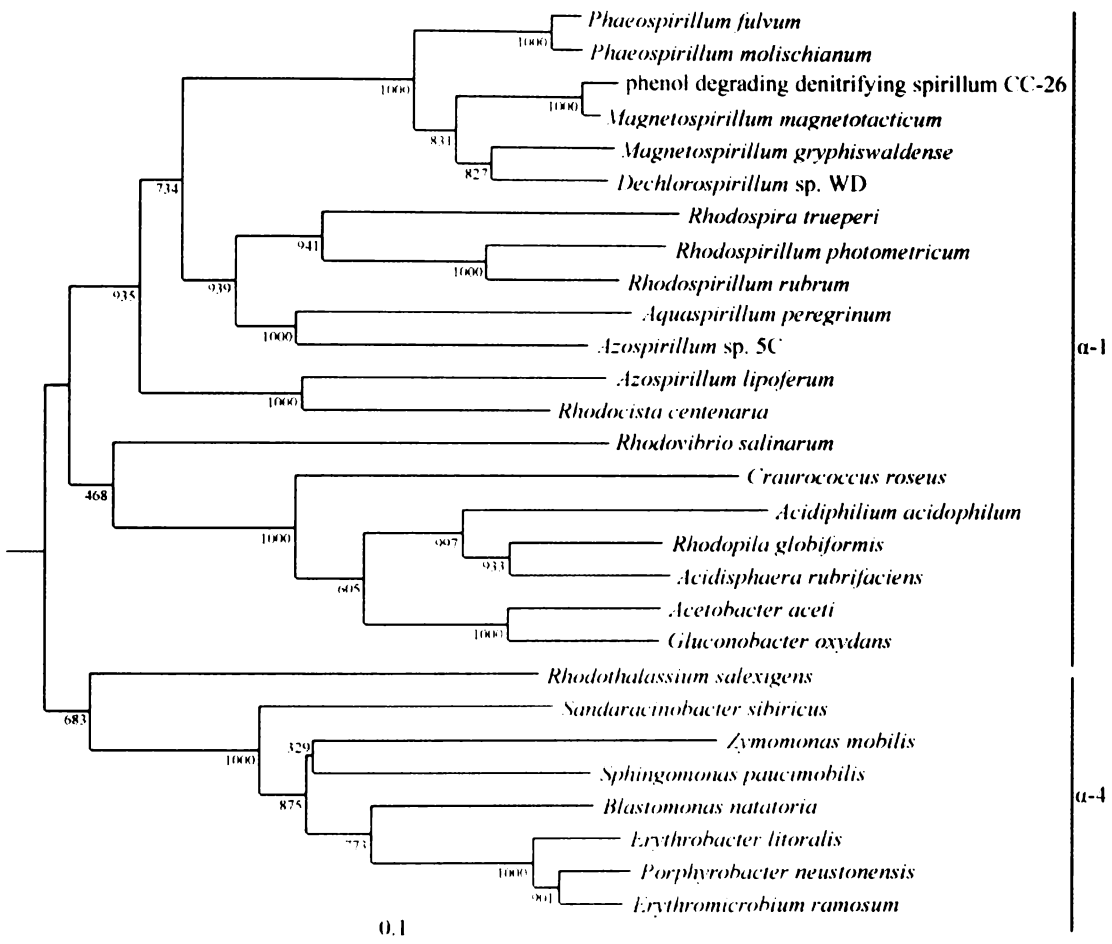


Figure 2.4 Neighbour-joining tree showing phylogenetic relationships between selected species of the α-1 and α-4 subdivisions, based on an alignment of 1348 nucleotides of the 16S rRNA gene, and rooted using the sequence of *Escherichia coli* as outgroup

Rsp. photometricum was also isolated many years ago (Molisch 1907; Giesberger 1947) and is the largest of the photosynthetic spirilla.

Rsp. centenum (Favinger *et al.* 1989) has been the subject of some taxonomic controversy (Gest and Favinger 1998; Gest and Favinger 2001; Tindall 2001). As this organism does not form a monophyletic clade with the other *Rhodospirillum* species, it is discussed under its alternate name, *Rhodocista centenaria* (Kawasaki *et al.* 1992) below. Throughout the remainder of the thesis, both names are found. *Rci. centenaria* is used by preference, but in phylogenetic trees, either name may appear, depending on the description of the strain from which the DNA sequence was obtained.

Phaeospirillum

The genus *Phaeospirillum* (Imhoff *et al.* 1998a) was created to contain two species of photosynthetic spirilla whose mass cultures are brown in colour due to the presence of the carotenoids lycopene and rhodopin and the absence of spirilloxanthin (Imhoff and Trüper 1989). The brown spirilla, including *Rsp. photometricum*, are markedly more sensitive to oxygen than the red spirilla, and will not grow in the dark at full atmospheric oxygen tension. *Phs. fulvum* (van Niel 1944) requires para-aminobenzoic acid as a vitamin, and is capable of growth on benzoate. *Phs. molischianum* (Giesberger 1947) cells are slightly larger, and the organism is incapable of benzoate degradation.

Rhodocista

Rhodocista centenaria is the sole representative of this genus. The first strain was isolated from a water sample taken from Thermopolis Hot Springs, Wyoming, USA (Favinger *et al.* 1989), and others were subsequently isolated from various Yellowstone springs and greenhouse soil (Nickens *et al.* 1996) and paper factory effluent in Japan (Kawasaki *et al.* 1992). All strains require biotin and cyanocobalamin for growth. Maximum growth temperature is 46-48°C with optimum growth occurring at 39-45°C (Stadtwald-Demchick *et al.* 1990a). Growth does not occur on the C_4 dicarboxylic acids malate, succinate or fumarate. Aerobic growth in darkness is possible. Unlike most PNSB species, synthesis of photopigments is not strongly inhibited by O_2 in this organism (Yildiz *et al.* 1991b). Under aerobic conditions during growth on butyrate, vegetative cells are converted into cyst forms which are strongly desiccation and heat resistant. This property may be related to accumulation of poly-β-hydroxybutyrate (Nickens *et al.* 1996). Some cells contain

refractile bodies of complex structure resembling the R-bodies observed in some non-photosynthetic bacteria, particularly the endosymbionts of paramecia (Pond *et al.* 1989). On agar, peritrichously flagellated swarmer cells are produced. Colonies of some strains on agar show a macroscopic phototactic response, migrating towards an incandescent light source, or away from a fluorescent light source (Ragatz *et al.* 1995). Individual swarmer cells are scotophobic but not phototactic. It has been suggested that the apparent phototactic behaviour is a colonial response due to self-shading within the colony (Sackett *et al.* 1997). This organism is currently the object of much research into the genetics of photosynthesis and phototaxis (Yildiz *et al.* 1991b).

Rhodospira

The single species of this genus, *Rhodospira trueperi* (Pfennig *et al.* 1997), was isolated from the Great Sippewissett Salt Marsh, Mass., USA, and grows optimally in the presence of 2% (w/v) NaCl. It is the only spirilloid purple nonsulfur bacterium known to contain bacteriochlorophyll *b*. Internal photosynthetic membranes are of the vesicular type. Mass cultures are beige to peach in colour.

Rhodovibrio

Two halophilic species are classified in the genus *Rhodovibrio* (Imhoff *et al.* 1998a). *Rvb. salinarum* is capable of growth over an extremely broad range of salt concentrations, from sea water salinities to over 24% (w/v) (Nissen and Dundas 1984) and optimally around 4-6% (Mack *et al.* 1993). Mass cultures are blood red. *Rvb. sodomensis* was isolated from the Dead Sea (Mack *et al.* 1993). Optimum growth occurs at 12% (w/v) NaCl and growth is possible over the range 4-20%. Mass cultures are pink. *Rvb. sodomensis* is incapable of N_2 fixation, and synthesises only light-harvesting complex I (B875).

Roseospirillum

Like *Rhs. trueperi*, *Roseospirillum parvum* was isolated from a microbial mat in the Great Sippewissett Salt Marsh. *Rss. parvum* contains bacteriochlorophyll *a*, but exhibits unusual long wavelength absorbance at 911 nm, which can be exploited in its enrichment. It is dependent on a reduced sulfur source for growth and grows optimally at 1-2% (w/v) NaCl. The phylogenetic position of this species remains uncertain. Glaeser and Overmann (1999) report its affiliation with the α-2 subdivision

genus *Rhodobium* together with *Rhodothalassium salexigens*, but other phylogenetic analyses place the latter organism as a member of the α-4 subdivision (Stackebrandt *et al.* 1996; Kawasaki *et al.* 1997), and analysis with the phylogeny program Clustal X (Thompson *et al.* 1997; see Section 3.9.6) places *Rss. parvum* at the very base of the α-1 cluster. Affiliation with the α-1 subdivision is more congruent with the organism's spirilloid morphology and possession of spirilloxanthin series carotenoids, which give mass cultures a rose-red colouration.

Roseospira

Rhodospirillum mediosalinum was transferred to the genus *Roseospira* as *Rsi. mediosalina* (Imhoff *et al.* 1998a). Though not included in Figure 2.4, this species is distantly related to *Rhs. trueperi*. Cells contain bacteriochlorophyll *a*, possess vesicular membranes, and anaerobic cultures range from pink to brownish-red. Sulfide may be used as photosynthetic electron donor. The species was isolated from a salty hot spring in Azerbaijan. Growth was optimal at 4-7% (w/v) NaCl and at 30-35°C. No growth was observed above 40°C, although the temperature of the hot spring was 43-51°C (Kompantseva and Gorlenko 1985).

Rhodopila

Rhodopila globiformis (Pfennig 1974; Imhoff *et al.* 1984) is also a member of the α-1 subdivision, though of a separate branch to the spirilloid species, and is the only purple nonsulfur bacterium to exhibit coccoid morphology. *Rpi. globiformis* was isolated from a warm sulfur spring at pH 3.0. Mass cultures are intensely purple-red, and develop best at pH 4.8-5.0 when grown on mannitol, and at 5.6 on fumarate. No growth occurs at 40°C. Biotin and para-aminobenzoic acid are required as growth factors. Thiosulfate or cysteine are required as reduced sulfur sources. *Rpi. globiformis* unusually is inhibited in the presence of normal levels of sulfate, due to apparent misregulation of sulfate assimilation enzymes (Imhoff *et al.* 1981).

Rhodothalassium

Rhodothalassium salexigens (Drews 1981) is the only known PNSB of the α-4 subdivision. It was the first obligately halophilic species of PNSB to be discovered. It is dependent on glutamate as nitrogen source (Rubin and Madigan 1986). Cells are similar in size to those of *Rsp. rubrum* and mass cultures are red.

2.4.2 The α-2 subdivision

The α-2 subdivision contains the genera *Rhodopseudomonas*, *Rhodoblastus*, *Blastochloris*, *Rhodoplanes*, *Rhodobium* and *Rhodomicrobium*. With the exception of *Rhodomicrobium*, long-established species were all formerly classified in the genus *Rhodopseudomonas*. As can be seen from Figure 2.5, none were specifically related to the type strain *Rps. palustris* to the exclusion of non-photosynthetic organisms. *Rps. palustris* instead shows a close relationship to the nodulating symbiont species of the genus *Bradyrhizobium* and to *Blastobacter denitrificans*, which was also recently shown to be capable of forming symbioses with a legume species (van Berkum and Eardly 2002). Several aerobic anoxygenic phototrophic bacteria have been classified in the genus *Bradyrhizobium* or related genera (Chaintreuil *et al.* 2000; Fleischman and Kramer 1998; Wong *et al.* 1994). *Nitrobacter* strains are also closely related to *Rps. palustris* (Orso *et al.* 1994; Seewaldt *et al.* 1982). Although their physiologies are dissimilar, morphological characteristics are shared between the group – namely, budding mode of division, formation of rosettes of cells, and intracellular membrane systems in lamellar structures.

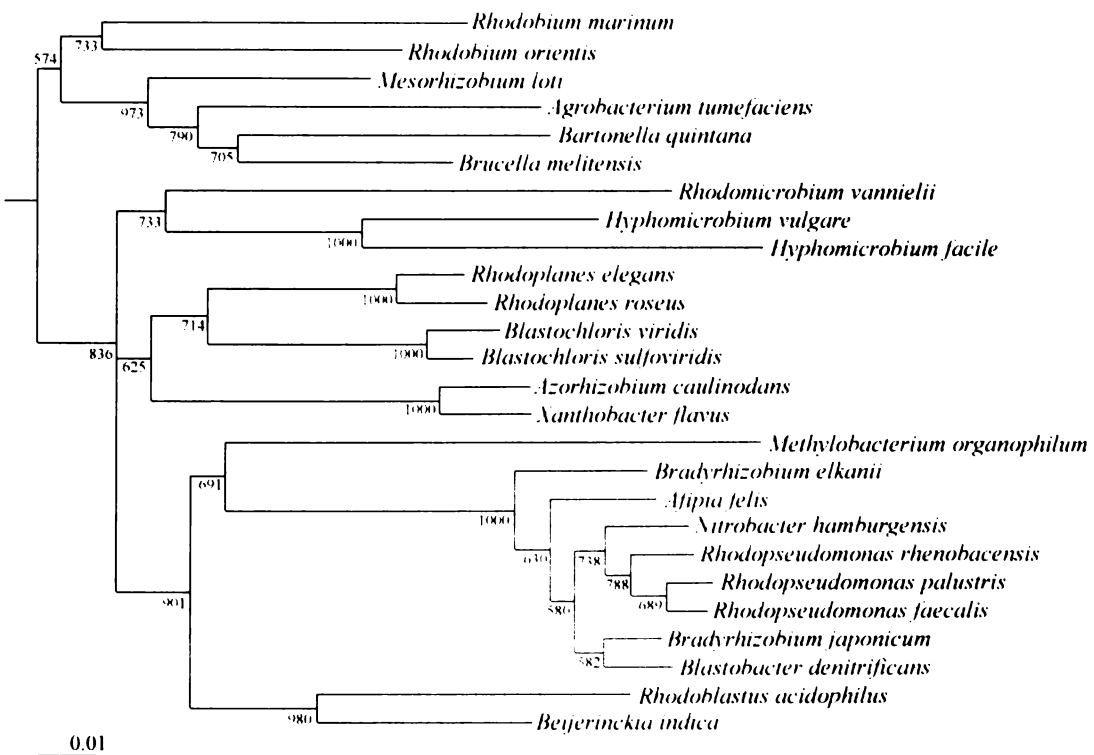


Figure 2.5 Neighbour-joining tree showing phylogenetic relationships between selected species of the α-2 subdivision, based on an alignment of 1351 nucleotides of the 16S rRNA gene, and rooted using the sequence of *Rhodobacter capsulatus* as outgroup

Blastochloris and *Rhodoplanes* are neighbouring genera, but show a relationship to *Azorhizobium caulinodans*, a free-living nitrogen-fixing organism. *Rhodomicrobium* is related to several organisms of similar morphology of the genus *Hyphomicrobium* (Stackebrandt *et al.* 1988).

Rhodopseudomonas

Purple nonsulfur bacteria with budding morphology and lamellar intracytoplasmic photosynthetic membranes were historically classified in the genus *Rhodopseudomonas*. Phylogenetic data have led to the reorganisation of the genus, which currently contains only three recognised species.

Rhodopseudomonas palustris is an archetypal "loose species" (Ambler 1996), consisting of many similar strains of slightly varying 16S rDNA sequence. The type strain is capable of growth on the widest range of carbon sources of any PNSB, including branched chain fatty acids and alcohols (Charlton 1997), many aromatic compounds (Rahalkar *et al.* 1993) and halogenated compounds (McGrath and Harfoot 1997; Oda *et al.* 2001). Other strains show similar versatility. *Rps. rutila* (Akiba *et al.* 1983) has an identical 16S rDNA sequence to *Rps. palustris* and therefore the two species are regarded as synonymous (Hiraishi *et al.* 1992).

Rps. rhenobacensis (Hougardy *et al.* 2000) and *Rps. faecalis* (Zhang *et al.* in press) are recently-described species whose 16S rDNA sequences are 98% and 99.4% similar to that of *Rps. palustris*. *Rps. rhenobacensis* is a nitrate-reducing organism with a pH optimum of 5.5. *Rps. faecalis* is mildly thermotolerant, growing between 28 and 45°C and optimally at 38°C, and was isolated from a bioreactor containing chicken faeces.

"*Rps. cryptolactis*" (Stadtwald-Demchick *et al.* 1990b) was isolated from the same location as the first strain of *Rhodocista centenaria*, Thermopolis Hot Springs in Wyoming. "*Rps. cryptolactis*" is restricted in the range of carbon compounds it can use for growth. Growth on lactate, which is more oxidised than cell carbon, is dependent on the presence of bicarbonate. Growth occurs at temperatures between 35 and 46°C and optimally between 38-40°C. "*Rps. cryptolactis*" requires nicotinic acid and cyanocobalamin as growth factors. Under strong illumination, "*Rps. cryptolactis*" produces both LH I (B875) and LH II (B800/850) complexes. Under

low light conditions, however, a different light-harvesting complex is produced with absorption maximum at 822 nm (Halloren *et al.* 1995). The name "*Rhodopseudomonas cryptolactis*" has not appeared in the Validation Lists published in the IJSEM, and thus has no standing in bacterial nomenclature.

Rhodoblastus

Rhodoblastus acidophilus is the sole species in this genus (Imhoff 2001), first described by Pfennig (1969b). Cells of this species are larger than those of other *Rhodopseudomonas*-like organisms. It has an optimum pH for growth of 5.5-6.0 and is unable to grow at pH 7.0.

Rhodoplanes

The type strain of this genus was isolated in New Zealand from a freshwater lake and described as *Rhodopseudomonas rosea* (Janssen and Harfoot 1991). *Rps. rosea* was transferred to the new genus *Rhodoplanes* after the isolation in Japan of a second species, *Rpl. elegans*, from activated sewage sludge (Hiraishi and Ueda 1994b). A characteristic feature of these strains is their inability to use medium or long-chain fatty acids as carbon sources. Photoautotrophic growth is possible with thiosulfate as electron donor. Growth in darkness is possible aerobically or anaerobically in the presence of nitrate. Complete denitrification is possible. A second strain of *Rpl. roseus* has been isolated from microbial mats at a Swiss alpine lake (Wiggli *et al.* 1998).

Blastochloris

The genus *Blastochloris* (Hiraishi 1997) was created to accommodate the species *Rps. viridis* (Drews and Giesbrecht 1966) and *Rps. sulfoviridis* (Keppen and Gorlenko 1975), which form a unique line of descent together with *Rhodoplanes*. The *Blastochloris* species contain bacteriochlorophyll *b* as their major pigment, and mass cultures are green in colour. Some strains of *Blc. sulfoviridis* are unable to assimilate sulfate and require reduced sulfur sources (Neutzling *et al.* 1982). Sulfide and thiosulfate may also be used as photosynthetic electron donors by *Blc. sulfoviridis*, but not by *Blc. viridis*. Resnick and Madigan (1989) isolated *Rhodopseudomonas* strain GI from a geothermal area in New Mexico, USA. Like *Blc. sulfoviridis* DSM 729, this strain requires a reduced sulfur source. Growth occurs to a maximum of 47°C.

Rhodomicrobium

The genus *Rhodomicrobium* contains the single species *Rmi. vannielii* which possesses unique morphological features (Duchow and Douglas 1949). Non-motile mother cells divide by budding with formation of filaments, leading to development of large dendroid aggregates. Smaller heat and desiccation-resistant exospores may also be formed in clusters of one to four at the ends of filaments (Gorlenko 1969). Motile peritrichously-flagellated "swarmer" cells are also observed. The vegetative cell cycle and morphology are similar to those of *Hyphomicrobium*. Cell suspensions are variable in colour, ranging from red to orange-brown to pink. No vitamins are required. *Rmi. vannielii* tolerates sulfide at 2-3 mM which is high relative to most PNSB. Use of Fe^{2+} as electron donor for photosynthesis has been observed in a *Rmi. vannielii* strain (Heising and Schink 1998).

Rhodobium

Two *Rhodobium* species have been isolated from marine environments. They grow optimally in the presence of 4-5% (w/v) NaCl. *Rbi. marinum* (Imhoff 1983; Mangels *et al.* 1986) has a similar absorption spectrum to *Rsp. rubrum* with low absorbance at 803 nm and a maximum at 883 nm. *Rbi. orientis* is capable of denitrification under dark anaerobic conditions. Freshwater *Rhodobium marinum* strains have been reported from sulfur springs in the Kurile Islands (Kompantseva and Panteleeva 1998), and these grow optimally at 1-3% (w/v) NaCl, but are also capable of growth in the absence of added salt. *Rps. julia* (Kompantseva 1989) was isolated from sulfur springs on the shores of the caldera lake of a Kamchatkan volcano, and was described as exhibiting a number of unusual characteristics. Photolithoautotrophic growth was possible on elemental sulfur. Intracellular sulfur droplets were observed when cells were grown in the presence of sulfide. Its 16S rDNA sequence is identical to that of *Rbi. marinum*.

2.4.3 The α-3 subdivision

Three genera of PNSB are located in the α-3 subdivision (Figure 2.6). These are *Rhodobacter* (Imhoff *et al.* 1984), *Rhodovulum* (Hiraishi and Ueda 1994a) and *Rhodobaca* (Milford *et al.* 2000). *Rhodobacter* strains are freshwater, *Rhodovulum* are marine, and the single species of *Rhodobaca* is slightly haloalkaliphilic.

Many aerobic anoxygenic phototrophic bacteria lie in the α-3 subdivision, including the pink-pigmented *Roseobacter* species commonly found on seaweed (Shiba 1991), the saline *Roseovarius tolerans* (Labrenz *et al.* 1999), ***Rubrimonas cliftonensis*** (Suzuki *et al.* 1999) and others.

Agrobacterium ferrugineum is a non-photosynthetic marine organism that forms star-shaped aggregates that was assigned to the genus *Agrobacterium* on the basis of unreliable genotypic studies (Rüger and Höfle 1992). Its DNA gyrase subunit B (*gyrB*) and 16S rRNA (Figure 2.6) gene sequences clearly show its close affiliation with *Rhodobacter* species. Transfer to the new genus *Pseudorhodobacter* has been proposed (GenBank sequence description, AB014904).

The nearest phylogenetic relative of *Rbc. bogoriensis* is "*Natronohydrobacter thiooxidans*". No description has yet been published, but the organism is apparently a facultatively autotrophic hydrogen-oxidising bacterium from a Kenyan soda lake (GenBank sequence description, AJ132383)

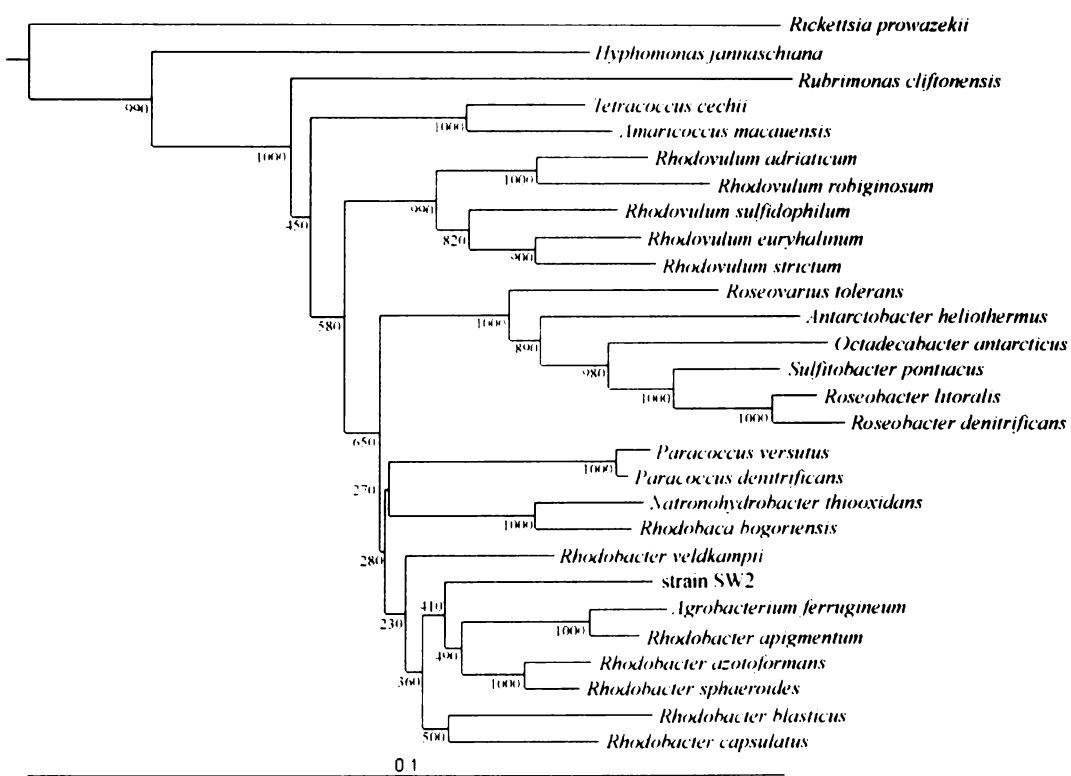


Figure 2.6 Neighbour-joining tree showing phylogenetic relationships between selected species of the α-3 subdivision, based on an alignment of 1328 nucleotides of the 16S rRNA gene, and rooted using the sequence of *Rhodopseudomonas palustris* as outgroup

Rhodobacter

The genus *Rhodobacter* was created for several species previously classified as members of *Rhodopseudomonas* (Imhoff *et al.* 1984). The genus currently contains five well-described species. Information regarding "*Rba. apigmentum*" is not available in the published literature. It is a non-photosynthetic phenanthrene-degrading organism from marine sediments described so far only by its 16S rDNA sequence (GenBank sequence description, AF035433), and is not included in the following discussion.

Cells are ovoid to rod-shaped. Cells of most species divide by binary fission and contain internal photosynthetic membranes of the vesicular type. Anaerobic cell suspensions range in colour from yellow to green-brown to brown due to the presence of carotenoids of the sphaeroidene group. On exposure to oxygen, cell suspensions turn red as these carotenoids are oxidised to keto-derivatives.

Physiologically, the *Rhodobacter* species are extremely versatile, and are perhaps the most versatile of all bacteria (Imhoff and Trüper 1989). All species require thiamine as a growth factor, and several require in addition nicotinate, biotin or para-aminobenzoic acid. They are all mesophilic and neutrophilic, having temperature optima from 30-35°C and growing in the pH range 6.0 to 8.5. They have no requirement for NaCl, though some species are able to tolerate salt concentrations as high as 3% (w/v).

Rba. capsulatus and *Rba. sphaeroides* (van Niel 1944) are extremely common species, readily enriched and isolated from environments suitable for PNSB. They are sometimes difficult to distinguish (Pellerin and Gest 1983). *Rba. capsulatus* has a tendency to produce zigzag chains of cells and is up to 10^5 times more sensitive to penicillin (Weaver *et al.* 1975). *Rba. azotoformans* is closely related to *Rba. sphaeroides*, but all known strains are capable of complete denitrification (Hiraishi *et al.* 1995b). *Rba. veldkampii* (Hansen and Imhoff 1985) is unable to assimilate sulfate as a sulfur source and is able to tolerate high levels of sulfide (~5 mM). In this it is similar to *Rhodovulum* species, but it has no sodium ion requirement.

Rba. blasticus (Eckersley and Dow 1980) differs from other members of the genus in that it undergoes sessile budding and has lamellar membranes. It therefore was

retained in the genus *Rhodopseudomonas* until molecular evidence showed its true phylogenetic affiliation (Kawasaki *et al.* 1993a). *Rba. blasticus* is much less common than other *Rhodobacter* species. The isolation of only two strains has been reported (Eckersley and Dow 1980; Yurkov and Gorlenko 1992a). A bacteriophage specific to *Rba. blasticus* has been found at the location from which the type strain was isolated (Gorham and Dow 1996).

"*Rba. marinus*" was described by Burgess *et al.* (1994). Its phylogenetic position remains undetermined, but as a marine organism sensitive to oxygen, it is likely to fall within the *Rhodovulum* radiation rather than with *Rhodobacter sensu stricto.* Nonetheless, its sensitivity to sulfide would indicate that it is not a typical *Rhodovulum* species either.

Strain SW2 has not yet been formally described, but is known to use ferrous iron ions as electron donors for photosynthesis (Ehrenreich and Widdel 1994). "*Rba. gluconicum*" is another undescribed species whose 16S rDNA sequence is most closely related to that of *Rba. blasticus* (GenBank sequence description, AB077986).

The presence of organisms related to *Rhodobacter* has been reported as the dominant population in the mesohyl compartment of the marine sponge *Halichondra panicea* (Althoff *et al.* 1998), which perhaps indicates that the full diversity of *Rhodobacter* species remains unexplored.

Rhodovulum

The species of *Rhodovulum* were previously grouped with those of *Rhodobacter* and share similar morphological and physiological properties, but are phylogenetically distinct (Hiraishi and Ueda 1994a; Figure 2.6). Moreover, the described *Rhodovulum* species are unified by their requirement for NaCl for optimal growth. *Rhodovulum* species have been found only in marine or hypersaline environments, and circumstantial evidence points to their absence from freshwater environments (Imhoff 2001). The type strain is *Rov. sulfidophilum*, which was the first PNSB to be shown to readily use sulfide as an electron donor for photosynthesis (Hansen and Veldkamp 1973). *Rov. adriaticum* (Neutzling *et al.* 1984), *Rov. euryhalinum* (Kompantseva 1985), *Rov. iodosum* and *Rov. robiginosum* (Straub *et al.* 1999) require a reduced sulfur source for biosynthesis, and all *Rhodovulum* species have a higher tolerance for

sulfide than most PNSB. *Rov. strictum* is mildly alkaliphilic, growing in the pH range 7.5 to 9.0 and optimally at 8.0 to 8.5 (Hiraishi and Ueda 1995). *Rov. iodosum* and *Rov. robiginosum* are notable for their ability to use Fe^{2+} as photosynthetic electron donor (Straub *et al.* 1999).

Rhodobaca

The sole species of the genus *Rhodobaca, Rbc. bogoriensis*, was isolated from alkaline soda lakes in the Rift Valley of eastern Africa (Milford *et al.* 2000). It forms a unique lineage distinct from *Rhodobacter* and *Rhodovulum.* Unlike these organisms, *Rbc. bogoriensis* is unable to fix N_2 and has not been observed to grow photoautotrophically. The growth parameters of *Rbc. bogoriensis* closely reflect its hot haloalkaline habitat. It grows optimally at 1-3% (w/v) NaCl, but is able to grow in the absence of NaCl. Its optimum pH is 9.0, making it the most alkaliphilic PNSB known. *Rbc. bogoriensis* has a temperature optimum of 39°C and a maximum of 43°C.

2.4.4 The β-subdivision

The β-subdivision contains only a few known PNSB species which are only slightly related to one another (Figure 2.7). *Rubrivivax gelatinosus* is related to *Ideonella dechloratans*, a chlorate metabolising organism (Stenklo *et al.* 2001).

Rhodoferax

The genus *Rhodoferax* contains two species which are phenotypically similar to *Rubrivivax gelatinosus* (Hiraishi *et al.* 1991). *Rfx. fermentans* is notable for its rapid fermentative growth in darkness on fructose. Some strains are also capable of denitrification (Hougardy and Klemme 1995). *Rfx. antarcticus* is the only known mildly psychrophilic PNSB, having an optimum temperature for growth of 15-18°C, and a maximum of 25°C (Madigan *et al.* 2000).

Rubrivivax

This genus contains the single species *Rvi. gelatinosus* (Willems *et al.* 1991), which has been reclassified a number of times, having previously resided in the genera *Rhodopseudomonas* and *Rhodocyclus*. Cells have a peach or brown colour due to sphaeroidene carotenoids, produce copious amounts of mucus, and have the ability to

liquefy gelatin, a property shared only by *Rfx. fermentans* and some strains of *Rba. capsulatus* among the PNSB.

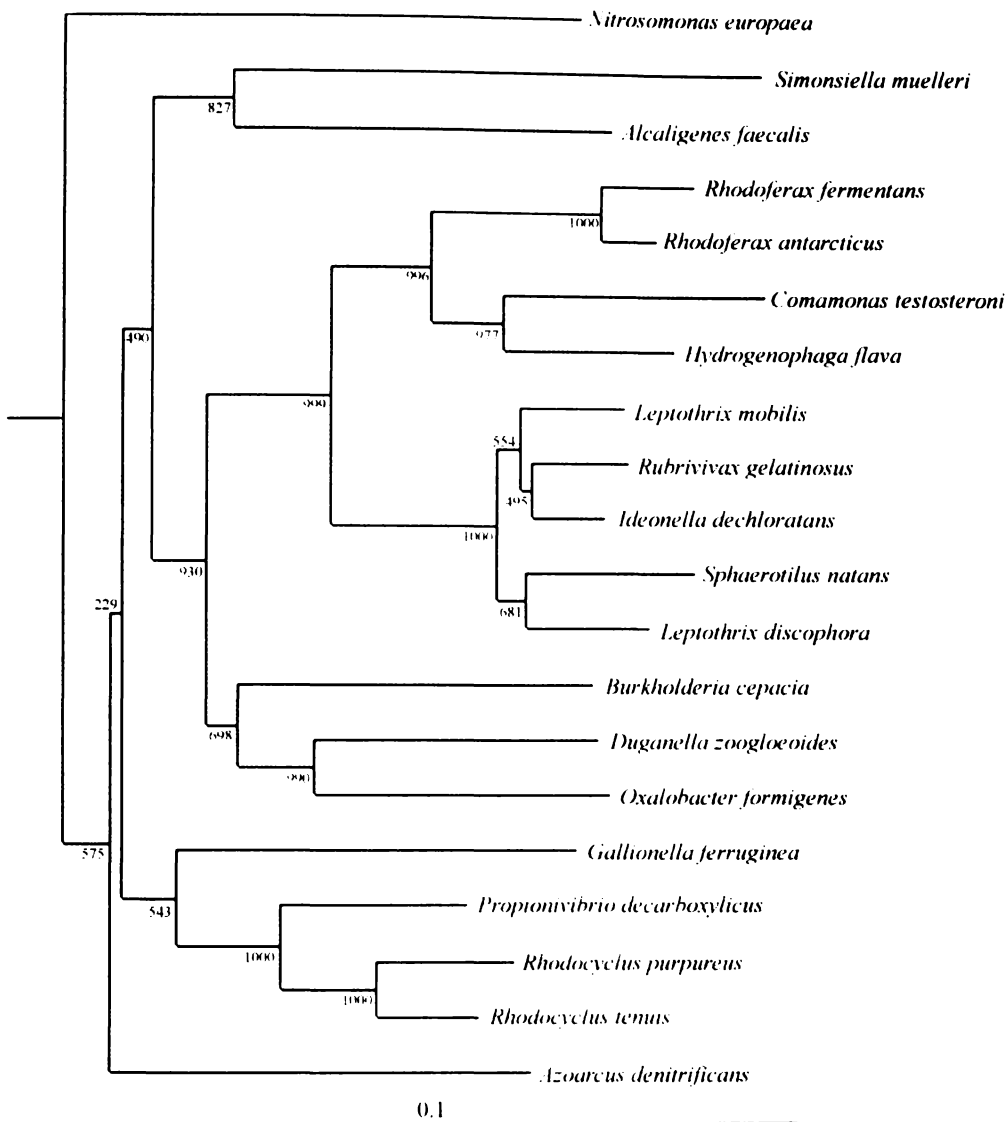


Figure 2.7 Neighbour-joining tree showing phylogenetic relationships between selected species of the β-subdivision, based on an alignment of 1369 nucleotides of the 16S rRNA gene, and rooted using the sequence of *Rhodopseudomonas palustris* as outgroup

Rhodocyclus

Rhodocyclus tenuis cells are small spirilla (Pfennig 1969a). The species is extremely common in sewage sludge and freshwater environments. *Rcy. purpureus* is known only from a single strain isolated from a pig sewage pond in Iowa, and is probably rare in nature (Pfennig 1978). It has a unique half-circle morphology, and is non-

motile. *Rcy. purpureus* is relatively limited in its use of carbon sources (e.g. Harfoot and Janssen 1985), but is capable of benzoate degradation.

2.5 Thermophilic photosynthetic organisms

Many species of cyanobacteria are thermophilic. *Synechococcus* strains from North America are the most thermophilic photosynthetic organisms known, able to grow to a maximum temperature of 73°C (Miller and Castenholz 2000). Thermophilic anoxygenic photosynthetic bacteria capable of growth above 50°C include the green nonsulfur bacteria *Chloroflexus aurantiacus*, *Cfl. aggregans* (Pierson and Castenholz 1974; Hanada *et al.* 1995) and *Heliothrix oregonensis* (Pierson *et al.* 1985), the green sulfur bacterium *Chlorobium tepidum* (Wahlund *et al.* 1991), *Heliobacterium modesticaldum* (Kimble *et al.* 1995), and the purple sulfur bacterium *Thermochromatium tepidum* (Madigan 1986; Imhoff *et al.* 1998b). This last is the only purple photosynthetic bacterium known to grow above 50°C. Other anoxyphototrophs are known which are mildly thermophilic, including several species of heliobacteria and members of the Ectothiorhodospiraceae.

Purple nonsulfur bacteria are the least thermotolerant of the photosynthetic bacteria. To date, only three mildly thermophilic purple nonsulfur bacteria isolated from geothermal environments have been described: *Rhodospirillum centenum* (*Rhodocista centenaria*), *Blastochloris* strain GI and "*Rhodopseudomonas cryptolactis*".

Rhodospirillum centenum was first isolated from Thermopolis Hot Springs, Wyoming (Favinger *et al.* 1989). Subsequently, strains have been isolated from other locations in the United States and Japan (Kawasaki *et al.* 1992). Its properties have been described above in Section 2.4.1.

Blastochloris strain GI, originally described as *Rhodopseudomonas* strain GI, was isolated from microbial mats at 47°C at a hot spring in New Mexico called Soda Dam (Resnick and Madigan 1989). Enrichment cultures contained acetate, CO_2 and low levels of sulfide (<1 mM). The strain grows optimally at 42°C, has a temperature maximum of 47°C, and requires a reduced sulfur source. A 16S rDNA sequence is available (Hoogewerf and Madigan, unpublished results, GenBank Accession Number

AF084510), which is highly similar to that of *Blc. sulfoviridis*. Unfortunately, this sequence is internally incomplete.

"*Rhodopseudomonas cryptolactis*" was isolated from a soil sample taken from the edge of Thermopolis Hot Springs in Wyoming after enrichment at 37°C on a medium lacking NH_4Cl but containing pyruvate and soytone (Stadtwald-Demchick *et al.* 1990b). Growth occurs optimally at 38-40°C to a maximum of 46°C.

In addition, *Rps. faecalis* grows optimally at 38°C and has a maximum temperature for growth of 45°C. Unlike other thermotolerant PNSB, it was not isolated from a natural geothermal area (Zhang *et al.* in press).

Several of the extremely halophilic purple nonsulfur bacteria species have temperature maxima for growth that approach those of the three freshwater thermophilic PNSB. *Rhodothalassium salexigens* grows optimally at 40°C and has a temperature maximum of 45°C (Drews 1981); *Rhodovibrio salinarum* has an optimum of 42°C; *Rvb. sodomense* grows optimally at 35-40°C, and its temperature maximum is 47°C (Mack *et al.* 1993). These high temperatures probably occur intermittently due to solar heating in the shallow pools and salterns from which these organisms were isolated. A correlation coefficient between temperature optimum and salinity optimum of 0.8 for a range of photosynthetic organisms was calculated by Kuntikov and Gorlenko (1998).

Several other species of PNSB were originally isolated from geothermal environments. These include *Blastochloris sulfoviridis*, *Roseospira mediosalina*, *Rhodopila globiformis* and *Rhodopseudomonas julia*. However each of these species have temperature optima in the normal range for PNSB, 30-35°C, and temperature maxima below 40°C.

Russian researchers, notably V.M. Gorlenko, have published a number of reports on the diversity of photosynthetic bacteria in hot spring environments. *Rba. capsulatus*, *Rps. palustris* and *Rubrivivax gelatinosus* were found over a remarkably wide temperature range, including 45-60°C in springs in the caldera of Uzon Volcano and near Lake Baikal (Gorlenko *et al.* 1986; Gorlenko *et al.* 1987). *Rsi. mediosalina* was detected at temperature up to 51°C. *Blc. sulfoviridis*, *Blc. viridis*, *Rba. blasticus*, *Rmi.*

vannielii and *Rps. palustris* were detected in alkaline springs near Lake Baikal at temperatures up to 54°C (Yurkov *et al.* 1992; Yurkov and Gorlenko 1992b). No novel purple nonsulfur bacterial species were discovered, but the most striking observation was that without exception all the PNSB that were cultivated were mesophilic, showing no differences in cardinal temperature values from the type strains. It was suggested that these organisms were transient members of the hot spring communities, recently washed in from locations of lower temperature, or that resting forms of cells are capable of survival at temperatures above the growth maximum.

2.6 Studies of photosynthetic organisms in the geothermal environments of New Zealand

The earliest study of New Zealand geothermal environments was carried out by F. von Hochstetter, in whose honour several geothermal features have been named, who around 1864 measured temperatures and collected and described specimens of algae, most of which were cyanobacteria. Little further work was carried out in the next hundred years.

Kaplan (1956) examined hot springs and fumaroles on White Island, in Rotorua and in Taupo. Besides examining algae, Kaplan attempted to culture representatives of major physiological groups, including sulfate-reducing bacteria, colourless sulfur bacteria, and photosynthetic sulfur bacteria. Two forms of the latter were described (see Figure 2.8).

Figure 2.8 Line drawings of organisms observed by direct microscopy. A: purple sulfur bacteria from Orakei Korako, B: green sulfur bacteria from Rotorua (drawings to same unspecified scale, reproduced from Kaplan 1956)

The purple sulfur bacterium was ovoid and non-motile, while the green sulfur bacterium was thread-like. The identity of these organisms in relation to currently described species is uncertain.

Brock and Brock (1971) surveyed many North Island thermal areas, but concentrated their studies on algae and cyanobacteria, and ephydrid flies. In other cases, they simply recorded presence or absence of microorganisms. The cyanobacteria *Mastigocladus laminosus* and *Phormidium* sp. were reported at Waimangu and Orakei Korako. A *Synechococcus* species was also present at Orakei Korako. Extensive accumulations of "orange flexibacteria" were reported at various locations. These are presumably related to the organism that would later be described as *Chloroflexus aurantiacus* (Pierson and Castenholz 1974).

Shiea *et al.* (1991) reported the presence of *Chloroflexus aurantiacus* and a species of the cyanobacterium *Chlorogloeopsis* in the microbial mats of Orakei Korako. The failure to observe purple bacteria by microscopic analysis was noted, though this doubtless refers only to morphologically conspicuous purple sulfur bacteria, not to rod-shaped PNSB which would be largely indistinguishable from non-phototrophic organisms.

The thermophilic green sulfur bacterium *Chlorobium tepidum* was first observed in a sulfidic stream (45°C, pH 5.9, 1 mM sulfide) in Rotorua (Castenholz *et al.* 1990). It was later isolated in sulfide/CO_2 enrichment culture. In the presence of acetate, purple sulfur bacteria resembling *Thermochromatium tepidum* dominated instead (Wahlund *et al.* 1991). The isolation of *T. tepidum*-like strains from New Zealand hot springs has also been reported. These strains differ from *T. tepidum* in that their maximum temperature for growth is lower, and they contain a B890 light-harvesting complex rather than the unusual B920 complex (Madigan 2001).

The only reference to the isolation of a purple nonsulfur bacterium from a geothermal site in New Zealand is to a strain designated OB5 and identified as *Rhodobacter sphaeroides*, which was isolated from an enrichment on acetate and thiosulfate of algal mat material from the overflow stream of a hot spring near Taupo. However the water temperature at the sample site was only 30°C (Janssen 1985) and the strain was typical of mesophilic *Rba. sphaeroides*.

Chapter 3
General Methods

3.1 Introduction

This chapter provides a general overview of the materials and methods used throughout this study. More detailed methodologies used in specific experiments are described in the relevant chapters.

3.2 List of laboratory bacterial strains

Strains of purple nonsulfur bacteria maintained in this laboratory were obtained as gift cultures from other workers, isolated in previous work, or purchased from type culture collections. Table 3.1 lists the species and strain number of PNSB used.

Blastochloris sulfoviridis DSM 729	*Rhodovulum sulfidophilum* DSM 1374
Blastochloris viridis DSM 133	*Rhodoplanes elegans* AS130
Blastochloris strain GI	*Rhodoplanes roseus* 941
Rhodobacter azotoformans KA25	"*Rhodopseudomonas cryptolactis*"
Rhodobacter blasticus DSM 2131	*Rhodopseudomonas julia* DSM 11549
Rhodobacter capsulatus DSM 1710	*Rhodopseudomonas palustris* DSM 123
Rhodobacter sphaeroides DSM 158	*Rhodopseudomonas rutila* ATCC 33872*
Rhodobacter veldkampii ATCC 35703	*Rhodopseudomonas* strain GI
Rhodoblastus acidophilus DSM 137	*Rhodospirillum centenum* ATCC 51521
Rhodobium marinum ATCC 35675	*Rhodospirillum photometricum* DSM 122
Rhodocyclus purpureus DSM 168	*Rhodospirillum rubrum* DSM 467
Rhodocyclus tenuis ATCC 25093	*Rhodothalassium salexigens* DSM 2132
Rhodoferax fermentans FR2	*Rhodovibrio salinarum* ATCC 35394
Rhodomicrobium vannielii DSM 162	*Rubrivivax gelatinosus* DSM 1709

* now *Rps. palustris*

Table 3.1 Purple nonsulfur bacteria strains used in this study

Rba. azotoformans KA25, *Rbi. marinum* ATCC 35675, *Rfx. fermentans* FR2 and *Rov. sulfidophilum* DSM 1374 were kindly provided by Prof. Akira Hiraishi, Toyohashi University of Technology, Japan. *Rcy. purpureus* DSM 168 and *Blastochloris* (*Rhodopseudomonas*) strain GI were kindly provided by Prof. Michael Madigan, Southern Illinois University, USA. *Rps. cryptolactis* and *Rsp. centenum* were kindly

provided by Prof. Carl Bauer, Indiana State University, USA. *Rpl. roseus* was isolated in this laboratory, and has been maintained here. All other strains were originally obtained from the DSM or ATCC culture collections.

3.3 Materials

Commonly used materials such as salts and simple organic compounds were of analytical grade and were obtained from Sigma-Aldrich Chemical Company (Sydney, Australia), Invitrogen (Auckland, NZ) or BDH (Dorset, UK). All gases used were obtained from BOC Gases (Auckland, NZ). Yeast extract was obtained from BBL (Fort Richard, NZ). SeaKem agarose was obtained from FMC Bioproducts, BioLab Scientific, NZ. Purified agar was obtained from Oxoid (Hampshire, UK).

Custom-made oligonucleotide primers were purchased from Life Technologies, Auckland, NZ. Taq polymerase was obtained from Boehringer-Mannheim, Germany or Perkin Elmer, Australia. Deoxynucleotide triphosphates (dNTPs) were obtained from Boehringer-Mannheim. RNAse, proteinase K and pronase E were obtained from Sigma-Aldrich, NZ. Restriction enzymes were obtained from Boehringer-Mannheim, Sigma or Roche. FastPrep-Soil Kit was obtained from Bio101. Topo-TA Cloning Kit was obtained from Invitrogen.

3.4 Growth media

The following sections list the composition of various growth media and growth media components used in standard procedures in this work.

3.4.1 Trace element solution

The following salts were dissolved in 6.5 mL of 25% (v/v) hydrochloric acid: 60 mg H_3BO_4, 100 mg $MnCl_2.4H_2O$, 120 mg $CoCl_2.6H_2O$, 70 mg $ZnCl_2$, 25 mg $NiCl_2.6H_2O$, 15 mg $CuCl_2.2H_2O$ and 25 mg $NaMoO_4.2H_2O$. This solution was made up to 1 L with distilled water, and sterilised by autoclaving.

3.4.2 Bicarbonate solution

A 7.5% (w/v) bicarbonate solution was prepared by dissolving 7.5 g $NaHCO_3$ to a total volume of 100 mL in carbon dioxide saturated distilled water.

The water was saturated by alternately flushing the head space of the flask with CO_2, and shaking the stoppered vessel vigorously. This process was continued until no negative pressure was felt on removal of the stopper (Lee 1992). The solution was then either autoclaved in sealed universal bottles, or filter-sterilised through a 0.22 μM syringe-tip filter (Millipore Corporation, Bedford, Massachusetts) under CO_2 into a sterile serum phial. In general, 0.1 mL of sterile bicarbonate solution was added aseptically to a final volume of 10 mL of complete growth medium, bringing the final pH to 6.8.

3.4.3 Vitamin solutions

Vitamin solutions were added to individual Hungate tubes rather than to bulk growth media. Working vitamin stocks were prepared from 100 times stock solutions and were added at 0.05 mL per Hungate tube. Individual working stock solutions contained: 20 μg/mL nicotinic acid, 10 μg/mL pantothenic acid, 10 μg/mL pyridoxamine, 2 μg/mL biotin, 20 μg/mL thiamine, 10 μg/mL para-aminobenzoic acid and 2 μg/mL cyanocobalamin.

Two stock solutions containing vitamin mixtures were also used. The first vitamin pool, V1, contained nicotinic acid, pantothenic acid and pyridoxamine at the above concentrations. The second pool, V2, contained biotin, thiamine and para-aminobenzoic acid at the above concentrations.

3.4.4 Ferric quinate solution

0.27 g $FeCl_3.6H_2O$ and 0.11 g quinic acid were dissolved in distilled water to a total volume of 100 mL. Ferric quinate solution was added to some growth media at 2 mL per litre.

3.4.5 Sulfide solution

5 g $Na_2S.9H_2O$ was dissolved in 90 mL of distilled water which had been degassed under vacuum for 15 minutes. The solution was neutralised by slow addition with stirring of 5 M HCl, and made up to 100 mL with more degassed distilled water. The resulting 5% (w/v) sulfide solution was autoclaved in a serum vial with the headspace flushed with oxygen-free nitrogen gas.

3.4.6 Medium MC

The following were dissolved in distilled water to a total volume of 1 L:

0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.4 g NaCl, 1.0 g NH_4Cl, 0.075 g $CaCl_2.2H_2O$, 0.05 g $Na_2S_2O_3.5H_2O$, 0.02 g yeast extract, 1 mL trace element solution (Section 3.3.1), 1 mL Vitamin B_{12} (cyanocobalamin) solution at 10 mg/L and 2 mL ferric quinate solution (Section 3.4.4). The medium was dispensed under nitrogen and sterilised by autoclaving.

3.4.7 Minimal salts medium

The following were dissolved in distilled water to a total volume of 1L:

0.5 g KH_2PO_4, 0.16 g $MgCl_2.6H_2O$, 0.4 g NaCl, 0.05 g $CaCl_2.2H_2O$, 1 mL trace element solution (Section 3.3.1), and 2 mL ferric quinate solution (Section 3.4.4). The medium was dispensed under argon and sterilised by autoclaving. Minimal salts medium (MSM) was augmented as required from stock solutions of 2% (w/v) $MgSO_4.7H_2O$, 4% (w/v) NH_4Cl, or vitamin solutions (see Section 3.4.3). MSM was used as the base medium for determination of the vitamin requirements and nitrogen and sulfur source utilisation of isolated organisms.

3.4.8 Thermotolerant enrichment medium

The following were dissolved in distilled water to a total volume of 1 L:

0.5 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.08 g $MgCl_2.6H_2O$, 0.4 g NaCl, 0.05 g $CaCl_2.2H_2O$, 1 mL trace element solution (Section 3.3.1), and 2 mL ferric quinate solution (Section 3.4.4). The medium was dispensed under nitrogen and sterilised by autoclaving.

3.4.9 Basal salts medium

The following were dissolved in distilled water to a total volume of 1 L:

0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.4 g NaCl, 0.4 g NH_4Cl, 0.05 g $CaCl_2.2H_2O$, 0.02 g yeast extract, 1 mL trace element solution (Section 3.3.1), 1 mL Vitamin B_{12} (cyanocobalamin) solution at 10 mg/L and 2 mL ferric quinate solution (Section 3.4.4). The medium was sterilised by autoclaving. Basal salts medium (BSM) was used for routine cultivation of mesophilic purple nonsulfur bacteria.

3.4.10 Rhodospirillaceae medium M-27

The following were dissolved in distilled water to a total volume of 1 L:

0.5 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.4 g NaCl, 0.05 g $CaCl_2.2H_2O$, 0.4 g NH_4Cl, 1 g yeast extract, 1 mL trace element solution, 1 mL Vitamin B_{12} (cyanocobalamin) solution at 10 mg/L, 5 mL ferric citrate solution at 1 g/L, 0.5 mL ethanol, and 1 g disodium succinate. The medium was sterilised by autoclaving.

3.4.11 Media for maintenance of stock cultures

Stock cultures of most strains were maintained in M-27 medium. Stock cultures of *Rcy. purpureus* which is incapable of growth on succinate were grown on basal salts medium containing 10 mM malate or pyruvate. Stock cultures of *Rsp. salinarum* were likewise maintained on 10 mM lactate.

Modifications of the standard growth media were required for several species, as shown in Table 3.2.

Species	Media Supplement
Blc. sulfoviridis	0.1 g/L $Na_2S_2O_3.5H_2O$
Rba. veldkampii	0.5 g/L $Na_2S.9H_2O$
Rbi. marinum	5% (w/v) NaCl
Rbl. acidophilus	pH of 5.6
Rov. sulfidophilum	2.5% (w/v) NaCl
Rth. salexigens	10% (w/v) NaCl

Table 3.2 Specific media requirements of PNSB

3.4.12 Preparation of anaerobic media

For most of the experiments in this study, bacterial cultures were grown in Hungate tubes (Bellco Glass Inc., New Jersey, USA) filled with 9.5 mL of growth medium. Serum vials were filled with 20 mL, 50 mL or 100 mL, depending on their size. Occasionally, medium was degassed directly in a serum vial by boiling in a microwave oven.

Large quantities of growth media were dispensed anaerobically by the following method, which is similar to that described by Patel *et al.* (1985). 1 litre of medium (typically, medium MC) was degassed in an Oxoid dispenser by boiling vigorously for 1 minute in a microwave oven at high power. Porcelain chips or glass beads were added to minimise bumping. The headspace of the dispenser was then continually

flushed with a stream of oxygen-free nitrogen gas directed through a bent wide-bore syringe needle. This flow was maintained to retain an oxygen-free headspace over the medium throughout the dispensing procedure. The dispenser was allowed to cool for 30 minutes, or until it was safe to handle. A second bent syringe needle carrying oxygen-free nitrogen was attached to the outlet pipe of the dispenser in such a fashion that both might be placed in the mouth of the transfer vessel upon dispensing. This allowed the headspace of the transfer vessel to be displaced with nitrogen, ensuring anaerobic transfer. The vessel was sealed with an *s*-butyl rubber septum or stopper immediately after dispensing.

3.4.13 Preparation of solid media

Double-strength growth medium and 2.4% (w/v) purified agar (Oxoid, Hampshire, England) in distilled water were autoclaved separately, and combined after cooling to approximately 45°C. Mixed medium was poured into plastic Petri plates.

3.4.14 Sterilisation of media

All media were sterilised by autoclaving using a time-temperature automatic autoclave (Mercer and Sons Ltd, Christchurch, New Zealand) at 121°C and one atmosphere overpressure for 20 minutes.

3.4.15 Substrates for carbon source utilisation tests

All substrates for carbon source utilisation experiments were prepared as stock solutions in degassed distilled water, and stored under oxygen-free nitrogen in serum vials or Bellco bottles sealed with an *s*-butyl rubber stopper. This procedure served to minimise the introduction of oxygen to anaerobic Hungate tubes when adding the substrate. Most stock solutions were sterilised by autoclaving, as described in Section 3.4.14. Stock solutions of volatile or heat sensitive substrates (ethanol, methanol, fructose, glucose etc.) were sterilised by filtration through a 0.22 μm syringe-tip filter into pre-autoclaved Bellco bottles.

3.4.16 Mixed-acid substrate

Mixed-acid substrate (MA) was used as a generic carbon source to allow high density growth of any PNSB species. MA was prepared according to the recipe given in Gest *et al.* (1985). 20 g disodium succinate hexahydrate, 8 g trisodium citrate dihydrate, 5 g sodium malate, 5 g sodium acetate trihydrate, and 5 g sodium lactate (as 60%

syrup) were dissolved in a minimum volume of distilled water. The pH of the solution was adjusted to 7.0 with sodium hydroxide pellets, and the volume adjusted to 100 mL. The solution was sterilised by autoclaving. 0.1 mL of the MA stock was added to 10 mL of growth medium.

3.5 Inoculation and sterile manipulation

Bacterial strains were routinely cultured in Hungate tubes containing 10 mL of anaerobic growth medium. All manipulations involving bacterial cultures, sterile media and substrates were performed in a sterile laminar air flow cabinet (Nuaire Ltd, Maine, USA). Stoppers and septa of vials and Hungate tubes were sterilised by briefly flaming with 95% (v/v) ethanol. Substances and bacteria were transferred between containers using sterile single-use 1 mL syringes fitted with 25 gauge hypodermic needles.

3.6 Incubation of cultures

After inoculation, cultures were incubated in the dark for a minimum of 7 hours to enable them to remove through respiration traces of oxygen that might otherwise on direct transfer to the light inhibit phototrophic metabolism or cause photooxidative damage to cells. Low levels of residual O_2 are of small concern in the culturing of PNSB, as most species have the ability to respire aerobically and all are at least microaerophilic (Imhoff and Trüper 1989). Cultures were incubated in the light at a constant temperature of either 30°C or 40°C. Continuous illumination of approximately 2000 lux was provided by lamps fitted with 75 W tungsten bulbs. Incubation in the dark was in a closed incubator at 30°C or 40°C. Cultures on plates were incubated anaerobically in GasPak anaerobic jars (BBL, Maryland, USA) that were pre-flushed with nitrogen gas.

3.7 Measurement of growth

Spectrophotometric measurement was used as a convenient and rapid means of assessing growth. Optical densities of cultures were determined at 660 nm, chosen since there is very little absorbance due to photosynthetic pigments at this wavelength, allowing a more accurate measure of turbidity due to cells alone. Optical

densities were read directly from Hungate tubes using a Sequoia-Turner Model 340 spectrophotometer fitted with a black plastic light-tight cover. A tube of uninoculated growth medium was used as a reference blank.

Specific growth rates were calculated from the exponential portions of growth curves according to the equation: $k = (\ln A_t - \ln A_0) / 0.693t$, where A_t and A_0 are the optical densities of the culture at times t and 0 respectively. Doubling times were calculated according to the equation $t_{gen} = 1 / k$.

When scoring utilisation of carbon sources, positive growth was indicated by a higher absorbance reading in the sample tube when compared to the control tube at 660 nm. Table 3.3 shows the relationship between OD_{660} and the qualitative measures of growth recorded in Chapter 4.

Code	Description of Growth	Absorbance at 660 nm with respect to control
I	Growth inhibited	<0.000
-	Equivalent to control tube	0.000 - 0.050
±	Marginal growth	0.050 - 0.100
+	More growth than control	0.100 - 0.200
++	Good growth on substrate	0.200 - 0.500
+++	Excellent growth on substrate	>0.500

Table 3.3 Protocol for assessment of substrate utilisation

Direct visual assessment of growth was used for species such as *Rubrivivax gelatinosus* which do not grow in uniform suspension and are thus difficult to monitor using the spectrophotometer. Positive growth was taken to be a more intense pigmentation in growth tubes than control tubes. The size of the undisturbed cell pellets which formed at the bottom of Hungate tubes was also compared.

3.8 Characterisation of bacterial strains

3.8.1 Light microscopy

All light microscopic examination was carried out using an Olympus BH-2 phase-contrast microscope (Olympia Optical Company Ltd., Shibuyaku, Japan). Procedures included observation of cell morphology, motility, mode of division, routine checks of

purity, and monitoring of enrichment cultures. Measurements of cell size were performed using a stage micrometer from Olympus. Phase contrast photomicrographs were taken using an Olympus camera. Cells were immobilised by addition of a drop of molten 1% (w/v) agarose to a drop of bacterial culture on a microscope slide heated to 40°C immediately prior to adding the cover slip.

3.8.2 Electron microscopy

Preparation of samples for electron microscopy was carried out by David Wild or Alf Harris at the Electron Microscope Unit, MIRINZ, Hamilton. Preparations were shadowed with uranyl formate.

3.8.3 Vitamin requirements

Vitamin requirements were determined by addition of 0.05 mL of stock vitamin solutions to cultures inoculated into Hungate tubes containing 9.5 mL of minimal salts medium supplemented with 4% (w/v) NH_4Cl and 2% (w/v) $MgSO_4.7H_2O$. MA was used as carbon source.

Vitamin requirement determination was a two-stage process. In the first stage, tubes were supplemented with two of three vitamin pools, V1, V2 and cyanocobalamin (see Section 3.4.3). In the second stage, cultures which showed growth on vitamin pools were subcultured on the same medium to confirm continued development, while cultures which failed to grow in the absence of a particular vitamin pool were tested in tubes of medium supplemented with all vitamins except one member of the pool. Failure to grow in any of these tubes was considered as proof of vitamin requirement.

3.8.4 Nitrogen source utilisation

For tests of nitrogen source utilisation, cultures were grown in minimal salts medium which had been dispensed into Hungate tubes in 9.5 mL volumes under an argon headspace. MSM was supplemented with 2% (w/v) $MgSO_4.7H_2O$, complete vitamin mixture and MA as carbon source. Cultures were initially grown on medium lacking any nitrogen source in order that they reach a nitrogen-starved condition. Subsequently, nitrogen-starved cells were inoculated into MSM tubes supplemented with a single nitrogen source – NH_4Cl, $NaNO_3$, or N_2. The N_2 tube was prepared by flushing the headspace for 30 seconds with filtered nitrogen gas via a hypodermic needle, using a second needle as an outlet to prevent pressure build-up. The ability to

use each nitrogen source was determined by comparison of growth with the amount obtained in a control tube containing no nitrogen source.

3.8.5 Sulfur source utilisation

For tests of sulfur source utilisation, cultures were grown in minimal salts medium supplemented with 4% (w/v) NH_4Cl, complete vitamin mixture and MA as carbon source. Cultures were initially grown on medium lacking any sulfur source for two successive transfers in order that they reach a sulfur-starved condition. Subsequently, sulfur-starved cells were inoculated into MSM tubes supplemented with a single sulfur source – $MgSO_4$, 0.5 mM Na_2S, or $Na_2S_2O_3$. The ability to use each sulfur source was determined by comparison of growth with the amount obtained in a control tube containing no sulfur source.

3.8.6 Nitrate reduction test

Use of nitrate at 10 mM as an electron acceptor for anaerobic growth in darkness was tested in Hungate tubes containing an inverted Durham gas collection tube. Production of nitrogen gas was detected by the presence of a bubble of gas trapped in the Durham tube. The presence of nitrite was determined as described by Holding and Collee (1971). 0.1 mL of 1% (w/v) sulfanilic acid in 5 N acetic acid and 0.1 mL of 0.6% (w/v) dimethyl-α-naphthylamine in 5 N acetic acid were added to 1 mL of culture supernatant. Development of a red colour indicated the presence of nitrite. In the case of a negative result, a small amount of zinc dust was added. Red colouration visible after centrifuging the zinc dust indicated the presence of residual nitrate.

3.8.7 Absorption spectra

In vivo spectra of photosynthetic pigments were determined as described by Imhoff and Trüper (1992) on a Pharmacia Biotech Ultrospec 3000 using quartz cuvettes. Briefly, cells were pelleted by centrifugation and resuspended in 60% (w/v) sucrose solution. Cell suspensions were scanned over the range 400 to 1000 nm for bacteriochlorophyll-*a* containing organisms, or from 400 to 1100 nm for those containing bacteriochlorophyll-*b*.

3.8.8 Quinone analysis

Quinone analyses were carried out by Prof. Akira Hiraishi (Toyohashi University of Technology, Toyohashi, Japan). Quinones were extracted with a chloroform-

methanol mixture, purified by thin-layer chromatography and then analysed by HPLC (Hiraishi and Hoshino, 1984)

3.9 Molecular biology methods

3.9.1 Isolation of DNA from bacterial cultures

DNA was isolated from bacterial cultures according to a modification of the Marmur method. 10 mL of exponential phase culture was harvested by centrifugation at 14000 g for 10 minutes. The pellet was washed and resuspended in 400 µL of DNA extraction buffer (20 mM Tris, 100 mM EDTA, pH 8.0), SDS was added to 0.1% (w/v), and lysozyme to 4 mg/mL, and the suspension was incubated at 37°C for 1 hour with occasional mixing. Proteinase K was added to a final concentration of 2 mg/mL, and the mixture was incubated for 2 hours at 65°C; alternatively, the mixture was digested with 2 mg/mL pronase E at 37°C for 2 hours. Pronase was preincubated in TE for 60 min at 37°C to destroy contaminating nuclease activity. Proteolysis was followed by extraction with phenol-chloroform-isoamyl alcohol (24:1:1, v/v) and chloroform. The organic solvents were added in equal volume to the aqueous phase. RNAse was added to the final aqueous phase which was then incubated at 37°C for 30 min. A further phenol-chloroform extraction was carried out. The DNA was then precipitated by addition of one-tenth volume 3 M sodium acetate, pH 5.6 and 1 volume of isopropanol. The DNA pellet was collected by centrifugation, washed with 70% ethanol, dried under vacuum in a desiccator, and finally redissolved in 50 µL of TE buffer. Isolated DNA was stored at -20°C.

3.9.2 Agarose gel electrophoresis

In general, agarose gels were prepared with 1% (w/v) agarose in 1x TAE or 1x TBE buffer containing 0.8 µg/mL ethidium bromide, and run at 30-100 V. Size markers were 100 bp ladders obtained from Gibco BRL.

3.9.3 Quantitation of DNA

Initially, and for critical purposes, DNA was quantified by fluorimetry. The fluorimeter (Hoefer Scientific Instruments, California, USA) was zeroed against Labarca-Paigen buffer (50 mM NaH_2PO_4, 2 mM, Na_2EDTA, 1 µg/mL Hoechst 33258 dye), and calibrated by addition of 2 µL of calf thymus DNA at 100 ng/µL. 2 µL of DNA sample solution was added to 2 mL of fresh Labarca-Paigen buffer. In most

cases, DNA concentration was determined approximately by visual estimation of the intensity under UV illumination of an ethidium bromide-stained band on an agarose gel. In general, PCR products had a concentration of 40-80 ng/μL.

3.9.4 Polymerase chain reaction and DNA sequencing

PCR reactions were set up in a final volume of 100 μL, with nucleotide triphosphates at a concentration of 200 μM each, primers at a concentration of 0.5 μM, 1 to 3 U of Taq polymerase, and 10-50 ng of template DNA. PCR was performed on an eppendorf Mastercycler gradient thermocycler or a Techne Genius thermocycler, with identical results from either machine.

PCR products were purified prior to further manipulation using the WizardPrep DNA Purification system (Promega), according to the manufacturer's instructions.

Direct sequencing of purified PCR products was achieved using the Prism™ Ready Reaction Dichlororhodamine Terminator Cycle Sequencing Kit (Perkin Elmer) following the manufacturer's protocol. Sequences were analysed at the Waikato DNA Sequencing Facility on the Applied Biosystems Model 377 automated DNA sequencer from Perkin Elmer.

3.9.4.1 Amplification and sequencing of 16S rRNA genes

PCR amplification of 16S rRNA genes was achieved using consensus eubacterial primers pA and pH* (Edwards *et al.* 1989). Primer pA (5'-AGA GTT TGA TCC TGG CTC AG-3') binds to positions 8-28 in the *E.* coli numbering system and pH* (5'-AAG GAG GTG ATC CAG CCG CA-3') to positions 1542-1522. The product of amplification is approximately 1500 bp long.

After an initial denaturation at 94°C for 3 minutes, PCR was carried out for 30 cycles, with 45 s denaturation at 94°C, 60 s annealing at 50.2°C, and 110 s extension at 72°C. A final extension at 72°C for 5 minutes was then performed.

Sequencing of 16S rRNA genes was carried out using 3 sequencing primers: the previously-mentioned pA, Sef1 (5'-CGT GCC AGC AGC CGC GGT AAT-3') and Sef2 (5'-GGA GCA TGT GGT TTA ATT CG-3'). Sef1 binds to positions 513-533, and Sef2 to positions 943-962.

The positions of the primer binding sites within the 16S rRNA gene are shown schematically in Figure 3.1.

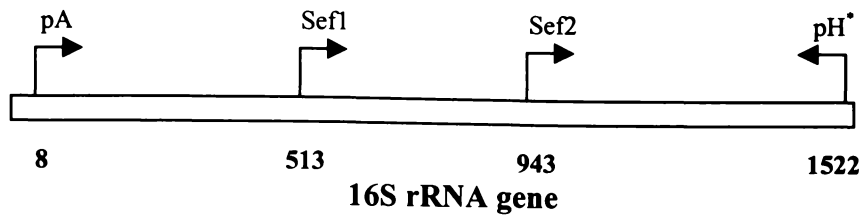


Figure 3.1 Schematic of primer binding sites on 16S rRNA gene

3.9.4.2 Amplification and sequencing of *pufL* and *pufM* genes

PCR amplification of *pufL* and *pufM* genes was performed using the forward primer pL2 (5'-CTG TTC GAC TTC TGG GTS GG-3'), based on a primer designed by Nagashima *et al.* (1995), which binds to bases 64-82, numbered according to the *Rhodospirillum rubrum* gene. When amplification of a portion of the *pufL* gene only was desired, then one of two reverse primers pLr4 and pLr5 (5'-AGG CCG AGG CGG TGR AT-3'; 5'-AGA CCC AGA CGA TGR AT-3'), both binding to positions 704-688, was used, yielding a product around 640 bp in length. The reverse primer pM* (5'-CCC ATS GTC CAG CGC CA-3') (Nagashima *et al.* 1995), binding 1610-1594 bases from the start of the *pufL* gene, was used to obtain an approximately 1540 bp product comprising a major portion of both *pufL* and *pufM*.

After an initial denaturation at 94°C for 3 min, PCR was carried out for 30 cycles, with 45 s denaturation at 94°C, 60 s annealing at 54.5°C, and extension at 72°C for 110 s with pL2/pM* or 50 s with pL2/pLr4 and pL2/pLr5. A final extension at 72°C for 5 min was then performed.

Sequencing of *pufL-pufM* PCR products was carried out using the primers pL2, pM* and pLf2 (5'-CCA CCT CGA YTG GGT-3'). pLf2 binds to positions 459-474.

The positions of the primer binding sites within the *pufL* and *pufM* genes are shown schematically in Figure 3.2.

3.9.5 DNA-DNA Hybridisation

DNA-DNA hybridisation studies were carried out by Dr Akira Hiraishi (Toyohashi University of Technology, Toyohashi, Japan), using quantitative dot-blot

hybridisation with photobiotin labelling and colorimetric detection (Hiraishi *et al.* 1991).

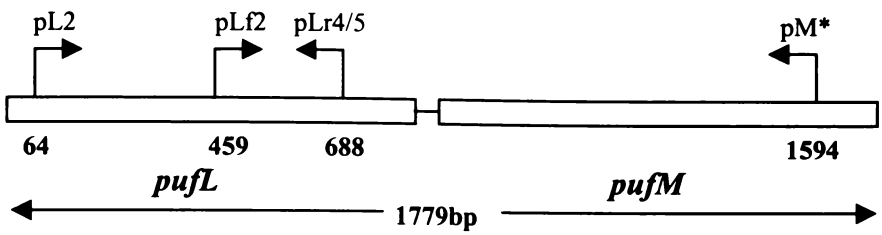


Figure 3.2 Schematic of primer binding sites on *pufL* and *pufM* genes

3.9.6 Sequence analysis

Sequences were compared with those in the GenBank database using the Blastn program (Altschul *et al.* 1997). Multiple alignments were created using Clustal X (Thompson *et al.* 1997). Aligned sequences were truncated and manually realigned where required. Phylogenetic trees were generated by Clustal X using the neighbour-joining method (Saitou and Nei 1996) and were in general bootstrapped with 1000 replicates. Phylogenetic trees figures were created using the program TREEVIEW (Page 1996). The GenBank accession numbers of all sequences used in phylogenetic analyses are given in Appendix 1.

Chapter 4

Enrichment and Isolation of Thermotolerant Purple Nonsulfur Bacteria

4.1 Introduction

Three freshwater purple nonsulfur bacteria exhibiting mild thermophily (*Blastochloris* strain GI, *Rhodocista centenaria* and *Rhodopseudomonas cryptolactis*) have previously been described (Resnick and Madigan 1990; Stadtwald-Demchick *et al.* 1990a and 1990b). In addition, there have been reports of the isolation of mesophilic purple nonsulfur bacteria from Russian hot pools (Gorlenko *et al.* 1985 and later papers). In the present work, samples collected from the geothermal areas of the North Island of New Zealand were used as inocula in a range of enrichment cultures with the aim of determining whether purple nonsulfur bacteria species were present, and of isolating a representative selection.

4.2 Materials and Methods

4.2.1 Sampling Sites

Samples were collected from eleven locations in the North Island of New Zealand over the period from September 1997 to January 2001. The locations of sampling sites are given in Table 4.1. Sample locations are described below.

4.2.1.1 Waimangu Volcanic Valley

This geothermal area was formed in June 1886 in the aftermath of the eruption of the volcano Mount Tarawera and underwent further development in the following years. No major alterations have occurred since 1917 when a hydrothermal eruption led to the formation of Echo Crater.

Wai1: Frying Pan Lake (Figure 4.1)

Frying Pan Lake formed in Echo Crater after an eruption in 1917. It now covers 38000 m^2 and has an average temperature of around 50°C. The water is acidic, pH 2.4, and CO_2 and H_2S bubbles constantly rise to the surface. The shore of Frying Pan Lake is accessible at one point along the main path. In this area, cyanobacterial mats

float on the surface of the water, and also lie beneath several inches of water immediately adjacent to terrestrial vegetation. Mat samples were taken at the lake edge, where the temperature ranged from 34-55°C and the pH was 6.2.

Location	Latitude	Longitude	NZMG Northing	NZMG Easting
Waimangu Volcanic Valley	-38.2884	176.3778	6318507.2000	2805570.0000
Orakei Korako	-38.4755	176.1440	6298500.0000	2784400.0000
Tokaanu	-38.9666	175.7539	6245154.4000	2748696.2000
Waiotapu	-38.3420	176.3548	6312641.8000	2803338.5000
Waitangi Soda Springs	-38.0394	176.5424	6345567.2000	2821055.7000
Kuirau Park	-38.1362	176.2412	6335837.0000	2794242.2000
Ohinemutu	-38.1296	176.2502	6336534.5000	2795062.5000
Tikitere	-38.0662	176.3511	6343243.6000	2804160.6000
Lake Rotowhero	-38.3239	176.3759	6314575.2000	2805259.6000
Magpie pool (approximate location)	-38.3245	176.3867	6314474.1000	2806202.9000
Kerosene Creek	-38.3122	176.3608	6315931.2000	2803990.1000

Data from http://www.linz.govt.nz/

Latitude: the degrees and decimals of a degree of latitude
Longitude: the degrees and decimals of a degree of longitude
NZMG Northing/NZMG Easting: The coordinates indicate the position of the feature in New Zealand Map Grid (NZMG) coordinates. The results are given in metres.

Table 4.1 Locations of major sampling sites

Wai2: Hot Water Creek

At this point, a hot stream flows from Frying Pan Lake after overflowing through a narrow rock notch at around 110 L/s and at 50°C. Mineral deposits and layers of cyanobacteria in loose sinter material can be seen. Sediment containing a thin emerald-green layer at 43°C was sampled. Microbial mats are present in the centre of the stream, but are all at high temperature.

Wai3: Nga Puia o te Papa

The Hot Springs of Mother Earth are a cluster of permanent boiling springs amongst small silica formations lying above the hot water stream. There are several cooler pools formed by collection of splash water from the boiling pools. A sediment sample was taken from a pool with a temperature of 41°C and pH 5 to 5.5.

Figure 4.1 Wai1 – the shore of Frying Pan Lake

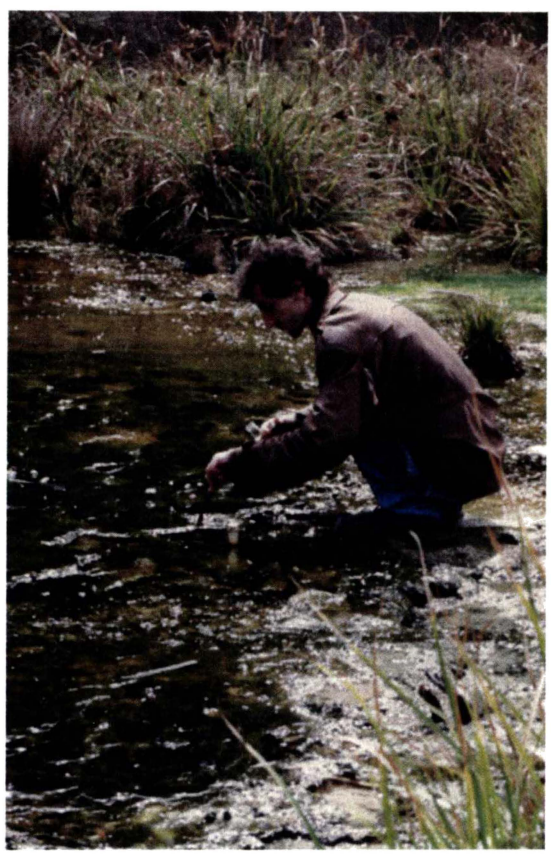


Figure 4.2 Wai5 – extensive microbial mats at the outflow of Warbrick Terrace

Wai4: Puara Terrace

A sample was taken from a thin cyanobacterial mat on sinter where the outflow from a boiling spring had cooled to 45°C at pH 6. The source spring was at 80°C, and contained profuse growth of brown filaments that were identified by molecular methods as *Hydrogenobacter* species (D. Götz, work carried out at Portland State University, Oregon, USA, personal communication).

Wai5: Warbrick Terrace (Figure 4.2)

Warbrick Terrace is a small silica terrace that has built up around a central spring. Streams flow from the terrace and spread over surrounding marshy ground that is not possible to cross without damaging the surface. In 1998, extensive mats covered this area. Samples were taken from the accessible edge where the temperature was 30°C and the pH was 6. On a later visit, the temperature was 33°C and the pH was alkaline, between 7.5 and 8. Subsequently, the flow of water diverted and the mats died off.

4.2.1.2 Orakei Korako

The Orakei Korako geothermal area was at one time more extensive, but in January 1961, damming of the Waikato River led to the raising of the water level by almost 20 m to create Lake Ohakuri, which has submerged around 70% of the thermal features. The activity of the remaining features in general increased. Access to Orakei Korako is by boat across Lake Ohakuri.

OK1: Emerald Terrace

Orange mats on the terrace that lies above Lake Ohakuri were sampled at a point where the temperature was 46°C, largely due to runoff water from the upper terrace and from the Diamond Geyser.

OK2: Ruatapu (Aladdin's) Cave

A green pool lies at the bottom of Ruatapu Cave. The water temperature is 47°C. Sediment and water samples were taken.

OK3: Devil's Throat (Figure 4.3)

The Devil's Throat is a rock formation spouting boiling water that flows along a drainage channel whose margins are lined with algal growth. At the position of sample site OK3, the temperature of the water is above 70°C in the centre of the

channel. Pink mat samples were taken at the edge of the channel where the temperature was 45°C, and orange samples where the temperature was 39°C. pH was around 9. On subsequent visits to site OK3, the mats were absent.

OK4: Tim and Terry (Figure 4.3)

This sample site lies downstream of OK3, where the water temperature in the channel is 53°C and microbial growth is more abundant. The temperature of the mat sample was 36°C.

OK5: Hot Springs Algae mat (Figure 4.4)

Water from the broad flat terrace labelled as Hot Springs Algae flows in a stream beneath a boardwalk at 60°C and near neutral pH. A substantial microbial mat was attached to a concrete support of the boardwalk and extends over the stream, partly submerged. Upper portions of the mat were sampled on several occasions. Bulk pH of the mat surface was around 7.5-8. Temperatures of samples were 38, 44 and 48°C. This mat was present in 1998, was completely absent at the time of a visit at the start of 1999, but had regrown with much the same shape at the time of a visit in November 1999.

OK6: Emerald Terrace

Downstream of the OK5 site, the mat material is brown-black in colour. A sample was taken at 41°C.

OK7: Lake Ohakuri shore

This sample site is located on the opposite side of Lake Ohakuri to the Orakei Korako thermal area. The lake shore is predominantly sandy. Hot water wells up in several locations. 30 cm beneath the sand surface the temperature varies from 60-80°C, but samples taken from the surface are at 45-50°C, overlain by tepid (30°C) lake water.

OK8: Danger sign

This sample site has no convenient name, but lies between OK5 and OK4, near a 'Danger' sign. Mud (that is, organic-rich soil and water mixtures, not geothermal mud) samples taken from beneath the sign were at a temperature of 45°C and a pH of 6.1. A later sample was taken from this site when higher water level had led to the development of microbial mats over the mud. The pH was then alkaline, at 9.

OK9, OK11, OK13: Mat samples

Three mat samples were taken from various points between sites OK5 and OK8, at locations chosen for their temperature of 42°C. Site OK12 was sampled for purposes unrelated to this research project.

OK10: Hochstetter view

A mat near the Hochstetter Pool was sampled at 45°C.

4.2.1.3 Tokaanu

The Tokaanu geothermal area is located on the south side of Lake Taupo near Turangi. The field has several sinter deposits, hot springs and pools, fumaroles, steam vents and seepages, but has been damaged by poorly planned developments, including the construction of a car park over much of the former field.

Tok1: Te Waihoto (Figure 4.5).

Mat samples were collected from this pool on two occasions. Temperature was 45-50°C and pH was 6-7.

Tok2:

This sample site was a muddy depression at the side of the path less than 10 cm in diameter that was formed by upwelling of heated water and gas. Temperature was 48°C and pH was 5.5. Activity had ceased at the time of a subsequent visit, and the precise location of the bubbler could no longer be determined.

Tok3-6:

These sample sites consisted of mud or mat samples at temperatures between 40 and 46°C and in the pH range 5.5-6.5.

Tok7:

This site was a small muddy hole containing heated water, very similar to Tok2 but with visible green growth around the edge. The temperature was 42°C and the pH was 5.5.

Figure 4.3 OK3 (foreground) and OK4 (by figure) – microbial mats at the edge of an outflow channel

Figure 4.4 OK5 – microbial mat growing above a thermal stream (30 cm ruler shows scale)

Tok8:

A sample was taken from a thin green layer overlying a clay surface, at 35°C and pH 7.8.

Tok9: Te Paenga (Figure 4.6)

Samples were taken from green and orange floating mats in the Te Paenga pool which was at 49°C and pH 7. At a later date, water samples were also taken (see Section 4.3.5).

4.2.1.4 Waiotapu

Waiotapu is a major geothermal area 30 km south of Rotorua, but provided few samples suitable for enrichment of purple nonsulfur bacteria. Microbial mats similar to those found at Orakei Korako or Waimangu were not present. Waiotapu includes an area of acid-sulfur springs and mud pools, a large silica terrace known as Primrose Terrace, and several thermal lakes.

WoT1-3: Small pools

These samples of sediment from small pools ranged from 36-46°C, were of very low pH, and contained very high levels of antimony or arsenic minerals.

WoT4: White filaments

In this area, water at 40°C from a lake yellow with colloidal sulfur flowed through a small channel that was filled with profuse growth of white filamentous bacteria, possibly *Beggiatoa*.

WoT5: Stream

At this location beneath the Bridal Veil Falls, cooled water originating from the Primrose Terrace mixed with the outflow of an 84°C muddy acidic pool to produce a stream at 36°C and pH 3.5. Sediment samples were taken.

WoT6: Algal bloom

A sample was taken from a location where a cold stream flows into an area of hot ground, and mass development of eukaryotic algae intermingled with purple growth had occurred. Temperature was however around 24°C.

Figure 4.5 Tok1 - microbial mats underlying water in Te Waihoto pool

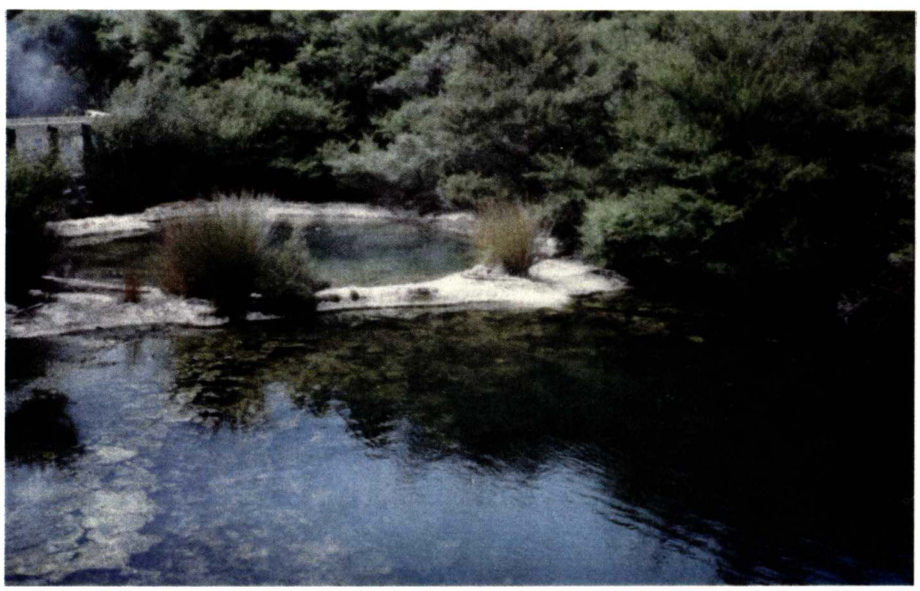


Figure 4.6 Tok9 – Takarea pool and Te Paenga pool (foreground), showing floating and submerged mats

4.2.1.5 Waitangi Soda Spring

Waitangi Soda Spring is found near Lakes Rotoehu and Rotoma. The springs are administered by members of the subtribe Ngati Pikiao of Te Arawa, who have plans to build a health spa at the site. The soda springs are fed by two hot springs and a cold stream; one source pool is inaccessible. The water flowing into the springs from the accessible pool is piped beneath a road and is rich in iron that precipitates around the inflow pipe and forms deposits on the sediments of the main pool.

WSS: Waitangi Soda Spring (Figure 4.7)

A sample of sediment was taken from a part of the springs that was accessible by wading. The temperature at this point was 42°C and the pH was 6.

W3S: Waitangi Soda Spring source pool

This 50°C, pH 5.3 pool is the single accessible source of thermal water to the main pool, located on the opposite side of the access road to the main pools. The spring water is turbulent due to rapid evolution of gas, and is at 50°C and pH 5.3.

4.2.1.6 Kuirau Park

Kuirau Park is a thermal area in the heart of the town of Rotorua, dominated by the thermal Kuirau Lake and containing acid or weakly alkaline water. Activity has greatly increased over the past several years and average temperatures have risen.

Kui1, Kui2:

These were samples of mud at the edges of pools. Kui1 was at 53°C and contained greenish water. Kui2 was at 46°C.

Kui3: Channel

At this location, water from a small spring was directed through an artificial channel. Profuse mat growth subdivided the 60°C water flow, producing a number of areas that were of lower temperature (32-45°C); a sample was taken at the higher end of this temperature range. This site was destroyed in a hydrothermal eruption that occurred on 26 January 2001.

Kui4, Kui5:

These were mat samples at 43°C taken from a stream that runs through Kuirau Park.

Kui6: Purple bloom (Figure 4.8)

A mass accumulation of purple sulfur bacteria was observed in a sheltered bay of Kuirau Lake where the temperature of 30-34°C was lower than that of the main water body. On a second inspection of the site some months later, the temperature of the bay had risen to that of the main lake, and the bloom had disappeared.

4.2.1.7 Individual sampling sites

A single sample was obtained from a number of sites which are described in this section.

OH3: Ohinemutu is a small settlement at the northern edge of Rotorua on the shore of Lake Rotorua that contains several clear alkaline hot springs in marshy ground. A sample was obtained by C.G. Harfoot near a septic tank drainage inflow. Temperature gradients were steep, but the mat sample was around 47°C and near neutral pH.

TKT: Tikitere (Hell's Gate) lies to the north-east of Rotorua and is a fairly extensive geothermal area characterised by high acidity and mud pots. Overall, it provided few suitable sample sites. A single sample was obtained opposite a feature called the Cooking Pool, where a tepid stream joined the overflow of two small bubbling vents among white filaments, black mineral precipitates and cyanobacterial growth (Figure 4.9). Temperature was 45-6°C, pH was less than 3.5.

LR: Lake Rotowhero

A sample of water and silty sediment was taken from the shore of this lake at 37°C. The water was green with algal growth.

MP: The Magpie Pool (Figure 4.10) is located at the edge of an unmarked back road in the vicinity of Rainbow Mountain and Lake Rotowhero. The source pool was at a temperature of 75°C and contained the decaying remains of an Australian magpie (*Gymnorhina tibicen*). The drainage channel from this pool was bordered by bacterial mats. A sample was taken in an area at 45°C.

KCr: Kerosene Creek (Hakereteke Stream) is located near Lake Rotowhero and Rainbow Mountain. The temperature of the stream remains constant at 40°C for

much of its course, presumably due to influx of warm water from springs present in the stream bed at various locations. The pH is 3.5. A sediment sample was taken at the edge of the stream.

Figure 4.7 WSS – iron-rich sediment at Waitangi Soda Springs

Figure 4.8 Kui6 – a purple bloom in a pool in Kuirau Park (15 cm pen shows scale)

Figure 4.9 TKT – drainage channel bordered by cyanobacterial growth (15 cm pen shows scale)

Figure 4.10 MP – drainage channel from a thermal pool containing a dead magpie

4.2.2 Enrichment cultures

Enrichment cultures were set up in Hungate tubes containing appropriate enrichment medium and various carbon sources. Tubes were routinely augmented with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of Photosystem II, to inhibit growth of cyanobacteria or eukaryotic algae. Water samples or watery mud were injected into Hungate tubes by hypodermic needle. Solid samples of mud or mat material were placed in Hungate tubes by briefly removing the cap and septum. After inoculation, tubes were placed in darkness for a period of at least four hours to allow time for removal of oxygen through bacterial metabolism. Tubes were then placed in water baths at an enrichment temperature close to that of the sample site, and placed under constant illumination of approximately 1000 lux. Enrichment cultures showing development of pigmented growth were examined microscopically to determine the dominant morphologies present, and isolation techniques described below were employed.

4.2.3 Direct isolation plates

Direct isolation plates were prepared as described in Section 3.4.13, with succinate as carbon source, and addition of DCMU. Mat material was gelatinous or rubbery, and could not be macerated to give a spreadable homogenous suspension, so portions of mat were rubbed over the surface of agar plates to transfer a proportion of cells that were present. Water or mud samples were spread evenly with a glass spreader. Plates were incubated anaerobically in GasPak anaerobic jars that were pre-flushed with nitrogen gas. Pigmented colonies were examined microscopically, and representative colonies were purified.

4.2.4 Isolation of strains

The agar shake tube technique was employed when a small number of cultures were to be purified. Thin-wall 10 mL glass tubes with plastic caps were filled with 3 mL of 2.2% (w/v) purified agar, and sterilised by autoclaving. A series of five tubes were microwaved briefly to melt the agar, cooled to 45°C in a water bath, and then supplemented with 6 mL of complete medium. The enrichment culture inoculum was then added to the first tube in the series and mixed thoroughly with the agar by rolling and tilting the tube. Several drops of the mixture were then poured into the next tube in the series, and this process was repeated to produce a dilution series of five tubes.

Inoculated tubes were placed in cold water to set the agar. When the agar had solidified, the tubes were sealed by addition of a molten 3:1 (v/w) mixture of liquid paraffin and paraffin wax to a depth of several centimetres. Tubes were placed in darkness for several hours before transfer to the light.

When coloured colonies developed in agar-shake tubes, a tube containing well-separated colonies was sterilised externally by washing with 70% ethanol, then broken open by a sharp blow with a metal rod to expose the agar surface. Several individual colonies were removed by hypodermic needle and syringe containing a small amount of sterile medium, and were transferred to a new agar-shake series, or streaked on agar plates.

When a large number of liquid cultures were to be purified, or when colonies from direct isolation plates were to be purified, anaerobic plating was employed in the first step of purification.

In general, colonies were restreaked or agar-shaked twice or three times. Several colonies were taken from the final tube or streak plate and transferred to Hungate tubes, to ensure that at least one culture grew. Purity was checked by microscopic examination, and in the course of growth tests.

4.2.5 Identification of isolates

Isolated strains were given a unique identifier. Although ultimately arbitrary, strain identifiers were generally composed of three elements: first, the abbreviation for the sample site from which the organism was isolated, sometimes followed by a lower case letter if the sample had been obtained on a return visit to the site; second, an element referring to a characteristic of the isolate or the enrichment culture, usually colour or carbon source; third, a number referring simply to the number of the colony picked from the final plate. Thus, the strain name Wai1bG3 refers to the third green colony isolated from an enrichment on the second Frying Pan Lake sample.

Isolates were tentatively identified on the basis of cell size and morphology, bacteriochlorophyll type, vitamin requirements and utilisation of distinguishing carbon sources such as benzoate, long-chain fatty acids, citrate and sugars. A partial 16S rDNA sequence was obtained for many isolates as a definitive verification of

affiliation. The results of preliminary identification tests are not given in Chapter 4, but can be found in chapter 5 together with the more full characterisation of isolates.

4.3 Results and Discussion

4.3.1. Initial enrichments

On the basis of the information available in the published literature, it was considered most likely that any PNSB present in the New Zealand samples that were capable of growth at elevated temperature would be related to the three previously-described thermophilic species (though the discovery of thermophilic representatives from other lineages was a possibility), and that most PNSB found would be mesophilic but thermotolerant organisms like the Russian isolates.

Initial enrichments were set up in medium MC (Section 3.4.6) with the pH adjusted to that of the sample site and at near-neutral pH. Enrichments were incubated in water baths at a range of temperatures above 37°C, at a temperature closest to that of the sample site. If incubation at elevated temperature failed to yield positive enrichments, then incubation at 30-35°C, in the range for mesophilic PNSB, was contemplated. No enrichments of pH less than 6 or greater than 8 gave rise to any visibly pigmented accumulations of photosynthetic bacteria (with one exception), so all future enrichments were set up at near neutral pH.

Samples were first obtained from Waimangu Volcanic Valley and Orakei Korako. No photosynthetic bacteria were observed in enrichments on samples Wai2 and Wai4. Enrichment cultures inoculated with mat material from Warbrick Terrace (Wai5) quickly gave rise to green-pigmented growth. Organisms resembling *Chlorobium* were observed microscopically and by spectroscopic examination of photosynthetic pigments. After one to two weeks, abundant reddish growth occurred on the surface of the mat material. When carbon sources such as acetate and succinate, which are commonly used by anoxygenic phototrophic bacteria, were supplied, these cultures were dominated by purple sulfur bacteria of the *Chromatium*-type. Enrichments inoculated with sediment from Nga Puia o te Papa (Wai3) also gave rise to cultures of purple sulfur bacteria. However, rod-shaped organisms were dominant in 43°C enrichments from these sites containing tartrate as carbon source, and were isolated

by the agar-shake technique. The resulting pink cultures were designated Wai5R4 and Wai3R3, and were identified as members of the genus *Rhodoplanes* on the basis of their inability to use long-chain fatty acids.

By contrast, many enrichments on succinate inoculated with material from Frying Pan Lake (Wai1) developed pink to red growth dominated by rod-shaped organisms, although *Chromatium*-type cells were also present. Strain Wai1S1, identified as an organism similar to *Rhodopseudomonas palustris*, was isolated from an enrichment of this type. An enrichment culture at 40°C of a vertical section of microbial mat from Frying Pan Lake gave rise to a reddish-brown culture containing large numbers of spirilloid cells. These spirilla failed to grow on solid media, and were not observed in the enrichment culture after a further three days. However, streaking of a spirillum-dominated culture produced green-brown colonies which were purified as the bacteriochlorophyll *b*-containing *Blastochloris* strain Wai1bG3.

Enrichment cultures on mixed acids and tartrate at 40°C of OK5 samples gave rise after 2 days to green gelatinous cultures dominated by rod-shaped motile cells 1 μm by 10 μm, and many small vibrioid cells, possibly sulfate-reducing bacteria. Whole cell suspensions showed an absorption peak at 790 nm, indicating the presence of bacteriochlorophyll *g*, identifying the large rods as heliobacteria. OK5 enrichments on succinate were dominated by purple sulfur bacteria, while OK6 enrichments contained a mixture of purple sulfur bacteria and heliobacteria.

Enrichment cultures that were dominated by purple sulfur bacteria had a distinct sulfidic odour when opened. Addition of 20 μL of saturated lead citrate solution to 1 mL of centrifuged culture supernatant led to the formation of a brown precipitate, also indicating the presence of sulfide. The dominance of PSB in many enrichment cultures was thus probably due to sulfate-reducing bacteria generating sulfide in the enrichment medium, which would be used as an electron donor for photosynthesis by PSB, and might also directly inhibit growth of PNSB. Gest and Favinger (1983) had hypothesised that their enrichment of *Heliobacterium chlorum* was a result of increased sulfide levels in their medium, due to inadvertent substitution of NH_4Cl with $(NH_4)_2SO_4$.

4.3.2 Thermotolerant enrichment medium and the use of molybdate

Two steps were taken to overcome the problem of overgrowth by heliobacteria and purple sulfur bacteria in initial enrichment cultures. The use of enrichment medium lacking a fixed nitrogen source has been suggested for the selective enrichment of anoxygenic photosynthetic bacteria (Gest *et al.* 1985), in addition to the use of light and non-fermentable substrates. Therefore, thermotolerant enrichment medium (Section 3.4.8), which contains no ammonium chloride and also half the amount of sulfate present in medium MC, was employed. This medium would not be useful in the enrichment of particular anoxyphototrophs, as most species are capable of nitrogen fixation. Many non-phototrophs are also diazotrophic, including several *Clostridium* and *Desulfovibrio* species, which were commonly observed microscopically in enrichments.

Development of photosynthetic bacteria occurred more rapidly in enrichments in medium MC than in thermotolerant enrichment medium. The use of the new medium appeared counterproductive in the enrichment of purple nonsulfur bacteria. Perhaps in the presence of ammonium salts, purple nonsulfur bacteria grew sufficiently rapidly that they often were able to exclude significant development of other organisms. In the absence of fixed nitrogen, their growth rate may have been reduced to an extent that other organisms were able to develop first, including bacteria that generated sulfide inhibitory to subsequent development of PNSB.

To inhibit the growth of sulfate-reducing bacteria, 1 mM sodium molybdate was added to enrichment cultures (Compeau and Bartha 1985). This strategy seemed very effective, and PSB were rarely observed in numbers in enrichment cultures containing molybdate. Occasionally, molybdate-augmented medium developed a pale blue colouration over several days. This occurred most commonly in enrichments of sulfidic samples or at low pH, and was attributed to conversion of molybdate to a reduced heteropolyanion species or "molybdenum blue" (Cotton and Wilkinson 1988).

Pink budding strains OK5R2b and OK3P1, similar to the *Rhodoplanes* strains from Waimangu, were isolated at 42°C using thermotolerant enrichment medium augmented with molybdate. An apricot slime-producing isolate consisting of small

ovoid cells was isolated from a 42°C mixed acids/molybdate enrichment of the pink 45°C mat seen at the Devil's Throat, and was identified as *Rubrivivax gelatinosus*. Several red rod-shaped organisms similar to *Rps. palustris* were isolated from molybdate-containing enrichments. Strain Wai1val1 was isolated from a 40°C enrichment in thermotolerant enrichment medium with molybdate and 5 mM valerate as carbon source. Kui6R2 was isolated from the purple sulfur bloom observed at Kui6, using succinate and molybdate in the enrichment. OK4bR1 was obtained from a succinate/molybdate enrichment, while OH3F2 was isolated from a 41°C enrichment on fumarate and molybdate.

No differences were apparent between enrichments on MC with molybdate and thermotolerant enrichment medium with molybdate. As development of photosynthetic bacteria was faster in MC, this medium in conjunction with molybdate was used for the majority of enrichments.

4.3.3 Enrichments in the presence of potassium tellurite

The heavy metal oxyanion tellurite (TeO_3^{2-}) is toxic to most bacteria at concentrations as low as 1 μg/mL. Purple nonsulfur bacteria exhibit a high level of resistance to tellurite and other oxyanions, generally fifty to several hundredfold higher under photosynthetic conditions. In most cases, resistance is associated with reduction of the oxyanion to the corresponding metal (Moore and Kaplan 1992).

The use of glucose as a carbon source in enrichment cultures in general led to the development of fermentative organisms in place of photosynthetic organisms, with acidification of the growth medium. Enrichments on glucose in the presence of low levels of potassium tellurite were attempted. A green-brown culture developed in a 42°C enrichment containing 5 mM glucose and 2 μg/mL potassium tellurite inoculated with sediment samples from Wai3. *Blastochloris s*train Wai3G1e was purified by sequential restreaking of plated colonies. A colony picked from an earlier streak plate was found to be contaminated with a bacteriochlorophyll *a*-containing organism, which was purified as *Rhodoplanes* strain Wai3P2. Strain OK10ST2, a red rod-shaped organism similar to *Rps. palustris*, was similarly isolated from a 41°C succinate-tellurite enrichment.

No organisms showing similarities to members of the genus *Rhodobacter* were isolated from any of several hundred enrichment tubes. As *Rhodobacter* species are among the most common PNSB in normal environments, attempts were made to specifically enrich for these organisms. Enrichments under photoautotrophic conditions with hydrogen as electron donor lead to the development of purple sulfur bacteria. Attempts to enrich on carbon sources such as citrate that are commonly used by *Rba. capsulatus* and *Rba. sphaeroides* were unsuccessful, though *Rhodomicrobium vannielii* strain OK9V1 and strain OK9C2, a beige-brown slime-producing organism, were isolated from a 42°C citrate enrichment. Strain OK9C2 was identified as *Rubrivivax gelatinosus*. *Rhodobacter* species, particularly *Rba. sphaeroides*, have the highest resistance to tellurite of any purple nonsulfur bacteria. Ovoid cells were observed in an OK4 enrichment, and this was subcultured into medium containing 500 μg/mL potassium tellurite. Enrichments on an OK4 sample rapidly became intensely black, as tellurite was reduced to tellurium metal. Strain OK4bTR3 (TR for tellurite resistant) was isolated by streaking, but was a red-pigmented budding rod, similar to *Rhodopseudomonas palustris*. In the course of this study, *Rhodobacter* species were not found in thermal environments. This is unexpected, given how common the genus is and how versatile many of its species are.

4.3.4 Other enrichment cultures

An OK3 enrichment produced a green culture that yielded only pink colonies from agar-shakes and platings, and all subcultures developed pink. The original enrichment was therefore serially diluted hundredfold to 10^8, but the highest dilutions showing growth were still pink. A repeat dilution series was illuminated behind an infra-red filter in a "filter chamber" consisting of an enclosed light-proof container with a front window made of a Schott bottle filled with a full-grown culture of *Rhodobacter azotoformans*. The IR filter, whose absorption spectrum is shown in Figure 4.11, served to block visible light of wavelength less than 800 nm, while the *Rhodobacter* culture absorbed wavelengths of light used by the pink organisms without absorbing those used by bacteriochlorophyll-*b* containing organisms. The early subcultures contained a mixture of pink and green organisms, but the 10^6 and 10^8 subcultures were dominated by a green organism. This organism however would not form colonies on plates or agar-shakes until titanium citrate was used as a reductant. Titanium (III) citrate solution was prepared by adding 5 mL of a 15% (w/v) titanium

(III) chloride solution to 50 mL of 0.2 M sodium citrate solution and neutralising with saturated sodium carbonate solution (Zehnder and Wuhrmann, 1976). This reagent was added at a 1 in 100 dilution. The final isolate was designated OK3G7.

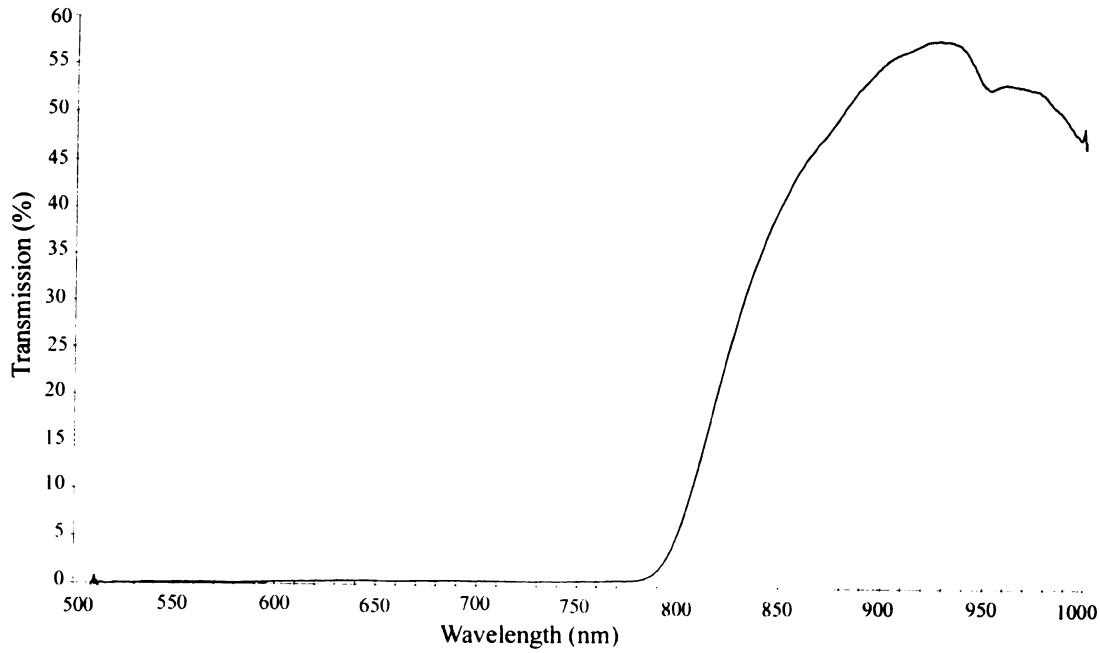


Figure 4.11 Transmission profile of the IR filter used in the enrichment chamber

An enrichment on succinate from Wai5 also gave rise to a green culture that upon subculture was dominated by pink-pigmented organisms. However, in this case, a mixture of pink and brown colonies was observed in agar shake tubes, and a green isolate designated Wai5bG1 was obtained by restreaking one of these.

A purple bloom observed at the shore of the thermal lake in Kuirau Park was sampled as Kui6. Microscopic examination revealed the presence of purple sulfur bacteria, probably *Allochromatium vinosum*, along with many morphologically unremarkable rod-shaped cells. The water sample was diluted sequentially tenfold to 10^{10} in enrichment medium with succinate as carbon source. Purple sulfur bacteria dominated the first 5 dilutions, but in the 10^{7} and 10^{8} dilutions, green-pigmented organisms developed. A green isolate was designated *Blastochloris* strain Kui6G1. The *Rhodopseudomonas palustris*-like strain Kui6R2 was also isolated from this bloom sample, by enrichment on succinate in the presence of molybdate, which seemed to successfully prevent the growth of the purple sulfur bacteria.

Organisms showing the distinctive morphology of *Rhodomicrobium vannielii* were observed as a minor component in a 40°C enrichment on mixed acids (Section 3.4.16) of a 38°C OK5 sample. The culture was subcultured onto medium lacking yeast extract and vitamins, with 5 mM benzoate as carbon source, and limiting levels of mixed acids. The resulting culture was dominated by *Rhodomicrobium*, and strain OK5M2 was isolated by agar-shake.

Mass accumulations of white filamentous bacteria, different in appearance to those found at lower temperature at WoT4, and perhaps related to sulfur-turf organisms (Yamamoto *et al.* 1998), periodically develop on Primrose Terrace, sometimes in a very short period of time. Filaments covering an area of 5 m^2 were observed to develop on previously bare sinter overlain by water at 55-60°C in the three days between two sampling trips. These mats were not sampled. Only one sample from Waiotapu gave rise to any photosynthetic organisms. A *Rhodomicrobium vannielii* strain designated WoT6V1 was isolated from an algal bloom. Strain TKT2, similar to *Rhodopseudomonas palustris*, was likewise the only organism isolated from the Tikitere sample.

Microscopic examination of samples taken from the dead magpie in the MP pool revealed heavy colonisation by organisms with the characteristic morphology of the thermophilic bacterium *Dictyoglomus* which is known for its proteolytic abilities. It is however unknown whether the decomposing magpie had any effect on the presence of purple nonsulfur bacteria in the outflow channel, though it had undoubtedly temporarily increased the levels of organic compounds in the pool. The use of amino acids as carbon source is reported to favour the development of *Rhodospirillum* and *Phaeospirillum* species in enrichments (Imhoff and Trüper 1992). Only two organisms were isolated from MP enrichments. A brown spirillum was isolated from an enrichment on acetate at 43°C, and designated MPA1. At the same temperature but on succinate, the red rod-shaped MPSR1 was obtained. These morphological types were also the only ones detected on direct isolation plates (Figure 4.13).

4.3.5 Direct plating of samples

Enrichment cultures in liquid medium are versatile, and allow the development of desired organisms from low initial numbers, but may introduce culture biases.

Numerically dominant or fast-growing organisms may routinely out-compete other members of the community. This problem can be avoided by direct plating of samples, though this is only feasible with samples containing high numbers of the organisms of interest or with water samples that may be concentrated by filtration. Colonies develop physically separated from one another, avoiding problems of direct competition.

Sediment and water samples from Waitangi Soda Springs were plated directly onto solidified succinate enrichment medium. Two green colonies and 15 red colonies were obtained, overlain by white filmy growth. *Blastochloris* strain WSSG4 was obtained by restreaking of a green colony. *Rhodopseudomonas palustris*-like WSSR4 was isolated from a red colony. Four of the red colonies were darker and leathery in appearance, and contained large (1.5 μm by 7 μm) vibrioid cells. One of these was restreaked to yield a culture that was brown-red in colour, which was later purified to yield a brown-pigmented strain designated WSSB3, which was however more spirilloid (Figure 5.30). Organisms of a similar morphology were also observed in a 40°C WSS enrichment with citrate as carbon source.

No anoxygenic photosynthetic organisms were obtained by direct platings or enrichments from Waitangi Soda Springs source pool (W3S). Plates contained tiny colourless lenticular colonies. Enrichments that contained low levels of thiosulfate or sulfide to facilitate photoautotrophic growth also did not yield photosynthetic organisms. In an acetate enrichment, however, a white veil-like growth composed of rod-shaped cells developed. These were not identified. Colourless lens-shaped colonies were also the only types observed in platings of Lake Rotowhero (LR) and Kerosene Creek (KCr) samples. A *Rhodopseudomonas palustris*-like organism, KCrS2, was however obtained from a KCr enrichment culture on succinate. No isolates were obtained from LR, perhaps due to the high density of algae in the water.

Rubrivivax gelatinosus strains OK8bDIB2 and OK13bO2 and *Rhodoplanes* strain OK10bDIP1 were purified from colonies observed on direct isolation plates. Similar organisms were observed in an OK10 enrichment on acetate, along with moderate numbers of heliobacteria. The OK10 plate also contained green filamentous organisms, probably *Chloroflexus*, which had spread from a small piece of mat

material on the agar surface. OK11 plates however contained only pale grass-green algal colonies, despite the use of DCMU in the medium.

Direct plating was carried out for several sites that had previously been examined through use of enrichment cultures. Figure 4.12 shows the range of colonies obtained on a plate inoculated with OK3 mat material. Yellow-green colonies were *Blastochloris*; orange colonies, visible mainly in the lower-right corner, were *Rubrivivax*; red colonies that were examined were all *Rhodoplanes*. Figure 4.13 shows colonies obtained from the Magpie Pool (MP) material. Most colonies had the same morphology as strain MPA1. Several colonies such as the one marked with the arrow in the figure contained budding rods, like MPSR1.

No organisms were detected on direct isolation plates that were not also seen in enrichment cultures. To this extent, the use of direct plating was a relative failure, in that it did not allow isolation of organisms that could not be enriched in liquid culture. On the other hand, it did enable rapid assessment of the diversity of purple nonsulfur bacteria present in a sample, and provide evidence that no readily culturable PNSB had been missed by enrichment culture.

4.3.6 Enrichments on Tokaanu samples

Only one isolate was obtained from the Tokaanu geothermal area, from a 40°C enrichment on tartrate of mud and water from the Tok2 sample. This isolate was designated Tok2tar1. Neither PNSB nor purple sulfur bacteria developed in any of the other enrichment cultures set up on the eight other Tokaanu samples. No pigmented colonies were observed on direct platings of samples.

The failure to culture purple photosynthetic organisms from the majority of samples taken from Tikitere and Waiotapu can be attributed to several factors. Few suitable sampling sites were available, due to the relative dryness of large parts of these geothermal areas, the absence of extensive microbial mats of the sort observed at Orakei Korako or Waimangu, and the apparent high mineral content of the pools.

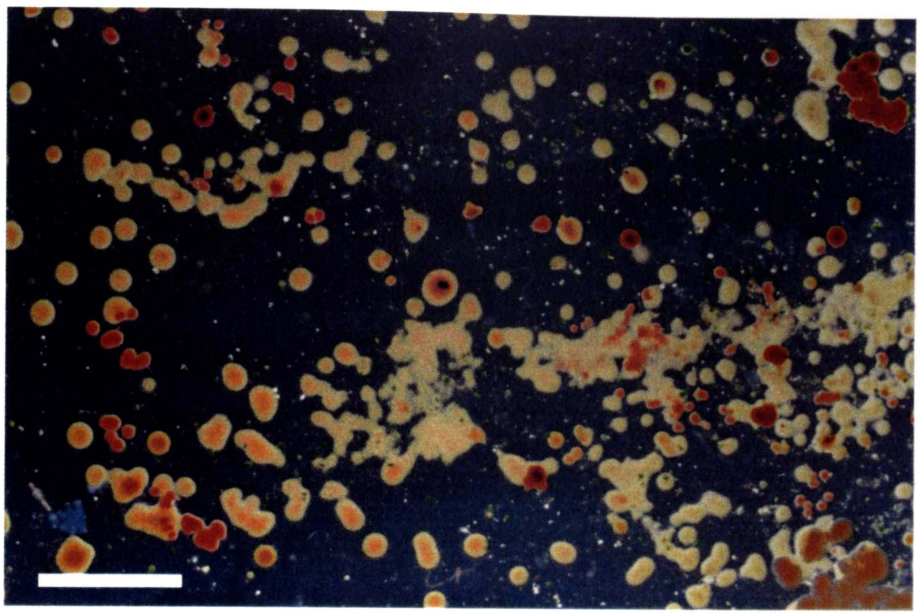


Figure 4.12 Colonies on direct isolation plate of OK3 sample. Bar, 2 mm

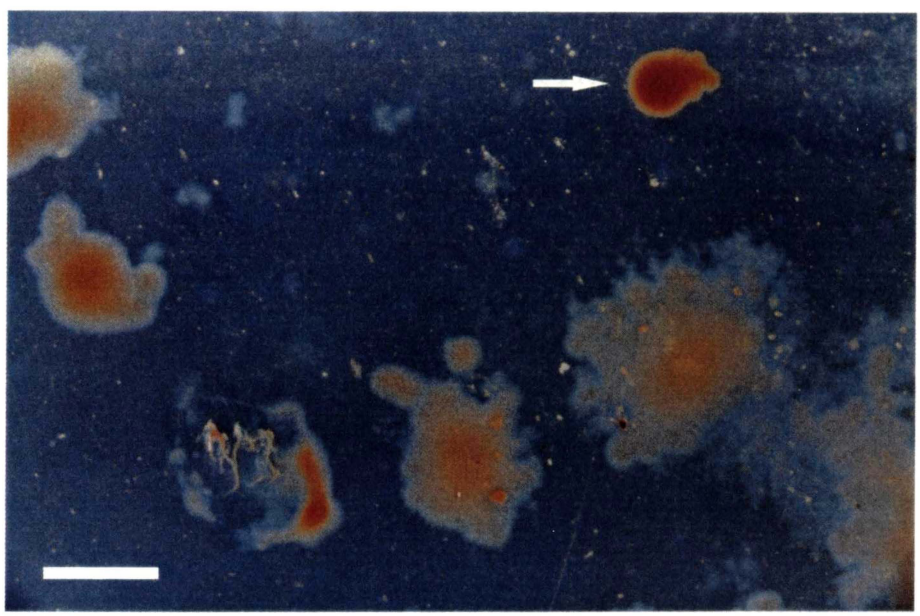


Figure 4.13 Colonies on direct isolation plate of MP sample. Bar, 2 mm. Arrow, colony containing budding rods

The pH of sediments and waters at these locations was also very low. However, the temperature and pH of sample sites at Tokaanu were appropriate for the development of purple photosynthetic bacteria. The odour of hydrogen sulfide was not detectable, and extensive mats of similar visual appearance to those found at Orakei Korako could be seen in many of the pools, indicating that cyanobacteria and *Chloroflexus* were present.

Hirner *et al.* (1998) carried out a survey of the mineral compositions of geothermal water samples from several New Zealand hot springs. Samples from five springs at Tokaanu contained high levels of arsenic (up to 6700 μg/kg), germanium (up to 137 μg/kg) and antimony (up to 707 μg/kg). By comparison, levels at Frying Pan Lake, Waimangu, were 200 μg/kg, 58 μg/kg and 22.6 μg/kg respectively. Perhaps these high levels of toxic metals are responsible for the apparent absence of phototrophic bacteria from Tokaanu pools.

Growth medium MC was made up using water taken from the Te Paenga pool (site Tok9; Figure 4.6) at Tokaanu, and a range of PNSB species were tested for growth in the medium. Most strains tested, including strain Tok2tar1, grew very slowly and to a lower cell density on this medium than on MC made with distilled water. Strain OK3O3 appeared unaffected. However, on subsequent subculture, all strains grew as well on the thermal water medium as on normal MC. The failure to enrich purple phototrophs from Tokaanu samples requires further explanation.

4.3.7 Attempts to enrich for *Rhodocista centenaria*

The thermophilic spirillum *Rhodocista centenaria* or *Rhodospirillum centenum* has been isolated from sites in the United States and Japan (Kawasaki *et al.* 1992; Nickens *et al.* 1996), which may indicate widespread geographic distribution. Organisms with spiral morphology were observed only in enrichments from sample sites Wai1, MP and WSS. Isolates from the latter two sites proved to be brown spirilla, but the unisolated Wai1 organisms were red in colour, so might have been *Rsp. rubrum* or *Rci. centenaria*. Nickens *et al.* (1996) report that strains of the latter organism are commonly resistant to 100 μg/mL kanamycin, an aminoglycoside antibiotic. The type strains of *Rba. capsulatus*, *Rsp. rubrum*, *Blc. viridis*, *Rmi. vannielii*, *Rbi. marinum* and a New Zealand isolate of *Marichromatium gracile* (obtained from an estuary at Oakleigh, Whangarei Harbour) were all completely

inhibited by 16 μg/mL kanamycin, while the type strain of *Rps. palustris* was inhibited by 64 μg/mL kanamycin (P.J. Charlton, J.E. McGrath, unpublished results). Enrichment cultures were therefore set up containing 100 μg/mL kanamycin using a variety of inocula, including Wai1, but cultures positive for photosynthetic bacteria contained only *Rps. palustris*-like organisms. *Rci. centenaria* also produces heat-tolerant cysts, but it was previously known that pasteurised enrichments were dominated only by heliobacteria or occasionally *Rmi. vannielii*. It is thus not known whether organisms resembling *Rci. centenaria* are present in New Zealand thermal areas (however, see Chapter 6.)

4.3.8 Enrichments from non-thermal environments

A small number of enrichment cultures were set up on samples taken from non-thermal areas, to determine whether thermotolerant or mildly thermophilic PNSB are restricted to locations of elevated temperature or are present in many environments. Samples were taken from several locations around the University of Waikato campus, and from cold muddy areas in Tokaanu and outside Kuirau Park. Mixed acid and tartrate enrichments at 30°C of the campus and Kuirau Park samples yielded within four to five days a wide range of photosynthetic bacteria, including *Rhodopseudomonas palustris*, *Rhodocyclus tenuis*, *Rhodobacter capsulatus* and *Rhodomicrobium vannielii* among the PNSB, and purple sulfur bacteria growing in chains of 5 to 8 cells, which were presumably *Thiocapsa roseopersicina*. No visible growth of phototrophs occurred in two weeks of incubation at 41°C. No photosynthetic bacteria were found in the cold Tokaanu sample at either incubation temperature.

4.3.9 Unusual organisms observed

A number of organisms were observed in enrichment cultures but were not successfully isolated, or failed to grow in subsequent subculture. Several of these organisms had unusual properties.

No growth of purple nonsulfur bacteria was observed in enrichments at 46-47°C, and growth of purple sulfur bacteria only occurred very occasionally at this temperature. Most enrichments at high temperature showed little visible bacterial growth. However, enrichments set up on sample sites OK1 and OK6 on the Emerald Terrace

and OK7, the shore of Lake Ohakuri, were dominated by heliobacteria, though purple sulfur bacteria were also present in the first two cultures. The heliobacteria observed were straight rods, approximately 1 μm by 10 μm in size, though longer and shorter cells were common. They were actively motile, exhibited a scotophobic response, contained subterminal endospores, and grew extremely rapidly at 43°C. Growth was not observed at 50°C.

Despite numerous attempts, these heliobacteria from Orakei Korako were not successfully isolated. Colonies could not be obtained on agar-shake tubes. Deep green smooth-edged colonies were observed on agar plates streaked under strict anaerobic conditions in an anaerobic hood, but only in the initial part of the streak, where many other colonies were found. Repeated restreaking did not lead to purification. Pasteurisation of the initial culture at 80°C for 15 minutes produced a culture that contained only two morphological types: heliobacteria and thin rod-shaped cells with large spherical endospores located terminally, which were presumably of the genus *Clostridium*. However, heliobacterial colonies free of contaminating clostridia could not be obtained. This simplified mixed culture could be stably transferred for several generations on unmodified medium MC. Heliobacterial cells showed a strong tendency to lyse during stationary phase. As reported by other authors, endospores were no longer observed after several transfers.

On the basis of the limited information available, the Orakei Korako strains cannot be assigned to a known species. Species of the genera *Heliorestis* and *Heliophilum* are alkaliphilic or have unusual morphologies, while *Heliobacterium gestii* is a spirillum (Bryantseva *et al.* 1999; Bryantseva *et al.* 2000; Ormerod *et al.* 1996). *H. chlorum* is has a strong tendency to form spheroplasts and lyse but is non-motile, and forms spreading colonies on agar (Beer-Romero and Gest 1987; Gest and Favinger 1983). *Heliobacillus mobilis* (Beer-Romero and Gest 1987) rarely lyses, and has a maximum temperature for growth of only 44°C. The thermophilic species *Heliobacterium modesticaldum* is capable of growth to a maximum of 56°C, and grows optimally at 50-52°C (Kimble *et al.* 1995), above the maximum growth temperature observed for the Orakei Korako strains. Thus it appears that these unisolated organisms represent a novel moderately thermophilic lineage, adapted to a temperature range between those of *H. mobilis* and *H. modesticaldum*.

Stevenson *et al.* (1997) noted that purple nonsulfur bacteria tended to out-compete heliobacteria in enrichments from moist soil, and that heliobacteria were not observed unless cultures were pasteurised. This observation is supported by the fact that no PNSB were isolated from sites OK1, OK6 or OK7. In the case of OK1, it may be supposed that the mats in this location were periodically subjected to large increases in temperature when the Diamond Geyser entered periods of continuous activity. Organisms present in the tepid surface layers of the OK7 lake shore may also be exposed occasionally to much higher temperatures due to the transfer of sand to deeper levels as a result of wave action which is enhanced by passing boats. The OK6 mats appeared to be subject to periodic drying and were partially moribund at the time of sampling. The ability of heliobacteria to form endospores would be of survival value under such conditions, which may also be responsible for the absence of PNSB.

Extremely rapidly moving spirilla that exhibited a strong scotophobic response were observed as numerically dominant organisms in early enrichments set up on Frying Pan Lake samples. These spirilla failed to grow on solid media, and disappeared from enrichment cultures within several further days of incubation. Such enrichment cultures showed deep red pigmentation, indicating that the organisms were probably not *Rhodospirillum photometricum* or *Phaeospirillum* species, similar to the two brown-pigmented spirilloid organisms that were later isolated from MP and WSS samples, although they were of similar size. Their identity remains unknown.

An initial culture from one of the four red colonies on the WSS direct isolation plate grew red-brown in colour. However, when mannitol, sorbitol or glutamate were used as carbon source, a pink culture developed instead. The culture was re-purified by streaking on plates to yield strain WSSB3, which did not grow on these compounds, but no pink colonies were observed alongside the brown ones. The mixed culture was transferred to tubes with 2 mM mannitol, 2 mM sorbitol and 1 mM glutamate as carbon sources, and developed pink colouration, but only brown colonies were observed when this culture was transferred to solid medium. After sequential subculture, low numbers of pink colonies were eventually obtained in an agar-shake tube. A pink spiral organism was obtained that was free of the contaminating WSSB3 type. However, a third spiral organism was present in very low numbers in this pink

culture (Figure 4.14), and the pink strain was never obtained free of this contaminant. It is unknown whether this organism was also photosynthetic, but no scotophobic behaviour of this cell type was observed. The mixed culture required nicotinate.

The ability to grow on mannitol and sorbitol is typical of the species *Rhodospirillum photometricum*, which however varies in colour from brown-red through brown-orange to dark brown. Pink strains have not been described. The unknown spirillum resembled *Rsp. rubrum* in its colour and size (1 μm by 4-7 μm), but not in its carbon source utilisation or vitamin requirement.

Figure 4.14 Unisolated pink-pigmented vibrioid organism, and contaminating spirillum, centre-left. Bar, 5 μm

An unusual isolate was obtained by agar-shake from a Wai5 enrichment on enrichment culture medium with succinate at 40°C. The organism contained bacteriochlorophyll *a*, was brown in colour, and exhibited a strikingly unusual morphology, with two ovoid rod shaped cells and a central regular coccus in a linear "bow-tie" or "propeller" arrangement, somewhat reminiscent of the cell arrangement seen in "*Gemmiger formicilis*" (Gossling 1975), which has however two central coccoid structures. Unfortunately, microscopic examination of this culture was not carried out until the culture was in stationary phase, so no conclusions can be drawn as to the mode of division. The culture failed to grow in subculture under any conditions tried. Attempts were made to grow the organism with added vitamins, additional yeast extract, soytone and peptone, a range of carbon sources, in diluted culture medium, under photoautotrophic conditions, and under microaerophilic conditions, with no success. Most of the culture was thereby used as inoculum for

subculture attempts. A further 1 mL was used for extraction of DNA in an attempt to obtain a 16S rRNA gene sequence, but no positive PCR product could be obtained. Cells in the final volume showed significant lysis, and the culture was presumed to be dead. Indeed, it is likely that the organism rapidly lost viability upon reaching stationary phase, and was non-culturable by the time it was first examined.

A 42°C enrichment culture from OK5 was carried through agar-shakes with the unusual result shown in Figure 4.15. Growth was observed only in the first tube of the series. Scattered white colonies were seen throughout the agar, but pigmented growth was restricted to a tight band about 1 cm beneath the agar surface. The agar-shake tube was sealed with paraffin wax, but the upper layers might have contained traces of oxygen, in which case the brown organism would appear to be a microaerophilic phototroph or aerobic anoxygenic phototroph with a very narrow range of oxygen tolerance. Disappointingly, it did not prove possible to culture this organism from the original enrichment or from the agar-shake tube, either under anaerobic conditions in the light, under fully aerobic conditions, or in Hungate tubes with varying proportions of air injected into the headspace.

On one occasion, growth of photosynthetic organisms was observed in an enrichment culture set up at extreme pH. This was the second OK8 sample, whose pH was measured as 9. This enrichment culture was dominated by purple sulfur bacteria of the *Chromatium* type. Transfer of this culture into tubes at different pH gave intriguing results. Growth was observed at a pH as low as 6.5, and as high as 9.2, but relatively poor growth was observed in tubes at weakly alkaline pH, with almost no growth at pH 7.7. This bimodal pH optimum curve strongly suggested that two purple sulfur bacteria were present in the culture, one with a pH range typical of PSB of approximately 6.5-7.5 (Pfennig and Trüper 1989), the other moderately alkaliphilic, with a range of 7.8-9.2. Colonies could not be obtained on solid medium, and despite the apparent non-overlapping ranges for growth, the neutrophilic organism could not be diluted from the culture by sequential transfer at pH 8.

Phase-contrast microscope examination of an enrichment culture from Warbrick Terrace mats (Wai5) revealed the presence of very small coccoid organisms physically associated with the majority of purple sulfur bacterial cells (Figure 4.16).

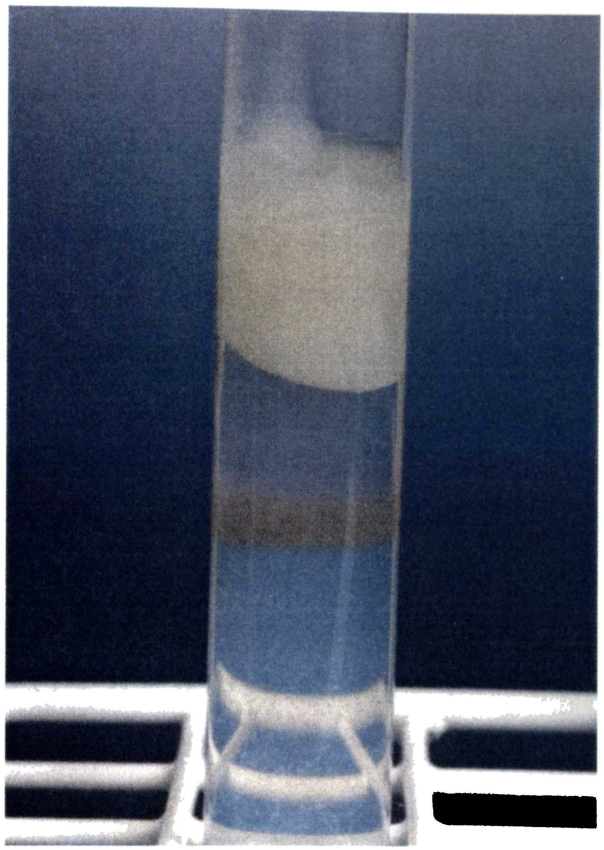


Figure 4.15 Agar-shake tube containing an unknown microaerophilic phototroph

Figure 4.16 Phase-contrast photomicrographs of epibionts parasitising purple sulfur bacterial cells. Bar = 2 μm

Scanning electron micrographs were taken of a sample from the Wai5 enrichment culture in order to examine the morphology of these unusual organisms in more detail, and representative micrographs are shown in Figures 4.17-4.20. The coccoid organisms commonly formed stacks of up to four cells. Division septa could be clearly seen, showing that reproduction occurs while attached to purple sulfur bacterial cells.

The epibionts appeared morphologically similar to *Vampirococcus*, a parasitic (or predatory) epibiont of purple sulfur bacteria that has resisted attempts at isolation (Esteve *et al.* 1983). *Vampirococcus* cells have been observed in plates of purple sulfur bacteria in karstic lakes in Spain, in association with *Allochromatium vinosum* and *Thiocystis minor* (formerly *Chromatium minus*), although they were not attached to a *Lamprocystis* species also present (Esteve *et al.* 1983; Guerrero *et al.* 1986). Another organism showing similarities to *Vampirococcus* was reported in a separate Spanish lake, in association with *Chromatium weissei*. This organism was suggested to be a symbiont, as *C. weissei* cells did not appear debilitated (Clarke *et al.* 1993). Characteristic droplet-shaped dispersal cells were observed at the ends of epibiont stacks.

Epibionts of purple sulfur bacteria have not previously been reported from geothermal areas (Isabel Esteve-Martinez, Universitat Autònoma de Barcelona, pers. comm.). All other *Vampirococcus* strains have been observed in dense purple sulfur bacterial plates in lakes, where they may be abundant (Esteve *et al.* 1992; Gaju *et al.* 1992). The Wai5 organism resembles the parasitic *Vampirococcus* rather than the symbiotic *C. weissei* epibiont. No cell forms showing similarities to dispersal cells were observed in SEM fields. In addition, the purple sulfur bacteria of the enrichment culture, most probably *Allochromatium vinosum*, showed signs of damage. Cells bearing epibionts were poorly mobile, and purple sulfur bacteria did not develop in subcultures, confirming that they were debilitated. At the time the epibionts were observed, the only actively growing purple sulfur bacterial culture available was, unfortunately, of the estuarine species *Marichromatium gracile*. Infection of this culture with the epibiont was attempted, but no *Vampirococcus* cells were observed.

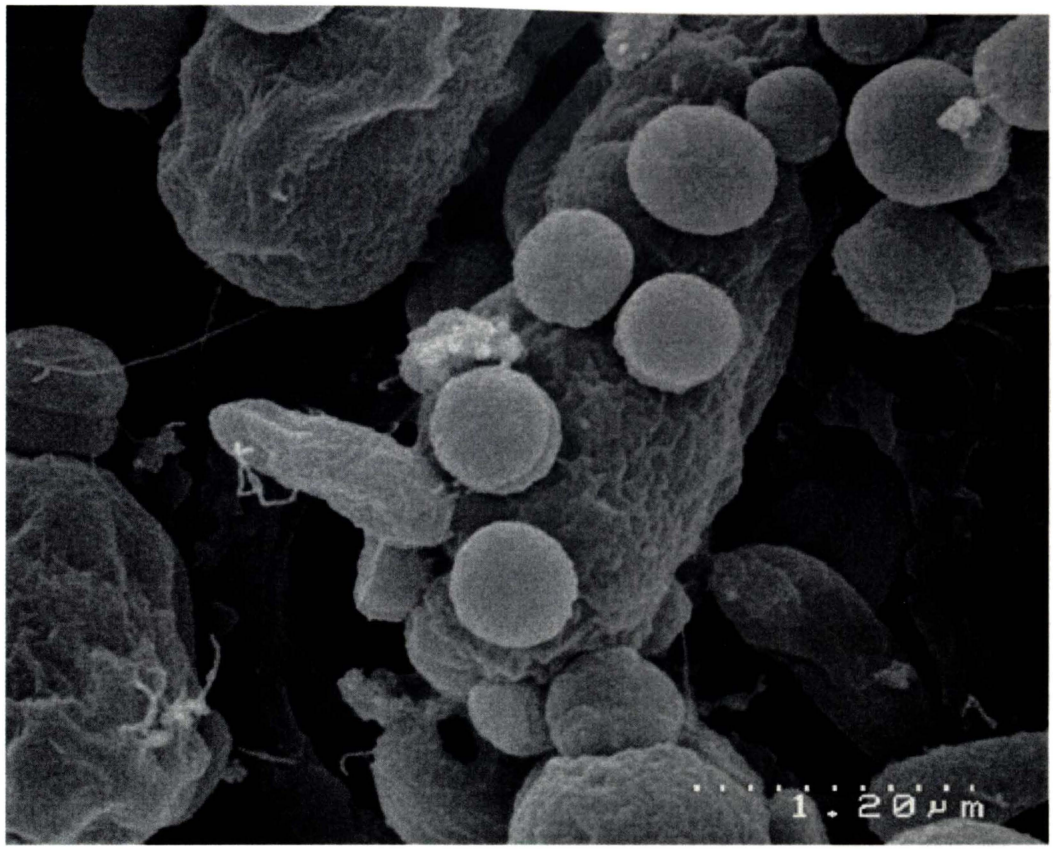


Figure 4.17 SEM of epibionts parasitising purple sulfur bacterial cells

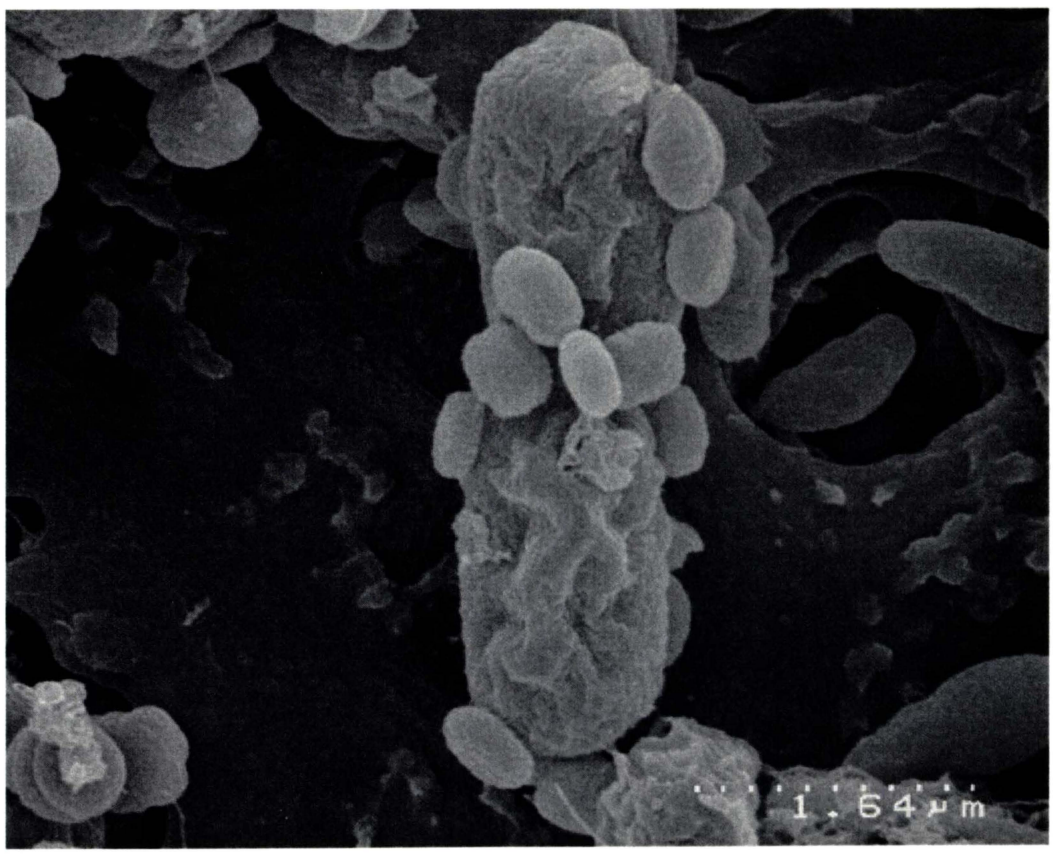


Figure 4.18 SEM of epibionts parasitising purple sulfur bacterial cells

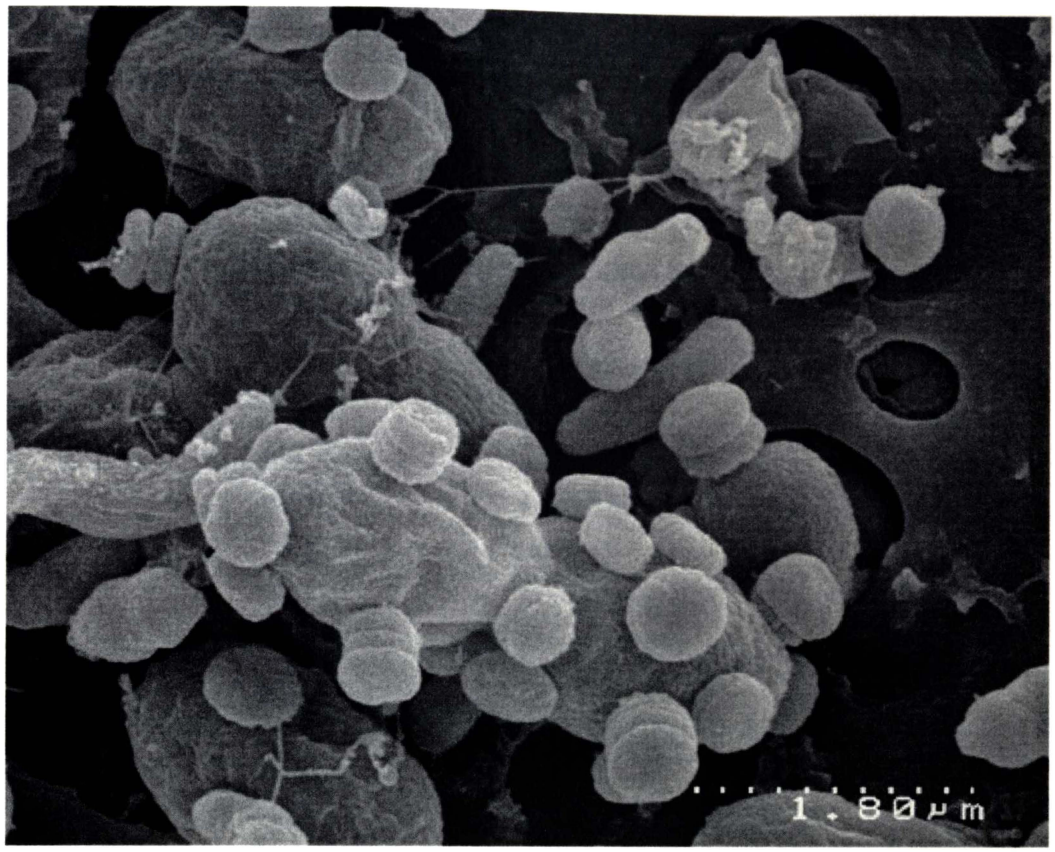


Figure 4.19 SEM of epibionts parasitising purple sulfur bacterial cells

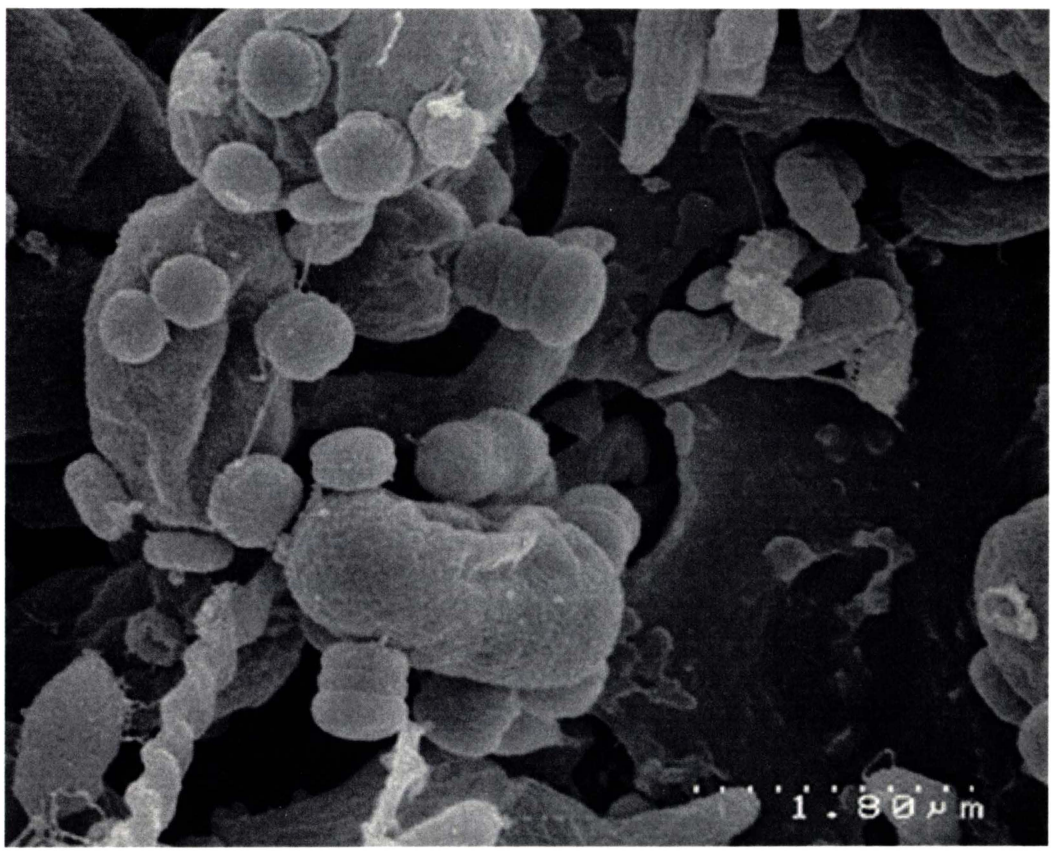


Figure 4.20 SEM of epibionts parasitising purple sulfur bacterial cells

4.3.10 List of Isolates

A complete list of isolates and their taxonomic assignment is provided in Table 4.2.

Sample site	Isolate	Taxonomic assignment
Wai1	Wai1bG3	*Blastochloris* sp.
	Wai1bR2	*Rhodoplanes* sp.
	Wai1S1	*Rhodopseudomonas palustris*
	Wai1val1	*Rhodopseudomonas palustris*
Wai3	Wai3G1e	*Blastochloris* sp.
	Wai3P2	*Rhodoplanes* sp.
	Wai3R3	*Rhodoplanes* sp.
Wai5	Wai5bG1	*Blastochloris* sp.
	Wai5R4	*Rhodoplanes* sp.
OK3	OK3G7	*Blastochloris* sp.
	OK3O3	*Rubrivivax gelatinosus*
	OK3P1	*Rhodoplanes* sp.
OK4	OK4bR1	*Rhodopseudomonas palustris*
	OK4bTR3	*Rhodopseudomonas palustris*
OK5	OK5bB3	*Rubrivivax gelatinosus*
	OK5M2	*Rhodomicrobium vannielii*
	OK5R2b	*Rhodoplanes* sp.
OK8	OK8bDIB2	*Rubrivivax gelatinosus*
OK9	OK9V1	*Rhodomicrobium vannielii*
	OK9C2	*Rubrivivax gelatinosus*
OK10	OK10bDIP1	*Rhodoplanes* sp.
	OK10ST2	*Rhodopseudomonas palustris*
OK13	OK13bO2	*Rubrivivax gelatinosus*
Tok2	Tok2tar1	*Rhodoplanes* sp.
Kui6	Kui6G1	*Blastochloris* sp.
	Kui6R2	*Rhodopseudomonas palustris*
WoT6	WoT6V1	*Rhodomicrobium vannielii*
WSS	WSSB3	*Rhodospirillum photometricum*
	WSSG4	*Blastochloris* sp.
	WSSR4	*Rhodopseudomonas palustris*
TKT	TKT2	*Rhodopseudomonas palustris*
OH3	OH3F2	*Rhodopseudomonas palustris*
MP	MPA1	*Phaeospirillum* sp.
	MPSR1	*Rhodopseudomonas palustris*
KCr	KCrS2	*Rhodopseudomonas palustris*

Table 4.2 Purple nonsulfur bacterial isolates and their taxonomic assignments

4.4 Summary

Purple nonsulfur bacteria capable of growth at temperatures greater than 40°C, the typical maximum for mesophilic species, were found to be common inhabitants of New Zealand thermal environments. As a consequence, mesophilic organisms persisting at elevated temperature, similar to those reported by Russian researchers, were not directly sought. Purple sulfur bacteria dominated many enrichments, but their development could be minimised by addition of sodium molybdate to enrichment cultures.

In total, 18 sample sites produced positive enrichment cultures from which purple nonsulfur bacteria were isolated. The widest diversity of species was found in samples from Frying Pan Lake (Wai1), Devil's Throat (OK3), Hot Springs Algae (OK5) and Waitangi Soda Springs (WSS). Strains assigned to three separate species were isolated from each of these.

Rhodopseudomonas palustris-like isolates were found in half of the positive samples, and both *Blastochloris* and *Rhodoplanes* strains were present in approximately one-third. In several cases, initial developments of *Blastochloris* strains were overgrown by *Rhodoplanes* in subsequent subcultures. The use of filtered light sources assisted isolation of *Blastochloris*.

Other species were less commonly encountered. *Rubrivivax gelatinosus* strains were readily isolated from samples taken at Orakei Korako, though failure to obtain them from other locations does not indicate that they are restricted to this location. *Rhodomicrobium vannielii* strains were found at two Orakei Korako sites and at Waiotapu. Photosynthetic spirilla were observed in several samples, but only two representatives were isolated.

4.5 Suggestions for further work

The enrichment culture work described in this chapter constituted a general survey of thermal environments in search of thermotolerant purple nonsulfur bacteria. Fairly standard enrichment culture and direct plating techniques were employed, although the addition of molybdate was used to control the development of sulfate-reducing

bacteria, and the high level resistance of purple nonsulfur bacteria to tellurite was exploited. Other cultivation techniques are available that were not used, or were used only rarely. Dilution culture can allow the isolation of numerically dominant but slow-growing organisms. Organisms isolated from liquid enrichment cultures were also the numerically dominant colony types seen by direct plating, so it is not clear that the dilution culture technique would be generally useful in the isolation of purple nonsulfur bacteria that could not obtained by other methods. It was however used in the isolation of a *Blastochloris* strain from a bloom of purple sulfur bacteria, and thus might prove useful. The efficacy of direct plating might be increased through the use of agar plates cast with single or two-dimensional chemical gradients, such that growth of different organisms would be favoured in different areas of a plate. Such plates, with perpendicular gradients in pH and NaCl concentration, have previously been used in the characterisation of purple nonsulfur bacteria (Wimpenny *et al.* 1986), but reports of their application as an aid to isolation have not been published. Imposition of a temperature gradient across a plate would be the most obvious method for the current work, but suitable equipment to maintain a stable gradient was not available.

More detailed studies could be carried out to explore the ecology of PNSB in a particular location, including the variation in species distribution with changes in physicochemical parameters. Such studies could involve sampling along gradients of temperature, although suitable sites with stable defined gradients in the temperature range of interest would be difficult to find. Examination of the distribution of purple nonsulfur bacteria within a mat sample by vertical sectioning would be more promising. The use of fluorescent in situ hybridisation techniques (FISH) could be employed, if suitable probes were available. Cultivation of artificial microbial mats in the laboratory, as reported by Fenchel (1998a, 1998b), could allow ecological studies of mat composition under changing conditions.

A surprisingly large number of organisms observed in enrichment cultures could not subsequently be isolated or maintained in culture. At least a partial characterisation of organisms of particular interest could have been carried out in mixed culture, though the unambiguous interpretation of results is substantially more difficult.

Chapter 5

Characterisation of Thermotolerant Purple Nonsulfur Bacteria

5.1 Introduction

This chapter consists of two parts. In the main part, the results of the characterisation of purple nonsulfur bacteria isolated from geothermal areas are presented. The methods used were given in Chapter 3, and a full list of isolates is available in Table 4.2. In the second part, the temperature relations of some mesophilic purple nonsulfur bacteria are examined.

5.2 *Rhodoplanes* isolates

5.2.1 Strains

Budding rod-shaped bacteria producing mass cultures pink in colour were the most common morphological type isolated in the course of this study. Isolates with a cell diameter of approximately 1 μm that were unable to use long-chain fatty acids as carbon sources were tentatively assigned to the genus *Rhodoplanes*, which was subsequently confirmed by sequencing of the 16S rRNA gene. Eight strains were obtained, which included four strains from Waimangu Volcanic Valley (Wai1bR2, Wai3P2, Wai3R3, Wai5R4), three strains from Orakei Korako (OK3P1, OK5R2b, OK10bDIP1), and the only isolate that was obtained from Tokaanu (Tok2tar1). Samples that yielded these strains were predominantly microbial mats, though Tok2tar1 was isolated from muddy water. Strain Wai5R4 was subsequently lost, and strain OK10bDIP1 was not characterised further.

5.2.2 Morphological properties

Cells of all strains were 1 μm wide and 1.8 to 3 μm long, and divided by budding with the formation of a division tube between mother and daughter cells (Figure 5.1). Rosettes were observed, particularly in older cultures. In this respect, the isolates were more similar to *Rpl. elegans* than *Rpl. roseus* which rarely forms rosettes or clusters. Cells were actively motile. Electron microscopy of thin sections of strain Wai3R3 showed intracytoplasmic membranes of the lamellar form typical of the genus *Rhodoplanes* in phototrophically grown cells (Figure 5.2).

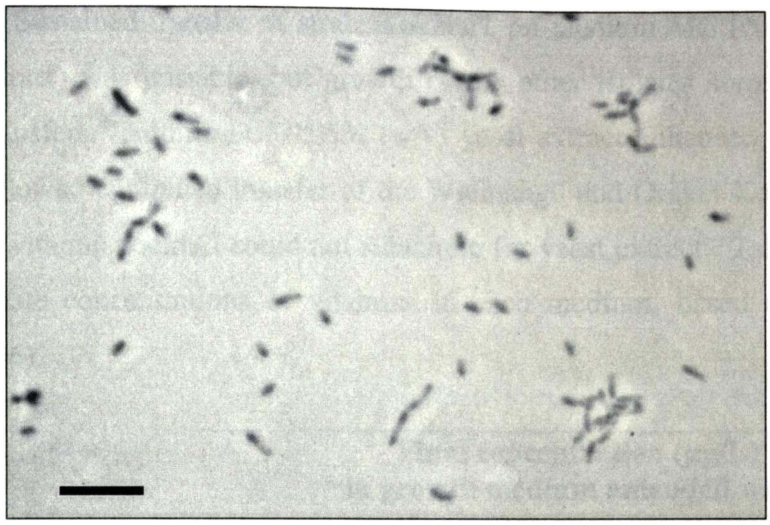


Figure 5.1 Phase-contrast photomicrograph showing the cell morphology of strain Wai3R3. Bar, 10 μm

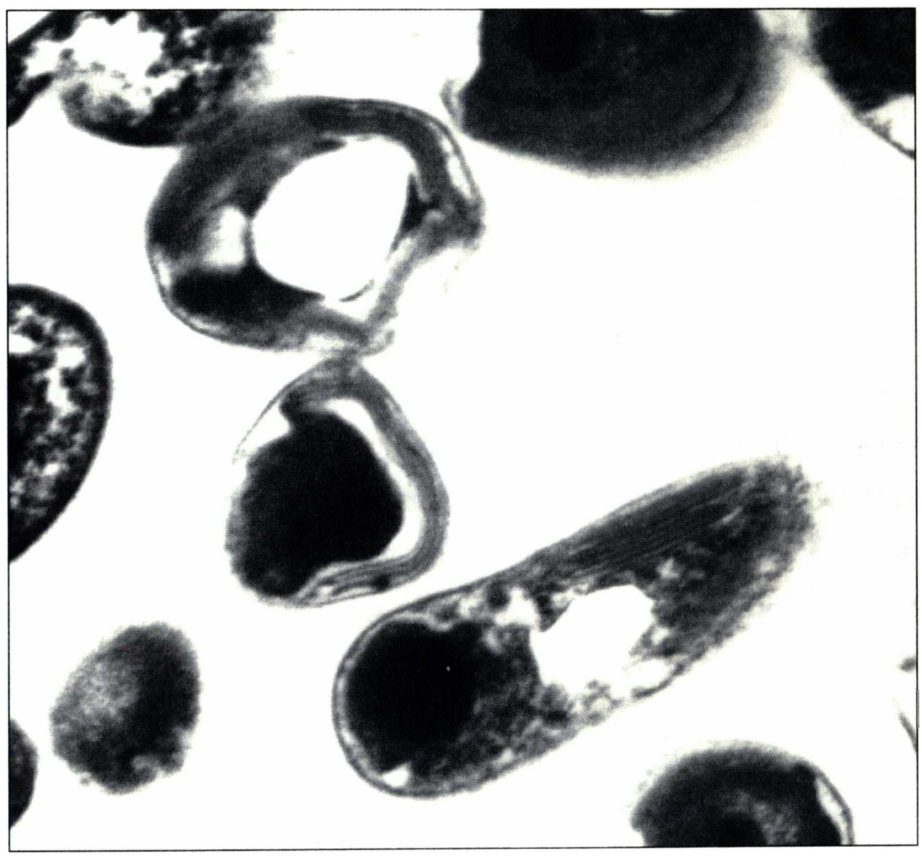


Figure 5.2 Electron micrograph of ultrathin sections of strain Wai3R3 showing the intracytoplasmic membranes (x20000)

5.2.3 Cultivation conditions

The *Rhodoplanes* isolates initially grew poorly in laboratory culture, achieving a low cell density. Sustained transfer of strain Tok2tar1 on medium MC (containing 0.02 g/L yeast extract) was possible, but growth of the other isolates sometimes ceased after three transfers. Addition of 0.05% (w/v) yeast extract enhanced growth of all strains and allowed continued transfer of the Waimangu and Orakei Korako isolates. The standard vitamin cocktail could not substitute for yeast extract. Table 5.1 shows the approximate concentrations of vitamins in each medium, based on data from Sommer (1996).

	Final concentration (µg/L) in growth medium amended with:	
Vitamin	**0.05% (w/v) yeast extract**	**vitamin cocktail**
nicotinate	354	114
pantothenate	156	56
pyridoxamine	12.0	50
biotin	1.3	10
thiamine	15.6	101
PABA	unknown	~50
B_{12}	unknown	~10

Table 5.1 Vitamin contents of supplemented growth media.

Only nicotinate and pantothenate were supplied in the yeast extract medium at higher levels than in the medium supplemented with the vitamin cocktail, suggesting that either of these components was the limiting factor for growth of these *Rhodoplanes* strains in standard medium. Vitamin test cultures of strain Wai3R3 that had shown no growth were supplemented with pantothenate and nicotinate. Supplementation with pantothenate to 150 µg/L did not stimulate growth, but addition of 350 µg/L nicotinate led to rapid culture development.

Vitamin tests were carried out according to the methods given in Section 3.8.3, using an increased amount of nicotinate, and showed that all the *Rhodoplanes* isolates, including strain Tok2tar1, required nicotinate as a growth factor. In addition, strains Wai3P2 and OK3P1 required vitamin B_{12}. The growth of strain Tok2tar1 however was not enhanced by addition of higher levels of nicotinate. *Rpl. roseus* is known to require nicotinate (Janssen and Harfoot 1991), but grew well on unsupplemented

medium. It thus appeared that the Waimangu and Orakei Korako strains of *Rhodoplanes* had an unusually high requirement for this vitamin.

Nicotinate requirement was examined by measuring the final culture density achieved by several *Rhodoplanes* strains on minimal salts medium supplemented with varying amounts of nicotinic acid. Inocula consisted of cells that had been starved of nicotinic acid by one transfer on MSM without vitamins. Mixed acids was used as carbon source. Results are shown in Table 5.2.

	Final optical density at 660 nm of strain:		
Concentration of nicotinate (µg/L)	**Wai3R3**	**OK3P1**	***Rpl. roseus***
0	0.028	0.019	0.076
50	-	-	0.975
100	0.200	0.132	1.034
200	0.561	0.188	0.976
500	0.784	0.321	1.046
1000	0.876	0.720	1.038
2000	1.127	1.047	-
3500	1.028	0.983	-
5000	1.238	1.143	1.019

Table 5.2 Response of *Rhodoplanes* strains to nicotinic acid supplementation

Rpl. roseus was able to reach a full growth yield on the minimum nicotinic acid concentration tested, 50 µg/L, which is less than half the amount present in medium MC. By contrast, growth of the thermotolerant strains was limited at concentrations below 2000 µg/L, and was approximately proportional to the amount of nicotinate available.

The Waimangu and Orakei Korako strains thus required up to 40 times the amount of nicotinate required by *Rpl. roseus* or other nicotinic acid-requiring purple nonsulfur bacteria, such as *Phaeospirillum photometricum*. This presented the possibility that these strains exhibited some abnormality in the metabolism of nicotinic acid. On the basis of this slight evidence, the *Rhodoplanes* strains were tested for growth on nicotinic acid as a carbon source, at a concentration of 2 mM. Unexpectedly, *Rpl. elegans* AS130 and the Waimangu and Orakei Korako strains grew well on nicotinate.

Rpl. roseus and Tok2tar1 failed to grow. The ability to use nicotinate as a carbon source is an extremely rare property among purple nonsulfur bacteria, which warranted further investigation. The results of this study are presented more fully in Chapter 7.

Other PNSB, including type strains and thermotolerant isolates, were screened for the ability to grow on nicotinate. "*Rhodopseudomonas cryptolactis*" was the only organism that was also able to use nicotinate as sole carbon source, which provided a strong indication that this thermotolerant organism is affiliated with *Rhodoplanes*, rather than *Rhodopseudomonas sensu stricto*. In further support, "*Rps. cryptolactis*" also requires B_{12} and nicotinate (at a regular concentration) as vitamins, and is reported to metabolise a restricted range of carbon sources (Stadtwald-Demchick *et al.* 1990b). Consequently, the properties of "*Rps. cryptolactis*" were re-examined alongside those of the *Rhodoplanes* strains.

With the routine addition of nicotinate to growth media at a concentration of 2 mg/L, reliable cultivation and further characterisation of the properties of the *Rhodoplanes* isolates were possible.

5.2.4 Photopigments

All strains produced pink colonies on solid medium and pink cell suspensions in liquid medium under anaerobic conditions in the light. Tokt2tar1 had a "chalky" appearance, similar to that of *Rhodobium marinum* var. *agilis* (Mangels *et al.* 1986). The absorption spectra of intact cells grown under phototrophic conditions resembled those of *Rpl. roseus* or *Rpl. elegans* (Figure 5.3), due to the presence of bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series, with peaks close to 800 and 855-865 nm. Under high light conditions, the *in vivo* absorbance spectrum of "*Rps. cryptolactis*" is similar. Under low light conditions, "*Rps. cryptolactis*" synthesises an alternate light-harvesting antenna system that absorbs maximally at 822 nm (Stadtwald-Demchick *et al.* 1990b; Halloren *et al.* 1995).

Rhodoplanes strains and "*Rps. cryptolactis*" were grown at 2000 lux and at approximately 50 lux behind a neutral density filter. The wavelengths of absorption maxima of *Rpl. elegans* and *Rpl. roseus* were unaltered by growth in low light (Figure 5.3). No peak at 822 nm could be seen.

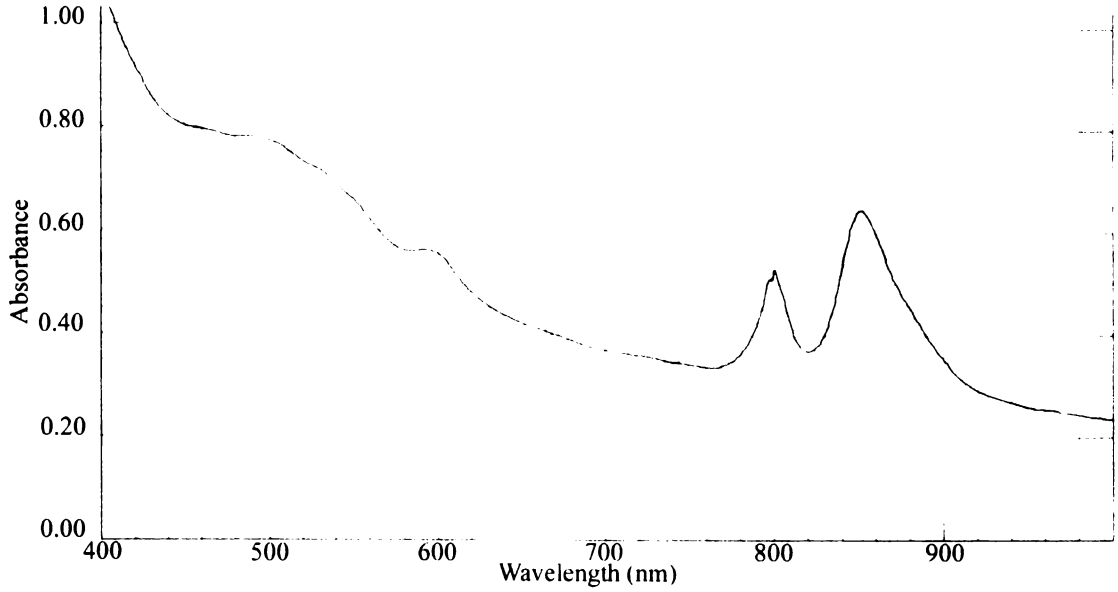


Figure 5.3 *In vivo* absorption spectrum of *Rhodoplanes elegans* AS130, grown under low light conditions

The development of a peak at 822 nm and reduction of absorbance at 857 nm under low light conditions was confirmed in "*Rps. cryptolactis*" (result not shown). Under low light conditions, the *Rhodoplanes* strains tested, Wai5R4, Wai3R3, Tok2tar1 and OK3P1, did not exhibit a peak at 822 nm. However, each exhibited an unusual "shoulder" on the 855 nm peak with an approximate wavelength maximum of 900 nm. The shoulder was most prominent for strains Wai5R4 and Wai3R3 (Figure 5.4), less obvious but certainly present in strains Tok2tar1 and OK3P1.

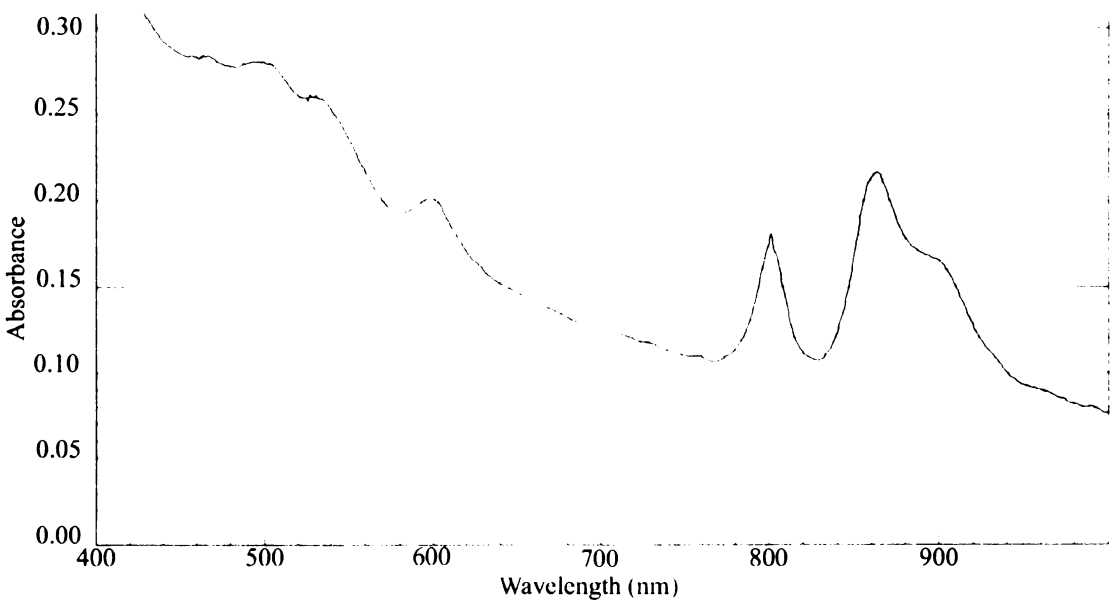


Figure 5.4 *In vivo* absorption spectrum of strain Wai3R3, grown under low light conditions

The cause of this change is not certain, but does not appear to be due to increased synthesis of light-harvesting complex I, which absorbs at 875 nm in *Rhodoplanes* (Gläser and Overmann 1999). It may conceivably signify the presence of an unusual long-wavelength light-harvesting antenna complex. In a microbial mat, shading by other organisms causes reduced light intensity and selective removal of particular wavelengths, which may lead to a selective advantage for organisms that are able to absorb longer wavelength infrared light that penetrates to deeper layers (Resnick and Madigan 1989). In this regard, the B920 light-harvesting complex that is produced by *Roseospirillum parvum* (Gläser and Overmann 1999) and some strains of *Thermochromatium tepidum* (though apparently not New Zealand strains) is of interest (Madigan 2001).

Strains tested – Wai5R4, Tok2tar1 and Wai3P2 – were colourless when grown under aerobic conditions in darkness.

5.2.5 Physiological characteristics

Rpl. roseus and *Rpl. elegans* have temperature maxima of 40 and 42°C respectively, but grow optimally at 32-37°C (see Figure 5.39 C and D). The thermotolerant *Rhodoplanes* strains were isolated from environments that ranged in temperature from 30 to 48°C, and developed in enrichment cultures at 42-43°C. The maximum temperatures for growth of these strains were determined to be 46.5°C for Wai3R3 and Wai5R4; 46°C for Wai3P2; 45°C for OK3P1 and OK5R2b and 44°C for Tok2tar1. The strain with the lowest maximum temperature for growth came from the sample of highest temperature, and vice versa, an irony that lacks explanation.

Figures 5.5 and 5.6 present graphs of specific growth rate versus temperature. Final growth yields were similar at each growth temperature, except at the maximum. At this temperature, cultures reached around one-third of the optical density achieved at lower temperature. Presumably, cells were forced to divert substrate for cell repair rather than division. Strain Wai3R3 had an optimum temperature of 40-44°C, but suffered a rapid reduction in growth rate beyond that. Similar curves were obtained for other Waimangu and Orakei Korako isolates, which had optima ranging from 40-43°C. Tok2tar1 grew optimally over a broad range from 36 to 42°C.

Tok2tar1 grew exceptionally slowly, even for a member of the genus *Rhodoplanes*, whose described members are noted for their relatively slow growth compared to other PNSB (Hiraishi and Ueda 1994b). The doubling time of Tok2tar1 was never less than 25 hours, although it is possible that optimal conditions for growth were not provided.

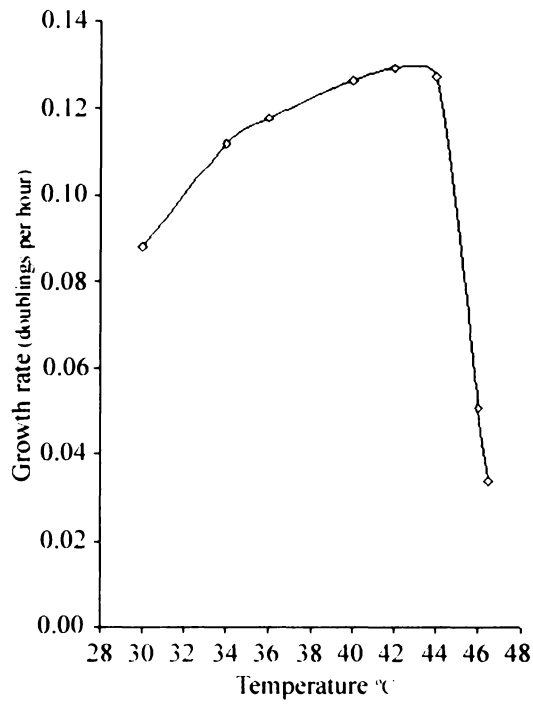


Figure 5.5 Specific growth rate of strain Wai3R3 as a function of temperature

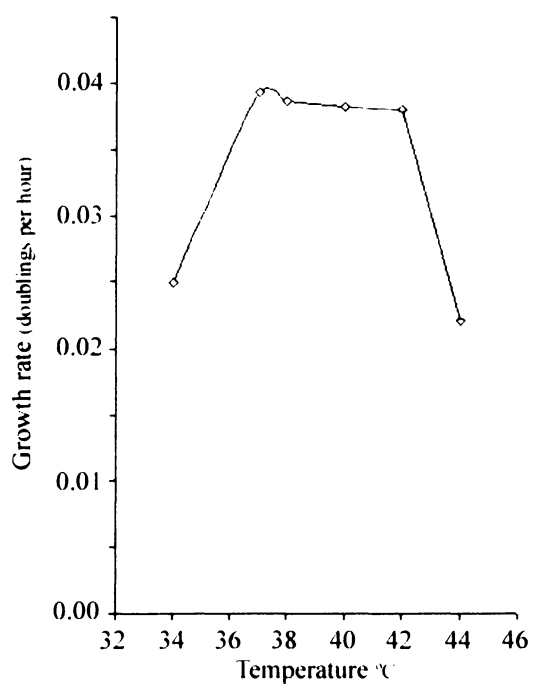


Figure 5.6 Specific growth rate of strain Tok2tar1 as a function of temperature

Strains Wai3P2, Wai3R3 and Tok2tar1 were capable of slow growth at 25°C, but showed no growth at 20°C after 10 days of incubation. Strain Wai5R4 grew even at 20°C. It was reported that "*Rps. cryptolactis*" did not grow phototrophically below 35°C, but in the present work, it too was found to be able to grow at 25°C.

The Waimangu and Orakei Korako strains of *Rhodoplanes* grew over the pH range 5.7-8.2. No previously described *Rhodoplanes* isolate is capable of growth below pH 6.0. *Rpl. elegans* grows in the range 6.0-8.5, and *Rpl. roseus* grows in the narrower range 6.0 to 8.0. Strain Tok2tar1 thus resembled a more typical *Rhodoplanes* strain in its pH range for growth of 6.2-8.5. "*Rps. cryptolactis*" has the most alkaline range, of 6.4-8.5. All strains, however, grew optimally around pH 7.0.

All strains were capable of growth using NH_4^+ or N_2 as nitrogen source. None required a reduced sulfur source. With the exception of Wai3R3 and OK3P1, all strains used thiosulfate as electron donor for photoautotrophic growth. Photoautotrophic growth did not occur with sulfide as electron donor at 0.5 mM. Growth on mixed acids was inhibited by sulfide at 1 mM. Like the established *Rhodoplanes* species, the thermotolerant isolates were inhibited by NaCl at a concentration of 1% (w/v).

In addition to growth anaerobically in the light and aerobically in darkness, the described *Rhodoplanes* species are able to grow anaerobically in darkness using nitrate as respiratory electron acceptor, producing nitrogen gas as end product. The Waimangu *Rhodoplanes* isolates and OK5R2b also were capable of complete denitrification, but were less active than the mesophilic species. Less nitrogen was produced and only small amounts of nitrite. Strains OK3P1 and Tok2tar1 carried out only limited nitrate respiration, producing a small amount of nitrite, with no visible accumulation of nitrogen gas in Durham tubes.

Compounds used as carbon sources or electron donors are listed in Table 5.3. For the novel isolates, growth was assessed according to the scheme given in Table 3.3. *Rpl. roseus* and *Rpl. elegans* are notable for their relatively restricted range of utilisable carbon sources, their inability to use fatty acids containing more than six carbon atoms, and their utilisation of citrate and tartrate (Hiraishi and Ueda 1994b).

All *Rhodoplanes* strains were capable of growth on acetate, butyrate, lactate, malate, pyruvate and succinate. None of the strains was able to use aspartate, benzoate, ethanol, fructose, glucose or glycerol. The majority of the new isolates were unable to use caproate, and several were unable to grow on valerate. None was able to grow on caprylate or pelargonate.

Carbon source/ electron donor	**Wai3R3**	**Wai3P2**	**Wai1bR2**	**OK3P1**	**OK5R2b**	**Tok2tar1**	***Rpl. elegans***	***Rpl. roseus***	***Rps. cryptolactis***
Acetate	++	+	+++	+++	+++	++	+	+	+
Arginine	-	-	-	-	-	-	0	0	-
Aspartate	-	-	-	-	-	-	-	-	-
Benzoate	-	-	-	-	-	-	-	-	-
Butyrate	+++	+++	+	+++	++	+	+	+	0
Caproate	-	+	-	+	-	-	+	-	0
Caprylate	-	-	-	-	-	-	-	-	-
Citrate	++	+++	-	+++	+++	-	+	+	+
Ethanol	-	-	-	-	-	-	-	-	-
Formate	-	-	-	-	(+)	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-
Fumarate	+	-	+	+	++	-	+	+	+
Gluconate	-	-	0	-	-	-	0	0	0
Glucose	-	-	-	-	-	-	-	-	-
Glutamate	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	+	-
Glycolate	-	-	-	-	-	-	-	-	-
Lactate	++	+	++	+++	++	+	+	+	+
Malate	++	+	++	++	++	++	+	+	+
Malonate	++	++	+	+	+	+	-	-	+
Mannitol	-	-	-	0	0	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-
Pelargonate	-	-	-	-	-	-	0	-	-
Propionate	(+)	++	+++	-	+++	+	+	+	+
Pyruvate	++	+	+++	+++	+++	++	+	+	+
Sorbitol	-	-	0	0	0	-	-	-	-
Succinate	++	++	++	++	++	+	+	+	+
Tartrate	-	-	-	-	-	+	+	+	+
Valerate	+	(+)	-	+	-	-	+	+	0
Yeast extract	++	+	+	-	+	+	+	+	+
Casamino-acids	++	+	+	+	+	+	+	+	-
Hydrogen	-	0	0	0	0	0	0	0	-
Thiosulfate	-	+	+	-	+	+	+	+	+
Sulfide	-	-	-	-	-	-	-	-	-

Table 5.3 Carbon sources and electron donors utilised by *Rhodoplanes* strains.
Data from current work, Hiraishi and Ueda (1994b), Janssen and Harfoot (1991), Stadtwald-Demchick *et al.* (1990b).
Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of four remaining strains is positive/negative only. 0 =not tested

The majority of new strains were able to assimilate citrate. Wai1bR2 and Tok2tar1 lacked this ability. The Waimangu and Orakei Korako strains were unable to use tartrate, though Tok2tar1 showed weak growth on this substrate. All the new isolates shared the ability to grow on malonate, an ability which the established *Rhodoplanes* species lacked. Malate and pyruvate were the only good carbon sources for growth of strain Tok2tar1, which was unable to use fumarate, an unusual property it shared with strain Wai3P2.

"*Rps. cryptolactis*" is unique in its inability to grow on lactate in the absence of bicarbonate. This peculiarity was not observed with any other thermotolerant isolates, which grew to approximately the same cell density in each of three consecutive transfers on lactate medium without bicarbonate. In general, however, the carbon source utilisation pattern of "*Rps. cryptolactis*" was consistent with membership of the genus *Rhodoplanes*, in absence of growth on long-chain fatty acids, and ability to use citrate, malonate and tartrate. Contrary to the results of Stadtwald-Demchick *et al.* (1990b), weak photoautotrophic growth on thiosulfate was observed.

5.2.6 Genetic relationships

16S rRNA gene sequences covering more than 1400 continuous nucleotide residues were determined for the majority of the isolates according to the methods given in Section 3.9. The 16S rRNA sequence of "*Rps. cryptolactis*" was kindly provided by Dr Akira Hiraishi of Toyohashi University of Technology, Toyohashi, Japan. The sequences of strains Tok2tar1, Wai3R3 and OK5R2b were submitted to the GenBank database under Accession Numbers AF077729, AF077730 and AF487437. The sequences of strains OK3P1 and OK5R2b were identical. The sequences of Wai1bR2 and Wai3P2 were more than 99.8% similar to that of Wai3R3. Binary sequence similarity values for *Rhodoplanes* strains and closely related organisms are given in Table 5.4.

The 16S rRNA genes of the thermotolerant isolates and "*Rhodopseudomonas cryptolactis*" showed high levels of similarity (>96.8%) to those of the established *Rhodoplanes* species, and much lower levels of similarity to *Rps. palustris*. The Waimangu and Orakei Korako isolates showed the greatest similarity to *Rpl. elegans*. The closest relative of "*Rps. cryptolactis*" was strain Tok2tar1, with a similarity of 99.6%.

No.	Species or strain	Sequence similarity (%) for 16S rRNA genes							
		2	3	4	5	6	7	8	9
1	*Rpl. elegans* AS130	99.3	99.0	97.7	97.5	98.6	98.4	94.2	91.9
2	OK5R2b		99.7	97.3	97.1	98.1	98.0	94.0	92.5
3	Wai3R3			97.0	96.8	97.8	97.7	93.7	92.2
4	*Rpl. roseus* 941				99.4	98.2	98.3	94.4	91.1
5	*Rpl. roseus* DSM13233					98.0	98.1	94.4	91.5
6	*Rps. cryptolactis* DSM 9987						99.6	93.8	91.3
7	Tok2tar1							94.0	91.8
8	*Blc. sulfoviridis* DSM 729								90.0
9	*Rps. palustris* ATCC 17011								

Table 5.4 Sequence similarities of 16S rRNA gene sequences of *Rhodoplanes* strains and their relatives

Sequences were used to construct a phylogenetic tree according to the methods given in Section 3.9.6 (Figure 5.7). The Waimangu and Orakei Korako strains form a cluster with *Rpl. elegans* that is well supported by bootstrap analysis. "*Rps. cryptolactis*" and strain Tok2tar1 cluster together with *Rpl. roseus* strains, with lower bootstrap support.

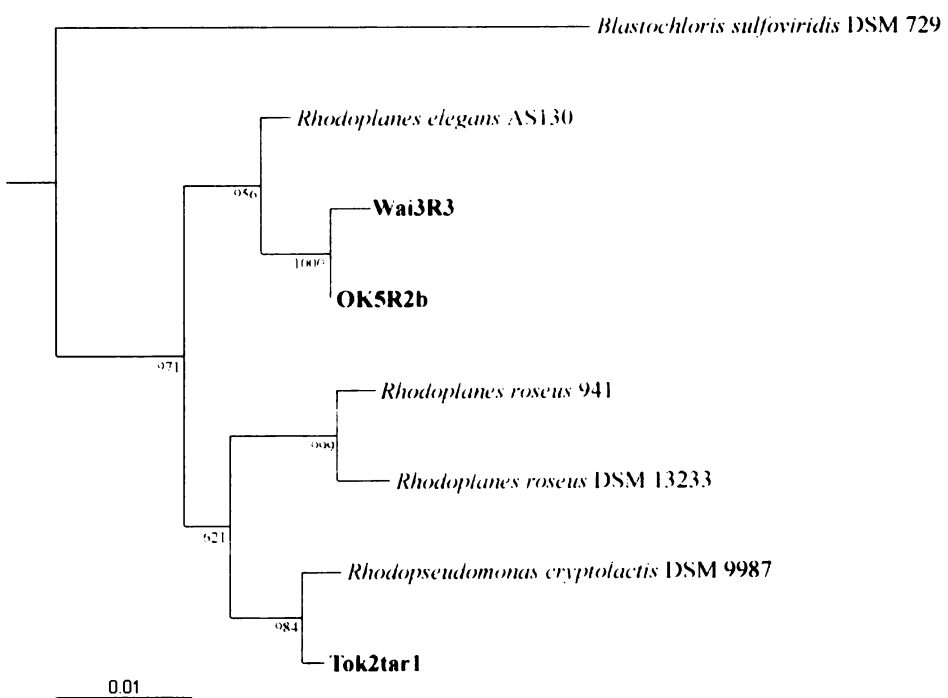


Figure 5.7 Distance matrix tree showing phylogenetic relationships of *Rhodoplanes* and *Blastochloris* strains, based on an alignment of 1401 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Rhodopseudomonas palustris*

Inspection of aligned sequences of the *Rhodoplanes* strains revealed structural differences in the stem-loop structure known as helix 6 that runs from bases 60-109 in the *E. coli* numbering system (see Appendix 2). These are shown in Figure 5.8.

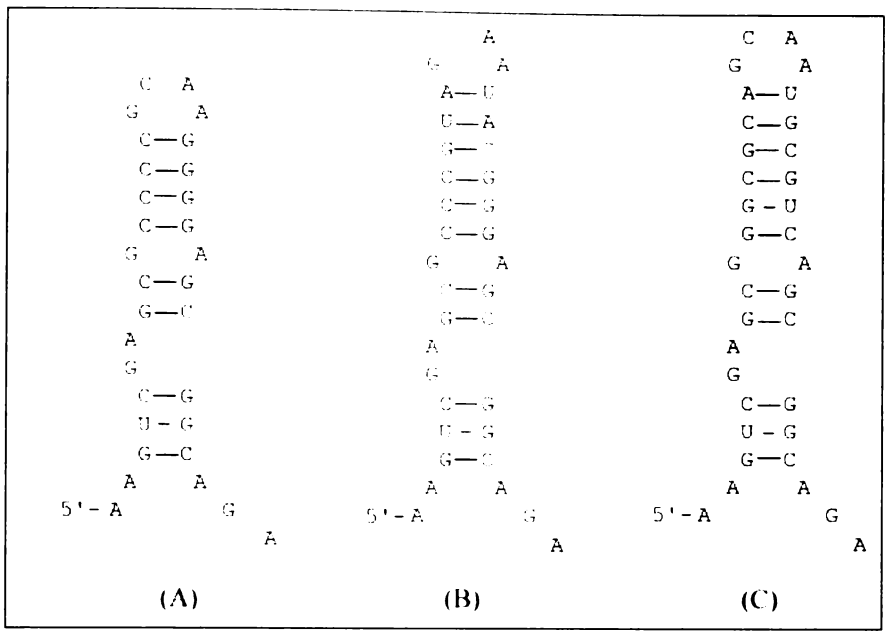


Figure 5.8 Deduced secondary structures of the stem-loop at *E. coli* positions 60-109 of the 16S rRNA molecules of: (A) *Rhodoplanes elegans*, "*Rhodopseudomonas cryptolactis*" and Waimangu and Orakei Korako strains; (B) *Rhodoplanes roseus*; (C) strain Tok2tar1

Hiraishi and Ueda (1994b) noted that the *Rpl. elegans* secondary structure (Figure 5.8 A) is unusual in having a stem that is two base-pairs shorter than the equivalent structure in several other *Rhodoplanes*, *Blastochloris* and *Rhodopseudomonas* species. This secondary structure was also found in Waimangu and Orakei Korako *Rhodoplanes* strains, and two isolates of the genus *Blastochloris* (see Figure 5.18 below). The sequence of *Rpl. roseus* (Figure 5.8 B) is similar to that of *Blc. sulfoviridis* DSM 729 or *Rps. palustris*. Strain Tok2tar1 (Figure 5.8 C) had the longer stem structure, but had a unique internal sequence. "*Rps. cryptolactis*", although overall 99.6% similar in 16S rDNA sequence to strain Tok2tar1, did not share this peculiarity. Instead, its sequence was similar to that of *Rpl. elegans* (Figure 5.8 A).

As preparation for research that is reported in Chapter 6, sequences were obtained of photosynthetic reaction centre genes from *Rpl. roseus*, *Rpl. elegans* and strain Tok2tar1. These sequences were deposited in the GenBank database under Accession

Numbers AF485345, AF485346 and AF159440. The Tok2tar1 sequence showed equal similarity to those of the two established species.

DNA-DNA hybridisation measurements were carried out by Dr Hiraishi, and are given in Table 5.5.

		% Hybridization to labelled DNA probe from:								
	Test Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	*Rpl. elegans* AS130	100	39	44	49	48	50	57	55	59
2	*Rpl. roseus* DSM5909	40	100	56	56	-	47	50	-	-
3	*Rps. cryptolactis* DSM9987	45	59	100	63	53	50	54	51	57
4	*Rhodoplanes* sp. Tok2tar1	43	57	65	100	52	54	55	51	56
5	*Rhodoplanes* sp. OK3P1	58	48	58	50	100	72	70	73	68
6	*Rhodoplanes* sp. OK5R2b	58	50	61	58	72	100	73	82	69
7	*Rhodoplanes* sp. Wai1bR2	57	48	58	55	70	73	100	98	93
8	*Rhodoplanes* sp. Wai3P2	61	51	60	55	72	72	98	100	97
9	*Rhodoplanes* sp. Wai3R3	59	45	59	60	63	67	95	94	100
10	*Blc. viridis* DSM133	13	11	14	16	15	16	16	15	15

Table 5.5 Genomic DNA relatedness among selected strains of budding phototrophic bacteria

Generally, strains that show a DNA similarity value of 70% or less are regarded as belonging to separate genospecies (Stackebrandt and Goebel 1994). *Rpl. roseus* and *Rpl. elegans* showed similarity of 39%, well below the species cut-off. Strain Tok2tar1 shows the highest degree of similarity to "*Rps. cryptolactis*", though at 65% this too is beneath the generally accepted value for species identity. The Waimangu strains Wai1bR2, Wai3P2 and Wai3R3 showed very high similarity, greater than 92%, indicating that they are closely related strains of a single genospecies. *Rpl. elegans*, the closest relative of these strains on the basis of 16S rDNA sequence similarity, showed however only 55-59% similarity with the Waimangu strains. Even lower similarity was observed between *Rpl. elegans* and Orakei Korako strains. OK3P1 and OK5R2b showed similarity values to each other and to the Waimangu strains of between 69 and 82%, indicating that they are genetically distinct, but still sufficiently similar that they fall within a single genospecies.

5.2.7 Taxonomy of *Rhodoplanes* strains

The results of the present work and research by Hiraishi show unequivocally that "*Rps. cryptolactis*" should be transferred to the genus *Rhodoplanes*. The question thus arises as to whether it or any of the other thermotolerant isolates should be described as novel species within the genus.

The new strains fall into two well-supported individual lineages according to neighbour-joining phylogenetic analysis of 16S rRNA genes (Figure 5.7). The overall degree of dissimilarity in their sequences is however relatively small, as low as 0.7% between *Rpl. elegans* and strain OK5R2b and 0.4% between "*Rps. cryptolactis*" and Tok2tar1. A commonly cited level of 16S rDNA gene sequence dissimilarity between species within a single genus is 3%. However, the degree of 16S rRNA sequence divergence between recognisable species may differ in different genera. Other phenotypically distinct organisms, including purple nonsulfur bacteria such as *Rpl. roseus* and *Rpl. elegans*, the two species of the genus *Phaeospirillum* and the two species of *Blastochloris*, show less than 3% divergence in 16S rDNA sequence. Fox *et al.* (1992) reported that three psychrophilic *Bacillus* strains showing low DNA-DNA relatedness exhibited more than 99.5% identity in 16S rDNA sequence, and commented that while 16S rRNA sequences can resolve relationships between genera and species, recently diverged species may not be recognizable.

One could perhaps speculate that the genetic variation between the Waimangu and Orakei *Rhodoplanes* strains has arisen since the year 1886, the earliest date at which colonisation of thermal sites in the newly-formed Waimangu valley by an ancestral Orakei Korako population could have occurred - if this were true, then species level genomic variation as measured by DNA-DNA hybridisation could arise in *Rhodoplanes* on a time scale of little more than a century. However, the existence of two Orakei strains that show a similar amount of difference in DNA similarity casts doubt on this idea.

DNA-DNA hybridisation measurements are regarded as the primary means of determining strain relatedness (Stackebrandt *et al.* 2002), and strains showing less than 70% homology may be defined as separate species where suitable distinguishing phenotypic properties are known.

The assignment of the Waimangu and Orakei Korako strains to a new species of *Rhodoplanes* is supported by phenotypic differences and the low DNA-DNA hybridisation values obtained with other members of the genus. Likewise, "*Rps. cryptolactis*" and strain Tok2tar1 share low homology with the other strains, and could form either a single new species, or two separate species; the latter option is favoured as there are substantial phenotypic differences between these strains.

It is therefore proposed:

- that the Waimangu and Orakei Korako strains be established as the novel species *Rhodoplanes venustus* sp. nov. with Wai3R3 as the type strain;
- that strain Tok2tar1 be established as the species *Rhodoplanes segnis* sp. nov;
- that "*Rps. cryptolactis*" be transferred to the genus *Rhodoplanes* as a novel species; and
- that the description of Genus *Rhodoplanes* be modified to reflect these changes.

Distinguishing phenotypic characteristics of the proposed *Rhodoplanes* species are given in Table 5.6 below.

Modified description of Genus *Rhodoplanes*. The description of Genus *Rhodoplanes* is as provided in Hiraishi and Ueda (1994b), with the following modifications: anaerobic growth is possible by nitrate respiration, with nitrite or nitrogen gas as end-products. Strains are mesophilic or mildly thermophilic. Habitats include freshwater, wastewater and geothermal environments.

Description of "*Rhodopseudomonas cryptolactis*". The description of "*Rhodopseudomonas cryptolactis*" is as provided by Stadtwald-Demchick *et al.* (1994b), except: photoautotrophic growth with thiosulfate as electron donor has been observed, and the organism is capable of growth on nicotinic acid. The species can be distinguished from other members of the genus *Rhodoplanes* by the presence of an alternate light-harvesting system absorbing at 822 nm, and the inability to grow phototrophically on lactate in the absence of bicarbonate. The species can in addition be distinguished from the closely related strain Tok2tar1 by its higher maximum temperature for growth, faster growth rate, ability to use citrate, fumarate and nicotinate, and requirement for vitamin B_{12}.

Characteristic	*Rpl. roseus*	*Rpl. elegans*	*Rpl. venustus*	*Rpl. segnis*	*"Rps. cryptolactis"*
Type of budding	sessile	tube	tube	tube	tube
Thermotolerance (high temperature optimum)	-	-	+	+	+
Maximum growth temperature °C	40	42	45-46.5	44	46
pH range	6.5-8.0	6.0-8.5	5.7-8.2	6.2-8.5	6.4-8.5
Alternate light harvesting system	none	none	900	900	822
Vitamin requirement	niacin	PABA, thiamine	niacin, some B_{12}	niacin	niacin, B_{12}
HCO_3^-dependent metabolism of lactate	-	-	-	-	+
End-product of nitrate respiration	N_2	N_2	N_2 or NO_2^-	NO_2^-	not tested
Utilisation of:					
nicotinate	-	+	+	-	+
citrate	+	+	v	-	+
fumarate	+	+	v	-	+
malonate	-	-	+	+	+
tartrate	+	+	-	+	+
Helix 6 stem	long	short	short	long	short

Table 5.6 Distinguishing characteristics of *Rhodoplanes* species

Description of *Rhodoplanes venustus* sp. nov. *Rhodoplanes venustus* (ve.nus'tus L. adj. *venustus*, attractive, charming). Cells are motile Gram negative rods, 1 by 2-3 μm, multiplying by budding. Rosette formation is found. Cells are facultative phototrophs, growing anaerobically in the light, aerobically in darkness, or anaerobically in darkness by nitrate respiration, with nitrogen gas or nitrite as end-product. Photosynthetic cultures are pink and contain bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series. Absorption maxima are at 465, 491, 527, 596, 800, 862 and, under low light, 900 nm. Growth is inhibited in the presence of 1% (w/v) NaCl. The pH range for growth is 5.7 to 8.2. Optimal temperature for growth is 40-44°C. No growth occurs above 46.5°C. Some strains do not grow above 45°C. Nicotinic acid is required as growth factor. Some strains require in addition cyanocobalamin (B_{12}). The following organic compounds are utilised for phototrophic growth by all strains: acetate, butyrate, lactate, malate, malonate, pyruvate, succinate, casamino acids, nicotinate. The following organic compounds are utilised for phototrophic growth by some strains: caproate, citrate, fumarate, propionate, valerate, yeast extract. Arginine, aspartate, benzoate, caprylate, ethanol,

formate, fructose, gluconate, glucose, glutamate, glycerol, glycolate, mannitol, methanol, pelargonate, sorbitol and tartrate are not utilised. Photoautotrophic growth may occur with thiosulfate but not sulfide as electron donor. Isolated from microbial mats and geothermal sediment in Waimangu Volcanic Valley and at Orakei Korako, New Zealand. The type strain is Wai3R3. The species can be distinguished from *Rpl. elegans* on the basis of the higher optimum and maximum temperature for growth, the ability to grow below pH 6.0, the presence of a shoulder on the absorption spectrum at 900 nm, vitamin requirement, ability to use malonate but not tartrate, and DNA-DNA homology. The species can be distinguished from *Rpl. roseus* by the mode of division, on the basis of the higher optimum and maximum temperature for growth, the ability to grow below pH 6.0, the presence of a shoulder on the absorption spectrum at 900 nm, ability to use malonate and nicotinate but not citrate or tartrate, 16S rDNA sequence and DNA-DNA homology. The species can be distinguished from strain Tok2tar1 (*Rhodoplanes segnis*, see below) on the basis of ability to grow above 44°C, pH range for growth, lower doubling time, and ability to use nicotinate. The species can be distinguished from "*Rps. cryptolactis*" by the ability to grow below pH 6.0, the presence of a shoulder on the absorption spectrum at 900 nm, the absence of an alternate light-harvesting system absorbing at 822 nm, the inability to grow on tartrate, the lack of requirement for bicarbonate for phototrophic growth on lactate.

Description of *Rhodoplanes segnis* sp. nov. *Rhodoplanes segnis* (se'gnis L. adj. *segnis*, slow, sluggish, in reference to the high doubling time). Cells are motile Gram negative rods, 1.0 by 2-3 μm, multiplying by budding. Rosette formation is found. Cells are facultative phototrophs, growing anaerobically in the light, aerobically in darkness, or anaerobically in darkness by nitrate respiration, with nitrite as end-product. Photosynthetic cultures are pink with a chalky appearance and contain bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series. Absorption maxima are at 801, 860 and, under low light, at 900 nm. Growth is inhibited in the presence of 1% (w/v) NaCl. The pH range for growth is 6.2 to 8.5. Optimal temperature for growth is 36-42°C. No growth occurs above 44°C. Nicotinic acid is required as growth factor. The following organic compounds are utilised for phototrophic growth: acetate, propionate, butyrate, malonate, lactate, malate, pyruvate, succinate, tartrate, yeast extract, casamino acids. The following compounds

are not utilised: arginine, aspartate, glutamate, benzoate, citrate, methanol, ethanol, formate, glycolate, fructose, gluconate, glucose, mannitol, sorbitol, nicotinate. Photoautotrophic growth occurs with thiosulfate but not sulfide as electron donor. Isolated from thermally-heated mud and water at Tokaanu, New Zealand. The type strain is Tok2tar1. The species can be distinguished from "*Rpl. venustus*" and "*Rps. cryptolactis*" as described above. It can be distinguished from *Rpl. elegans* and *Rps. roseus* by its higher temperature optimum and maximum, the presence of a shoulder on the absorption spectrum at 900 nm, and the ability to grow on malonate but not citrate or fumarate.

5.3 *Blastochloris* isolates

5.3.1 Strains

Six green and brownish-green pigmented strains were isolated in the course of this investigation from six separate sample sites that included microbial mats, pool sediment and a bloom of purple sulfur bacteria, at Waimangu Volcanic Valley (Wai1bG3, Wai3G1e, Wai5bG1), Orakei Korako (OK3G7), Kuirau Park (Kui6G1), and Waitangi Soda Springs (WSSG4). These were identified as *Blastochloris* strains. *Blastochloris* strains have previously been isolated from geothermal areas. Mesophilic strains have been isolated by Russian researchers, and a single thermotolerant isolate, *Blastochloris* strain GI, has been reported (Resnick and Madigan 1989).

5.3.2 Morphological properties

Cells were ovoid to rod-shaped, around 0.7 μm wide by 1.2 μm long, dividing by sessile budding. Rosettes, short chains of cells and other aggregates were observed (Figure 5.9 and Figure 5.10). Occasionally, cultures of strain OK3G7 were observed to contain giant aggregates of hundreds of cells, similar to the "hedgehog" type observed in *Rhodopseudomonas julia* (Kompantseva 1989), but the frequency of their occurrence diminished upon further laboratory culture. Young cultures contained actively motile cells. Electron microscopy of thin sections revealed intracytoplasmic membranes of the lamellar type (Figure 5.11).

5.3.3 Photopigments

Four of the isolates produced mass cultures that were brown-green in colour. Strains OK3G7 and Wai5bG1 were a brighter fresh green in colour, but older cultures of all strains became more brown. Absorption spectra of intact cells showed the presence of bacteriochlorophyll *b*, with a major absorbance peak at 1009-1012 nm, and carotenoid peaks at 451 and 482 nm, characteristic of *Blastochloris* species (Figure 5.12).

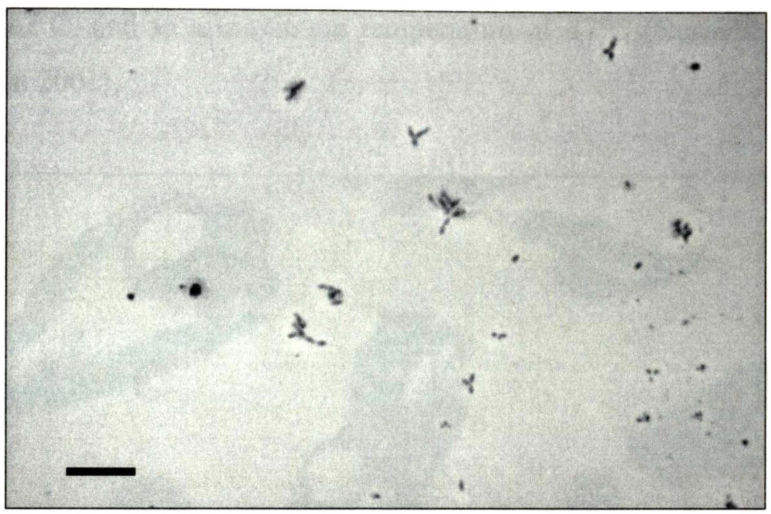


Figure 5.9 Phase-contrast photomicrograph showing the cell morphology of strain WSSG4. Bar, 10 μm

Figure 5.10 Phase-contrast photomicrograph showing the cell morphology of strain OK3G7. Bar, 10 μm

5.3.4 Physiological characteristics

The type strain of *Blastochloris*, *Blc. viridis*, grows optimally near 35°C (see Figure 5.39 B), and has a maximum temperature for growth of 39-40°C (Resnick and Madigan 1989; current work). *Blc. sulfoviridis* DSM 729 grows optimally at 32-36°C (see Figure 5.39 A) and is capable of slow growth at 40°C (current work), while strain ToP1 grows optimally at 34°C with a maximum of 36°C. *Blc. viridis* strains isolated from Bol'sherechenskii thermal springs were reported to grow optimally at 30°C (Yurkov and Gorlenko 1992b). The thermophilic *Blastochloris* strain GI grows optimally at 42°C, and to a maximum temperature of 47°C (Resnick and Madigan 1989; Madigan 2001).

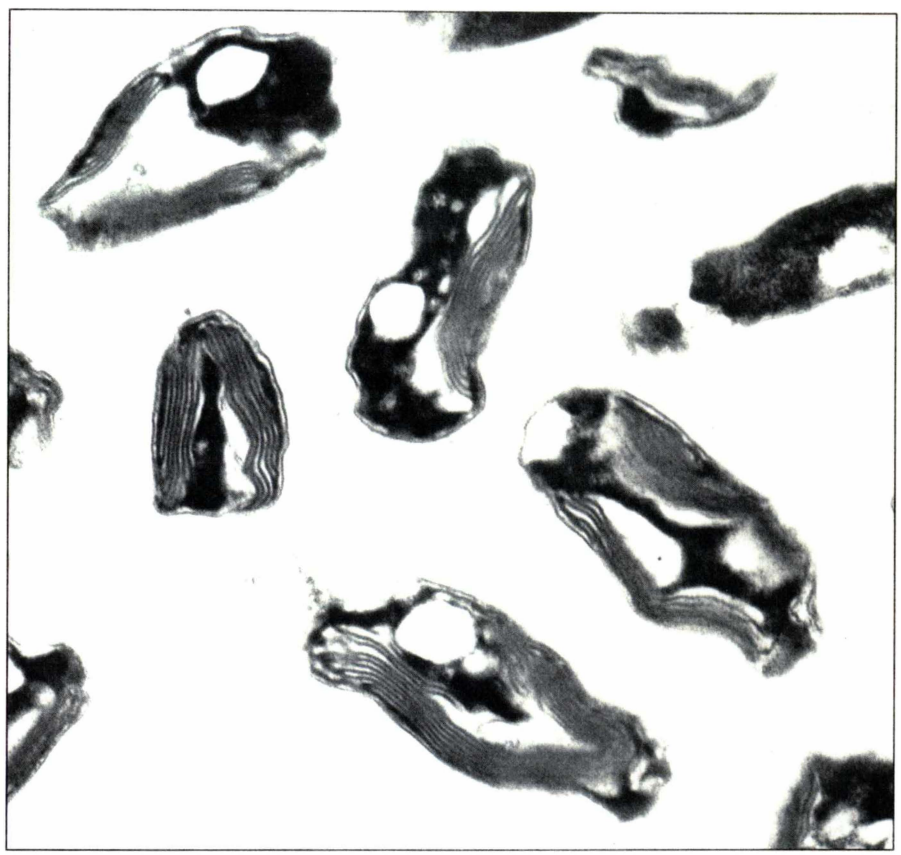


Figure 5.11 Electron micrograph of ultrathin sections of strain Wai3G1e showing the intracytoplasmic membranes (x 33000)

The six New Zealand *Blastochloris* isolates were isolated from sites that ranged in temperature from 33°C to 42°C, and developed in enrichment cultures at 40-42°C. Maximum temperatures for growth of these strains were determined to range from 46.5°C for strain Wai3G1e and 45°C for Wai1bG3, to 43-44°C for Wai5bG1 and OK3G7.

Figures 5.13-5.15 present graphs of specific growth rate versus temperature. Each of the isolates showed different temperature responses.

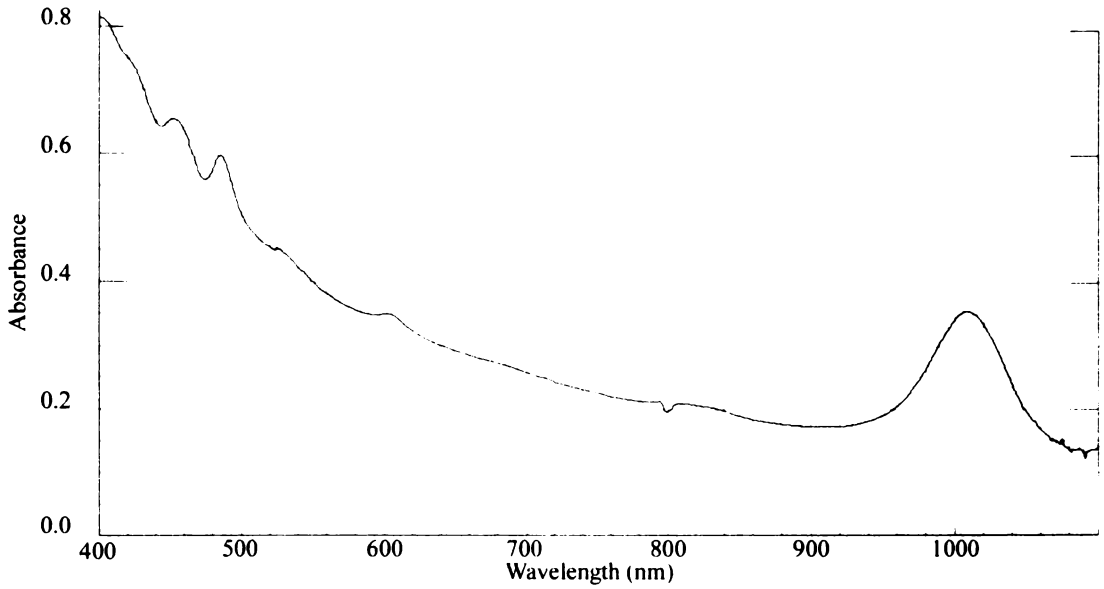


Figure 5.12 *In vivo* absorption spectrum of strain Wai3G1e

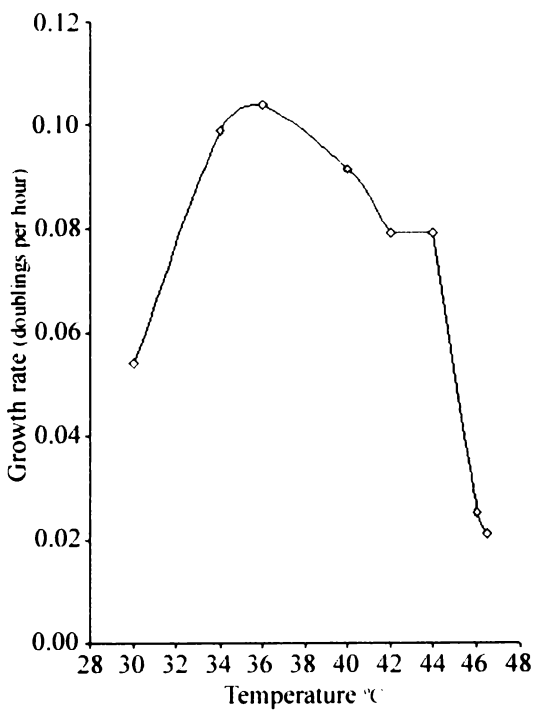


Figure 5.13 Specific growth rate of strain Wai3G1e as a function of temperature

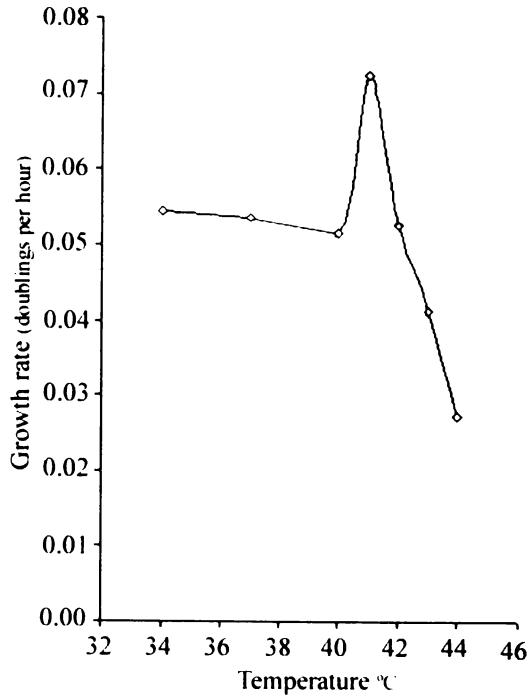


Figure 5.14 Specific growth rate of strain Wai5bG1 as a function of temperature

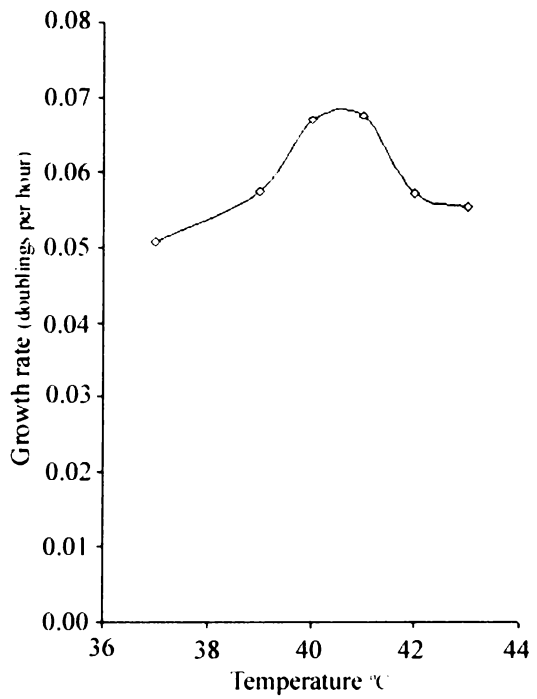


Figure 5.15 Specific growth rate of strain OK3G7 as a function of temperature

Although strains Wai1bG3 and Wai3G1e had the highest maximum growth temperature of the isolates, comparable with that of strain GI, their temperature optimum was lower. Optimal growth was observed at 36°C, and even lower for Wai1bG3, though reasonable growth rates were still observed up to 44°C. Strains

Wai5bG1 and OK3G7 achieved most rapid growth around 41°C, though Wai5bG1 had a narrower optimum, growing much less well at 40 or 42°C than OK3G7. Growth rates under optimal conditions were relatively slow, with doubling times ranging from 12-15 hours. As with *Rhodoplanes* isolates, lower growth yields were achieved at the maximum growth temperature.

Blastochloris strain GI and strain Wai3G1e showed growth at 20°C after 10 days of incubation. This contradicts the results of Resnick and Madigan (1989).

pH ranges for growth are shown in Table 5.7, with growth assessed according to the schedule in Table 3.3. *Blastochloris* strain GI grew over the pH range 6.1-8.2. Identical growth ranges were observed for strains Wai3G1e and Wai1bG3. Strain OK3G7 however showed no growth at pH 6.3. Good growth was obtained over the range pH 6.4 to 8.2. Strain Wai5bG1 grew well over the same range, but marginal growth was also obtained at pH 6.3. *Blc. sulfoviridis* ToP1 grows in the range 6.5-8.3 (Zengler *et al.* 1999).

	Growth at pH:								
***Blastochloris* strain**	**5.9**	**6.1**	**6.3**	**6.4**	**6.6**	**7.0**	**7.5**	**8.2**	**8.5**
GI	-	+	++	++	+++	+++	+++	+	-
Wai1bG3	-	+++	+++	+++	+++	+++	+++	+++	-
Wai3G1e	-	++	+++	+++	+++	+++	+++	+	-
Wai5bG1	-	-	+	++	+++	+++	+++	++	-
OK3G7	-	-	-	++	+++	+++	+++	++	-

Table 5.7 pH ranges for growth of thermotolerant *Blastochloris* strains

Blc. viridis strains are able to assimilate sulfate, while *Blc. sulfoviridis* DSM 729 has a requirement for a reduced sulfur source for biosynthesis. *Blc. sulfoviridis* strain ToP1, however, is capable of sulfate assimilation (Zengler *et al.* 1999). Medium MC, used for routine cultivation of the *Blastochloris* isolates, contained both sulfate and thiosulfate, but in tests of sulfur source requirement in minimal salts medium, it was found that none of the New Zealand isolates had a requirement for a reduced sulfur source. Sulfate, thiosulfate, sulfide and cysteine could be used as sulfur sources. Strains appeared to grow more rapidly in the presence of low levels of sulfide, which was presumably due to its effect as a reductant. Strain Wai3G1e tolerated sulfide at a

concentration of 1 mM, but showed no growth at 2 mM. Strain OK3G7 was able to grow in the presence of 2 mM sulfide, but not at 3 mM. All strains were capable of growth using NH_4^+ or N_2 as nitrogen source. NaCl was inhibitory at 2.5% (w/v).

Strains Wai3G1e, Kui6G1 and WSSG4 required biotin as growth factor. Strains Wai1bG3, Wai5bG1 and OK3G7 required biotin and PABA. Most *Blc. viridis* strains require both these vitamins, but some require only one or neither; *Blc. sulfoviridis* DSM 729 requires in addition pyridoxine (Imhoff and Trüper 1989). *Blastochloris* strain GI requires only biotin.

Strain Wai3G1e was capable of growth in darkness at 46°C at full oxygen tension. None of the other five isolates were able to grow aerobically in darkness. *Blastochloris* strain GI, *Blc. viridis* DSM 133 and *Blc. sulfoviridis* ToP1 are also unable to grow aerobically in darkness, but the type strain of *Blc. sulfoviridis* grows well. None of the New Zealand isolates was able to carry out anaerobic respiration with nitrate as electron acceptor.

Compounds used as carbon sources or electron donors by strains of *Blastochloris* are listed in Table 5.8.

The carbon utilisation patterns of the new strains did not clearly match those of either *Blc. viridis* or *Blc. sulfoviridis*. Whereas the type strains of both *Blc. viridis* and *Blc. sulfoviridis* are able to grow on ethanol, none of the other strains possessed this trait. Waimangu and Orakei Korako strains were unable to grow on glucose, which is a good substrate for *Blc. sulfoviridis*. Some strains, however, showed good growth on fructose, which is not used by *Blc. viridis*, though Wai5bG1 and OK3G7 showed little or no growth. Wai1bG3 was unusual in its ability to grow on citrate. Resnick and Madigan (1989) reported that strain GI differed from *Blc. viridis* in its ability to use butyrate, but they used a test concentration of 0.4% (w/v), at which level many fatty acids are inhibitory to purple nonsulfur bacteria. *Blc. viridis* grows well on 0.05% (w/v) butyrate (Charlton 1997).

Carbon source/ electron donor	Wai1bG3	Wai3G1e	Wai5bG1	OK3G7	Kui6G1	WSSG4	GI	DSM 729	ToP1	*Blc. viridis*
Acetate	++	+++	+++	+++	++	+++	+	+	+	+
Arginine	-	-	-	-	0	0	0	-	-	-
Aspartate	-	-	-	-	-	-	-	0	-	+
Benzoate	+++	+++	-	-	+	++	-	-	+	-
Butyrate	++	++	++	++	0	++	+	+	0	+
Caproate	+	+	++	0	+++	+++	0	+	+	+
Caprylate	+	+	++	-	0	0	0	-	-	+
Citrate	+	-	(+)	-	-	-	0	-	0	-
Ethanol	-	-	-	-	-	-	-	+	-	+
Formate	0	-	0	-	0	0	0	-	-	v
Fructose	+++	+++	-	+	0	0	+	+	+	-
Fumarate	++	++	++	++	0	0	+	+	-	+
Gluconate	-	-	0	-	0	0	0	0	0	+
Glucose	-	-	-	-	+	(+)	+	+	-	+
Glutamate	-	-	-	(+)	-	-	-	0	-	+
Glycerol	-	-	-	-	-	-	0	+	-	-
Glycolate	-	-	-	-	0	0	0	0	0	0
Lactate	+++	++	++	+	++	++++	-	+	+	v
Malate	++	++	++	+	+++	+++	+	+	-	+
Malonate	-	-	-	-	-	-	0	-	0	-
Mannitol	-	-	-	++	0	0	0	-	0	v
Methanol	-	-	-	-	0	0	-	-	0	-
Pelargonate	-	-	-	-	-	-	0	-	0	+
Propionate	-	+	(+)	(+)	-	(+)	-	-	+	-
Pyruvate	++	+++	+++	+++	++	+++	+	+	+	+
Sorbitol	-	-	-	-	0	0	0	+	0	+
Succinate	+++	++	+++	+++	0	0	+	+	+	+
Tartrate	-	-	-	-	-	-	0	-	0	v
Valerate	+++	++	++	+++	0	0	0	-	0	+
Yeast extract	0	0	0	0	0	0	0	0	0	0
Casamino-acids	0	+	0	0	0	0	0	0	0	+
Hydrogen	0	0	0	0	0	0	0	-	+	-
Thiosulfate	-	-	-	-	0	(+)	0	+	+	-
Sulfide	-	-	-	-	0	(+)	0	+	+	-

Table 5.8 Carbon sources and electron donors utilised by *Blastochloris* strains
Data from present work, Charlton (1997), Imhoff and Trüper (1992), Resnick and Madigan (1989), Zengler *et al.* (1999). Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of four remaining strains is positive/negative only. 0 =not tested, v =variable by strain

Several strains were found to use the aromatic compound benzoate as sole carbon source, which at the time was a novel property for members of the genus. Figure 5.16 shows growth of strain Wai3G1e on benzoate together with disappearance of the substrate. Doubling time was around 34 hours. Growth on benzoate has since been reported for *Blastochloris sulfoviridis* strain ToP1, which also grows on toluene as sole carbon source (Zengler *et al.* 1999). The New Zealand isolates were not tested for the ability to grow on toluene.

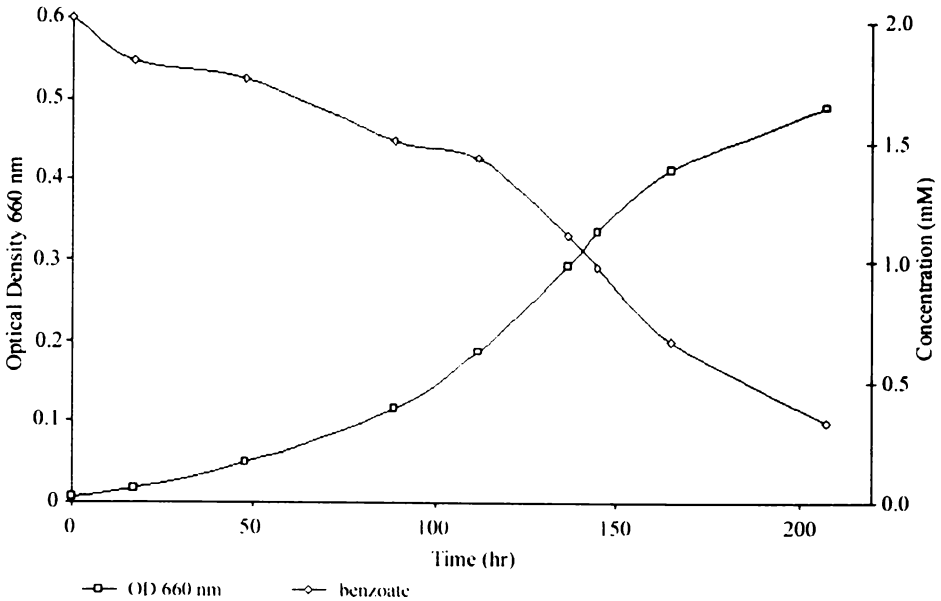


Figure 5.16 Growth of *Blastochloris sulfoviridis* strain Wai3G1e on 2 mM benzoate, and disappearance of substrate

The quinone content of strain GI and some New Zealand strains was determined by Dr Hiraishi according to the method given in Hiraishi and Hoshino (1984). Results are shown in Table 5.9.

	Quinone content (mol%)					
	Ubiquinones			Menaquinones		
Test strain	**Q-7**	**Q-8**	**Q-9**	**MK-6**	**MK-7**	**MK-8**
Blastochloris sp. Wai1bG3	tr	51	1	2	46	0
Blastochloris sp. Wai3G1e	tr	38	1	2	59	0
Blastochloris sp. Wai5bG1	4	50	1	2	42	1
Blastochloris sp. OK3G7	1	59	1	1	38	0
Blastochloris sp. GI	tr	54	0	2	43	1

tr= trace amount (<0.5 mol%)

Table 5.9 Quinone content of selected *Blastochloris* isolates

The strains contained Q-8 and MK-7 as primary components, which resembles the quinone composition of *Blc. sulfoviridis* rather than *Blc. viridis*, which contains Q-9 and MK-9 as major quinones (Kato *et al.* 1985).

5.3.5 Genetic relationships

16S rRNA gene sequences covering more than 1400 continuous nucleotide residues were determined for the four of the isolates according to the methods given in Section

3.9. In addition, the sequence of *Blastochloris* strain GI was redetermined. The sequences of strains OK3G7, Wai1bG3 and Wai3G1e were submitted to the GenBank database under Accession Numbers AY117148, AY117149 and AY117150. The sequences of strains OK3G7 and Wai5bG1 were identical.

Binary sequence similarity values for *Rhodoplanes* strains and closely related organisms are given in Table 5.10. The 16S rRNA genes of the thermotolerant isolates and *Blastochloris* strain GI showed high levels of similarity (>97.7%) to *Blc. viridis*, and 98.7-99.3% to *Blc. sulfoviridis* DSM 729, and lower levels of similarity (94-94.9%) to *Rhodoplanes*, the closest relative of *Blastochloris*. Strain Wai3G1e was most similar to *Blastochloris sulfoviridis* GN1, which was isolated from a lighted upflow anaerobic sludge blanket reactor (Sawayama *et al.* 2000a).

The sequences were used to construct a phylogenetic tree according to the methods given in Section 3.9.6 (Figure 5.17). The isolates and strain GI formed three clusters associated with *Blc. sulfoviridis*, with strain Wai1bG3 as the immediate neighbour of strain GI. As with *Rhodoplanes* isolates, variation was seen in the sequence of the helix 6 stem-loop structure at *E. coli* positions 60-109 (Figure 5.18).

The sequence at positions 60-109 exhibited by *Blastochloris* strains Wai1bG3 and Wai3G1e is also found in *Blc. sulfoviridis* GN, *Rhodopseudomonas* strain GI and many strains of *Blc. viridis*; the sequence of the type strains DSM 729 and DSM 133 is also found in *Blc. sulfoviridis* ToP1. However, it is notable that *Blastochloris* strains OK3G7 and Wai5bG1 have the same sequence at these positions as *Rhodoplanes elegans* AS130, "*Rps. cryptolactis*" and the thermotolerant *Rhodoplanes* strains isolated in this current work from Waimangu and Orakei Korako.

No.	Species or strain	Sequence similarity (%) for 16S rRNA genes											
		2	3	4	5	6	7	8	9	10	11	12	13
1	*Blc. viridis* DSM 133	99.6	99.4	98.8	98.8	98.4	98.2	98.0	97.8	98.0	98.0	93.7	90.4
2	*Blc. viridis* G3		99.8	98.5	98.5	98.2	97.9	97.8	98.2	97.8	97.8	93.4	90.0
3	*Blc. viridis* UN			98.4	98.4	98.1	97.8	97.7	98.1	97.7	97.7	93.5	90.1
4	*Blc. sulfoviridis* DSM 729				100.0	99.5	99.3	99.1	98.7	99.0	99.0	94.4	90.8
5	*Blc. sulfoviridis* ToP1					99.5	99.3	99.1	98.7	99.0	99.0	94.4	90.8
6	*Blc. sulfoviridis* GN						99.8	99.6	99.2	99.3	99.3	94.1	90.4
7	Wai3G1e							99.5	99.1	99.0	99.0	94.0	90.2
8	Wai1bG3								99.6	99.2	99.2	94.2	90.5
9	*Blc.* strain GI									98.8	98.8	93.8	90.1
10	OK3G7										100.0	94.9	90.4
11	Wai5bG1											94.9	90.4
12	*Rpl. elegans* AS130												92.8
13	*Rps. palustris* ATCC 17011												

Figure 5.10 Sequence similarities of 16S rRNA genes of *Blastochloris* strains and related organisms

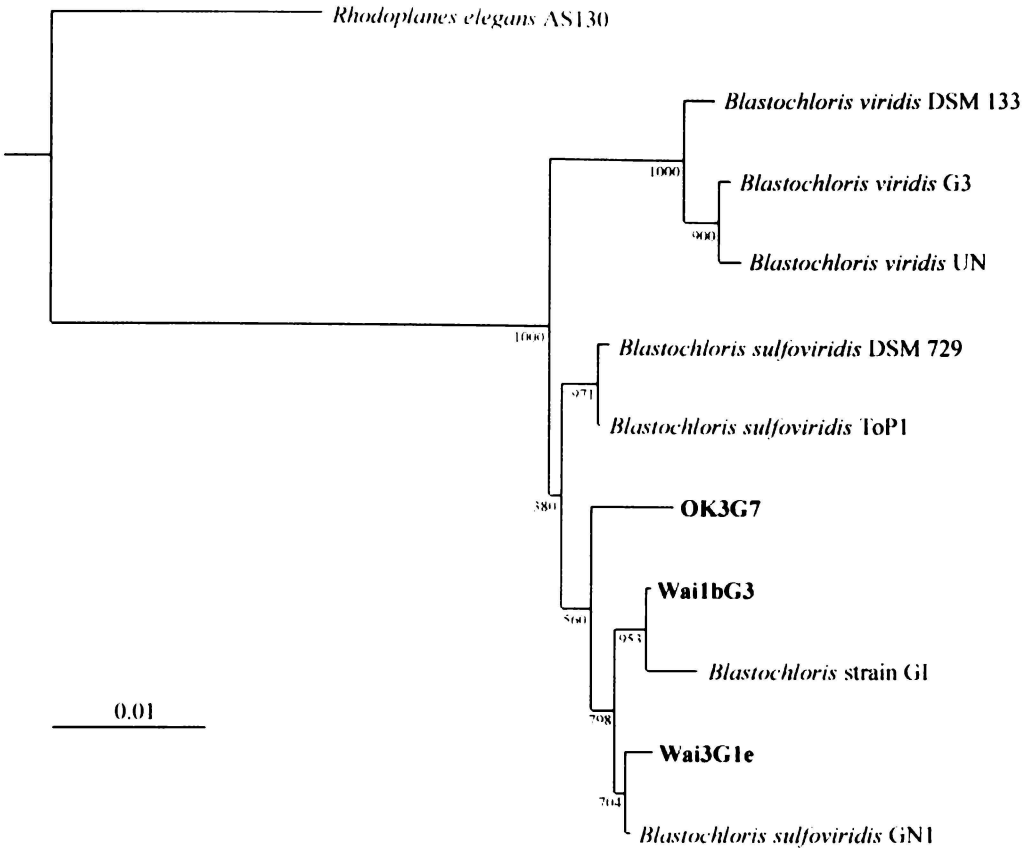


Figure 5.17 Distance matrix tree showing phylogenetic relationships of *Blastochloris* strains and *Rhodoplanes elegans*, based on an alignment of 1395 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Rhodopseudomonas palustris*.

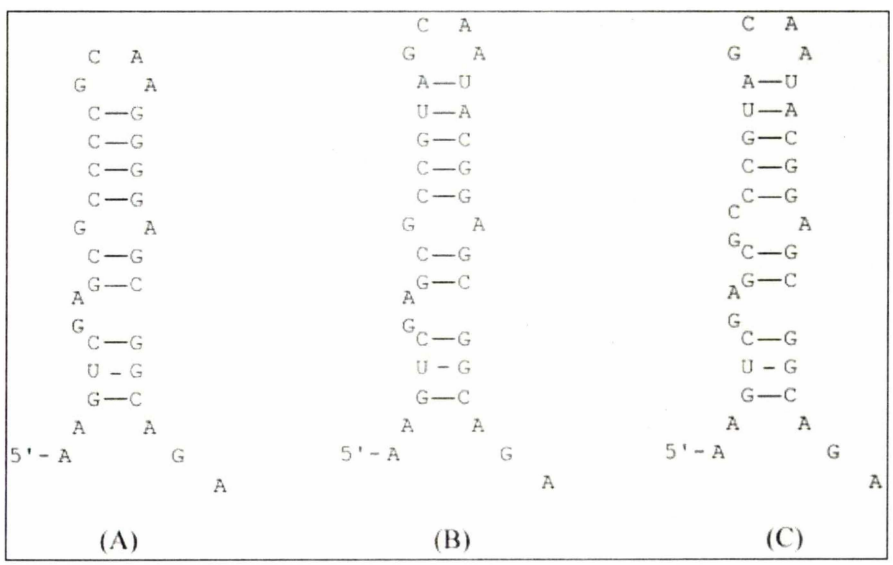


Figure 5.18 Deduced secondary structures of the stem-loop at *E. coli* positions 60-109 of the 16S rRNA molecules of: (A) *Blastochloris* strains OK3G7 and Wai5bG1; (B) *Blastochloris* strains Wai1bG3 and Wai3G1e, and *Rhodopseudomonas* strain GI; (C) the type strains *Blastochloris viridis* DSM 133 and *Blc. sulfoviridis* DSM 729

The relatedness of *Blastochloris* strains was also examined by comparison of partial sequences of the photosynthetic reaction centre L subunit (*pufL*) genes (Figure 5.19). The phylogeny of these genes was in broad agreement with that inferred from 16S rRNA sequences. *pufL* genes of strain GI and New Zealand isolates clustered with *Blc. sulfoviridis* to the exclusion of *Blc. viridis*.

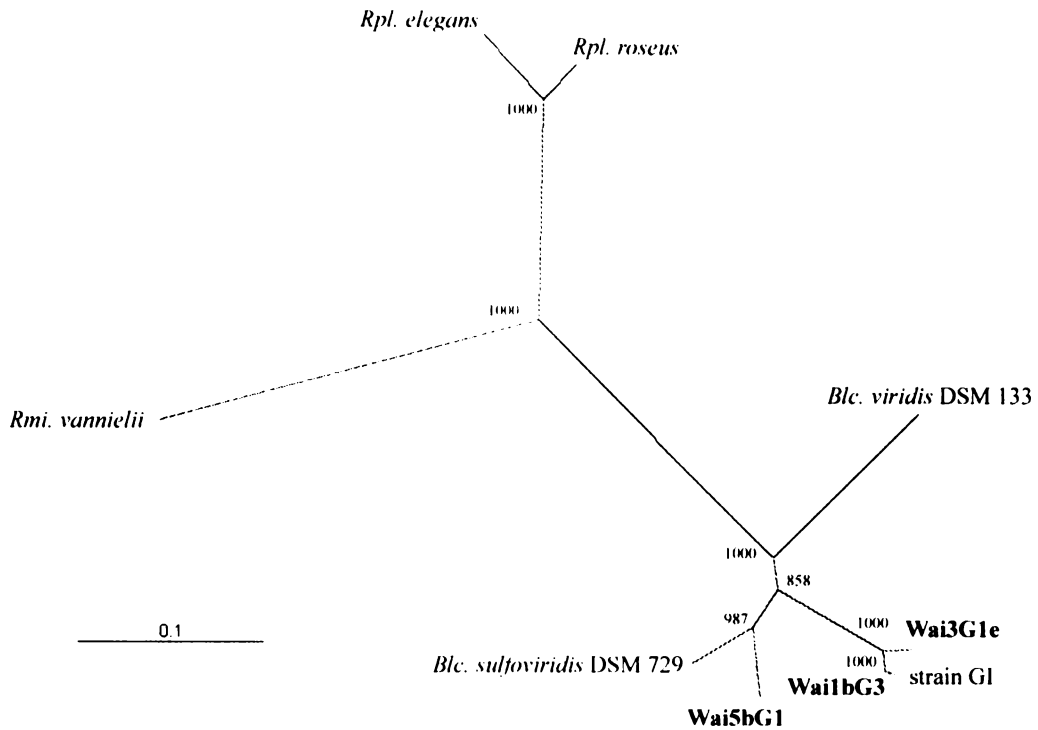


Figure 5.19 Phylogenetic relationships of *pufL* genes of *Blastochloris* isolates and selected phototrophic organisms, based on an alignment of 569 nucleotide positions

The isolates shared 85.9 to 92.4% similarity with the *Blc. sulfoviridis pufL* gene fragment, compared with 79.2 to 81.4% similarity with *Blc. viridis*. The sequences of strains Wai1bG3 and GI were 99.7% similar, an extremely close relatedness for a functional gene.

DNA-DNA hybridisation measurements were also carried out for *Blastochloris* isolates by Dr Hiraishi, and are given in Table 5.11. Zengler *et al.* (1999) reported that *Blastochloris sulfoviridis* strain TopP1 showed greater than 78% DNA-DNA similarity with *Blc. sulfoviridis* DSM 729, confirming its status as a strain of this species. By contrast, strain GI and the New Zealand isolates tested showed DNA-DNA relatedness values to *Blc. sulfoviridis* that were markedly less than 70%. Strains OK3G7 and Wai5bG1, which shared identical 16S rDNA sequences and a helix 6

structure unique within *Blastochloris*, showed a DNA similarity of 84%, which would indicate they are strains of a single species. Strains Wai1bG3, Wai3G1e and strain GI showed similarity values of around 62%, consistent with their formation of monophyletic subclusters in both 16S rRNA and *pufL*-based phylogenetic analysis. However, relatedness between this cluster and that formed by OK3G7-Wai5bG1 is relatively low.

	% Hybridization to labelled DNA probe from:			
Test Strain	**DSM 133**	**DSM 729**	**Wai3G1e**	**Wai5bG1**
Blc. viridis DSM 133	100	40	33	25
Blc. sulfoviridis DSM 729	36	100	51	39
Blastochloris sp. Wai1bG3	25	55	61	53
Blastochloris sp. Wai3G1e	34	58	100	51
Blastochloris sp. Wai5bG1	20	44	49	100
Blastochloris sp. OK3G7	22	46	45	84
Blastochloris sp. GI	28	47	62	42
Rpl. roseus DSM 5909	8	6	7	7

Table 5.11 Genomic DNA relatedness among selected strains of budding phototrophic bacteria

5.3.6 Taxonomy of *Blastochloris*

The *Blastochloris* strains can be divided on the available data into four relatedness groups, corresponding to genospecies: (1) *Blc. viridis* strains, (2) *Blc. sulfoviridis* DSM 729 and ToP1, (3) strains OK3G7 and Wai5bG1, and (4) strains GI, Wai1bG3, and Wai3G1e (presumably together with strain GN1). The designation of new species would be possible if substantial phenotypic differences between the groups could be found. Table 5.12 lists characteristics of these groups.

Kompantseva et al. (1998) also reported the examination by DNA-DNA hybridisation of eight *Blastochloris* strains. They fell into three clusters, corresponding to *Blc. viridis*, *Blc. sulfoviridis* and a novel genospecies. Members of this third cluster, represented by two strains, divided by sessile budding, were capable of photoautotrophic growth on sulfide and thiosulfate, and required a reduced sulfur source. As such, they were phenotypically indistinguishable from *Blc. sulfoviridis*, and dissimilar to the strains of groups 3 and 4 above. They were isolated from 40-

45°C neutral sulfur springs, but grew well at 25°C. They are thus representatives of the mesophilic purple nonsulfur bacteria reported on several occasions from hot springs by Russian researchers.

Characteristic	Group 1 DSM 133	Group 2 DSM 729, ToP1	Group 3 OK3G7, Wai5bG1	Group 4[1] GI, Wai1bG3, Wai3G1e
Type of budding	tube	sessile	sessile	sessile
Thermotolerance (high temperature optimum)	-	-	+	+
Maximum growth temperature	40	36-42	43-44	46-47
pH range	not tested	6.5-8.3	6.3-8.2	6.1-8.2
Sulfate assimilation	+	v	+	v
Aerobic growth in darkness	-	v	-	v
Photoautotrophy on sulfur compounds	-	+[2]	-	-[2]
Vitamin requirement	any of biotin, PABA	biotin, PABA	biotin, PABA	biotin, some PABA
Utilisation of:				
aspartate	+	-	-	-
benzoate	-	v	-	v
caprylate	+	-	-	+
citrate	-	-	-	v
ethanol	+	v	-	-
fructose	-	+	v	+
glucose	+	v	-	v

[1] little data on strain GN1 is available; [2] no data for ToP1 or GI available; v = variable

Table 5.12 Characteristics of members of the genus *Blastochloris*

A case could be made for the designation of strains OK3G7 and Wai5bG1 as members of a novel species of the genus *Blastochloris*. They form a separate line of descent with relatively low DNA-DNA relatedness to other strains, assimilate a more restricted range of carbon sources, and while their temperature optimum approaches that of strain GI, their maximum temperature for growth is only 44°C. They also contain measurable amounts of the quinone Q-7.

The phenotypic differentiation of Group 4 strains from *Blc. sulfoviridis* is more difficult, given that sulfate assimilation, vitamin requirements, carbon source utilisation patterns and ability to grow aerobically in darkness are of no diagnostic value, as strains within Groups 2 and 4 differ from one another in these traits. The

cardinal temperatures for growth of strain GN1 have not been determined (Shigeki Suwayama, AIST, Onogawa, Japan, pers. comm.). It was isolated from a reactor vessel operated at 35°C. This is close to the optimum temperature for growth of strains Wai1bG3 and Wai3G1e, and it is at least possible that strain GN1 too is a thermotolerant strain with a temperature maximum near 47°C. If not, then mesophilic and thermotolerant strains are intermingled in this cluster of closely related organisms. This group could perhaps be characterised as consisting of thermotolerant strains capable of growth below pH 6.5. A definitive answer must await the discovery of other distinguishing phenotypic properties or the isolation of more strains.

The topology of the 16S rRNA and *pufL* trees shown in Figures 5.17 and 5.19 would require exclusion of Group 4 isolates from *Blc. sulfoviridis* in the event that strains OK3G7 and Wai5bG1 were described as a novel species. In the absence of sufficient distinguishing features for Group 4, this cannot yet be done.

The primary physiological characteristic separating *Blc. viridis* and *Blc. sulfoviridis* was the dependence of the latter on reduced sulfur sources for biosynthesis. Neutzling and Trüper (1982) reported that the inability of *Blc. sulfoviridis* strain DSM 729 to grow on sulfate was due the absence of detectable activity of the enzymes sulfate permease, adenylylsulfate sulfotransferase and thiosulfonate reductase. Regardless, this cannot be a universal trait of *Blc. sulfoviridis*-like strains, as strain ToP1 and all of the New Zealand isolates are capable of growth on sulfate as sole sulfur source. The description of the species *Blastochloris sulfoviridis* should be updated to take account of the ability of some strains to assimilate sulfate as sulfur source, whether the thermotolerant isolates are eventually assigned to this species or not. *Blc. viridis* and *Blc. sulfoviridis* can still be distinguished on the basis of morphological and biochemical characteristics, including their markedly different quinone content.

5.4 *Rhodopseudomonas* isolates

5.4.1 Strains

A total of 11 budding rod-shaped isolates producing mass cultures that were pink to red in colour that could not be assigned to the genus *Rhodoplanes* were obtained from geothermal samples. Many strains were able to use long-chain fatty acids or benzoate

as carbon sources. They were assigned to the genus *Rhodopseudomonas*, as presumptive *Rps. palustris*-like organisms. *Rps. palustris* was confirmed as a close relative by 16S rDNA sequencing of some strains. They could be distinguished from *Rhodoblastus acidophilus* by their relatively small cell size and ability to grow at pH 7.0.

Two isolates, Wai1S1 and Wai1val1, were obtained from a single Frying Pan Lake sample, and two more, OK4bR1 and OK4bTR3, from a "Tim and Terry" sample. A third isolate, OK10ST2, was obtained from Orakei Korako, and other strains were obtained from Kuirau Park (Kui6R2), Waitangi Soda Springs (WSSR4) and the Magpie pool (MPSR1). *Rhodopseudomonas palustris*-like organisms were the only strains to be isolated from Tikitere (TKT2), Ohinemutu (OH3F2) and Kerosene Creek (KCrS2).

5.4.2 Morphological properties

Cells of all strains were rod-shaped, approximately 0.7 μm wide by 1.5-2.0 μm long, but variable, dividing by budding with formation of a division tube (Figure 5.20). Rosettes were commonly observed. Cells were actively motile, with sub-polar flagella (Figure 5.21).

5.4.3 Photopigments

All strains produced red colonies on solid medium and pink to red cell suspensions in liquid medium under anaerobic conditions in the light. The absorption spectra of intact cells grown under phototrophic conditions were typical of organisms containing bacteriochlorophyll *a*, with peaks close to 805 and 870 nm, and carotenoids of the normal spirilloxanthin series (Figure 5.22).

5.4.4 Physiological characteristics

The type strain *Rps. palustris* DSM 123 was found to be capable of growth to a maximum of 39°C, although growth above 36°C was sometimes irregular. As "*Rhodopseudomonas cryptolactis*" is phylogenetically a member of the genus *Rhodoplanes*, no thermotolerant members of the genus *Rhodopseudomonas sensu stricto* have been described. The New Zealand *Rhodopseudomonas* isolates were obtained from sites that ranged in temperature from 30°C to 46°C, and developed in enrichment cultures at 40-43°C. With the exception of the 46°C TKT and the 47°C

OH3 sites, the sites that yielded *Rhodopseudomonas* isolates were at the lower end of the temperature range sampled. The maximum temperatures for growth of these strains were determined to range from 46.5°C for strain Wai1S1, 46°C for OK4bR1, OK4bTR3 and KCrS2, 44°C for WSSR4 and Kui6R2, 43°C for OH3F2 and TKT2, to 42°C for Wai1val1.

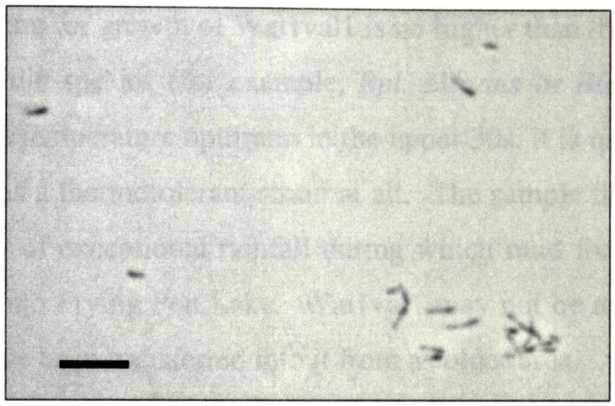


Figure 5.20 Phase-contrast photomicrograph showing the cell morphology of strain Wai1S1. Bar, 10 μm

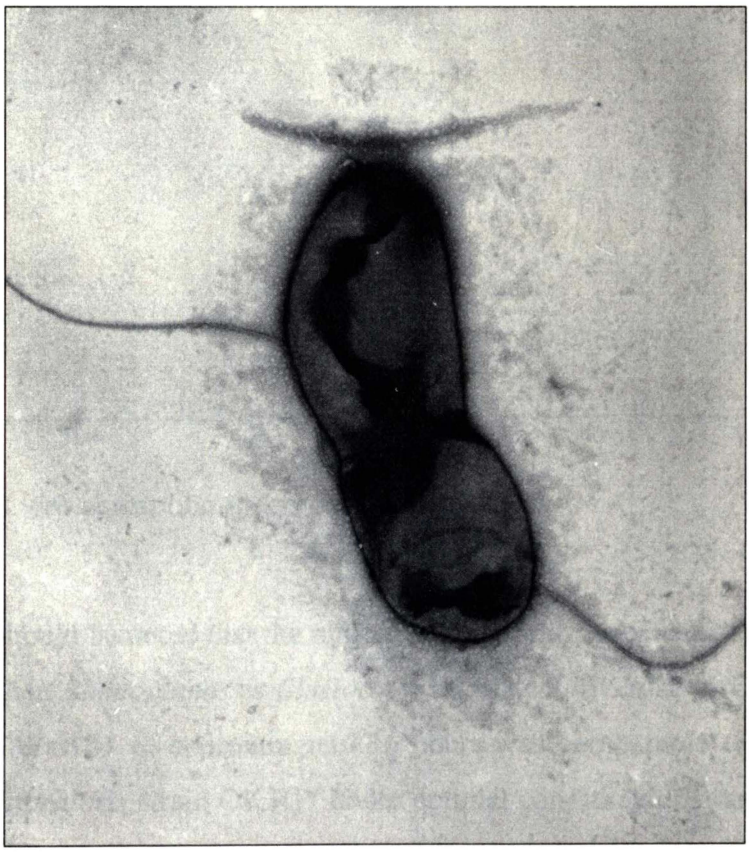


Figure 5.21 Electron micrograph of negatively stained dividing cells of strain Wai1S1, also showing flagella (x 43000)

Figures 5.23-5.25 present graphs of specific growth rate versus temperature for three strains. Strain Wai1S1 grew optimally at 38°C, but maintained rapid growth up to 44°C. Strain KCrS2 had a temperature optimum of only 36°C, but maintained a good specific growth rate to 42°C, before specific growth rate declined steeply to its maximum of 46°C. Strain Wai1val1 also grew optimally at a similar temperature, but showed a steep decline in specific growth rate to its maximum of 42°C. The maximum temperature for growth of Wai1val1 is no higher than the maxima observed for several mesophilic species (for example, *Rpl. elegans* or *Blc. sulfoviridis*). As Wai1val1 also has a temperature optimum in the upper 30s, it is questionable whether it can be regarded as a thermotolerant strain at all. The sample that yielded Wai1val was taken on a day of exceptional rainfall during which mud from the hillsides and paths was washed into Frying Pan Lake. Wai1val1 may not be autochthonous to the lake, but instead have been transferred into it from a colder area.

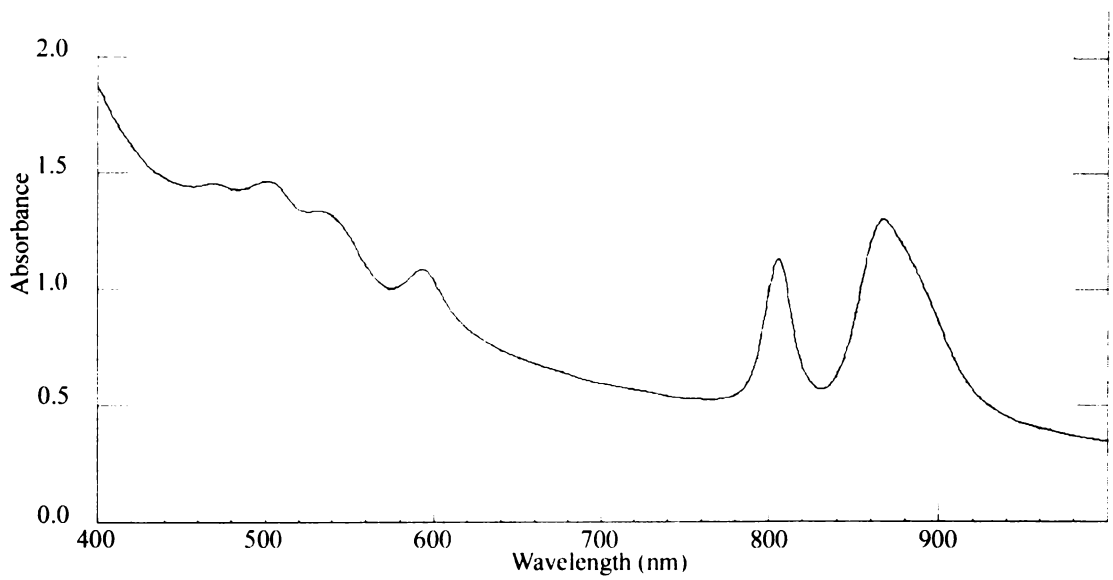


Figure 5.22 *In vivo* absorption spectrum of strain Wai1S1

In general, it should be noted that the majority of *Rhodopseudomonas* strains grew far more rapidly than *Rhodoplanes* or *Blastochloris* isolates. Even at 46°C, the doubling time of strain Wai1S1 of approximately 16 hours was comparable with the rate of growth of *Blastochloris* strain OK3G7 under optimal conditions. At any temperature other than their maxima, *Rhodopseudomonas* strains grew more rapidly than *Rhodoplanes* strain Tok2tar1.

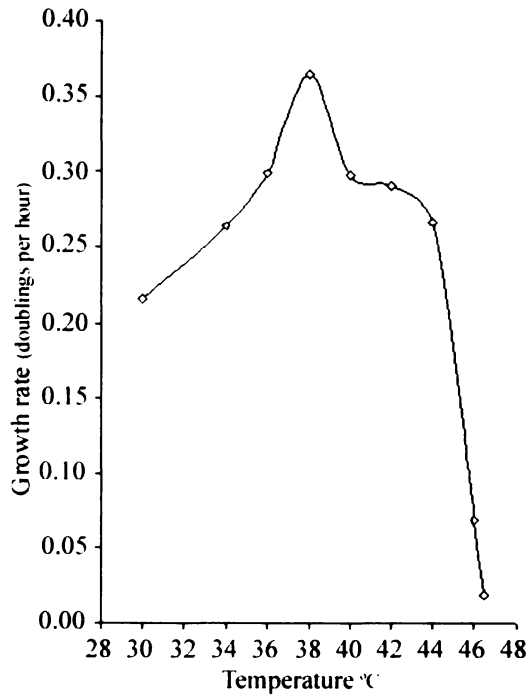


Figure 5.23 Specific growth rate of strain Wai1S1 as a function of temperature

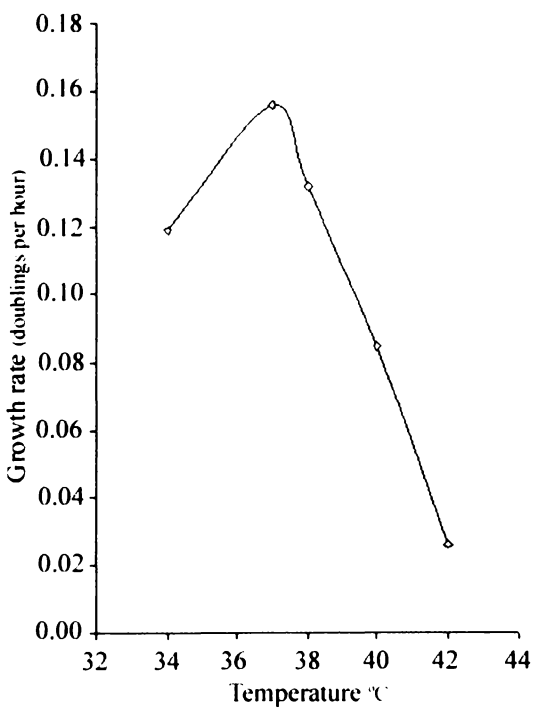


Figure 5.24 Specific growth rate of strain Wai1val1 as a function of temperature

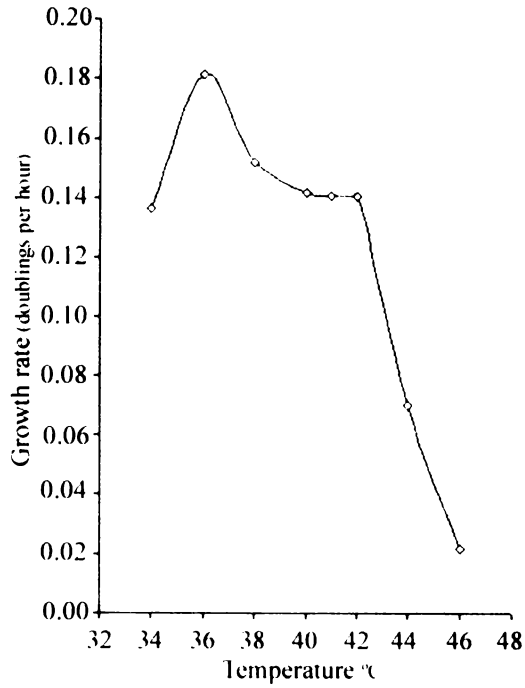


Figure 5.25 Specific growth rate of strain KCrS2 as a function of temperature

Rhodopseudomonas strains WaiS1 and Wai1val1 showed growth after 10 days of incubation at 20°C. None of the isolates was able to grow anaerobically in darkness with nitrate as electron acceptor. No production of nitrite or nitrogen gas was detected. Several isolates were tested for the ability to grow aerobically in darkness under full oxygen tension. Under these conditions, cultures of the type strain *Rps. palustris* DSM 123 and strains OH3F2 and Wai1val1 were colourless. By contrast, cultures of strains Wai1S1 and OK4bR1 were noticeably pink, and contained appreciable amounts of bacteriochlorophyll (Figure 5.26). Attenuated oxygen repression of pigment synthesis has been reported for *Rci. centenaria* (Yildiz *et al.* 1991b). Strain Wai1S1 was only observed to grow aerobically at a maximum temperature of 42°C. All strains grew optimally around pH 7, including strains KCrS2 and TKT2 which were isolated from samples with a bulk pH of 3.5. Growth was possible over the range 5.5-8.5. Strain OH3F2 was able to grow in the absence of vitamins. Strain Wai1S1 required both biotin and PABA. Strains from Orakei Korako, MPSR1 and Wai1val1 required only PABA. The vitamin requirements of strains Kui6R2, TKT2 and KCrS2 were not determined.

Carbon sources used by *Rhodopseudomonas* isolates are given in Table 5.13. Five of the 11 isolates were able to grow on benzoate, regarded as a diagnostic substrate for *Rps. palustris*. The same five strains only also grew on ethanol. All strains grew well

on most fatty acids, including the nine carbon compound pelargonate. None was able to grow on glucose or fructose, or nicotinic acid. Only Wai1val1 could grow on formate.

Carbon source/ electron donor	**Wai1S1**	**Wai1val1**	**OK4bR1**	**OK4bTR3**	**OK10ST2**	**TKT2**	**OH3F2**	**WSSR4**	**MPSR1**	**Kui6R2**	**KCRS2**	***Rps. palustris***	***R. rhenobacensis***	***Rps. faecalis* g-c**
Acetate	+++	++	++	+++	+++	+++	+++	+	0	++	0	+	+	+
Arginine	-	-	-	-	-	-	-	0	0	0	0	-	-	0
Aspartate	-	-	-	-	-	-	-	0	0	-	-	v	0	-
Benzoate	-	+++	-	-	-	+++	+++	-	-	+++	+++	+	-	-
Butyrate	+++	+++	+++	+++	+++	+++	0	0	0	0	0	+	+	+
Caproate	+++	+++	+++	+++	+++	+++	0	0	0	0	0	+	0	0
Caprylate	+++	+++	+++	+++	-	+++	+++	0	0	0	0	+	0	0
Citrate	(+)	-	-	-	-	-	-	-	-	-	-	v	-	-
Ethanol	-	+++	-	-	-	+++	+++	-	0	+++	+++	+	+	-
Formate	-	+	-	-	-	-	-	0	0	0	0	+	+	-
Fructose	-	-	-	-	-	-	-	0	0	0	0	v	-	0
Fumarate	+	+++	++	+	++	++	++	+	+	0	++	+	+	0
Gluconate	-	-	-	-	0	-	-	0	0	0	0	0	+	-
Glucose	-	-	-	-	-	-	-	-	-	-	-	v	-	0
Glutamate	+	-	-	-	-	-	-	0	-	-	-	+	+	-
Glycerol	++	++	-	-	-	++	++	+	+	++	+++	+	0	0
Glycolate	-	-	++	-	-	0	-	0	0	0	0	+	0	0
Lactate	+++	+++	-	-	+++	+++	+++	0	+++	0	++	+	+	+
Malate	+++	+++	-	-	+	+++	+++	0	0	+++	+++	+	+	+
Malonate	+	+	-	-	-	+	+	0	0	0	0	+	0	0
Mannitol	-	-	-	0	-	-	-	0	0	-	-	v	0	-
Methanol	-	-	+++	-	-	-	-	0	0	0	0	v	-	-
Pelargonate	+++	+++	+	+++	+++	+++	+++	++	0	0	0	+	0	0
Propionate	+	+	+++	-	-	0	++	+++	0	0	0	+	-	0
Pyruvate	++	++	0	+++	++	+++	+++	+++	0	+++	+++	+	+	+
Sorbitol	-	-	+++	0	-	0	-	0	0	0	0	+	0	-
Succinate	++	+++	-	+++	-	+++	+++	0	0	+++	+++	+	+	+
Tartrate	+	-	+++	-	-	-	-	-	0	-	-	-	+	-
Valerate	+++	+	+++	+++	+++	+++	++	+++	0	++	+++	+	0	0
Yeast extract	+	-	-	-	-	0	0	0	0	0	0	+	0	0
Casamino-acids	-	-	0	-	-	0	0	0	0	0	0	+	0	0
Hydrogen	++	++	-	0	0	0	++	0	0	0	0	+	0	0
Thiosulfate	++	+	-	-	-	+	+	-	0	-	+	+	-	-
Sulfide	+	-	0	0	-	-	-	-	0	-	-	*	0	-

Table 5.13 Carbon sources and electron donors utilised by *Rhodopseudomonas* strains
Data from current work, Imhoff and Trüper (1992); Hougardy *et al.* (2000); Zhang *et al.* (in press). Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of two remaining strains is positive/negative only. 0 =not tested, v =variable by strain

Strain OK4bR1 showed several peculiarities in substrate utilisation with respect to the other isolates. Excellent growth was obtained on the somewhat unusual substrates

methanol, sorbitol and tartrate; malate, lactate and succinate, compounds that are widely used by PNSB, failed to support growth. OK4bTR3 also failed to grow on lactate or malate, and OK10ST2 could not grow on succinate. This is unusual, but not unknown – *Rci. centenaria* also shows poor growth on four-carbon acids. In particular, the carbon source utilisation patterns of the three Orakei Korako isolates differed markedly, indicating that different strains of *Rhodopseudomonas* inhabited different Orakei Korako sample sites.

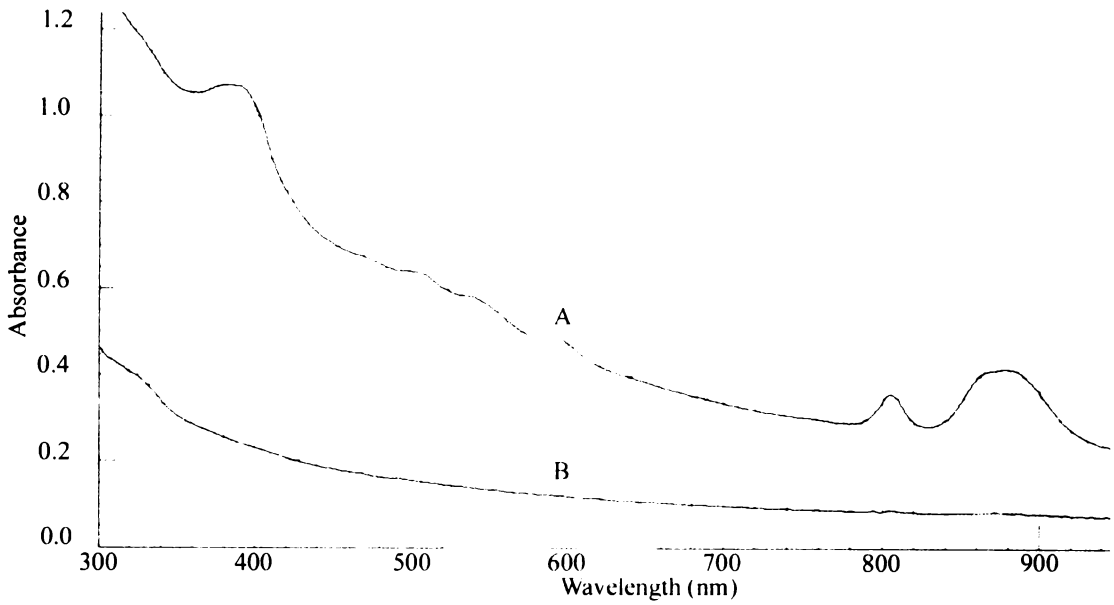


Figure 5.26 *In vivo* absorption spectra of (A) strain Wai1S1 and (B) strain Wai1val1 grown under dark aerobic conditions

5.4.5 Genetic relationships

16S rRNA gene sequences covering more than 1400 continuous nucleotide residues were determined for strains Wai1S1 and Wai1val1 according to the methods given in Section 3.9. Partial 16S rRNA sequences covering a little more than 500 bases were in addition obtained for OK4bR1, OK4bTR3, OH3F2, TKT2, and MPSR1. Some sequences were submitted to the GenBank database under Accession Numbers AF487428 to AF487432 (see Appendix 1). Binary sequence similarity values for Wai1S1, Wai1val1 and closely related organisms are given in Table 5.10. Incidentally, the 16S rRNA sequence of *Rhodopseudomonas julia* DSM 11549 was also determined and was found to be identical with that of *Rhodobium marinum* DSM 2698 (P. Charlton, J. McGrath, unpublished results), which has been independently confirmed by Hiraishi (GenBank Accession Number AB087720).

No.	Species or strain	Sequence similarity (%) for 16S rRNA genes														
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Wai1val1	99.7	98.6	98.6	98.6	98.0	98.3	99.1	98.9	99.2	98.9	97.7	98.7	98.6	97.9	97.3
2	*Rhodopseudomonas* strain T		98.8	98.9	98.9	98.3	98.6	99.4	99.2	99.5	99.2	98.0	99.0	98.9	98.2	97.6
3	Wai1S1			99.9	99.7	98.5	99.2	99.1	98.9	99.2	99.2	97.7	98.1	98.0	96.9	96.9
4	*Rhodopseudomonas* strain RN1				99.8	98.5	99.3	99.2	99.0	99.3	99.2	97.8	98.2	98.1	97.0	96.9
5	*Rps. faecalis* g-c					98.8	99.7	99.4	99.4	99.4	99.3	98.2	98.2	98.2	97.2	97.1
6	*Rps. rhenobacensis*						98.6	98.0	98.1	98.1	98.1	98.3	98.2	98.1	97.2	96.9
7	*Rhodopseudomonas* sp. v-1							98.9	99.1	99.1	99.0	97.7	97.9	97.9	97.1	96.8
8	*Rps. palustris* ATCC 17011								99.7	99.9	99.5	97.6	98.4	98.3	97.6	96.9
9	*Rps. palustris* S55									99.7	99.5	97.7	98.2	98.2	97.6	97.1
10	*Rhodopseudomonas* strain F7-1										99.7	97.6	98.6	98.5	97.7	97.1
11	*Rhodopseudomonas* sp. B29											97.6	98.5	98.4	97.6	97.0
12	*Nitrobacter hamburgensis*												98.1	98.0	97.1	97.3
13	*Bradyrhizobium japonicum*													99.9	98.7	98.1
14	*Bradyrhizobium lupini*														98.6	98.0
15	*Blastobacter denitrificans*															97.1
16	*Afipia felis*															

Table 5.14 Sequence similarities of 16S rRNA genes of *Rhodopseudomonas* strains and related species of the α-2 subdivision of the Proteobacteria

Both strains were extremely closely related to other strains of *Rhodopseudomonas palustris*, with sequence similarities ranging from 98.6 to 99.9%. Strain Wai1S1 was most similar to *Rps. palustris* strain RN1, which was isolated from the same lighted upflow anaerobic sludge blanket reactor as *Blastochloris* strain GN1 (Sawayama *et al.* 2000a). Strain Wai1val1 showed closest similarities to *Rps. palustris* T.

The full-length sequences were used to construct a phylogenetic tree according to the methods given in Section 3.9.6 (Figure 5.27). Low bootstrap values indicated that the precise branching order and in particular the position of *Rps. rhenobacensis* could not be determined with confidence, due to the high overall similarity of *Rhodopseudomonas* strain 16S rDNA sequences. Other *Rhodopseudomonas* strains formed a monophyletic cluster consisting of three subclusters (containing Wai1val1, Wai1S1 and ATCC 17001).

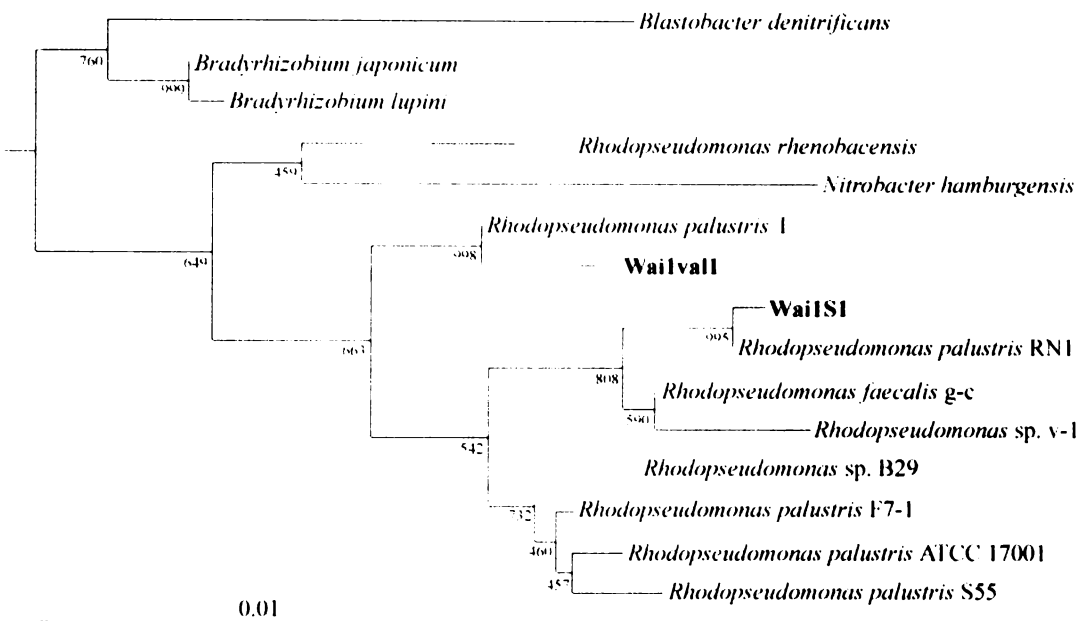


Figure 5.27 Distance matrix tree showing phylogenetic relationships of the thermotolerant isolates Wai1S1 and Wai1val1 among *Rhodopseudomonas* strains and selected members of the α-2 subclass of the Proteobacteria, based on an alignment of 1311 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Afipia felis*

A second phylogenetic tree incorporating the shorter sequences of other New Zealand isolates was constructed (Figure 5.28). In this analysis, the sequences of strains S55/No7 and TKT2 were identical, as were F7-1, T and ATCC 17001, and Wai1S1 and RN1. The precise branching order of *Rps. rhenobacensis*, *Rps. palustris* T and

Wai1val1 differed between the trees, due to the lower number of positions in the analysis. Strains OH3F2 and TKT2 clustered with Wai1val1, while MPSR1, OK4bR1 and OK4bTR3 formed a cluster together with Wai1S1 and RN1 that was well-supported by bootstrap value.

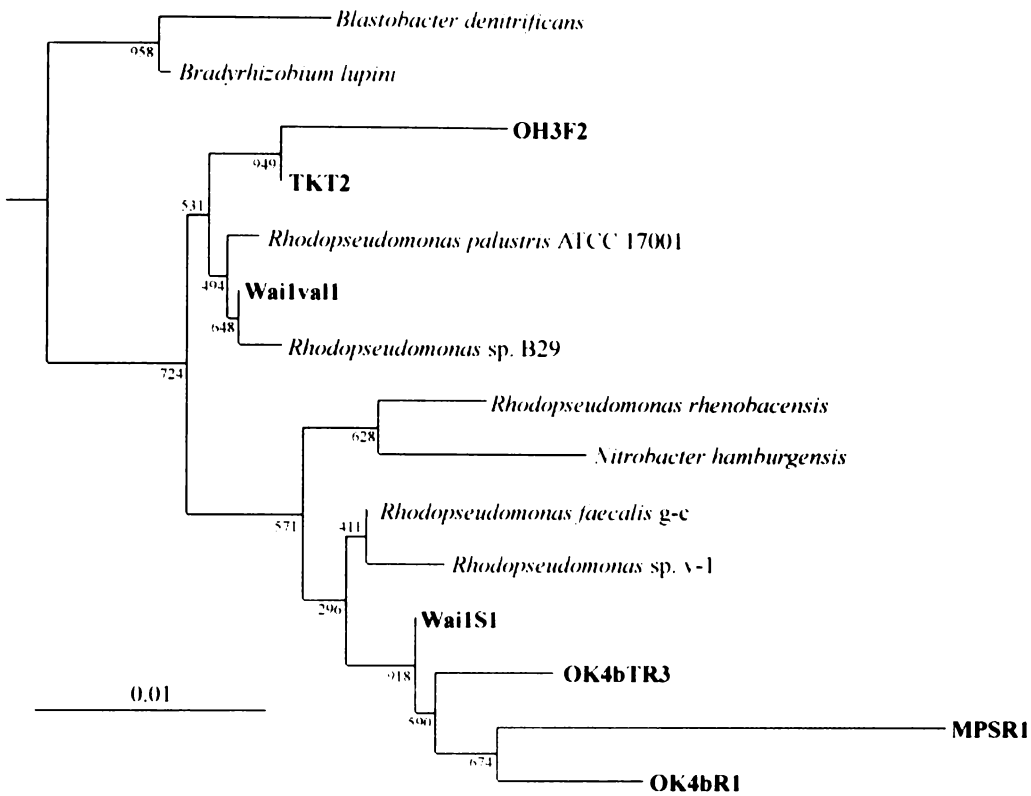


Figure 5.28 Distance matrix tree showing phylogenetic relationships of *Rhodopseudomonas* strains and selected members of the α-2 subclass of the Proteobacteria, based on an alignment of 522 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Afipia felis*

Inspection of aligned sequences revealed that all strains in the ATCC 17001 cluster in Figure 5.28 have the sequence AC at positions 153-154 and GU at positions 165-166. These residues form base pairs in the stem of helix 8 (see Appendix 2). The members of the other cluster, including *Rps. rhenobacensis* and *Nitrobacter hamburgensis*, have UG and, correspondingly, CA (Figure 5.29). A New Zealand isolate from a non-thermal area designated CCLRSP3 (McGrath 2000) would cluster with strains v-1 and g-c, while strain YBS3 falls in the "AC" cluster (unpublished results).

5.4.6 Taxonomy of *Rhodopseudomonas*

Rhodopseudomonas palustris-like strains comprise a vast assemblage that differ only slightly in 16S rDNA sequence and show broad similarities in phenotypic properties,

while differing in details of carbon source utilisation, vitamin requirements or other features. Insufficient information is contained in their 16S rDNA sequences to allow a definite determination of branching order. *Rps. faecalis* g-c was reported to share only 17% DNA-DNA similarity with *Rps. palustris* ATCC 17001 (Zhang *et al.* in press). Kompantseva *et al.* (1996) isolated many *Rps. palustris*-like strains, whose 16S rDNA sequences are not available, and found that they fell into 13 separate clusters on the basis of DNA-DNA relatedness. It seems that the majority of independent isolates represent novel genospecies.

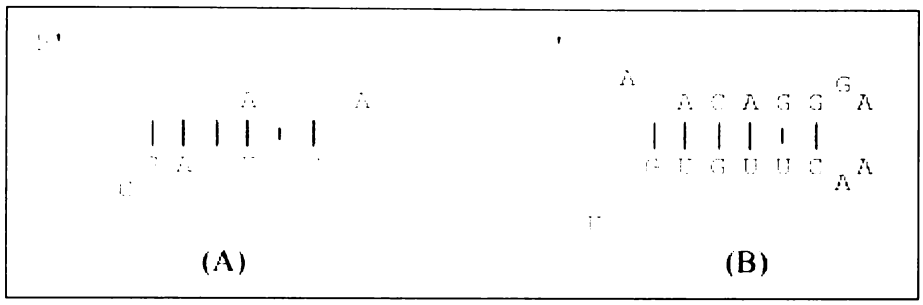


Figure 5.29 Deduced secondary structures of the stem-loop at *E. coli* positions 150-169 of the 16S rRNA molecules of strains in: (A) the Wai1S1 cluster, and (B) the *Rps. palustris* ATCC 17001^T cluster in Figure 5.28

Patterns of carbon source utilisation vary greatly even between isolates that form single 16S rDNA relatedness pairs (such as OK4bR1 and OK4bTR3). It may often be possible to distinguish genospecies on the basis of ability or failure to use particular sets of carbon sources, but it is doubtful whether such differences in patterns would be more than coincidental. There is a risk of proliferation of *Rhodopseudomonas* species that contain only single strains. While this may reflect some element of evolutionary reality, a major underlying principle of a taxonomic classification must be usefulness for researchers. It would be sensible to postpone description of novel *Rhodopseudomonas* species until whole genome data is available, or to limit description to those instances where several geographically separated strains can be assigned to a single taxonomic unit.

The signature sequence in the helix 8 structure perhaps indicates that known strains form two separate lines of descent, as seen in Figure 5.28. Thermotolerant isolates are represented in both clusters, which, if they indeed mark separate lines of descent, would indicate a polyphyletic origin for thermotolerant strains of *Rhodopseudomonas*.

However, all the New Zealand isolates with lower maximum temperatures (42-43°C) fall in the "AC" cluster, while all the New Zealand strains with higher maximum temperatures for growth (45-46°C) form a monophyletic group within the "UG" cluster, together with strain RN1. No information is available regarding the temperature relations of strain RN1. It was isolated from the same anaerobic reactor operated at 35°C as *Blastochloris* strain GN1 (Sawayama *et al.* 2000a), which also clustered with thermotolerant strains. One might predict that these strains would show thermotolerance, and perhaps be capable of growth at 45°C or higher. *Rps. faecalis* g-c, also a member of the "UG" cluster, is thermotolerant, growing optimally at 38°C and to a maximum of 45°C. The ability to use benzoate is rare among those organisms affiliated with the "UG" cluster. *Rps. palustris* RN1, *Rps. rhenobacensis*, *Rps. faecalis* and the four affiliated New Zealand isolates do not use benzoate (Sawayama *et al.* 2000b; Hougardy *et al.* 2000; Zhang *et al.* in press). Strain CCLRSP3 is the only known exception. The three New Zealand isolates and the type strain in the "AC" cluster use benzoate as sole carbon source. It would be interesting to examine the genes of the benzoate degradation pathway in *Blastochloris sulfoviridis*, *Phaeospirillum fulvum* and non-photosynthetic proteobacteria, which might shed light on the origin of benzoate degradation genes in *Rhodopseudomonas* (Egland *et al.* 1997).

Attenuated repression by oxygen of pigment synthesis, observed in strains Wai1S1 and OK4bR1, is not limited to the "UG" cluster. Strain B29, a member of the "AC" cluster, is reported to grow pink under aerobic conditions (Kiwamu Minamisawa, Tohoku University, Sendai, Japan, pers. comm.).

5.5 *Phaeospirillum* and *Rhodospirillum* isolates

5.5.1 Strains

Spiral organisms showing scotophobic behaviour were observed on many occasions in red or brown-pigmented enrichment cultures containing bacteriochlorophyll *a*, but in most cases these organisms rapidly disappeared as the cultures developed. Only three spiral organisms were successfully maintained in laboratory culture, and of these one strain could not be purified (see Section 4.3.9 and Figure 4.14). The two isolates

obtained in pure culture were WSSB3, from Waitangi Soda Springs, and MPA1, from a minor thermal feature designated the Magpie Pool.

5.5.2 Morphological properties

Cells of strain WSSB3 were large spirilla, measuring approximately 1.0-1.5 μm wide by 6-8 μm long, and normally completing approximately one spiral turn. Polar flagella were visible under the light microscope (Figure 5.30). Cells were rapidly motile and exhibited a strong scotophobic response. No cell aggregations were observed. Cells of strain MPA1 showed similar properties, but were vibrios to short spirilla, measuring 0.6-0.7 μm wide by 2.5-4 μm long, in a similar size range to *Phs. fulvum* or *Phs. molischianum* among brown spirilla (Figure 5.31).

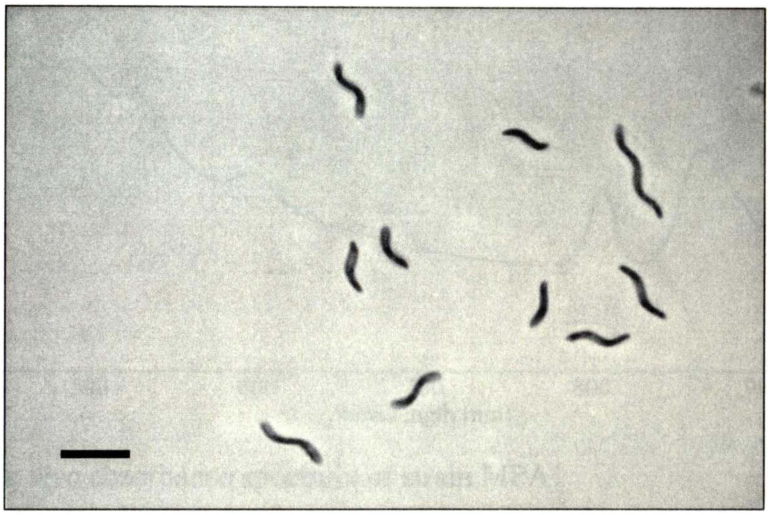


Figure 5.30 Phase-contrast photomicrograph showing the cell morphology of strain WSSB3. Bar, 10 μm

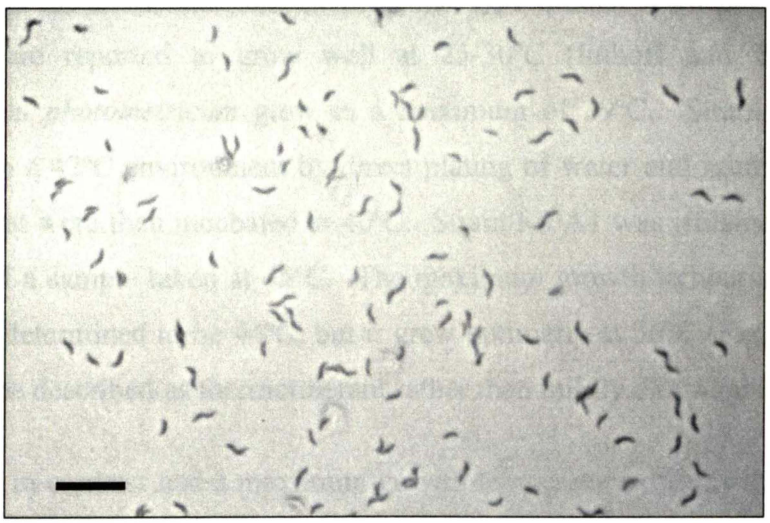


Figure 5.31 Phase-contrast photomicrograph showing the cell morphology of strain MPA1. Bar, 10 μm

5.5.3 Photopigments

Cultures of the unpurified spirillum were light pink in colour, but cultures of the morphologically similar WSSB3 were red-brown. Strain MPA1 produced light brown cultures. Absorption spectra of intact cells of strains MPA1 and WSSB3 had maxima at 458, 495, 525, 595, 803 and 860 nm, indicating the presence of bacteriochlorophyll *a* and similar carotenoids to those of brown photosynthetic spirillum species, that is lycopene and rhodopinal, but not spirilloxanthin (Figure 5.32).

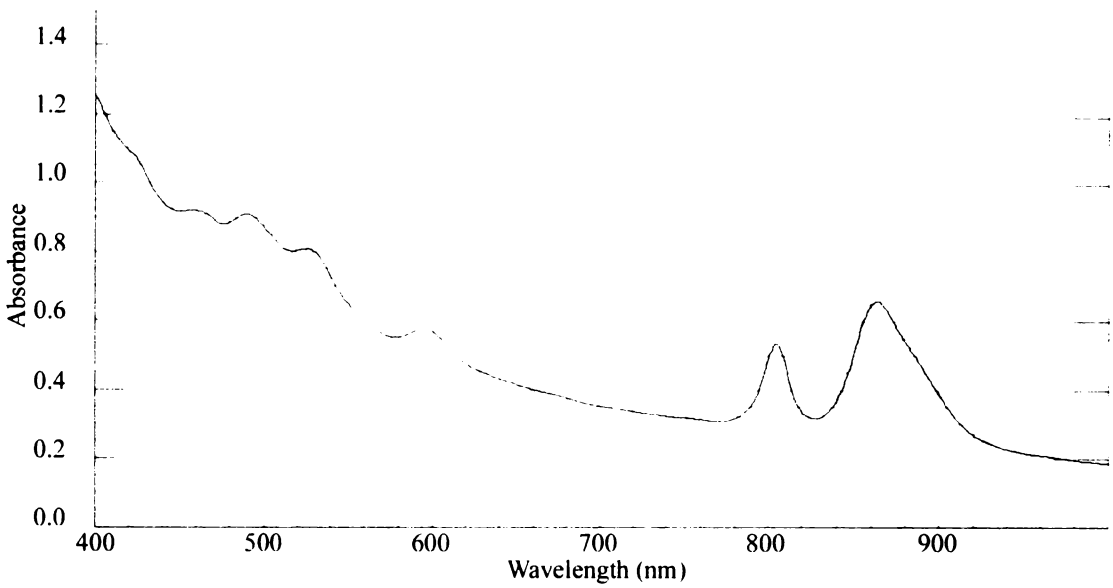


Figure 5.32 *In vivo* absorbance spectrum of strain MPA1

5.5.4 Physiological characteristics

Phaeospirillum molischianum and *Phs. fulvum* type strains were not available for testing, but are reported to grow well at 25-30°C (Imhoff and Trüper 1989). *Rhodospirillum photometricum* grew to a maximum of 39°C. Strain WSSB3 was obtained from a 42°C environment by direct plating of water and mud samples onto agar plates that were then incubated at 40°C. Strain MPA1 was isolated from a 43°C enrichment of a sample taken at 45°C. The maximum growth temperature for strain WSSB3 was determined to be 44°C, but it grew optimally at 36°C (Figure 5.33). As such, it may be described as thermotolerant rather than mildly thermophilic.

Strain MPA1 in contrast had a maximum growth temperature of only 42°C, but grew optimally at 40°C, an optimum that compares favourably with the previously

described thermotolerant or mildly thermophilic purple nonsulfur bacteria (Figure 5.34).

Growth rates were among the fastest observed of the New Zealand isolates, with minimum doubling times around 3-4 hours. Growth of both isolates occurred over the pH range 6.0-8.5. Neither isolate was able to grow aerobically in darkness.

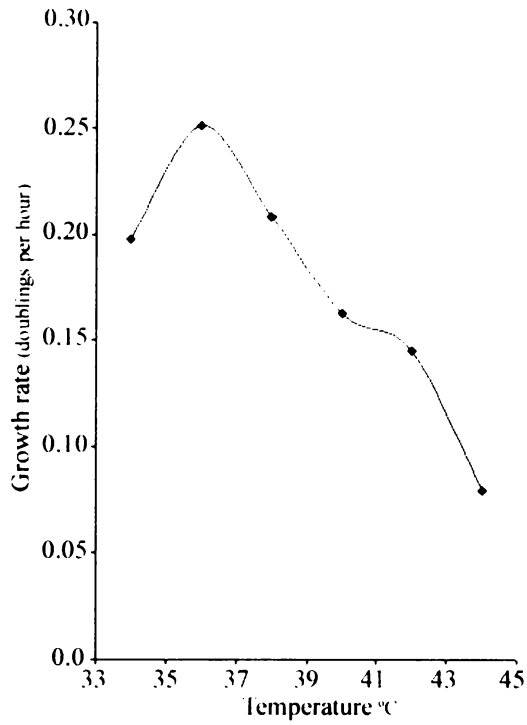


Figure 5.33 Specific growth rate of strain WSSB3 as a function of temperature

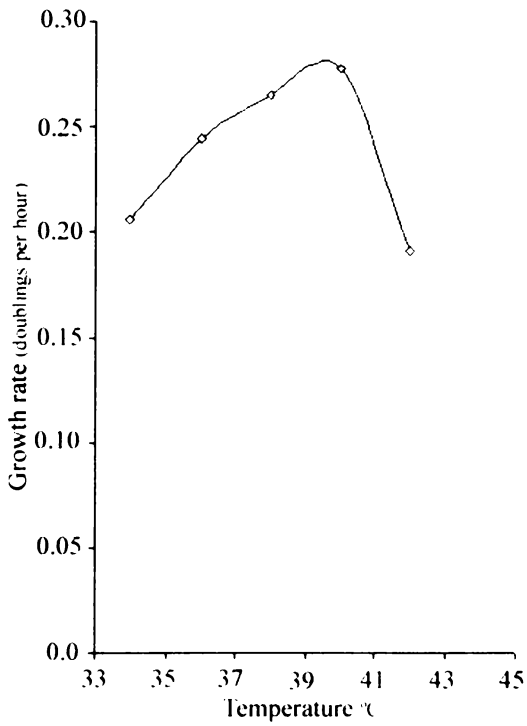


Figure 5.34 Specific growth rate of strain MPA1 as a function of temperature

Strains WSSB3 and MPA1 both used N_2 or NH_4^+ as nitrogen sources, and could assimilate sulfate or thiosulfate as sulfur sources. Strain WSSB3 required nicotinate as growth factor, which is also required by *Rsp. photometricum*. MPA1 required PABA, which is also required by *Phs. fulvum*.

Compounds used as carbon sources or electron donors by spiral-shaped purple nonsulfur bacteria are listed in Table 5.15.

Carbon source/ electron donor	**MPA1**	**WSSB3**	***Phs. fulvum***	***Phs. molischianum***	***Rsp. photometricum***	***Rsp. rubrum***	***Rci. centenaria***
Acetate	+++	+++	+	+	+	+	+
Arginine	-	-	-	-	-	+	-
Aspartate	-	-	v	v	v	+	+
Benzoate	+++	-	+	-	-	-	-
Butyrate	+++	+++	+	+	+	+	+
Caproate	+++	+++	+	+	+	+	+
Caprylate	-	+++	+	+	+	+	+
Citrate	-	-	-	-	-	-	-
Ethanol	-	+++	+	+	+	+	-
Formate	-	0	0	-	-	-	-
Fructose	-	+	-	-	+	v	-
Fumarate	++	++	+	+	+	+	-
Gluconate	0	0	0	0	0	-	-
Glucose	-	-	v	-	+	-	-
Glutamate	-	-	0	0	+	+	+
Glycerol	-	-	-	-	-	-	-
Glycolate	0	0	0	-	0	0	-
Lactate	-	++	-	v	+	+	+
Malate	+++	+++	+	+	+	+	-
Malonate	-	-	0	-	0	0	-
Mannitol	-	-	-	-	+	-	-
Methanol	-	0	-	-	-	v	-
Pelargonate	++	+++	+	+	v	+	+
Propionate	-	-	+	+	v	+	+
Pyruvate	++	++	+	+	+	+	+
Sorbitol	-	-	0	0	+	-	-
Succinate	+++	+++	+	+	+	+	-
Tartrate	-	-	0	-	-	-	-
Valerate	++	+++	+	+	+	+	+
Yeast extract	0	+	+	+	+	+	+
Casamino-acids	0	-	-	-	+	+	+
Hydrogen	0	0	+	0	+	+	0
Sulfide	-	-	+	-	0	*	0
Thiosulfate	-	-	0	0	-	-	0

Table 5.15 Carbon sources and electron donors utilised by spirilloid strains
Data from present work, Favinger *et al.* (1989) and Imhoff and Trüper (1992). Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of other species is positive/negative only. 0 =not tested, v =variable by strain, * = used at low concentrations

Strain MPA1 resembled *Phs. fulvum* in its ability to grow on benzoate and inability to grow on lactate, though it is also unable to use ethanol. Strain WSSB3 did not grow on glucose, glutamate, mannitol or sorbitol, substrates that support good growth of *Rsp. photometricum*. The pink spirillum in the mixed culture was maintained on a mixture of mannitol, sorbitol and glutamate for many transfers, and so appeared to have a carbon source utilisation pattern more similar to the *Rsp. photometricum* type strain.

5.5.5 Genetic relationships

16S rRNA gene sequences covering 1418 and 1428 continuous nucleotide residues were determined for strains MPA1 and WSSB3 according to the methods given in Section 3.9. Sequences were submitted to the GenBank database under Accession Numbers AF487433 and AF487434. Binary sequence similarity values for these strains and closely related organisms are given in Table 5.16.

The 16S rDNA sequence of strain WSSB3 was 99.4% similar to that of *Rsp. photometricum* and 99.0% similar to *Rhodospirillum* E-12 (Kawasaki *et al.* 1993b), and showed lower similarities to all other photosynthetic spirilla.

The sequences were used to construct a phylogenetic tree according to the methods given in Section 3.9.6 (Figure 5.35). As expected, strain WSSB3 was affiliated with *Rsp. photometricum* and *Rhodospirillum* strain E12, and strain MPA1 with the *Phaeospirillum* species, with excellent bootstrap support.

The 16S rRNA gene sequence of a strain identified as *Phaeospirillum fulvum* S3 was recently submitted to the GenBank database as Accession Number AF508113 by S.-H. Baek, K.H. Kim and S.-T. Lee. Whatever the physiological properties of this organism, it is too dissimilar to be regarded as a *Phaeospirillum* species. The sequence is only 84-88% similar to all of the species in Figure 5.35, and branched at the base of the tree.

No.	Species or strain	Sequence similarity (%) for 16S rRNA genes																	
		2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	WSSB3	99.0	99.4	95.5	89.3	89.9	89.1	87.5	87.0	86.8	87.3	87.3	87.8	88.2	88.1	87.6	85.3	86.0	82.0
2	*Rhodospirillum* sp. E12		99.0	95.0	89.0	89.4	88.4	87.2	86.9	86.6	87.0	86.8	87.4	87.6	87.5	87.0	85.3	86.1	81.7
3	*Rsp. photometricum*			95.1	88.8	89.4	88.5	87.1	86.6	86.4	86.9	86.9	87.5	87.7	87.5	87.0	84.8	85.7	81.7
4	*Rsp. rubrum* DSM467				91.1	89.9	89.8	87.9	87.2	87.1	86.8	86.7	87.2	87.3	87.0	87.0	84.8	86.7	82.5
5	*Rhodospira trueperi*					87.2	87.1	86.1	86.4	86.5	85.3	85.1	85.7	85.9	85.8	85.4	84.4	84.9	83.0
6	*Azospirillum* sp. 5C						90.8	88.5	88.2	88.4	87.9	87.7	88.3	88.3	88.2	88.0	86.8	86.7	85.3
7	*Aquaspirillum peregrinum*							88.2	87.5	87.5	87.2	87.1	87.6	87.7	87.4	87.6	85.2	86.3	83.8
8	MPA1								96.7	97.2	95.0	94.6	95.4	92.9	94.5	94.0	87.9	87.5	85.6
9	*Phaeospirillum fulvum*									99.0	94.5	93.9	94.7	93.5	95.1	93.3	88.1	87.4	85.9
10	*Phs. molischianum*										94.8	94.2	95.0	93.7	95.4	93.6	87.5	86.8	86.0
11	Spirillum CC-26											98.3	99.1	94.6	95.4	95.0	87.9	86.6	84.9
12	*Magnetospirillum* sp. MGT-1												99.2	95.1	94.8	95.1	87.3	86.2	85.0
13	*M. magnetotacticum*													95.6	95.6	95.5	87.9	86.9	85.3
14	*M. gryphiswaldense*														97.0	96.1	87.9	86.6	85.1
15	*Magnetospirillum* sp. MSM-4															96.5	88.6	86.8	86.2
16	*Dechlorospirillum* sp. WD																88.8	86.9	85.0
17	*Azospirillum lipoferum*																	91.2	85.5
18	*Rhodocista centenaria*																		84.8
19	*Sphingobium chlorophenolicum*																		

Table 5.16 Sequence similarities of 16S rRNA genes of photosynthetic spirilla and related organisms of the α-1 subdivision of the Proteobacteria

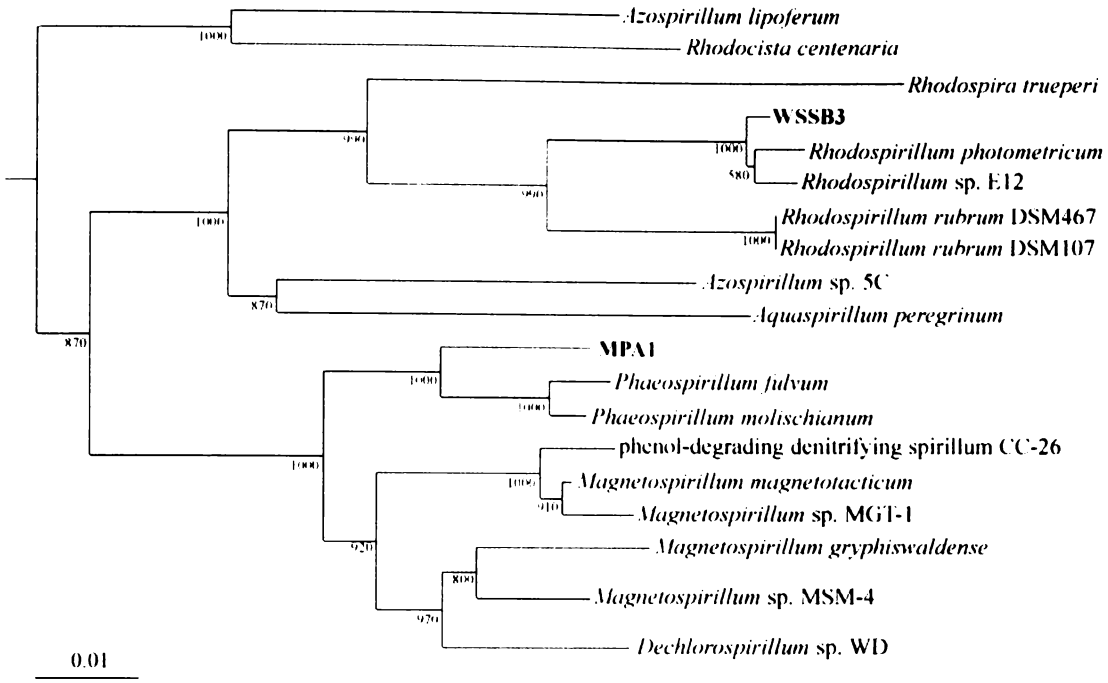


Figure 5.35 Distance matrix tree showing phylogenetic relationships of thermotolerant isolates WSSB3 and MPA1 among selected members of the α-1 subdivision of the Proteobacteria, based on an alignment of 1277 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Sphingobium chlorophenolicum*.

5.5.6 Taxonomy of *Phaeospirillum* and *Rhodospirillum*

Strain WSSB3 does not grow on mannitol, sorbitol, glucose or glutamate, and has a higher maximum temperature for growth than *Rsp. photometricum*. Strain WSSB3 is however closely related to *Rsp. photometricum* according to 16S rRNA gene sequence analysis, and evidence for substantial genetic difference must await DNA-DNA hybridisation data. Strain WSSB3 is therefore for the time being assigned to *Rsp. photometricum*.

The 16S rDNA sequence of strain MPA1 was 97.2% identical to that of *Phs. molischianum* and 96.7% to that of *Phs. fulvum*. These are lower similarities than between these two closely related species (99%). Assignment of strain MPA1 to either existing *Phaeospirillum* species would therefore require the conflation of the two existing species in order to preserve monophyly of the lineage. However, strain MPA1 is physiologically distinct from *Phs. molischianum*, and the similarity between strain MPA1 and *Phs. fulvum* is too low to warrant classification of strain MPA1 as a

strain of *Phs. fulvum*. Strain MPA1 should therefore be described as a new species of the genus *Phaeospirillum*. The mild thermophily of strain MPA1 is the only major physiological difference between strain MPA1 and *Phs. fulvum*. In addition, there are minor differences in carbon source utilisation pattern – strain MPA1 failed to grow on propionate, caprylate or ethanol. Strain MPA1 is therefore proposed as a new species, *Phaeospirillum tepidarium*, sp. nov.

Description of *Phaeospirillum tepidarium* sp. nov. *Phaeospirillum tepidarium* (tep.i.dar'i.um. L. n. *tepidarium*, a warm thermal spring bath; M.L. adj. *tepidarius*, warm bathing). Cells are motile Gram negative vibrios or spirilla, 0.7 μm wide by 2.5-4.0 μm long, multiplying by binary fission. Cells are facultative phototrophs, growing anaerobically in the light. Aerobic growth in darkness at full oxygen tension does not occur. Photosynthetic cultures are light brown and contain bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series. Absorption maxima are at 458, 495, 525, 595, 803 and 860 nm. The pH range for growth is 6.0 to 8.5. Optimal temperature for growth is 40°C. No growth occurs above 42°C. PABA is required as growth factor. The following organic compounds are utilised for phototrophic growth: acetate, benzoate, butyrate, caproate, fumarate, malate, pelargonate, pyruvate, succinate, valerate. The following compounds are not utilised: arginine, aspartate, caprylate, citrate, ethanol, formate, fructose, glucose, glutamate, glycerol, lactate, malonate, mannitol, methanol, propionate, sorbitol, tartrate. Photoautotrophic growth with sulfide and thiosulfate as electron donors has not been observed. Isolated from microbial mats near Rotorua, New Zealand. The type strain is MPA1. The species can be distinguished from *Phaeospirillum fulvum* and *Phs. molischianum* by its optimal growth temperature. It can in addition be distinguished from *Phs. molischianum* by its ability to use benzoate but not ethanol, lactate or propionate as carbon sources. It can in addition be distinguished from *Phs. fulvum* by its inability to use ethanol or propionate, and its inability to grow photoautotrophically on sulfide as electron donor.

5.6 *Rubrivivax* isolates

5.6.1 Strains

Five apricot to beige-brown strains were isolated during this study, all from samples taken at Orakei Korako. These were designated OK3O3, OK5bB3, OK8bDIB3, OK9C2 and OK13bO2. They were initially identified as *Rubrivivax gelatinosus* on the basis of their copious slime production. Strain OK8bDIB3 was isolated after the others, and was not characterised.

5.6.2 Morphological properties and photopigments

All strains consisted of short ovoid to rod-shaped cells. Young cultures of strain OK3O3 were apricot-orange and contained extremely rapidly moving cells. Cultures of strains OK5bB3 and OK9C2 were beige-brown, while OK13bO2 cultures were more orange in colour. All strains produced slime which led to clumping of cells into macroscopic smooth sheets. Cultures of strain OK13bO2 formed both tiny particulates and gelatinous sheets (Figure 5.36). All strains contained spheroidene carotenoids and bacteriochlorophyll *a*.

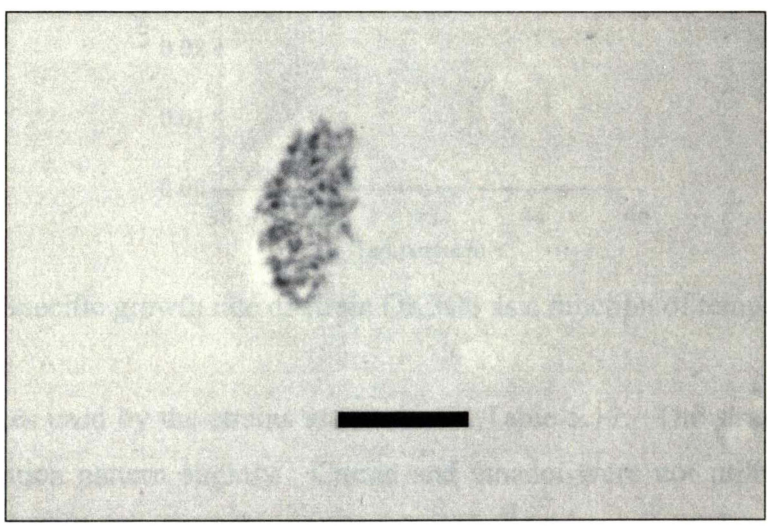


Figure 5.36 Phase-contrast photomicrograph showing a clump of cells of strain OK13bO2. Bar, 10 μm

5.6.3 Physiological properties

The strains were isolated from samples at temperatures of 45°C (OK3O3, OK8bDIB3) and 42°C. All strains grew optimally at 42-43°C and maximum temperature for growth was 45°C (Figure 5.37). Measurement of growth rates was difficult due to the

tendency of cells to clump. The type strain of *Rvi. gelatinosus* grew only to a maximum of 42°C.

NH_4^+, N_2 and amino acids were used as nitrogen sources. Sulfate was assimilated. The strains required only biotin as growth factor. Other strains of *Rvi. gelatinosus* are reported to require biotin and thiamine and occasionally pantothenate. Growth was possible in the dark under aerobic conditions.

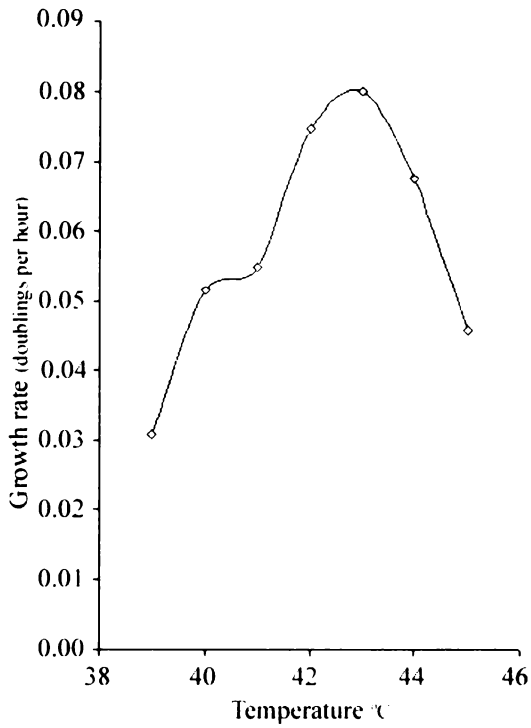


Figure 5.37 Specific growth rate of strain OK3O3 as a function of temperature

Carbon sources used by the strains are shown in Table 5.17. The strains differed in carbon utilisation pattern slightly. Citrate and ethanol were not utilised by strains OK3O3 and OK13bO2. OK9C2, which was isolated from an enrichment containing citrate as carbon source, grew well on citrate. OK3O3 failed to grow on pelargonate, and OK5bB3 did not grow on propionate. OK3O3 was the only strain which achieved measurable growth photoautotrophically with sulfide as electron donor.

Carbon source/ electron donor	OK3O3	OK5bB3	OK9C2	OK13bO2	*Rubrivivax gelatinosus*
Acetate	+++	+++	+++	+++	+
Arginine	+++	++	+	++	0
Aspartate	++	++	+	++	0
Benzoate	-	-	-	-	-
Butyrate	+++	+++	+++	+++	v
Caproate	+++	+++	+	++	+
Caprylate	++	+++	++	++	+
Citrate	-	+++	++	-	+
Ethanol	-	++	++	-	+
Formate	+	0	0	0	v
Fructose	+++	+	++	++	0
Fumarate	+++	++	++	++	+
Gluconate	0	0	0	0	0
Glucose	+++	+++	+++	+++	+
Glutamate	++	+	+	0	0
Glycerol	-	-	-	0	-
Glycolate	-	0	0	0	0
Lactate	+++	+++	+++	+++	+
Malate	+++	+++	++	++	+
Malonate	-	-	-	0	0
Mannitol	-	-	-	0	v
Methanol	-	-	-	0	v
Pelargonate	-	++	++	0	+
Propionate	+	-	+	+	v
Pyruvate	+	++	++	++	+
Sorbitol	-	-	-	-	0
Succinate	+++	+++	+++	+++	+
Tartrate	-	0	0	0	v
Valerate	+++	+++	+++	+++	0
Yeast extract	0	0	0	0	+
Casamino-acids	0	0	0	0	0
Hydrogen	0	0	0	0	v
Sulfide	+	-	-	-	0
Thiosulfate	0	0	0	0	-

Table 5.17 Carbon sources and electron donors utilised by *Rubrivivax* strains
Data from current work and Imhoff and Trüper (1992). Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of type strain is positive/negative only. 0 =not tested, v =variable by strain

5.6.4 Genetic relationships

A 16S rRNA gene sequence covering 1464 continuous nucleotide residues was determined for OK3O3, according to the methods given in Section 3.9. Partial sequences were obtained for OK13bO2 and OK9C2. The sequence of OK13bO2 matched that of OK3O3 over the first 593 residues, and showed no differences to that of OK9C2. The sequence of strain OK3O3 was deposited in the GenBank database

under Accession Number AF487435. The sequences were used to construct a phylogenetic tree according to the methods given in Section 3.9.6 (Figure 5.38).

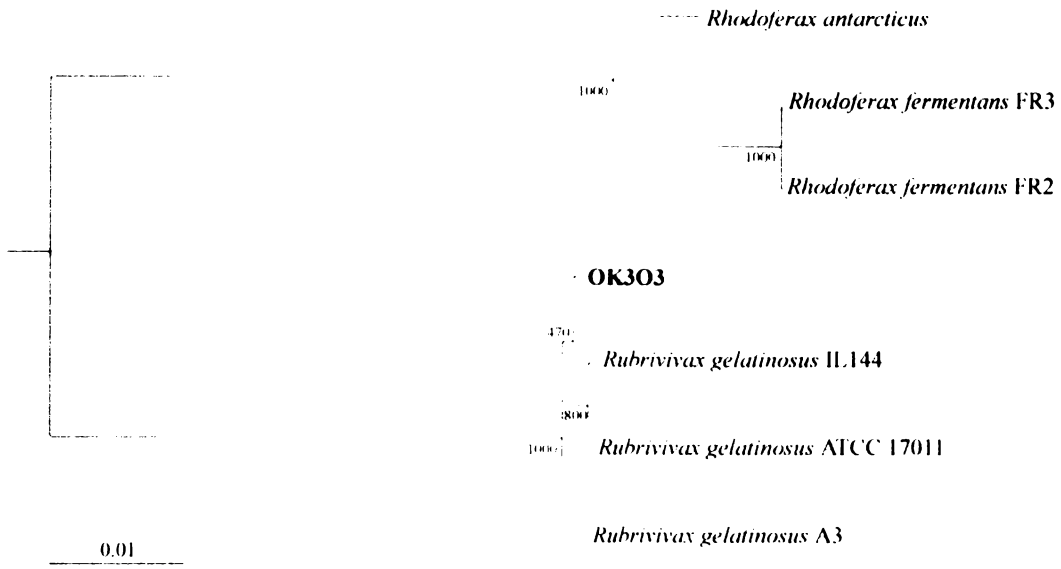


Figure 5.38 Distance matrix tree showing phylogenetic relationships of the thermotolerant isolate OK3O3 to purple nonsulfur bacteria of the β-subdivision of the Proteobacteria, based on an alignment of 1435 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Rhodocyclus tenuis* DSM 109

The sequence of OK3O3 was 99.7-99.8% similar to those of other *Rvi. gelatinosus* strains. In this organism, thermotolerance is not reflected in 16S rDNA dissimilarity. This may be because thermotolerance has arisen recently, or because *Rubrivivax gelatinosus*-like strains are a naturally "tight" species (Ambler 1996), showing little variation in 16S rRNA with alteration in physiological properties.

5.7 *Rhodomicrobium* isolates

5.7.1 Strains

Three isolates were obtained that showed the conspicuous morphology of *Rhodomicrobium vannielii*. Two strains, OK5M2 and OK9V1, were isolated from sampling sites at Orakei Korako. Strain Wot6V1 was the only purple nonsulfur bacterium to be isolated from Waiotapu, but was isolated from a location where a coldwater stream flowed into a thermal area.

5.7.2 Morphological properties and photopigments

Morphologically, the New Zealand isolates were typical of *Rhodomicrobium vannielii*. Variation in morphological properties such as length of division filaments or proportion of filaments bearing exospores appears to depend on culture conditions and not be strain related, though no rigorous study has been undertaken. Cultures were red-brown, containing bacteriochlorophyll *a* and carotenoids. Strain WoT6V1 was generally a darker brown than the other strains.

5.7.3 Physiological properties

Strain OK9V1 was enriched at 42°C from a 42°C sample. Strain OK5M2 was enriched at 40°C from a 38°C sample. It was observed as a minor component under the microscope and was isolated from a subculture into medium lacking vitamins with benzoate as carbon source.

At 41°C, strains OK5M2 and OK9V1 had a doubling time of 6 to 7 hours, compared with around 20 hours for the type strain and strain WoT6V1. Specific growth rates were comparable at 37°C, and maximum temperature of all strains was 42°C.

None of the strains required vitamins. All grew aerobically in the dark, could grow at pH 5.5, used NH_4^+ and N_2 as nitrogen sources, and assimilated sulfate.

Carbon sources used for growth by the two isolates from warm sites are shown in Table 5.18. Only a small range of carbon sources was tested. Minor differences were observed. Strain OK5M2, despite being enriched in the presence of benzoate, could not use this substrate. It also failed to grow on caprylate and propionate. Strain OK9V1 used benzoate, which has been observed in some other *Rhodomicrobium* strains (Wright and Madigan 1991). Neither strain used methanol, which is also used by some strains. Neither strain showed photoautotrophic growth with sulfide as electron donor.

5.7.4 Genetic relationships

A partial 16S rRNA gene sequence was determined for strain OK5M2, according to the methods given in Section 3.9. The sequence was identical to that of *Rmi. vannielii*.

Carbon source/ electron donor	OK5M2	OK9V1	*Rhodomicrobium vannielii*
Benzoate	-	+++	v
Butyrate	+++	+++	+
Caproate	+++	+++	+
Caprylate	-	+++	+
Ethanol	+++	+++	+
Lactate	+++	+++	+
Malonate	-	+++	+
Methanol	-	-	v
Propionate	-	+++	+
Succinate	+++	+++	+
Sulfide	-	-	+

Table 5.18 Carbon sources and electron donors utilised by *Rhodomicrobium* strains
Data from current work and Imhoff and Trüper (1992). Note: growth of New Zealand isolates is scored according to the protocol in Table 3.3; growth of other species is positive/negative only. 0 =not tested, v =variable by strain

5.8 Temperature Relations of Type Strains of Purple Nonsulfur Bacteria

5.8.1 Introduction

It is probable that thermotolerant purple nonsulfur bacteria arose from mesophilic ancestors. Other than *Rhodocista centenaria*, all thermotolerant PNSB have close mesophilic relatives. The mechanism of adaptation to a new set of environmental parameters is unknown. Development of thermotolerant and in particular mildly thermophilic PNSB may be a rare event that occurs on an evolutionary timescale; it is also possible that adaptation of mesophilic strains to growth at high temperature is readily achieved and occurs wherever suitable environments occur. This is discussed further in Section 5.9. If the latter is true, then adaptation of mesophilic type strains of PNSB may be observable in the laboratory over a short time period.

The most extensive series of studies of acclimation and adaptation of bacteria to altered environmental conditions have been carried out by the research group of R. Lenski who have maintained *Escherichia coli* populations under particular temperature regimes for 2000 generations. This might represent a minimum of a month of continuous growth under optimal conditions for *E. coli*; for PNSB, continuous growth for a period in excess of a year might be required. Adaptation to

high temperature growth was not always correlated with a loss of fitness at lower temperatures. Extension of the upper thermal limit as a result of selection at high temperature was a rare event (Bennett and Lenski 1993). However, exposure to high but nonlethal temperatures outside the organism's thermal niche occasionally produced so called "Lazarus" mutants with an extended thermal range (Bennett and Lenski 1993). Prior selection at elevated but nonlethal temperatures predisposed populations to develop more extreme thermotolerance (Mongold *et al.* 1999).

The evolution of thermotolerance of *Synechococcus* found in hot spring environments was examined by Miller and Castenholz (2000). Evidence was obtained to suggest that more thermotolerant lineages developed from less thermotolerant ancestors, and that extension of the thermal limit was associated with a reduction in the temperature range for growth.

An exploration of the adaptation of purple nonsulfur bacteria to higher temperature was intended to form a substantial part of the current research. However, it quickly became apparent that an individual researcher could not over an extended period take growth measurements of the exponential phase of organisms with widely varying doubling times without risk to health. The research was thus limited to that reported below.

5.8.2 Materials and Methods

The methods unique to this chapter that were used in selection experiments are given below. Other methods are as described in the appropriate sections of Chapter 3.

The type strains of *Blastochloris sulfoviridis*, *Blc. viridis*, *Rhodobacter capsulatus*, *Rhodoplanes elegans* and *Rpl. roseus* were used in a series of selection experiments. Each strain had been maintained in the laboratory for the past several years on basal salts medium, M27, MC or similar growth medium. The type strains were used as inocula for two sets of experimental lines. The strains of the first line, referred to as "**unselected**", were grown on medium MC at a temperature of 30°C. The strains of the second line, referred to as "**selected**", were grown at the maximum temperature known to support growth of the type strain (see Table 5.19). All cultures were allowed to grow to stationary phase and were then subcultured for 15 successive transfers.

The cultures that were the product of 15 successive transfers at either 30°C or their respective growth temperature maxima were then tested for their ability to grow at a range of temperatures between 25 and 42°C. Cultures were grown in duplicate at each temperature and the optical density at 660 nm of each culture was measured at hourly intervals throughout the exponential phase. Doubling times were calculated from these growth curves. Significance of differences was calculated using Student's t-test.

5.8.3 Results and Discussion

Initially, the maximum growth temperatures of mesophilic type strains of several species of purple nonsulfur bacteria were determined by incubating cultures in temperature-controlled waterbaths under constant illumination. Results are shown in Table 5.19. Inconsistent results were obtained for *Rps. palustris*. Growth was obtained initially up to 36°C, and the strain was then tested at higher temperatures. Most cultures failed to grow, but weak growth was occasionally seen at up to 39°C. Transfers of these cultures failed to continue to grow. Microscopic examination revealed enlarged and distorted cells. It is probable that cell wall synthesis is defective above 36°C leading to progressive weakening of daughter cells. Consistent subculture could only be achieved at 36°C.

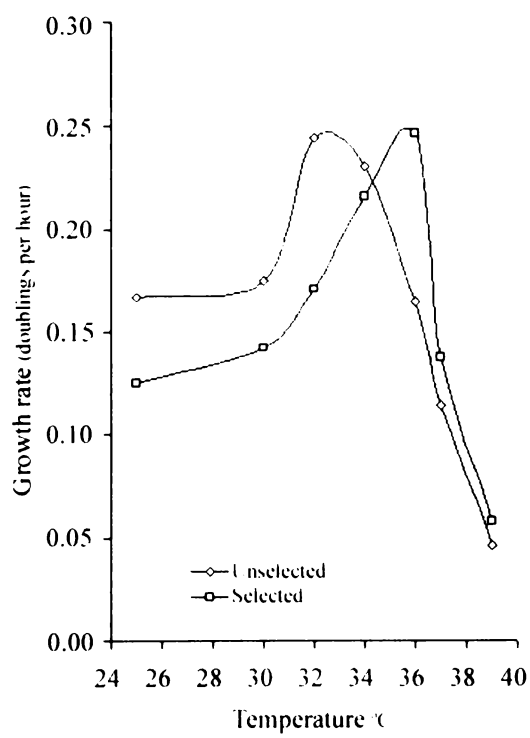
Species	Maximum growth temperature °C
Blastochloris sulfoviridis	40
Blastochloris viridis	40
Rhodobacter azotoformans	41
Rhodobacter capsulatus	38
Rhodocyclus tenuis	42
Rhodoplanes elegans	42
Rhodoplanes roseus	40
Rhodopseudomonas palustris	36
Rhodospirillum rubrum	39
Rubrivivax gelatinosus	42

Table 5.19 Maximum growth temperatures of type strains

The maximum tolerance temperatures (MTT) of a range of mesophilic type strains and thermotolerant strains isolated in the present study were determined by completely immersing inoculated tubes in water baths of set temperature for 64 hours,

under constant illumination. After 64 hours, the cultures were placed at their normal growth temperatures for one week. All strains, whether mesophilic or thermotolerant, were found to survive at temperatures up to 52°C, but none survived at 53°C, with the exception of the cyst-forming *Rhodomicrobium vannielii*, which was not challenged by pasteurisation for 15 mins at 60°C. At 60°C, the decimal reduction time of thermotolerant *Rhodoplanes* strain Wai3R3 was approximately 2.5 minutes, as determined by plating of time course samples.

The results of the selection experiments described in Section 5.8.2 are given in Figure 5.39. Variation in specific growth rate between replicates was between 2 and 4%. *Rba. capsulatus* (Figure 5.39 A) showed the most dramatic alteration to temperature response. In the selected line, growth was faster at 36°C and above than in the unselected line, and slower at lower temperatures. Most rapid growth was now achieved at 36°C rather than 32°C. Specific growth rates of the selected and unselected lines at these temperatures were significantly different ($p<0.01$, in each case). The selected line also grew significantly more slowly at 25°C and 30°C ($p<0.05$).



(A) *Rba. capsulatus*

Figure 5.39 Specific growth rates of unselected and selected cell lines as a function of temperature

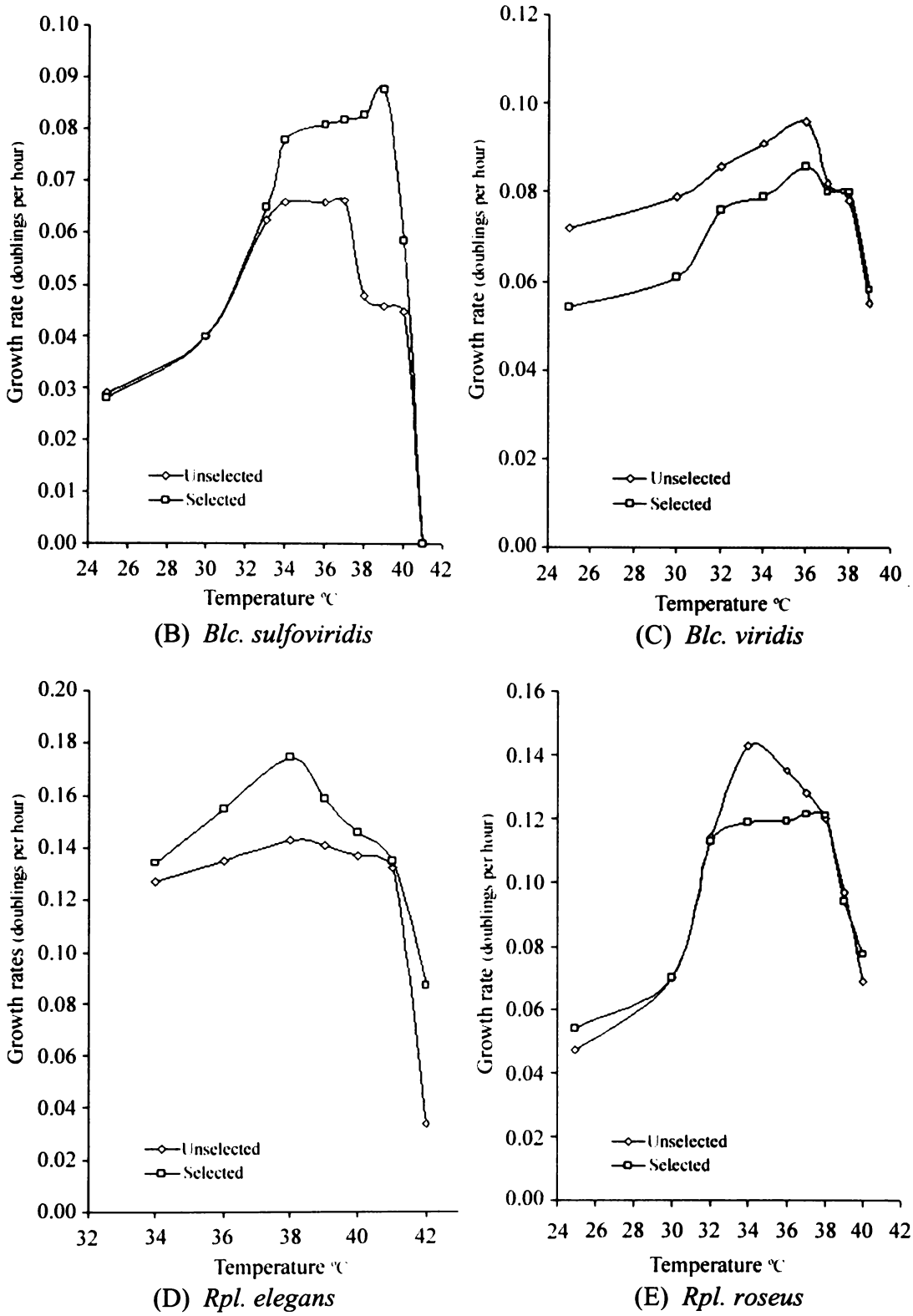


Figure 5.39 (cont.) Specific growth rates of unselected and selected cell lines as a function of temperature

The selected line of *Blc. sulfoviridis* (Figure 5.39 B) grew at the same rate as the unselected at low temperatures (25-33°C), and grew faster at higher temperatures, although the increase was not statistically significant at 34°C. In contrast, the selected *Blc. viridis* line (Figure 5.39 C) grew more slowly than the unselected at all temperatures up to 36°C and no significant change in specific growth rate was observed at higher temperatures.

The selected line of *Rpl. elegans* (Figure 5.39 D) grew faster at all temperatures than the unselected line, particularly at the maximum growth temperature, 42°C ($p<0.01$). No change in optimum temperature was observed. By contrast, no improvement in performance at high temperature was found in the selected *Rpl. roseus* line (Figure 5.39 E). In fact, growth at 34°C was significantly slower.

Selected lines were tested for the ability to grow at temperatures higher than the maximum temperature previously determined. No alteration in maximum temperature for growth was found in any of the selected lines. In tests of maximum growth temperature for the mesophilic strains, and also for the thermotolerant isolates, cultures that failed to show immediate growth were left under illumination for several weeks. No sudden delayed development of these cultures – due to appearance of a "Lazarus" mutant (Bennett and Lenski 1993) capable of growth at the temperature extreme - was observed. Development of the inocula occurred when tubes were removed from water baths and stored, showing that prolonged survival at non-permissive temperatures was possible.

In summary, *Blc. sulfoviridis*, *Rpl. elegans* and *Rba. capsulatus* selected lines showed improved growth rates at high temperatures. The entire temperature response curve of *Rba. capsulatus* was shifted in the direction of higher temperature. Improved performance at high temperatures was not associated with a decrease in growth rate at the lower temperatures tested for *Rpl. elegans* and *Blc. sulfoviridis*, although such a trade-off might have occurred at even lower temperatures. *Blc. viridis* and *Rpl. roseus* showed decreased growth rates at low temperature without experiencing any benefit at the other extreme.

For *Rpl. elegans*, the most dramatic increase in growth rate occurred at the temperature of selection (42°C). For *Rba. capsulatus* and *Blc. sulfoviridis*, selection

at the maximum growth temperature led to a more marked increase in growth rate at lower temperatures that were still suboptimal for the unselected line.

These experiments do not indicate whether alterations in growth responses in the selected line were due to physiological acclimation of the cells or to genetic adaptation of the selected population by differential growth of mutant organisms arising in the course of the serial transfers. The marked decrease in growth rate at low temperatures exhibited by some of the strains would indicate that genetic adaptation that cannot easily be reversed played a role. In one brief experiment, selected *Rba. capsulatus* cells were subcultured for one transfer at 30°C before being transferred to 38°C when growth rate was measured again. The growth rate was slightly lower (0.135 doublings per hour) than in the selected line that had been maintained at 39°C continually (0.147 doublings per hour), but not significantly so. Genetic adaptation therefore appeared to be largely responsible for the alteration in temperature response, though physiological acclimation played a part.

The implications of this series of experiments are intriguing. The thermotolerant *Blastochloris* strains that were isolated in this present study showed closer affiliations to *Blc. sulfoviridis* than *Blc. viridis*. The Waimangu and Orakei Korako *Rhodoplanes* strains were closer to *Rpl. elegans* than *Rpl. roseus* (although strain Tok2tar1 and "*Rps. cryptolactis*" form a separate line of descent marginally more closely related to *Rpl. roseus*). In the selection experiments, *Blc. viridis* and *Rpl. roseus* showed little ability to adapt to higher temperature over the short time scale of the selection regime compared with organisms such as *Blc. sulfoviridis* and *Rpl. elegans*. It may be that the latter organisms are "pre-adapted" to growth at higher temperature and more able to adjust to these conditions. One could also imagine that in an environment of fluctuating temperature, such as the margin of a hot pool with irregular activity, populations of organisms like *Blc. sulfoviridis* and *Rpl. elegans* could accumulate beneficial mutations during periods of elevated temperature without as a consequence suffering a reduced growth rate at low temperature that could lead to selective disadvantage during cold periods. Such a population could thereby "ratchet" itself into a new ecological niche.

Rba. capsulatus was the only organism that showed a shift in the optimum growth temperature after serial transfer at its temperature maximum. Nevertheless,

Rhodobacter strains were not detected in thermal environments. It may be that although *Rba. capsulatus* shows promise in adapting to higher temperature, its maximum growth temperature is initially too low to allow descendent populations to survive at the predominant temperatures of the sites sampled.

5.9 Discussion

In the course of this study, 35 isolates were obtained from geothermal areas. No strains resembling *Rhodocista centenaria* (Favinger *et al.* 1989; Kawasaki *et al.* 1992) were obtained, either because unsuitable enrichment methods were used, or because the organism is not present in New Zealand environments. Six isolates assigned to the genus *Blastochloris* were obtained that showed similarities to the previously described *Blastochloris* strain GI (Resnick and Madigan 1989). Other commonly-isolated strains were assigned to the genus *Rhodoplanes*. The previously described "*Rhodopseudomonas cryptolactis*" (Stadtwald-Demchick *et al.* 1990b) was found to resemble these isolates. A range of thermotolerant strains of the genus *Rhodopseudomonas sensu stricto* were isolated in the current work, and were found to lie in at least two clusters as part of the *Rps. palustris* strain complex. Thermotolerant representatives were also found for the genera *Phaeospirillum*, *Rhodospirillum* and *Rubrivivax*.

Some *Blastochloris* and *Rhodoplanes* isolates had temperature optima of 40-42°C and maximum growth temperatures of up to 46.5°C, and by the standards of the purple nonsulfur bacteria, at least, may be classed as mildly **thermophilic**. *Rubrivivax gelatinosus* strains also showed this pattern. Although several *Rhodopseudomonas* isolates were capable of growth at 46°C or slightly higher, the temperature optima of most isolates fell in the range 36-38°C. As such, these organisms represent **thermotolerant** purple nonsulfur bacteria. Other strains of *Rhodopseudomonas* had maximum growth temperatures of only 42-43°C, which is high relative to the type strain DSM 123, but not exceptional among PNSB species in general. Strain Wailvall could have been a transient inhabitant of Frying Pan Lake recently transferred by heavy rainfall.

Rubrivivax isolates had almost identical 16S rDNA sequences to the type strain, and strain WSSB3 was also closely related to *Rsp. photometricum*. Where more than one isolate was available, other thermotolerant strains showed a tendency to form individual lineages. That is, the Waimangu isolates clustered together with the independent Orakei Korako isolates, and "*Rps. cryptolactis*" clustered with strain Tok2tar1; mildly thermophilic *Blastochloris* strains clustered with the geographically remote strain GI; the most strongly thermophilic *Rhodopseudomonas* isolates also formed what is likely to be a monophyletic group. Taken together, these data suggest that thermotolerance is a phylogenetically-relevant trait, which in turn implies that emergence of a thermotolerant lineage is an infrequent event. However, the rapid adaptation in laboratory culture to growth at elevated temperature of some mesophilic type strains may indicate that thermotolerant lineages can arise independently from local mesophilic populations wherever suitable environments are to be found. Thermotolerant isolates from widely geographically separated locations are badly needed to examine to what extent each event occurs.

Rmi. vannielii strains from thermal areas showed no difference in optimum or maximum temperature for growth from mesophilic *Rhodomicrobium* isolates. The Orakei Korako strains had a higher specific growth rate at the temperature maximum, which is a response reminiscent of that observed for some of the 15 transfer selected lines of mesophilic type strains discussed in the previous section. Perhaps more thermotolerant *Rhodomicrobium* strains exist that were not isolated. Possibly the Orakei Korako strains have only recently adapted to high temperature environments. A third explanation is more speculative. On transfer to a high temperature environment, dividing *Rhodomicrobium* cells may produce predominantly exospores in the subsequent generation. Whether exospore formation is directly triggered by exposure to high temperature, or secondarily as cell functions cease to operate optimally, the result would be that *Rhodomicrobium* cells transform to heat resistant dormant cysts that would not be subject to selection for mutants capable of improved growth at high temperature. Some aspects of this hypothesis could be tested in the future.

Previously described mildly thermophilic purple nonsulfur bacteria grow no higher than 47°C. The characterisation of New Zealand isolates did not lead to the discovery

of any that were capable of growth above 47°C, reinforcing the idea that this temperature represents a thermal limit for PNSB. Other photosynthetic organisms, notably heliobacteria and cyanobacteria, grow to much higher temperatures. Among purple bacteria, the γ–proteobacterium *Thermochromatium tepidum* is able to grow well at 55°C (Madigan 1986), and this may reflect differences in the structure and function of the purple sulfur bacterial variant of the photosynthetic apparatus. Several aerobic anoxygenic phototrophic organisms of the α-4 subdivision genus *Porphyrobacter* are capable of growth at 50°C (Hanada *et al.* 1997; Silva *et al.* 1998), but these organisms may not be carrying out photosynthesis at this temperature. *Rhodobacter blasticus* RB-5, isolated from a Russian hot spring, was reported to have a temperature optimum of 25°C under photoheterotrophic conditions, but a broad optimum of 25-40°C and a maximum of 54°C in darkness (Yurkov and Gorlenko 1992a). Nonetheless, the thermotolerant PNSB isolated in the current work were not able to grow at temperatures higher than 47°C (or their respective maxima for photosynthetic growth) under non-photosynthetic conditions. Indeed, dark aerobic growth of *Rhodopseudomonas* strain Wai1S1 was achieved at 42°C but not 46°C.

The presence of PNSB in hot springs has been reported at temperatures up to 54°C (Yurkov *et al.* 1992; Yurkov and Gorlenko 1992b), which is several degrees higher than the maximum tolerance temperature of all mesophilic and thermotolerant strains that were tested (Table 5.19). This suggests either that the PNSB at higher temperatures were recently washed in from locations of lower temperature and would not have persisted, or that resting forms of cells are capable of survival at temperatures above the MTT of log phase cells, which is not unlikely. The maximum tolerance temperatures of stationary phase cells were not determined in the current work.

Rhodoplanes strain Tok2tar1 had an extremely slow growth rate, far lower than that of other thermotolerant *Rhodoplanes* strains at all temperatures. It is possible that suitable cultivation conditions were not obtained in the laboratory, and that more rapid growth could occur under truly optimal conditions. It is commonly stated that in the natural environment most bacteria exist under starvation conditions and for most of the time do not actively grow. This carbon limitation probably applies less to photosynthetic organisms, particularly autotrophs, although anoxygenic phototrophs

must still obtain an electron donor much less common than water. Under conditions of active growth, though, it would seem likely that Tok2tar1 would be rapidly out competed in the presence of other thermotolerant PNSB. It is perhaps notable then that Tok2tar1 was obtained from a geothermal area from which no other purple phototrophs were observed, and where there would therefore be few other organisms in direct competition with it.

5.10 Summary

Thirty-five isolates from New Zealand geothermal areas were characterised. Mildly thermophilic and thermotolerant strains were found, with maximum growth temperatures up to 46.5°C. Some *Rhodoplanes* isolates produced an unusual long-wavelength absorption shoulder centred on 900 nm. Some had a high requirement for nicotinic acid as a growth factor that was several ten-fold higher than that of close relatives. *Rhodoplanes* species were found to degrade nicotinic acid as a carbon source. It was suggested that the isolates represent two new thermotolerant species, proposed as *Rhodoplanes venustus* sp. nov. and *Rhodoplanes segnis* sp. nov. *Blastochloris* isolates formed two genospecies, but unambiguous differentiation of these strains from *Blc. sulfoviridis* was not possible. *Rhodopseudomonas palustris*-like strains fell into two relatedness groups, distinguishable by differing sequences in the 16S rRNA helix 8. More thermotolerant strains formed a monophyletic group that did not include mesophilic New Zealand isolates. Thermotolerant strains were intermingled with other *Rps. palustris* strains in the other cluster. By contrast, *Rhodomicrobium* isolates were not particularly thermotolerant. *Rvi. gelatinosus* isolates, while mildly thermophilic, were indistinguishable from the type species by 16S rDNA sequence. Two thermotolerant spirilla were found. One, strain MPA1, is proposed as the type strain of a novel species, *Phaeospirillum tepidarium* sp. nov.

Mesophilic strains of PNSB were found to show markedly different patterns of adaptation to higher temperature that had interesting parallels with the identities of the organisms isolated from natural thermal environments.

5.11 Suggestions for further work

Some speculative comments that could provide the basis for further work have been made in the discussion (Section 5.9) above. The characterisation of a new isolate can almost never be said to be complete. The discovery of more thermotolerant strains of particular species, notably of *Rhodospirillum* and *Phaeospirillum*, would be of value in expanding our view of these organisms. Examination of acclimation and adaptation, both of mesophilic strains, and of thermotolerant isolates to examine whether they have reached the limits of adaptation, could provide research material for many years, for a suitably large group. Stress responses of *Rhodobacter sphaeroides* have been examined by Nepple and Bachofen (1997) and Nepple *et al.* (1999). On 2-D electrophoresis gels, 34 heat-shock related proteins could be detected. Examination of the heat shock responses of other mesophilic and thermotolerant PNSB could be of interest.

Chapter 6

Detection of Photosynthetic Bacteria in Environmental Samples using Molecular Techniques

6.1 Overview

It has been known since the earliest days of microbiology that direct microscopic counts of organisms exceed counts obtainable by culture-based techniques by at least several orders of magnitude (Amann *et al.* 1995). This phenomenon has been referred to as the "great plate count anomaly" (Staley and Konopka 1985). The uncultured majority consist of known or unknown species that have entered a viable but non-culturable state or unknown species for which no suitable culture conditions have been devised. In addition, under usual culture conditions the same restricted set of species is often repeatedly enriched from a given sample. Such culture bias can partly be avoided by direct plating of samples, but rare colonies may be visually indistinguishable from the dominant type and so be overlooked.

In order to examine the diversity of unculturable microorganisms, techniques that avoid the need for cultivation have been developed. While chemotaxonomic markers such as extractable lipids and carotenoids (Shiea *et al.* 1991) and quinones (Hiraishi *et al.* 1999) have been used to examine changes in species composition between environmental samples, most non-culture based methods involve analysis of nucleic acids extracted from the environment. These studies were pioneered by Stahl *et al.* (1985) using 5S ribosomal RNA and Ward *et al.* (1990) using 16S ribosomal RNA. These researchers isolated and reverse-transcribed these ribosomal RNA molecules from hot springs in Yellowstone National Park and cloned and sequenced the cDNA. The development of PCR greatly facilitated the methods used for obtaining 16S rRNA sequences from the environment. 16S rRNA genes have become the target of choice for molecular diversity studies, since (a) they are present in all organisms, (b) originated from a common ancestor, (c) are long enough to provide sufficient sequence information, (d) contain both conserved and variable regions, and, finally, are thought to have a negligible frequency of horizontal transfer so that, unlike many functional genes, they represent the overall lineage of the organism (Rosado *et al.* 1997; Woese 2000).

16S rDNA primers can be designed to be specific for groups at many different phylogenetic levels, from domain specific primers (such as pA and pH* used in the present work, which are specific to Bacteria) to division level, such as cyanobacteria-specific primers used by Nübel *et al.* (1997), to subdivision level, such as primers specific for δ-Proteobacterial sulfate-reducing bacteria (Teske *et al.* 1996). Primers specific for a very broad group are certain to miss some representatives of the population, leading to an underestimation of the diversity present. Other biases may be due to differential lysis of cells, variable efficiency of nucleic acid extraction, biased amplification in PCR steps due to selectivity of the primers used or variable amplification rates.

Moreover, a 16S rRNA sequence by itself does not necessarily provide any information on the physiological properties of the organism from which it was obtained. Many 16S rRNA-defined phylogenetic groups are comprised of bacteria that share common physiological properties. For instance, all known members of the green sulfur bacteria division are photosynthetic, all members of the Aquificales are thermophilic hydrogen-oxidisers, and the distinctive spirochaete morphology is shown by all members of that division. However, this correspondence of physiological type with phylogenetic position is not universal. Within the *Bradyrhizobium* branch of the α-2 division of the Proteobacteria are found the photoheterotroph *Rhodopseudomonas palustris*, nitrite-oxidising lithoautotrophs such as *Nitrobacter*, symbiotic N_2-fixers such as *Bradyrhizobium*, and the causative organism of cat-scratch fever, *Afipia felis*. An uncultured group of organisms from Obsidian Pool in Yellowstone National Park was assumed to consist of sulfate-reducing bacteria on the basis of 16S rRNA affinities until the first cultured isolate proved to be an Fe(III)-reducing autotroph that was unable to reduce sulfate, indicating that these organisms are as likely to be Fe(III)-reducers (Kashefi *et al.* 2002).

When specific and unambiguous information is desired on the diversity in an environment containing organisms capable of carrying out a particular physiological process, that information must be obtained by amplifying genes involved in that particular physiological process. Amplification of functional genes is especially useful when the physiological trait is found in organisms of diverse phylogenetic

origin, or when organisms possessing and lacking the trait are intermingled in a particular 16S rRNA-defined group.

The environmental diversity of a number of physiological groups of organisms has been examined by analysis of functional gene sequences. Some of these studies are listed in Table 6.1. This list is by no means exhaustive.

Gene	Gene description	Targeted organisms	References
hydB	[NiFe] hydrogenase	*Desulfovibrio*	Wawer *et al.* 1997
amoA	Ammonia monooxygenase alpha subunit	Ammonia-oxidising bacteria	Rotthauwe *et al.* 1997; Purkhold *et al.* 2000
mxaF	Methanol dehydrogenase	*Hyphomicrobium*, methylotrophs	Fesefeldt and Gliesche 1997
nirK, *nirS*	Nitrite reductases	Denitrifying bacteria in forested and marsh soil	Braker *et al.* 1998; Priemé *et al.* 2002
various	Multi-component phenol hydroxylases	Phenol-degraders in activated sludge	Watanabe *et al.* 1998
dsr	Dissimilatory sulfate reductase	Sulfate-reducing bacteria in hypersaline mats	Minz *et al.* 1999
nosZ	Nitrous oxide reductase	Denitrifying bacteria in marine sediments	Scala and Kerkhof 1999
pmoA	Particulate methane monooxygenase subunit	Methylotrophs in water, peat soil and rice roots	Fode-Vaughan *et al.* 2001; Horz *et al.* 2001
mmoX	Soluble methane monooxygenase subunit	Methylotrophs on rice roots	Horz *et al.* 2001
cbbL, *cbbM*	RuBisCo subunits	CO_2-fixing bacteria in deep sea samples	Elsaied and Naganuma 2001
tet cluster	Tetracycline resistance genes	Swine effluent organisms	Aminov *et al.* 2002

Table 6.1 Functional genes targeted in environmental diversity studies

Most commonly, environmentally amplified gene fragments are cloned into plasmid vectors to enable sequence analysis. Direct electrophoretic analysis methods that allow rapid examination of community composition, have been developed recently. Denaturing gradient gel electrophoresis (DGGE) relies on separation of PCR products according to sequence-dependent differences in their melting behaviour (Muyzer *et al.* 1993). Single strand conformation polymorphism (SSCP) analysis separates products according to the differential mobility of sequence-dependent secondary structures formed by separated DNA strands (Widjojoatmodjo *et al.* 1995; Schwieger and Tebbe 1998). In T-RFLP (Liu *et al.* 1997; Clement *et al.* 1998), PCR products amplified with fluorescent dye-labelled primers are subjected to restriction analysis. The terminal fragments only are detected by their fluorescence. In a complex sample

containing many organisms, the RFLP profile obtained is simplified and the ease of associating particular fragments with known organisms is increased.

As described in Chapter 4, a range of species of purple photosynthetic bacteria was isolated from many of the samples taken from geothermal sites. *Rubrivivax gelatinosus*, *Rhodomicrobium vannielii*, *Blastochloris*, *Rhodoplanes* and *Rhodopseudomonas palustris*-like strains were repeatedly isolated from Orakei Korako microbial mats or observed in enrichment cultures, while purple sulfur bacteria were observed frequently in enrichments. The strains that were isolated may be the only PNSB species in the sampled environment, or they may be numerically dominant, or they may have a selective advantage over other PNSB species under the culture conditions employed. It is possible that other species of PNSB are present which are routinely outcompeted, or even that there are species present which are incapable of growth under the enrichment and culture conditions that were employed.

The "purple nonsulfur bacteria" are a group of organisms related by an aspect of their physiology; they are phylogenetically incoherent. Representatives are found throughout the α- and β-subdivisions of the Proteobacteria, intermixed with non-photosynthetic relatives. A similar though less severe situation holds for the purple sulfur bacteria, which, while restricted to the γ-subdivision, have a small number of known non-photosynthetic relatives, such as *Arhodomonas aquaeolei* (Adkins *et al.* 1993) and *Achromatium oxaliferum* (Head *et al.* 1996). This phylogenetic diversity and specific relatedness to non-photosynthetic organisms prevents the design of PCR primers targeted at the 16S rRNA gene that would amplify the 16S rRNA genes of all members of the purple nonsulfur bacteria exclusively, that is without also amplifying those of non-photosynthetic species. If this physiologically-defined set of organisms is to be examined through molecular methods, it is necessary to target genes responsible for the photosynthetic process, which are by definition present in all members of the group regardless of phylogenetic affiliation. The genes should also be absent from non-photosynthetic organisms, which rules out for example the RuBisCo gene which is also present in many autotrophic organisms. The photosynthetic operons of a wide range of purple photosynthetic bacteria have been sequenced completely or partially (*Rba. sphaeroides*, Naylor *et al.* 1999; *Rubrivivax gelatinosus*, Nagashima *et al.* 1994; *Allochromatium vinosum*, Corson *et al.* 1999;

Thermochromatium tepidum, Fathir *et al.* 1997). The widest range of available sequences are those of the *pufL* and *pufM* genes that encode the L and M subunits of the purple photosynthetic reaction centre; the *pufL* and *pufM* genes of a majority of the purple nonsulfur bacteria and a smaller number of purple sulfur bacteria have been sequenced (Nagashima *et al.* 1995; Nagashima *et al.* 1997a; and many papers relating to individual organisms).

It was decided therefore to attempt to examine the diversity of purple nonsulfur bacteria using primers specific for one or both of these genes, unique to purple phototrophs, and present universally throughout the group.

When the present research was begun, no reports on the use of *puf* genes to examine the diversity of photosynthetic organisms had been published. Three studies that involve the amplification from environmental samples of genes involved in the photosynthetic process in purple bacteria have been published since the completion of the research reported in this chapter.

Achenbach *et al.* (2001) described the design and testing of primers specific for each of the groups of photosynthetic bacteria. Primers targeting 16S rDNA sequences were used in the study of the phylogenetically coherent groups of heliobacteria, green nonsulfur and green sulfur bacteria. To study purple photosynthetic bacteria, primers were designed to amplify 229 bp of the *pufM* gene. The primer set also amplified the homologous gene from *Chloroflexus*. An amplification product from purple photosynthetic bacteria was successfully obtained from samples from two antarctic lakes and a Yellowstone spring.

Fode-Vaughan *et al.* (2001) amplified a 191 bp fragment of the *pufM* gene directly from a lake water sample without prior DNA extraction, but no further analysis of the amplification product was carried out.

Beja *et al.* (2002) carried out the first published study to report the sequences of environmentally-derived *pufM* genes, amplified from surface marine water samples. Sequences approximately 600 bp in length were obtained by PCR. A wide diversity of sequences showing general similarities to aerobic phototrophic bacteria was obtained.

6.2 Materials and Methods

Methods involved in the construction of a clone library of *pufL* fragments amplified by PCR from environmental DNA are given below.

6.2.1 Extraction of DNA from microbial mat material

Samples of mat material from site OK5 were immediately frozen in liquid nitrogen in the field, and stored at –70°C in the laboratory. At a later date, DNA was extracted from an approximately 150 mg portion of finely-chopped mat material using the FastDNA SPIN Kit for Soil (Bio101, California, USA) with some minor modifications of the manufacturer's instructions. Briefly, mat material was placed in Tissue Matrix Tubes containing silica and ceramic beads of various sizes; Sodium Phosphate and MT kit extraction buffers were added and the tubes were shaken on an orbital shaking device at full speed for four 30 second periods interspersed with cooling on ice; the tubes were then centrifuged in a bench-top centrifuge at 14000g for 30 s to pellet solid material, and the supernatant transferred to an eppendorf tube; PPS reagent was added to precipitate protein, and the tubes were centrifuged for a further 5 min; Binding Matrix Suspension was added to the transferred supernatant; after mixing for 2 minutes to allow binding of DNA to the matrix, the suspension was allowed to settle for 3 minutes, before discarding of 500 µL of the supernatant; the resuspended Binding Matrix Suspension was loaded in two portions onto SPIN filters which were centrifuged at 14000g for 1 minute; SEWS-M ethanol salt wash solution was then spun through the filter at 14000g for 1 minute; filters were dried of residual ethanol by spinning for a further 2 minutes and by air-drying for 5 minutes; finally, 50 µl DES (DNAse free water) was added to the filter tube, the pelleted binding matrix was stirred by gently tapping of the tube, and the eluted DNA transferred to a clean catch tube by centrifugation at 14000g for a further minute. The eluted DNA solution was at this point slightly discoloured, perhaps with phenolic compounds or pigments. Polyvinylpolypyrrolidone (PVPP) binds humic acid without appreciable loss of DNA (Steffan *et al.* 1988). One-third volume of PVPP previously fully hydrated in TE buffer was added to the tube, mixed and allowed to stand for 10 minutes, and then removed by centrifugation. Finally, the DNA solution was purified using the Wizard DNA Purification system (Promega, Sydney, Australia). The final yield of DNA from 150 mg of mat material was approximately 8 µg.

6.2.2 PCR of *pufL* gene fragment

Two different primer pairs were used in the amplification of an approximately 640 bp fragment of the *pufL* gene (see Section 3.9.4.2). The forward primer used was pL2. Either of two reverse primers, pLr4 and pLr5, each binding to the same position of the *pufL* gene but designed to target a different set of organisms, was used. PCR conditions were identical for each primer pair.

After an initial denaturation at 94°C for 3 minutes, PCR was carried out for 30 cycles, with 45 s denaturation at 94°C, 60 s annealing at 54.5°C, and extension at 72°C for 50 s. A final extension at 72°C for 5 minutes was then performed.

The PCR product was purified on a 1% (w/v) agarose gel; DNA was recovered from the excised gel slice using spin columns made from eppendorf tubes. DNA eluted from the spin column was finally purified using the WizardPrep system.

6.2.3 Cloning of PCR products into the plasmid vector pCR®2.1-Topo® and transformation of *E. coli* Top10

The PCR product obtained by amplification of OK5 mat DNA with primers pL2 and pLr4 was cloned using topoisomerase cloning into the vector pCR®2.1-Topo®. This plasmid is supplied linearised with single 3'-thymidine (T) overhangs for TA Cloning® of PCR products amplified using Taq polymerase, which non-specifically adds adenosine residues to the 3'-ends of amplification products. The enzyme Topoisomerase I from a vaccinia virus is bound to the vector (referred to as "activated" vector) by a covalent bond between the enzyme and the 3'-phosphate of the linearised vector. The 5'-hydroxyl group of a PCR product can attack the covalent bond, releasing the topoisomerase and completing ligation. A map of the cloning vector and a schematic of the ligation reaction of the activated vector are shown in Figure 6.1.

The Top10 strain of *E. coli* is used for blue/white screening without ITPG. Top10 cells were supplied chemically competent. The strain has the following genotypic description:

F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

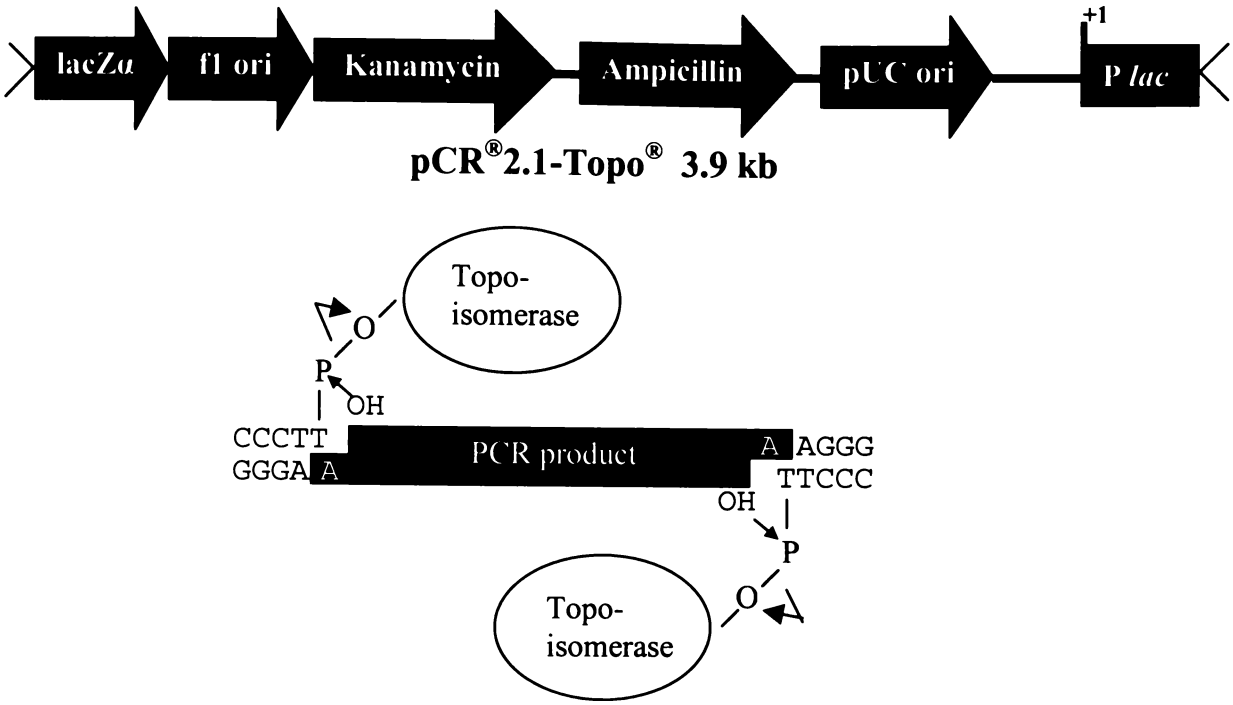


Figure 6.1 Map of the linearised cloning vector pCR®2.1-Topo and schematic of the ligation reaction

*Lac*Za fragment: bases 1-547; M13 reverse priming site: bases 205-221; Multiple cloning site: bases 234-357; T7 promoter/priming site: bases 364-383; M13 Forward (-20) priming site: bases 391-406; f1 origin: bases 548-985; Kanamycin resistance ORF: bases 1319-2113; Ampicillin resistance ORF: bases 2131-2991; pUC origin: bases 3136-3809

The cloning reaction was set up as shown in Table 6.2.

Reagent	Volume (µL)
Fresh PCR product	2
Salt solution	1
Sterile water	2
TOPO® Vector	1
Final Volume	**6**

Table 6.2 Composition of cloning reaction mixture

After incubation at room temperature for 15 minutes, 2 µL of the cloning reaction was added to a tube of chemically competent Top10 *E. coli* cells, which were then incubated on ice for 20 minutes. The cells were heat-shocked in a water bath at 42°C without shaking for 30 s, then immediately returned to ice. 250 µL room-temperature SOC medium was added and the tube was then shaken horizontally at 200 rpm at

37°C for 1 hour. After this period, 20 and 50 µL portions of the culture were plated out onto two LB plates containing 50 µg/mL kanamycin that had previously been spread with 40 µL of 40 mg/mL X-gal and dried at 37°C. Plates were incubated at 37°C overnight, and examined for white colonies the following day.

6.2.4 Isolation of plasmid from white colonies

White colonies were pricked out and restreaked in defined positions on LB plates containing 50 µg/mL kanamycin which were also incubated overnight and then stored at 4°C. Portions of streaks were used to inoculate tubes containing 3 mL of LB broth containing 50 µg/mL kanamycin, which was then incubated at 37°C overnight with shaking in an orbital incubator. 1.5 mL of each overnight culture was transferred to eppendorf tubes and cells were harvested by centrifugation in a bench-top centrifuge. Plasmid was extracted using the Wizard Plus Minipreps DNA Purification system (Promega) according to the manufacturer's instructions, or by standard SDS-alkaline lysis procedures.

6.2.5 PCR of insert from purified plasmid DNA

PCR of insert from purified plasmid was carried out as detailed in Section 6.2.2. 1 µL of plasmid DNA was used as template. PCR products were purified using WizardPreps, and were eluted in sterile water.

6.2.6 Screening of inserts by RFLP

In initial screening of clones, PCR-amplified inserts were digested with the restriction enzyme MvaI. Inserts yielding MvaI restriction fragments of identical length were also digested with HaeII. Reactions were set up as shown in Table 6.3. Digestions were carried out at 37°C for 2 hours. 10 µL of each restriction digest reaction was run on a 2% (w/v) TAE agarose gel.

Reagent	Volume (µL)
PCR product	16
Restriction enzyme buffer	2
Restriction enzyme (1 U/µL)	2
Final Volume	**20**

Table 6.3 Composition of restriction digest mixture

6.2.7 Sequencing of inserts and sequence analysis

DNA sequencing of inserts of interest was carried out as described in Section 3.9.4 using pL2 as the sequencing primer. Theoretical restriction digest patterns were determined using the online DNA analysis tools available at http://darwin.bio.geneseo.edu/~yin/WebGene/RE.htm. Phylogenetic analysis was carried out as described in Section 3.9.5. In one instance, a phylogenetic tree was constructed by the quartet-puzzling maximum likelihood method (Strimmer and von Haeseler 1996) using a web-based version of the program Tree-Puzzle 5.0 available at http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle-simple.html.

6.3 Results and Discussion

6.3.1 Extraction of DNA from samples

In preliminary attempts, DNA was extracted from microbial mat samples using variations on the Marmur method, that is sequential lysozyme and proteinase digestion followed by phenol extraction. PVPP extraction and WizardPrep column purification steps to remove contaminating material were necessary; without these steps, the extracted DNA was not amplifiable. Amplification was tested using the primers Sef1 and pH* which are specific for 16S rRNA genes.

The Bio101 FastDNA Spin Kit is designed to enable rapid extraction of PCR-ready genomic DNA present in soil samples. Cells present in the sample are lysed by bead-beating with a mixture of ceramic and silica particles of varying sizes, designed to efficiently lyse all microorganisms, in the presence of a chaotropic DNA stabilizing solution which is a proprietary mixture of detergents and salts. Extraction of microbial mat DNA using this kit was faster than by the Marmur method. The eluted DNA was however still discoloured, and PVPP and WizardPrep purification steps were retained.

6.3.2 Use of reverse primers pLr4 and pLr5

Selection of a reverse primer for amplification of a portion of the *pufL* gene took into account several requirements. It was thought desirable that the product to be amplified should be at least 400 bp long in order to provide sufficient sequence data for accurate phylogenetic placement; however, it might prove difficult to amplify too long a sequence from the generally highly fragmented DNA isolated from

environmental samples. In addition, it was necessary that digestion of the amplified fragment by a single restriction enzyme should lead to production of restriction fragments of differing length in most of the species thought likely to be found in microbial mat samples.

By simultaneous inspection of an alignment of purple nonsulfur and purple sulfur *pufL/pufM* sequences and restriction maps of each sequence, a location for a reverse primer which fulfilled the above criteria was selected, at positions 704-688 in the *Rsp. rubrum* sequence. The primer set would amplify 641 base pairs from most species; 644 bp would be amplified from *Rsp. centenum*, and 656 bp from *Allochromatium vinosum*.

Purple photosynthetic bacteria could be divided into two groups depending on whether a C or T was found at position 693. Therefore, two reverse primers pLr4 and pLr5 were designed, differing at this position, which should differentially amplify organisms from each group. Figure 6.2 shows the degree of matching of selected species with the pLr4 and pLr5 primer sequences.

	pLr4		pLr5
	ATYCACCGCCTCGGCCT		**ATYCATCGTCTGGGTCT**
Blc. viridis	ATTCACCGCCTCGGCCT	*All. vinosum*	ATCCATCGTCTGGGTCT
Rba. capsulatus	ATCCACCGCCTCGGCCT	*Blc. sulfoviridis*	ATCCATCGTCTTGGCCT
Rvi. gelatinosus	ATCCACCGCCTGGGTCT	*Rps. palustris*	ATCCATCGTGTGGGTCT
Rfx. fermentans	ATTCACCGCCTTGGTTT	*Rpl. elegans*	ATTCATCGCGTCGGCCT
Rsp. photometricum	ATTCACCGCCTTGGTGT	*Rmi. vannielii*	ATTCATAGACTCGGTGT
Phs. molischianum	ATTCACCGCATCGGCCT	*Rba. blasticus*	ATCCACCGTCTGGGCCT
Rsp. rubrum	ATCCACCGCGTGGGTCT	*Rcy. tenuis*	ATTCACCGTCTCGGTCT
Rba. blasticus	ATCCACCGTCTGGGCCT	*Blc. viridis*	ATTCACCGCCTCGGCCT
Rcy. tenuis	ATTCACCGTCTCGGTCT	*Rba. capsulatus*	ATCCACCGCCTCGGCCT
Rpl. elegans	ATTCATCGCCTCGGCCT	*Rfx. fermentans*	ATTCACCGCCTTGGTTT
Blc. sulfoviridis	ATCCATCGTCTTGGCCT	*Rsp. photometricum*	ATTCACCGCCTTGGTGT
All. vinosum	ATCCATCGTCTGGGTCT	*Rvi. gelatinosus*	ATCCACCGCCTGGGTCT
Rps. palustris	ATCCATCGTGTGGGTCT	*Rsp. rubrum*	ATCCACCGCGTGGGTCT
Rmi. vannielii	ATTCATAGACTCGGTGT	*Phs. molischianum*	ATTCACCGCATCGGCCT

Figure 6.2 Comparison of the reverse complement of primers pLr4 and pLr5 with the corresponding portions of the gene sequences of selected species

pLr5 should be more successful at amplifying gene fragments from *Blc. sulfoviridis*, *Rps. palustris* and *Allochromatium vinosum*, the three species that were most often observed in microbial mat samples. pLr4 therefore might be more successful in amplifying sequences of less common community members.

6.3.3 Development of an RFLP screening system for *puf* fragments

The restriction enzyme MvaI was selected as the primary enzyme for discrimination of amplified *puf* fragments, after inspection of restriction maps of the *pufL* and *pufM* genes in conjunction with selection of PCR primer locations. MvaI has the recognition sequence CC/WGG, where the position of the cut site is shown by the forward slash. The sizes of fragments generated by MvaI digestion of the *pufL* fragment of several purple phototrophs are shown in Table 6.4.

	Size of restriction fragments after digestion with enzyme:	
Species or strain	**MvaI**	**HaeII**
Blastochloris sulfoviridis DSM 729	540, 102	324, 176, 142
Blastochloris strain Wai3G1e	315, 210, 104, 11	413, 228
Blastochloris strain Wai5bG1	403, 134, 104	465, 176
Blastochloris viridis	527, 115	324, 176, 142
Phaeospirillum molischianum	331, 148, 93, 37, 32	227, 142, 137, 135
Rhodobacter blasticus	201, 163, 153, 91, 33	249, 174, 136, 70, 12
Rhodobacter capsulatus	314, 178, 149	272, 227, 142
Rhodobacter sphaeroides	278, 153, 70, 49, 47, 20, 16, 8	244, 143, 126, 118, 10
Rhodocyclus tenuis	400, 241	241, 136, 102, 98
Rhodoferax fermentans	256, 237, 68, 58, 22	414, 227
Rhodomicrobium vannielii	355, 278, 8	244, 148, 131, 118
Rhodoplanes elegans	168, 123, 114, 113, 110, 13	278, 244, 119
Rhodoplanes roseus	278, 123, 114, 113, 13	337, 279, 25
Rhodopseudomonas palustris	278, 123, 114, 94, 32	397, 244
Rhodospirillum centenum	281, 200, 70, 49, 44	348, 127, 98, 71
Rhodospirillum photometricum	400, 241	397, 141, 103
Rhodospirillum rubrum	316, 226, 52, 47	279, 175, 118, 69
Rubrivivax gelatinosus	318, 93, 79, 74, 70, 7	543, 98
Rubrivivax gelatinosus OK3O3	318, 246, 70, 7	279, 146, 118, 98
Allochromatium vinosum	278, 252, 126	335, 142, 137, 42

Table 6.4 Theoretical sizes of fragments generated by restriction digests of pL2/pLr4 *pufL* amplification product.

In general, it was predicted that the amplification product would be cut to produce two or three fragments greater than 100 bp in size (and thus readily resolvable by agarose gel electrophoresis). Two species, *Rsp. photometricum* and *Rcy. tenuis*, were predicted to give identical MvaI banding patterns. *Rpl. roseus* and *Rps. palustris* were

also predicted to give very similar patterns. The restriction enzyme HaeII, which recognises the sequence RGCGC/Y, was selected to allow discrimination of amplification products from these species. The fragments generated by HaeII digestion of the *pufL* amplification product are also shown in Table 6.4.

PCR products amplified from pure cultures using primers pL2/pLr4 or pL2/pLr5 were digested with MvaI or HaeII, confirming that the theoretically predicted RFLP patterns were obtained. An example is shown in Figure 6.3. *Blastochloris* strain Wai3G1e was predicted to give HaeII restriction fragments of 413 and 228 bp, *Blastochloris* strain Wai5bG1 fragments of 465 and 176 bp, and *Rvi. gelatinosus* strain OK3O3 fragments of 279, 146, 118 and 98 bp.

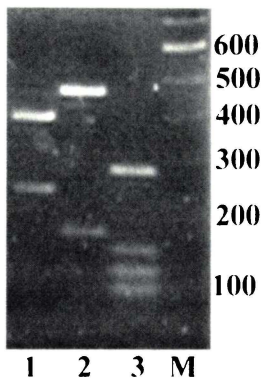


Figure 6.3 HaeII digests of 641 bp *pufL* fragments of *Blastochloris* strain Wai3G1e (lane 1), *Blastochloris* strain Wai5bG1 (lane 2) and *Rvi. gelatinosus* strain OK3O3 (lane 3), confirming theoretical restriction fragment size predictions. M=DNA size marker.

It would also be possible in theory to largely determine the species composition of a mixture of purple nonsulfur bacteria by a combination of MvaI and HaeII restriction fragment analysis of amplification products. The bands present on the gel in Figure 6.3 for example are well resolved and could readily be distinguished in a mixture of all three. The MvaI/HaeII RFLP system could also have been usefully employed to examine more complicated mixtures using the T-RFLP technique with fluorescent primer-labelled PCR products. However, examination of T-RFLP fragments would have required dedicated use of an automatic DNA sequencer run, which was not possible.

However, it should also be noted that organisms affiliated with *Blc. sulfoviridis* and *Rubrivivax gelatinosus* produced strain specific MvaI and HaeII RFLP patterns (Table 6.4 and Figure 6.3). On the one hand, this allows for finer level discrimination; on the other hand, if a similar situation were true for most species, then positive assignment of an RFLP pattern to a particular species on the basis of the RFLP patterns of type strains would not be possible. *pufL* sequences from multiple strains of a single species are available in too few cases to know how much intraspecific variation is present.

6.3.4 PCR analysis of OK5, WSS and Tok1 samples

Amplification of DNA purified from OK5 microbial mat samples using the Marmur method was achieved using both primers pL2 and pLr4, and pL2 and pLr5. However, at this point, an unexpected difficulty arose – the purified PCR products could not be digested by restriction enzymes, could not be sequenced and could not be cloned, although PCR products generated from pure cultures using the same methods were amenable to further manipulations. Presumably, a contaminant from the mat material was present which, although it had no effect on the action of Taq polymerase in the PCR reaction, was responsible for inhibition of restriction and sequencing, even after extensive purification of the DNA and dilution in the PCR reaction. Several authors have discussed the difficulties posed by inhibitory contaminants in environmental DNA preparations (e.g. Moreira 1998), but the methods of overcoming these problems generally refer only to steps preceding successful PCR amplification. However, occasional failure to clone PCR products amplified from rhizosphere soil samples has been reported (Head *et al.* 1998).

DNA was successfully isolated using the Bio101 FastDNA SPIN Kit from an OK5 mat sample obtained in January 2001. PCR amplification of this DNA was successful using the primer pair pL2 and pLr4 (see for example Figure 6.5). No product was obtained from this sample using pLr5. Two explanations are possible for this: the different DNA isolation methods may have resulted in differential isolation of nucleic acid from organisms falling under different reverse primer groups, or, more likely, the species composition of each OK5 mat sample was different.

DNA was also isolated from sediment at Waitangi Soda Springs (WSS) and from microbial mat material from site Tok1. The results of PCR amplification of this DNA

are shown in Figure 6.4. A product of the expected size was obtained only from the WSS sample using the reverse primer pLr4. No product was obtained from this sample using pLr5. No products of the expected size were obtained from the Tokaanu sample, supporting the suggestion that purple nonsulfur and purple sulfur bacteria are absent from this geothermal area.

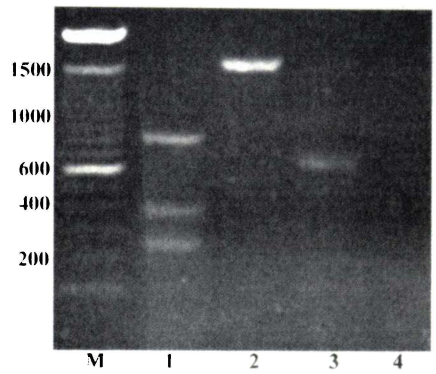


Figure 6.4 Amplification products obtained by PCR of Tok1 (lanes 1 and 2) and WSS (lanes 3 and 4) DNA using primer pairs pL2/pLr4 (lanes 1 and 3) and pL2/pLr5 (lanes 2 and 4). M=marker.

6.3.5 Estimation of the proportion of purple phototrophs in the OK5m sample

To gain some idea of the sensitivity of the pL2/pLr4 PCR amplification, PCR was employed on serially diluted DNA isolated from a pure culture of *Rhodobacter azotoformans* and on OK5m DNA. Serial dilutions were amended with *E. coli* DNA. Each 100 µL PCR reaction contained the same total amount of DNA, consisting of the appropriate amount of *Rhodobacter* or OK5m DNA and sufficient *E. coli* DNA to bring the total to 50 ng. If DNA is simply serially diluted, the abundance of the target sequence as a proportion of the total DNA remains constant. Successful amplification is more readily achieved than when the target sequence makes up a progressively smaller proportion of the total DNA, which is a more reasonable equivalent to the situation in an environmental sample, and allows for a better estimate of the sensitivity limit of the amplification as applied to experimental samples. Results of the serial dilution PCR are shown in Figure 6.5.

Ideally, most probable number techniques would be used on multiple PCR reactions containing target DNA diluted to extinction. Such an analysis, which would allow a more accurate estimation of phototroph numbers in the OK5m sample, was not

performed, both to conserve the OK5m DNA and because of the cost of materials that would be consumed.

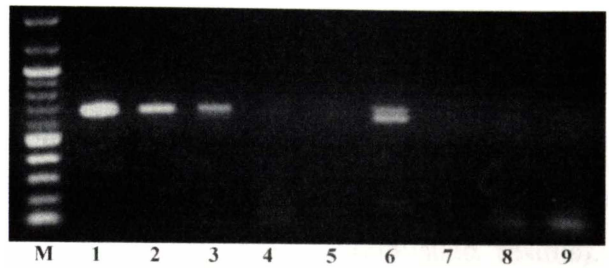


Figure 6.5 Amplification of *pufL* gene fragment from serially diluted DNA. Lanes 1-5, *Rhodobacter azotoformans* (10^0, 10^1, 10^2, 10^3 and 10^4 dilutions); Lanes 6-8, OK5m DNA (10^1, $5x10^1$ and 10^2 dilutions); Lane 9 *Escherichia coli* (50ng).

An amplification product was observed from the thousandfold dilution of *Rhodobacter* DNA, containing 50 pg of target (though this is difficult to see in Figure 6.5). Assuming a genome size of approximately 4 Mbp for *Rba. azotoformans* (Suwanto and Kaplan 1989), 50 pg of DNA would represent 11000 to 12000 copies of the genome. Amplification of OK5m DNA was observed at a one-tenth dilution (5 ng), but not at a five-fold further dilution. Thus, 1 ng of OK5m DNA must contain less than 50 pg of DNA bearing the target sequence; however, the intensities of the amplification products in lane 3 (500 ng) and lane 6 of Figure 6.5 are quite similar, so the amount is unlikely to be much less than 50 pg. Organisms containing the *pufL* gene therefore contribute around 5% of the total amount of isolated DNA from the OK5 sample, and presumably make up a similar proportion of the total number of cells present. 8 μg of DNA were isolated from 150 mg of mat material; a very rough estimate of the total count of *pufL* containing organisms would therefore be $5x10^8$ cells per gram of mat material. Small weight should be attached to this estimate, due to the inherent inaccuracy of the PCR experiment performed, the lack of knowledge of the genome sizes of the organisms present, and not least the incomplete extraction of DNA from the mat sample.

The slightly larger band in lane 6 of Figure 6.5, presumably consisting of an amplification product derived from purple sulfur bacteria, was observed only in the PCR reaction run on the gel shown in the figure, and was not observed in a repeated

amplification under the same conditions, or in any other OK5m amplifications. Unfortunately, it was not retained, so was unavailable for later cloning.

6.3.6 Attempts to detect *Rhodobacter* and *Rhodocyclus* in the OK5m sample

Rba. capsulatus, *Rba. sphaeroides* and *Rcy. tenuis* have been found to be ubiquitous and readily enriched from many samples, by other researchers (Burke *et al.* 1974; Guyoneaud *et al.* 1996; Imhoff and Trüper 1992) and in this laboratory (Janssen 1985; Lee 1992; McGrath 2000; Yuan 2000; unpublished results). It was therefore unexpected, considering the range of purple nonsulfur bacterial species isolated in the course of the current investigation, that no organisms belonging to the genera *Rhodobacter* or *Rhodocyclus* were observed in any enrichment set up on samples for geothermal areas. The organisms may indeed be absent from the sample sites; alternatively, they may be present in low numbers, such that they were not observed on direct isolation plates or they may be outcompeted by other nonsulfur purple bacteria that are faster-growing under the conditions of the enrichment cultures set up.

If *Rhodobacter* and *Rhodocyclus* species are present, it may be possible to detect their presence by specifically amplifying marker genes from these species. Accordingly, four PCR primers were designed that were specific for fragments of the *pufL* and *pufM* genes of these species.

Primers specifically amplifying *Rhodobacter* species were designated pRba and pRba-r. The primers targeting *Rhodocyclus* were designated pRcy and pRcy-r. The locations of these primer pairs with respect to the *pufL-pufM* suboperon and other PCR primers used are shown in Figure 6.6. pRba lies immediately downstream of the primer pLf2, which was not used for PCR.

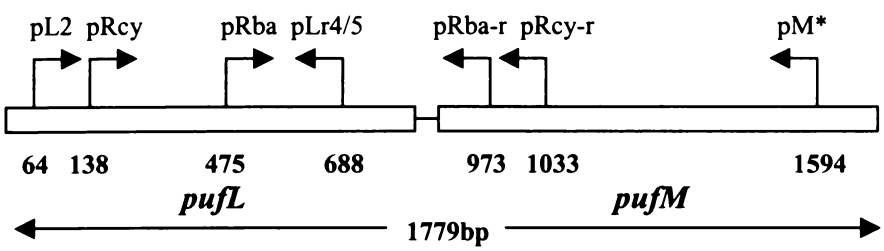


Figure 6.6 Schematic of primer binding sites on *pufL* and *pufM* genes

The design of *Rhodobacter*-specific PCR primers was achieved by inspection of an alignment of the *pufL* and *pufM* genes of three *Rhodobacter* species and 11 other purple sulfur and purple nonsulfur bacteria. Positions 475-492 were highly conserved between members of the genus *Rhodobacter* and divergent in other species, and were selected as the location for the forward primer binding pRba; in other species, three or four bases at the 3' end of the primer were mismatched (Figure 6.7), which would be more than sufficient to prevent priming. The reverse primer designated pRba-r was selected to bind opposite positions 973-990. This primer showed fewer mismatches with species outside *Rhodobacter*, although it would mismatch with the majority of species at the 3' base (Figure 6.8).

Rba. blasticus	CTCGATTGGGTCTCGAACACCGGCTACACCTACGGCAACTTC
Rba. capsulatus	CTCGACTGGGTGTCGAACACCGGCTACACCTACGGTAACTTC
Rba. sphaeroides	CTCGACTGGGTGTCGAACACGGGCTACACCTACGGCAACTTC
Rov. sulfidophilum	CTCGACTGGGTGTCGAACACCGGCTACCACTATCTGCACTTC
Blc. sulfoviridis	CTCGACTGGGTGAACAATTTCGGGTTCCAGTATCTCAACTGG
Rpl. elegans	CTCGACTGGGTGTCGAACGTCGGGTATCAAAACCTGCACTTC
Rps. palustris	CTCGATTGGGTCTCTAACACCGGCTATCAGTACGGCCAGTTC
Rmi. vannielii	CTAGATTGGGTATCCAACACCGGATATCAATATCTGCACTTC
Rsp. rubrum	CTCGACTGGGTGTCGAACACCGGTTACCAGTACGCCAACTTC
Phs. molischianum	CTGGATTGGGTGTCGAACGTCGGCTACCAGTTCCTGCACTTC
Rvi. gelatinosus	CTCGACTGGGTGTCGAACGTCGGTTACCAGTACCTGCACTTC
Rcy. tenuis	CTGGACTGGGTGTCGAATACCGGCTACCAGTACCTGCACTTC
All. vinosum	CTGGACTGGGTGTCGAATGTGGGGTATCAGTTCCTGAATTTC

Figure 6.7 Alignment of the portion of the *pufL* gene of selected PNSB species which contains the sequence of primer pRba, shaded (bases 475-492).

Rba. blasticus	GGCTGGTTCGGCAACGCCCAGATCGGACCGATCTATCTTGGC
Rba. capsulatus	GGCTGGATGGGGAACGCCCAGATCGGGCCGATCTATCTCGGC
Rba. sphaeroides	GGCTGGTTCGGCAACGCCCAGCTCGGCCCGATCTATCTCGGC
Rov. sulfidophilum	GGTTGGCTCGGCAATGCCCAGCTCGGCCCGATCTATCTGGGC
Blc. sulfoviridis	GGCCGTATCGGCGACGCTCAGATCGGTCCCGTCTATCTTGGT
Rpl. elegans	GGCAAGCTCGGTGATGCACAGGTCGGCCCGATCTATTTGGGT
Rps. palustris	GGTAAGATCGGTGACGCGCAGATCGGTCCGATCTACCTCGGT
Rmi. vannielii	GGCATCATCGGTGACGCGCAGATCGGCCCCATCTATCTCGGC
Rsp. rubrum	GGTAAGATCGGCGATGCGCAGATCGGCCCCATCTATCTGGGA
Phs. molischianum	GGCAAGGTCGCCAATGCGCAGATCGGGCCGATCTATCTCGGT
Rvi. gelatinosus	GGTCGCCTGGGCATGGCGCAGATCGGCCCGATCTACCTGGGC
Rcy. tenuis	GGCAAGATCGGCGACGCGCAGATTGGTCCGCTGTACCTGGGC
All. vinosum	GGCAAGATCGGTGACGCGGAGATCGGTCCGATCTACCTCGCC

Figure 6.8 Alignment of the portion of the *pufM* gene of selected PNSB species which contains the reverse complement of the sequence of primer pRba-r, shaded (bases 990-973).

The primer set pRba/pRba-r would theoretically amplify 514 bp from *Rba. capsulatus*, and 517 bp from *Rba. blasticus* and *Rba. sphaeroides*.

The design of a primer pair that would amplify *Rhodocyclus* was more straightforward. The *Rcy. purpureus puf* gene sequences were not available, so the design of the primers was based on the sequence of *Rcy. tenuis* DSM 109, in an alignment with the genes of 10 other species (Figures 6.9 and 6.10). This was not considered a particularly important limitation, as *Rcy. purpureus* is known from only a single strain, and is probably a rare species in nature (Imhoff and Trüper 1989).

Rcy. tenuis	CGCCCTGATGGGGACGCTGCTGATCGTACTGGGCGCTTCGTG
Rvi. gelatinosus	CTCGGTCCTGGGAACCGCGTTGATCATCTGGGGCGCTTCGCA
Blc. sulfoviridis	CGCCTCCCTCGGCATTTGTTTCATCGGCTATGCGGCCTCTCA
Rpl. elegans	TACCTTCCTCGGCACGGCACTCATCATTTGGGGCGCGGCCCT
Rps. palustris	CGCGCTTATGGGAACCGCGCTGATCATCTGGAACACGGCTCT
Rmi. vannielii	TGCTATCCTTGGAACGGCTTTGATCGTTTGGGGGGCTGCCCA
Rsp. rubrum	TACCGTTCTTGGCACCGCGCTGATCGTTTGGGGGGCCGCTCT
Phs. molischianum	CACCTTGCTCGGCGTCGCCCTCATCGTTTTGGCCGCGTCGCA
Rba. blasticus	CGCGGCGCTTGGCACACTTCTGATTCTTTACGGAACGGCGAT
Rba. capsulatus	CGCCACGCTCGGCTTTTTGCTTATCCTTTGGGGAGCCGCGAT
All. vinosum	TGCATTGTTGGTTGTGCTCCTGATCGTCTGGGGTGCAACCAT

Figure 6.9 Alignment of the portion of the *pufL* gene of selected PNSB species which contains the sequence of primer pRcy, shaded (bases 138-155)

Rcy. tenuis	TCACCGGTGTTGCATCACTGGTATGTGGTTTCATCGCCATCG
Rvi. gelatinosus	TCGGCCCGATCTACCTGGGCGCGCTGGGCATCCTGTCGATCG
Blc. sulfoviridis	CCTCCGGCATCGGTGGCATCACGTTCGGCTTGGTCGCGCTGC
Rpl. elegans	GGCTCGGCATCTTCTCGCTGCTCTGCGGCTTCATCGCCTTCG
Rps. palustris	TCACAGGTGTGGTGTCGGCGATCTTCTTCGCTTTTGCGATGC
Rmi. vannielii	TGACCGGCGTCGCTTCGATCATCTTCGGGTTCATCGCCATCG
Rsp. rubrum	CCACCGGTGTCCTGTCGCTGGTCTTCGGCTTCTTCGCCATCG
Phs. molischianum	GTTGGGGCGTGGCCTCGGCGCTGTGCTTCGGCATCGCGGCGG
Rba. blasticus	GCTGGGGCACTGTCTCGCTCATCTCGGGCGTGCTGTGGTTCA
Rba. capsulatus	TCGCCGGCACCGTCTCGCTGGCCTTCGGCGCGGCCTGGTTCT
All. vinosum	TCACCGGCACTCTGTCGATCATCTTTGGCTTCATGGCCATCT

Figure 6.10 Alignment of the portion of the *pufM* gene of selected PNSB species which contains the reverse complement of the sequence of primer pRcy-r, shaded (bases 1033-1016)

As *Rcy. purpureus* and *Rcy. tenuis* are closely related, it is possible that primers pRcy and pRcy would successfully amplify the target gene fragment from *Rcy. purpureus*,

but this was not attempted. pRcy bound to positions 138-155, while pRcy-r bound to the complement of positions 1033-1016, to amplify a theoretical 896 bp fragment of the *pufL* and *pufM* genes.

The *Rhodobacter* primer set was tested against DNA isolated from *Rba. azotoformans* and *Rba. blasticus*. *Rhodoplanes elegans* served as a negative control for the *Rhodobacter*-specific primers. Amplification using the universal primers pL2 and pLr4 was also performed. Results of the analysis are given in Figure 6.11. Products of the expected size were obtained from the *Rhodobacter* species, while no amplification product was observed from *Rpl. elegans* using the specific primers. The *Rhodocyclus* primers were similarly tested with the expected results (data not shown).

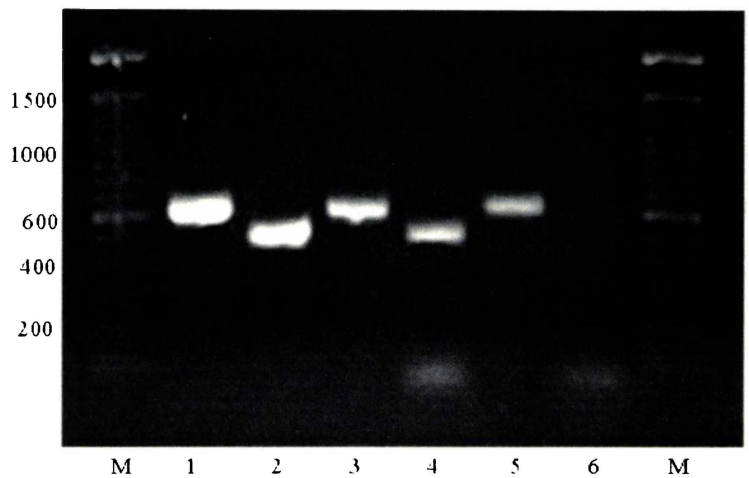


Figure 6.11 Amplification products obtained using the *Rhodobacter* specific primer set; Lane 1 *Rba. azotoformans* (pL2/pLr4); Lane 2; *Rba. azotoformans* (pRba/pRba-r); Lane 3 *Rba. blasticus* (pL2/pLr4); Lane 4 *Rba. blasticus* (pRba/pRba-r); Lane 5 *Rpl. elegans* (pL2/pLr4); Lane 6 *Rpl. elegans* (pRba/pRba-r). M=marker

PCR of OK5m DNA using the *Rhodobacter* and *Rhodocyclus* primer sets failed to produce any products, confirming that *Rhodobacter* and *Rhodocyclus* strains are present in low numbers at, or absent from, this sampling location.

6.3.7 Cloning of the OK5m PCR product

The OK5m *pufL* amplification product was successfully cloned into *E. coli* Top10 in the plasmid pCR®2.1-Topo® as described in Section 6.2.3. Approximately 150 white colonies were obtained on two plates.

6.3.8 Examination of the OK5m clone library

45 clones were picked for screening according to the methods given in Sections 6.2.4-6.2.6. PCR of the clone inserts gave from the majority of clones products of 640 bp; however as shown in Figure 6.12 below, a PCR product of almost 700 bp was produced from clone 9. It was thought that this was derived from a purple sulfur bacterium.

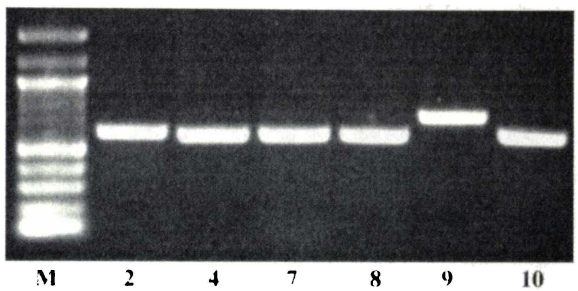


Figure 6.12 PCR products amplified from clones (as numbered) using primers pL2 and pLr4

The 45 clone inserts were subjected to digestion by MvaI and run on a series of 2% (w/v) TAE agarose gels. Composite gel photographs are shown in Figure 6.13 overleaf. Each distinct MvaI restriction digest pattern was designated by a letter code. The percentage contribution of each restriction pattern type to the overall composition of the clone library is shown in Table 6.5 and Figure 6.14 below.

Clone Group	Number of clones	Percentage of library
A	3	6.7
B	4	8.9
C	10	22.2
D	10	22.2
E	1	2.2
F	1	2.2
G	1	2.2
H	1	2.2
K	1	2.2
L	2	4.4
M	1	2.2
N	2	4.4
P	1	2.2
R	2	4.4
S	1	2.2
T	1	2.2
V	2	4.4
W	1	2.2

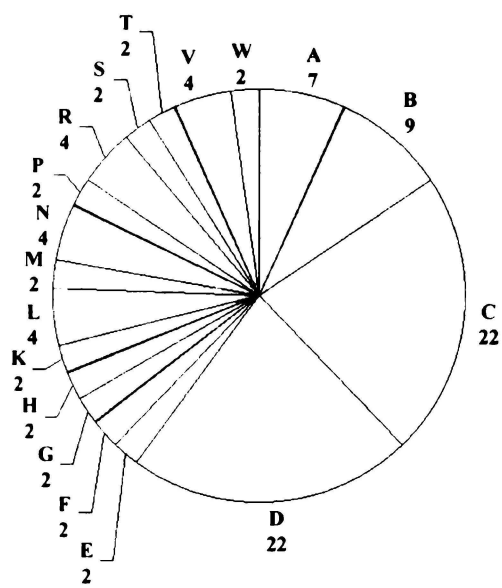


Table 6.5 and Figure 6.14 Percentage contribution of restriction digest pattern types to the clone library

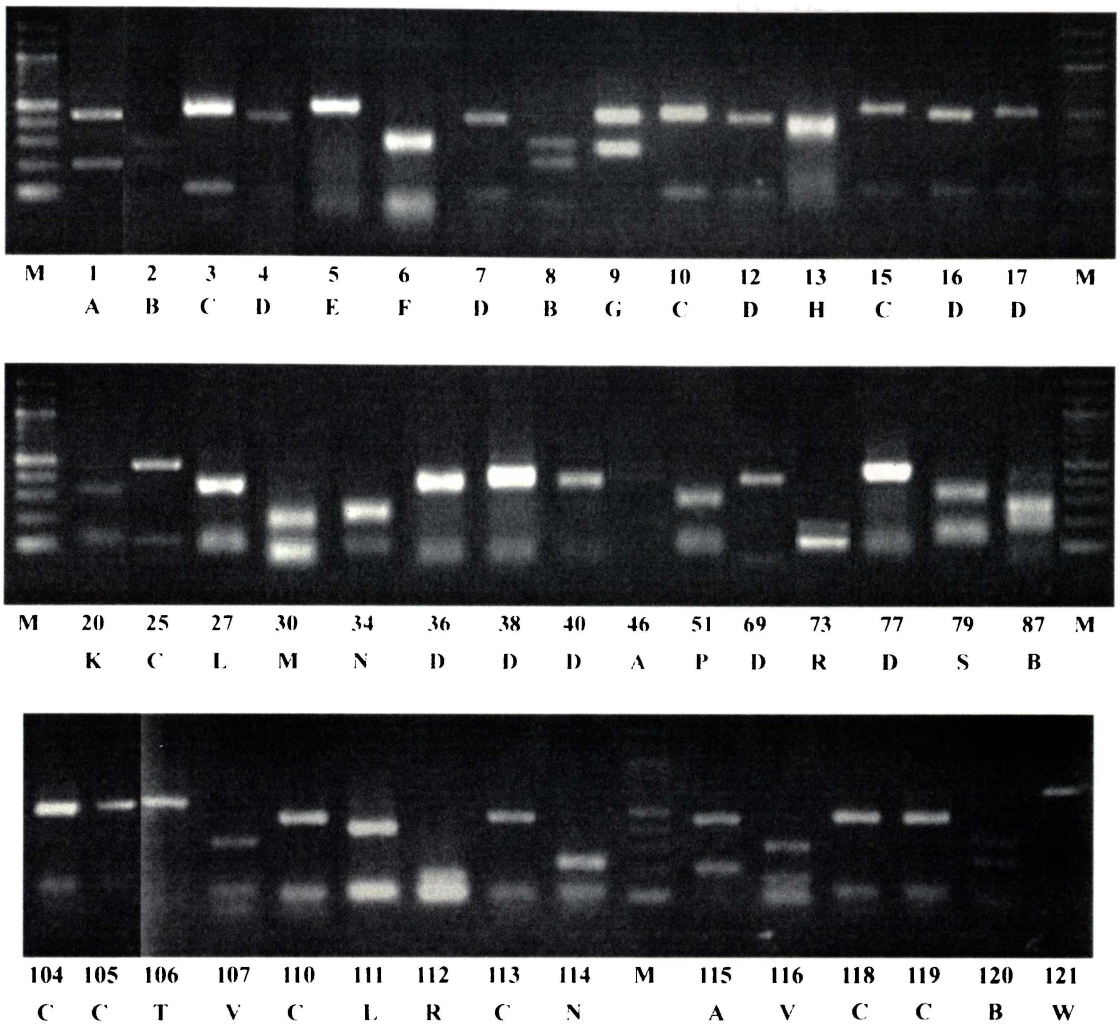


Figure 6.13 Composite gel photographs of MvaI digests of pL2/pLr4 PCR products amplified from OK5m clones. Legend: (top line) clone number, M=marker; (bottom line) letter code of restriction pattern type.

The clone library was dominated by clones which yielded the MvaI restriction digest patterns designated as types C and D. Moreover, the two patterns were difficult to distinguish. Therefore, several PCR products of type C and D clones were subjected to HaeII digestion (Figure 6.15). Each C type clone tested (clones 10, 110, 118) gave the same HaeII digest pattern of two bands approximately 400 and 240 bp in size. Each of the D type clones (clones 4, 7, 12 and 104) yielded 2 fragments of approximately 500 and 140 bp.

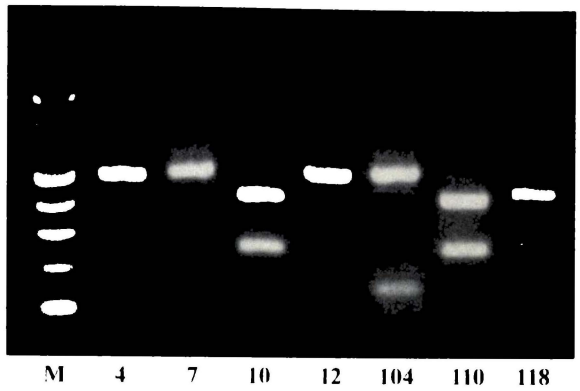


Figure 6.15 HaeII restriction digest of pL2/pLr4 PCR products amplified from OK5m clones. Legend: clone number; M=marker.

6.3.9 Rarefaction analysis

Rarefaction analysis was carried out using the program Analytic Rarefaction 1.3 (Holland 2001). The rarefaction curve shown in Figure 6.16 does not quite achieve saturation, indicating that analysis of more clones would be likely to reveal several more unique restriction patterns.

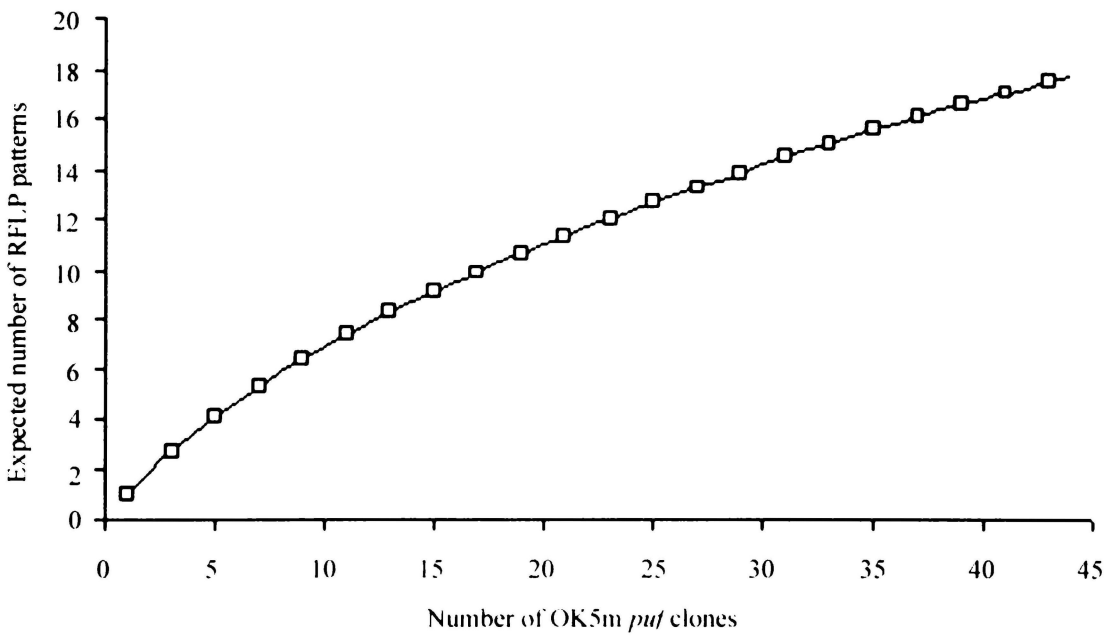


Figure 6.16 Rarefaction analysis of the *pufL* clone library. Grey lines mark 95% confidence intervals

6.3.10 Sequences of clones

PCR products that produced each of the unique restriction fragment patterns and several representatives from the major RFLP type groups (A, B, C, D) were sequenced in the forward direction using primer pL2. Very poor or no sequence was

obtained from sequencing of PCR products amplified from clones 1, 46 and 115 (group A), 5, 6, 9, 13 (E, F, G, H), 79, 106, 107 and 121 (S, T, V, W). High quality sequences were obtained from 15 clones, representing nine of the observed RFLP types. These sequences were initially examined by BLAST search against the GenBank database, and were all identified as showing closest similarities to *pufL* genes. However, none of the sequences was identical to any previously described *pufL* gene. Pairwise percentage homologies of DNA and deduced amino acid sequences of *pufL* fragments for clones and selected species are given in Tables 6.6 and 6.7. The sequence of clone 120 was identical to that of clone 87. The sequences have been deposited in the GenBank database under Accession Numbers AF484108-AF484110, AY048651-AY048656 and AY048658-AY048662. No chimeric sequences were found by visual inspection of sequence alignments. An alignment of *pufL* deduced amino acid sequences is given in Appendix 3 for reference.

6.3.11 Phylogenetic analysis

A neighbour-joining phylogenetic tree was constructed with Clustal X (Thompson *et al.* 1997) based on an alignment of 553 nucleotide positions of the *pufL* gene (Figure 6.17). 14 clone sequences and 35 *pufL* sequences from photosynthetic proteobacteria were used, including BAC 65D09, a sequence from an uncultured organism obtained from a bacterial artificial chromosome (Beja *et al.* 2002). The GenBank Accession Numbers of the sequences used are given in Appendix 1.

A neighbour-joining phylogenetic tree was also constructed using Clustal X on the basis of the deduced amino acid sequences of the *pufL* genes used to construct the nucleotide-based tree in Figure 6.17, and is shown in Figure 6.18. The amino acid sequences of clones 3 and 10 were identical, as were those of clones 4 and 69. The two phylogenetic trees show broad agreement, but there are some differences in the deeper branching orders and in the locations of certain deep-branching clone sequences.

It is immediately obvious that none of the 15 sequenced clones shows a close phylogenetic relationship to previously cultured organisms. Most of the clone sequences show strongest affiliations with aerobic phototrophic organisms. The clones fall into six phylogenetic clusters.

No.	Species or strain	Sequence similarity (%)																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	OK5m20		90.7	77.7	70.1	64.2	59.7	66.9	67.2	67.8	65.1	67.3	67.4	70.6	64.9	57.1	62.1	56.7	53.2
2	OK5m111	90.5		75.4	69.8	62.6	58.6	65.1	65.7	65.7	63.5	68.7	67.4	70.0	60.8	56.0	58.3	53.3	51.4
3	*Bradyrhizobium* ORS278	80.9	78.7		75.9	70.5	68.2	70.3	72.3	70.6	66.6	71.6	70.1	72.8	72.0	63.8	63.7	61.5	59.2
4	*Rhodopseudomonas palustris*	78.1	77.0	80.9		64.7	63.5	66.8	67.4	65.4	59.8	65.1	62.1	64.5	66.6	60.7	64.7	50.6	52.8
5	*Rhodospirillum rubrum*	73.0	73.0	75.3	73.0		64.9	67.1	65.7	68.3	64.5	64.2	65.4	66.3	67.7	63.2	61.4	52.5	53.6
6	*Rhodocyclus tenuis*	72.5	73.0	73.0	72.5	75.8		76.1	72.2	73.0	65.0	72.6	67.0	69.7	72.4	68.2	61.1	59.1	56.7
7	*Rubrivivax gelatinosus*	73.0	71.4	71.9	73.6	72.5	80.9		76.7	75.4	71.6	73.8	73.0	74.6	74.3	64.2	64.6	59.2	54.5
8	OK5m87	74.7	74.7	75.8	73.6	75.3	78.1	80.9		75.45	69.8	76.2	70.6	72.8	68.6	62.2	62.7	59.9	54.2
9	*Roseateles depolymerans*	72.5	73.0	71.4	71.4	71.9	81.5	82.0	83.7		71.4	79.0	70.1	72.0	70.3	63.9	68.3	58.6	57.6
10	*Rhodospirillum centenum*	71.9	70.8	68.5	68.0	65.7	71.9	73.6	81.5	79.2		74.6	69.1	71.1	68.0	64.1	62.4	51.6	53.6
11	OK5m51	71.9	75.3	69.7	70.2	70.2	76.4	76.4	82.0	81.5	84.3		74.1	76.4	70.8	65.5	66.5	59.8	54.4
12	*Rhodoplanes elegans*	71.4	71.9	70.2	69.7	73.6	74.2	77.5	79.8	71.9	74.7	80.3		92.1	71.5	65.4	63.0	54.3	51.4
13	*Rhodoplanes roseus*	73.0	73.6	71.9	71.9	74.7	77.0	78.1	82.0	75.3	77.0	82.6	92.7		73.0	66.5	63.3	58.7	53.8
14	*Phaeospirillum molischianum*	71.9	71.9	73.6	73.0	70.8	78.7	79.8	77.5	75.8	73.0	75.3	78.7	78.1		72.9	65.3	60.8	58.5
15	*Rhodospirillum photometricum*	64.6	64.6	67.4	66.9	67.4	71.9	70.8	70.8	68.5	68.0	70.8	77.5	77.5	77.0		56.6	52.7	54.2
16	*Rhodomicrobium vannielii*	69.7	68.5	68.0	71.4	70.2	74.7	74.7	75.8	76.4	73.0	74.2	70.8	73.6	74.7	70.2		54.0	51.1
17	*Blastochloris sulfoviridis*	61.8	60.7	60.7	57.3	55.6	63.5	61.2	62.4	65.7	61.2	62.4	59.6	60.7	64.6	58.4	61.2		82.0
18	*Blastochloris viridis*	64.0	61.8	64.0	60.1	57.3	65.7	62.9	64.0	66.3	61.2	63.5	61.8	62.4	66.3	62.4	64.0	90.5	

Table 6.6 Sequence similarities of *pufL* gene fragments from clones and related organisms (DNA similarity top right, deduced amino acid similarity bottom left)

No.	Species or strain	Sequence similarity (%)																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	OK5m03		99.3	71.7	68.1	68.1	68.6	68.6	70.3	70.6	73.6	69.4	64.9	64.6	61.5	64.3	61.5	52.4	65.2
2	OK5m10	100		72.0	67.8	67.8	68.4	68.3	70.6	70.6	73.6	69.1	65.5	64.3	61.2	64.3	62.2	52.0	64.9
3	*Blastomonas* NT12	84.3	84.3		68.3	69.2	68.9	68.6	71.0	73.3	75.9	74.3	68.6	64.6	63.6	65.2	61.0	56.9	62.9
4	OK5m36	80.9	80.9	84.8		99.1	99.1	99.1	83.5	82.6	77.8	73.3	66.4	59.0	58.0	57.7	56.3	50.2	59.6
5	OK5m77	80.3	80.3	84.3	99.4		99.3	99.3	83.8	82.6	78.0	73.6	66.6	60.0	58.3	58.7	56.6	50.9	59.9
6	OK5m69	80.9	80.9	84.8	100	99.4		99.6	84.0	82.6	78.0	73.9	67.5	60.0	59.0	58.3	57.2	50.9	60.9
7	OK5m04	80.3	80.3	84.3	99.4	98.9	99.4		83.8	82.6	78.0	73.6	67.2	59.3	58.7	58.0	56.9	50.1	60.6
8	OK5m114	80.3	80.3	83.2	93.3	92.7	93.3	92.7		79.7	75.6	71.1	65.5	60.9	59.6	60.9	60.2	52.0	59.6
9	OK5m73	80.3	80.3	82.0	93.3	92.7	93.3	92.7	88.2		77.5	71.5	65.8	59.9	59.9	61.8	60.6	52.7	56.6
10	*Blastomonas natatoria*	84.8	84.8	84.3	88.8	88.2	88.8	88.2	87.1	87.1		78.7	67.2	62.5	64.9	64.0	62.1	56.9	63.4
11	OK5m30	82.6	82.6	82.6	83.7	83.2	83.7	83.2	81.5	84.8	88.2		70.8	65.8	65.2	65.5	64.2	55.2	66.8
12	OK5m27	71.9	71.9	75.3	76.4	75.8	76.4	75.8	75.3	75.8	78.1	79.2		64.0	61.5	64.5	66.0	53.1	62.7
13	*Agrobacterium sanguineum*	69.7	69.7	71.4	67.4	66.9	67.4	66.9	68.0	68.0	69.7	71.9	70.8		86.9	84.9	81.2	70.1	79.1
14	*Porphyrobacter tepidarius*	68.5	68.5	70.8	66.9	66.3	66.9	66.3	66.9	65.7	69.1	70.2	69.7	92.1		86.5	83.1	67.6	77.7
15	*Porphyrobacter neustonensis*	67.4	67.4	70.2	65.7	65.2	65.7	65.2	66.3	67.4	68.5	70.2	69.1	91.0	91.0		87.6	64.8	74.7
16	*Erythromicrobium ramosum*	69.1	69.1	70.8	66.9	66.3	66.9	66.3	67.4	66.9	68.5	69.1	69.7	88.8	89.9	95.0		65.4	70.9
17	*Erythrobacter longus*	65.2	65.2	69.1	65.7	65.2	65.7	65.2	65.7	65.7	68.5	67.4	67.4	82.6	81.5	78.7	78.7		70.2
18	*Erythrobacter litoralis*	70.8	70.8	70.8	66.9	66.3	66.9	66.3	66.9	66.9	70.2	73.6	70.8	86.5	83.7	82.0	82.0	86.0	

Table 6.7 Sequence similarities of *pufL* gene fragments from clones and related organisms (DNA similarity top right, deduced amino acid similarity bottom left)

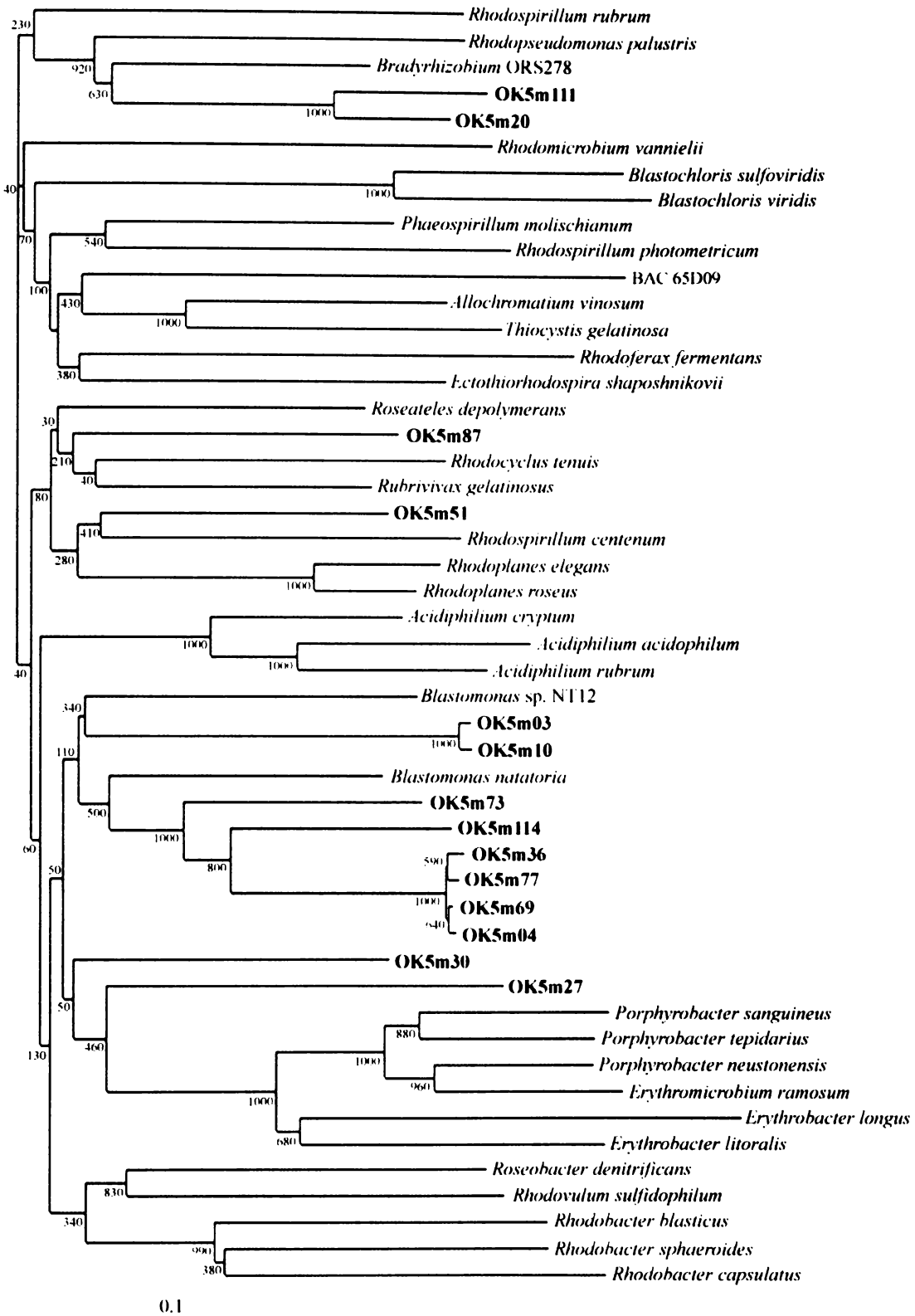


Figure 6.17 Phylogenetic relationships of *pufL* genes of OK5m clones and selected phototrophic and quasi-phototrophic organisms, based on an alignment of 553 nucleotide positions

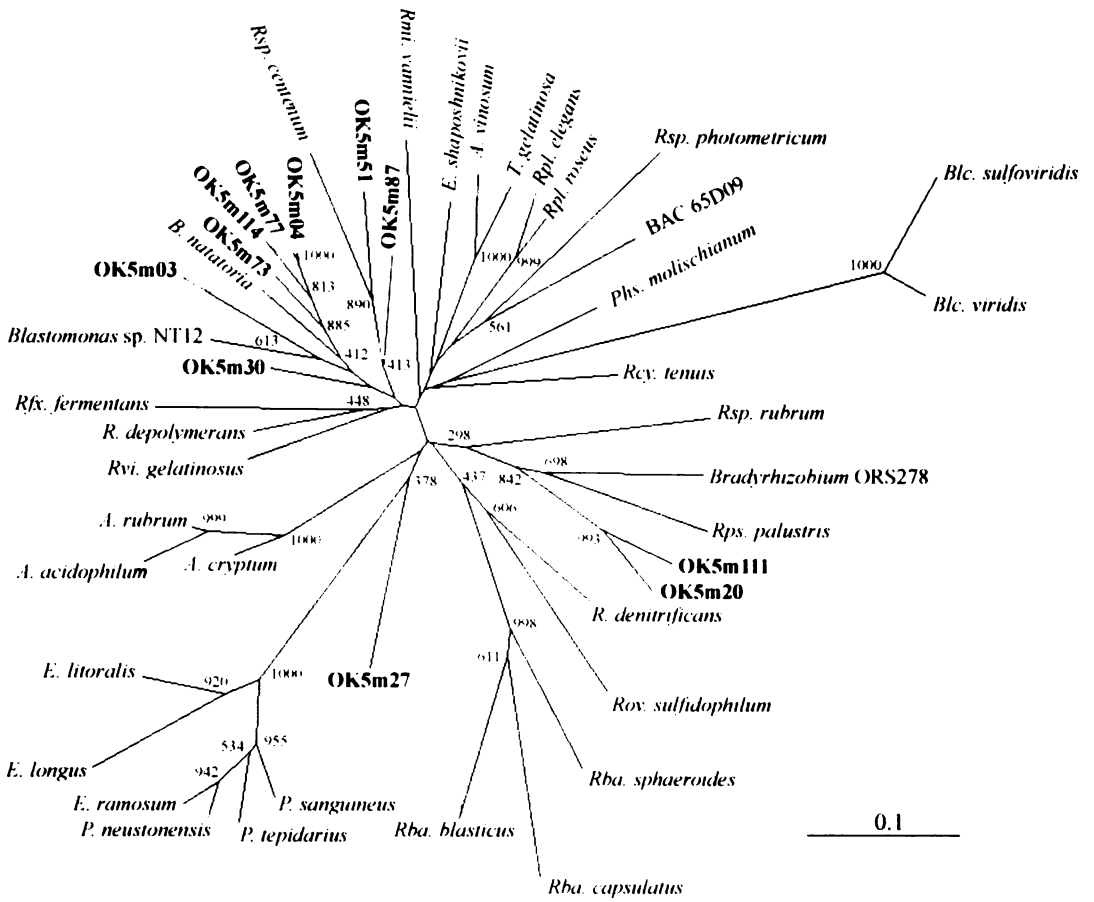


Figure 6.18 Phylogenetic relationships of deduced *pufL* amino acid sequences of OK5m clones and selected phototrophic and quasi-phototrophic organisms, based on an alignment of 178 amino acids, calculated by the neighbour-joining method, with 1000 bootstrap replicates

One major cluster is made up of clones 4, 36, 69 and 77 as a tight grouping, with clones 114 and 73 at a greater distance, with *Blastomonas natatoria* as their closest cultured relative. Clones 3 and 10 form a second cluster with *Blastomonas* sp. NT12 as their immediate relative. Together these sequences form a supercluster consisting of *Blastomonas* species. The branching order of these sequences is identical in Figures 6.17 and 6.18 and is supported by relatively high bootstrap values.

In Figure 6.17, clones 27 and 30 branch deeply at the base of a group containing aerobic phototrophs such as *Porphyrobacter*, *Erythromonas* and *Erythromicrobium* species. In the amino acid tree, however, clone 30 is found at the base of the *Blastomonas* cluster. The low bootstrap values for this branching indicate that the sequence cannot be placed with confidence.

On the basis of their phylogenetic position, only clones 51, 87, 20 and 111 are likely to be derived from anaerobic anoxygenic phototrophs, i.e. purple nonsulfur bacteria. Clones 20 and 111 show closest affiliation to a photosynthetic *Bradyrhizobium* strain in Figure 6.17, though the order of branching differs in Figure 6.18. As there are unlikely to be any nitrogen-fixing symbionts of plant species as active members of a microbial mat community, these clones are most probably derived from an organism similar to *Rhodopseudomonas palustris*. It is conceivable that they are derived from a *Methylobacterium* species (Kato 1978), as this genus, which contains aerobic phototrophic representatives, is a member of the α-2 subdivision, with close affiliations to *Rhodopseudomonas* (Hiraishi *et al.* 1995a); it is unfortunate that *pufL* sequences from *Methylobacterium* species are not yet available. According to the phylogenetic analysis shown in Figure 6.17, clone 87 is affiliated with *Rvi. gelatinosus* and *Rcy. tenuis*, though the β-subdivision aerobic phototroph *Roseateles depolymerans* is also a close relative. In Figure 6.18, clone 87 is affiliated rather with *Rsp. centenum*. In Figure 6.17, clone 51 is most similar to *Rsp. centenum* and *Rhodoplanes* species, but in Figure 6.18, the *pufL* genes of *Rhodoplanes* species do not form a monophyletic cluster with those of *Rsp. centenum*.

None of the clones clustered with members of the genus *Rhodobacter*, or with the cluster that contains purple sulfur bacteria and α-1 spirilla. No clones showing similarity to species of the genus *Acidiphilium* were found; this was not unexpected as the bulk pH of the sample site was around 7.5.

In an effort to clarify the phylogenetic position of some of the clones, a tree was constructed using the quartet puzzling maximum likelihood method (Strimmer and von Haeseler 1996) using the program Tree-Puzzle 5.0. This method is thought to give superior results to the neighbour-joining method in many cases, but is computationally intensive. The resulting phylogenetic tree is shown in Figure 6.19. Only branches supported by a bootstrap value greater than 50% are retained. Other branches are collapsed to produce multifurcations.

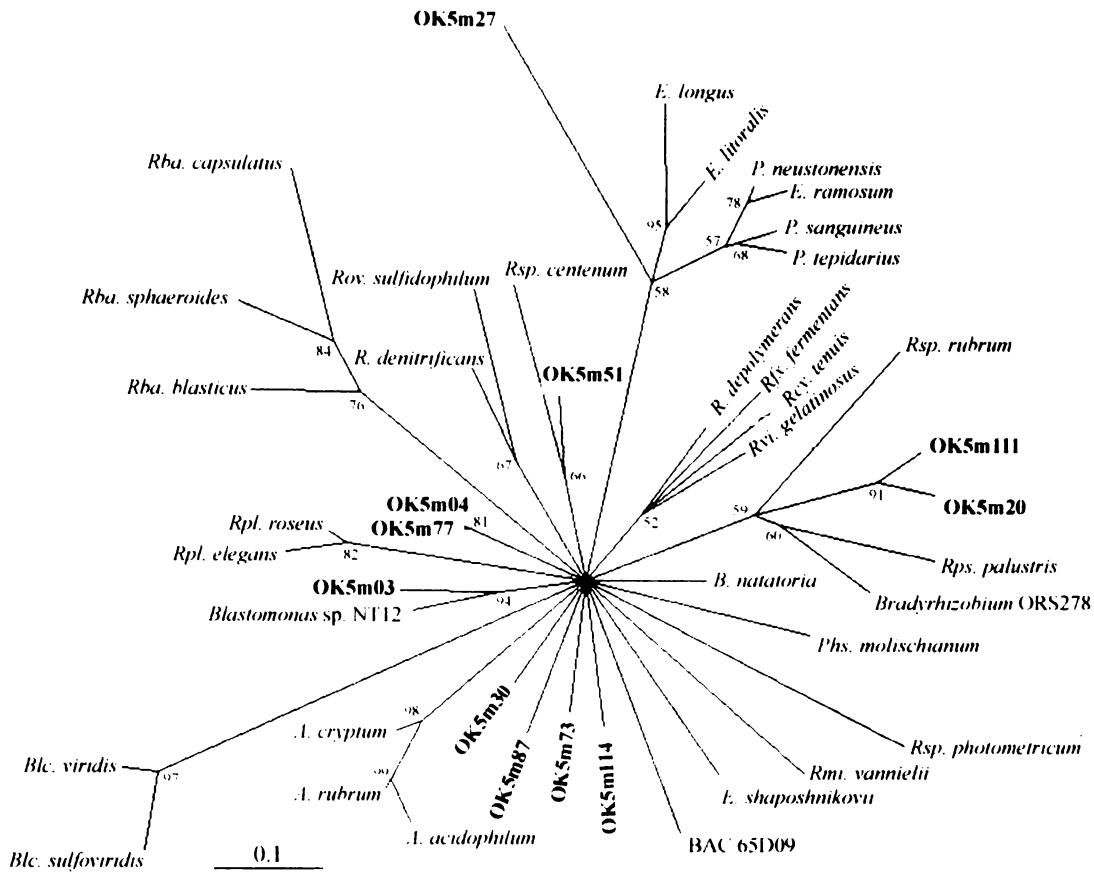


Figure 6.19 Phylogenetic relationships of deduced *pufL* amino acid sequences of OK5m clones and selected phototrophic and quasi-phototrophic organisms, based on an alignment of 178 amino acids, calculated by the quartet-puzzling maximum likelihood method, with 100 bootstrap replicates

The maximum-likelihood analysis supported the associations of clone 27 with the *Porphyrobacter-Erythrobacter* cluster, of clone 51 with *Rsp. centenum*, of clones 20 and 111 with *Rps. palustris*, and of clones 3 and 10 with *Blastomonas* sp. NT12. Other branching orders were not resolved.

6.3.12 Theoretical clone RFLP patterns

The theoretical MvaI and HaeII restriction digest fragment patterns obtained from the sequenced clones were determined using the WebGene restriction enzyme analysis tool at http://darwin.bio.geneseo.edu/~yin/WebGene, and are given in Table 6.8. The theoretically determined RFLP patterns matched those observed on the gels shown in Figure 6.13. Theoretical RFLP patterns of aerobic phototrophic species are given in Table 6.9 for comparison.

		Size of restriction fragments after digestion with enzyme:	
Clone number	**Restriction digest pattern**	**MvaI**	**HaeII**
3	C	478, 114, 40, 9	397, 244
4	D	466, 114, 52, 9	499, 142
10	C	478, 114, 40, 9	397, 244
20	K	313, 123, 114, 91	397, 244
27	L	404, 123, 114	305, 172, 109, 55
30	M	280, 124, 123, 114	255, 244, 142
36	D	466, 114, 52, 9	499, 142
51	P	325, 123, 114, 79	261, 97, 82, 69, 66, 39, 21, 6
69	D	464, 114, 54, 9	499, 142
73	R	176, 126, 123, 114, 102	279, 129, 118, 98, 17
77	D	466, 114, 52, 9	499, 142
87	B	325, 237, 79	266, 148, 129, 98
111	L	404, 123, 114	397, 244
114	N	240, 226, 114, 52, 9	157, 142, 137, 135, 70

Table 6.8 Theoretical MvaI and HaeII restriction digest patterns predicted from clone sequences

Clones giving type D restriction patterns also produced identical HaeII digest patterns, and formed a phylogenetically coherent group. Type C clones which had identical HaeII RFLPs also formed a phylogenetic cluster. For these clones, RFLP analysis was a good guide to phylogenetic affiliation. However, clones 27 and 111 which were defined as L type clones, and indeed give identical MvaI restriction patterns according to their sequences (Table 6.8), are phylogenetically distant from one other. Digestion with HaeII would have allowed the differentiation of these clones. Clone 27 would produce fragments of 55, 109, 172 and 305 base pairs, while clone 111 would produce fragments of 244 and 397 base pairs. Since only two L type clones were observed in the library and both were sequenced, HaeII digestion was not required.

Species or strain	Size of restriction fragments after digestion with enzyme: MvaI	HaeII
Acidiphilium acidophilum	527, 114	397, 244
Acidiphilium cryptum	278, 126, 123, 114	278, 124, 118, 82, 21, 17
Acidiphilium rubrum	278, 126, 123, 114	279, 244, 118
Blastomonas NT12	280, 124, 114, 114, 9	255, 162, 142, 82
Blastomonas ursincola	518, 114, 9	273, 244, 124
Bradyrhizobium ORS278	313, 237, 84, 7	397, 141, 82, 21
Erythromonas litoralis	154, 127, 124, 123, 113	641
Erythromonas longus	518, 123	434, 142, 65
Erythromicrobium ramosum	518, 123	576, 65
Porphyrobacter neustonensis	539, 102	576, 65
Porphyrobacter sanguineus	267, 127, 124, 103, 20	412, 280, 82
Porphyrobacter tepidarius	267, 250, 124	559, 82
Roseateles depolymerans	232, 126, 123, 102, 37, 12, 9	155, 118, 98, 97, 77, 69, 27
Roseobacter denitrificans	518, 123	255, 136, 129, 98, 17, 6

Table 6.9 Theoretical sizes of fragments generated by restriction digests of pL2/pLr4 *pufL* amplification product

None of the clone sequence RFLP patterns matched those predicted for aerobic phototrophic organisms. Clone 20 and the closely related *Bradyrhizobium* ORS278 shared a 313 bp fragment. Clone 30 showed a very similar MvaI pattern to that of the *Acidiphilium* species *A. cryptum* and *A. rubrum*, but the three would be distinguished by their HaeII-generated patterns. Incidentally, *Erythromonas litoralis* is unique among the available organisms in that no recognition sequence for HaeII occurs within the amplified portion of the *pufL* gene.

6.3.13 Unusual clone sequences

The sequence of clone 36 contained a single base deletion (at position 304 in the *Rsp. rubrum* numbering) that destroyed the sense of the gene and introduced several premature stop codons (Figure 6.20 A). Sense could be restored by insertion of a guanine residue at position 304 (*Rsp. rubrum* numbering), after the TGG-codon that codes for the conserved tryptophan (arrowed in Figure 6.20 A). The clone was sequenced in the reverse direction using primer pLr4, which confirmed the single-base pair deletion (Figure 6.20 B).

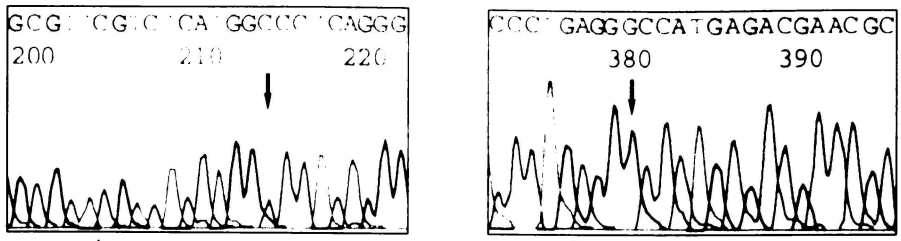


Figure 6.20 Electropherograms of sequencing reactions of clone 36 in the forward direction (A) and reverse direction (B), with arrows indicating the location of a single-base deletion

It remains unknown whether the observed sequence of clone 36 is indeed derived from an OK5 microbial mat organism which possesses a defective *pufL* gene (and is presumably therefore non-photosynthetic), or is rather due to an error introduced during the PCR amplification process. The latter is more likely true, as the sequence of clone 36 is otherwise very similar to that of clone 77. It might be expected that a non-functional *pufL* gene would have accumulated more mutations throughout its length. It is of course possible that the frameshift mutation occurred recently in evolutionary time and no other mutations have occurred since.

The phylogenetic position of clone 87 is uncertain. The DNA sequence shows the greatest similarity to *Rubrivivax gelatinosus* (76.7%) but the amino acid sequence is more similar to *Roseateles depolymerans* (83.7%) and *Rhodoplanes roseus* (82.0%) and to clone 51 (82.0%) than to *Rvi. gelatinosus* (80.9%). Particular care was taken to examine the sequence of clone 87 for possible chimeric origin. However, nucleotides matching those of *R. depolymerans* and *Rpl. roseus* are distributed evenly through the length of the sequence. The DNA sequence of clone 87 was also quite closely related to that of *Rcy. tenuis* (72.2%). However, the *pufL* gene found in clone 87 would not have been amplified directly from the OK5m DNA with the *Rhodocyclus* specific primers. Figure 6.21 shows that although there is a region of identity within the primer pRcy, the final three bases at the 3' end are different, which would preclude priming.

```
Rcy. tenuis     CGCCCTGATGGGGACGCTGCTGATCGTACTGGGCGCTTCGTG
OK5m87          TTCGTTGCTCGGCACCGCGCTGATCGTCTATGGCGCCGCGCT
                 *  ** * ** **   *********     *****  **
```

Figure 6.21 Alignment of the portion of the *pufL* gene of selected PNSB species which contains the sequence of primer pRcy, shaded (bases 138-155); matching bases asterisked

Clone 51 was placed in a clade with *Rsp. centenum* in each of the phylogenetic analyses, and in a cluster that included the *Rhodoplanes* species according to the analysis shown in Figure 6.17. Chimera analysis also indicated that nucleotide positions matching these organisms were evenly distributed through the sequence.

6.3.14 Identification of putative species level OTUs

The average dissimilarity of nearest neighbour pairs of the currently described species of α-4 subdivision phototrophs in the portion of the *pufL* gene contained in the clone sequences is around 17%. A 16S rDNA dissimilarity of 3% is normally taken to be the average difference required for definition of a new species. However, the average difference in the 16S rDNA sequences of these organisms is around 2%.

A scatter plot of the relationship between 16S rRNA divergence and *pufL* divergence was constructed from the 28 possible pairwise comparisons of the eight species of the α-4 subdivision for which both 16S rRNA and *pufL* gene sequences are available (Figure 6.22). Within this group, the relationship between 16S rRNA and *pufL* dissimilarities was described well by a linear curve (R^2 = 0.816). Pairwise comparison of the *pufL* and 16S rDNA sequences of other organisms, such as those of the two *Blastochloris* and two *Rhodoplanes* species, showed that their dissimilarity values also fall close to the curve shown in Figure 6.22. However, when these organisms were included in the full analysis (i.e. compared with each of the α-4 sequences), a logarithmic curve provided a better fit to the data. Presumably, over this larger genetic distance, a saturating level of mutation events at non-conserved codon positions is reached. β- and γ-Proteobacteria cannot be included in such an analysis as their *pufL* and *pufM* genes were obtained by horizontal transfer and as such are disjoint from the phylogenetic position of their 16S rRNA genes (Nagashima *et al.* 1997a). As the majority of clone sequences obtained showed affiliations with the α-4 subdivision species, the analysis was limited to the sequences of these organisms.

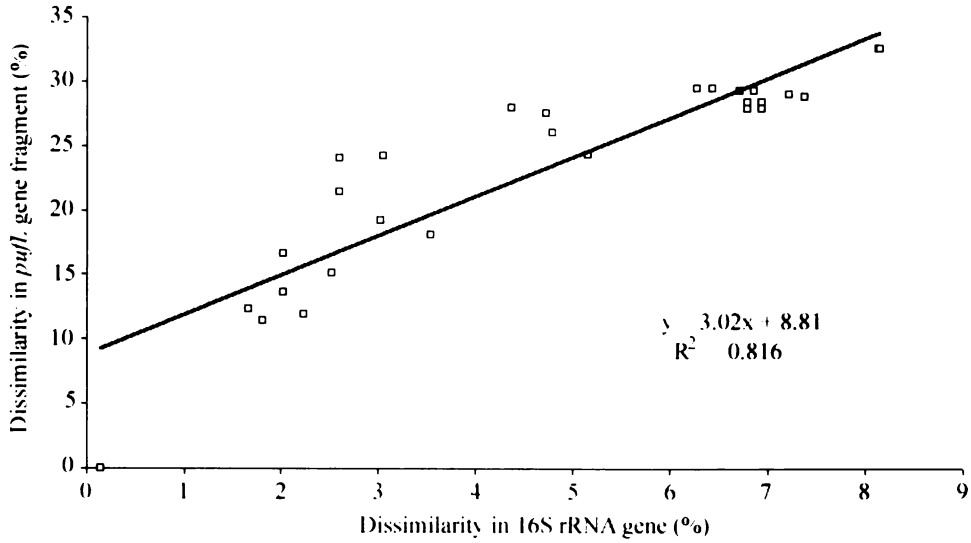


Figure 6.22 Scatter-plot of percentage dissimilarities in 16S rRNA and *pufL* gene fragments of aerobic phototrophs of the α-4 subdivision, based on 1394 bases of the 16S rRNA gene and 535 bases of *pufL*

From the Figure 6.22 curve, it is possible to estimate that a 2% difference in 16S rRNA sequence corresponds to an approximate 15% dissimilarity in *pufL* sequence. It is also possible to estimate the 16S rDNA dissimilarities of the uncultured organisms from which the clone sequences were derived with respect to presently cultured organisms. These dissimilarity values are shown in Table 6.10.

Clone	**compared with:**	***pufL* fragment dissimilarity (%)**	**Predicted 16S rDNA dissimilarity (%)**
3, 10	*Blastomonas* sp. NT12	23.6	4.9
73	*Blastomonas natatoria*	19.4	3.5
114	*B. natatoria*	20.8	4.0
4, 77	*B. natatoria*	19.1	3.4
73	114	17.8	3.0
73	4, 77	15.5	2.2
114	4, 77	14.6	1.9
27	*Porphyrobacter tepidarius*	30.1	7.0
30	*P. tepidarius*	27.9	6.3
27	30	25.1	5.4

Table 6.10 Dissimilarity values of *pufL* clone fragment and 16S rDNA sequences

Clones 73, 114 and the cluster made up of clones 4, 36, 69 and 77 showed *pufL* dissimilarities of 19-20% to *Blastomonas natatoria*, corresponding to estimated 16S

rDNA dissimilarities of around 3.5-4%, which is above the level normal for species cutoff. Clone sequences 73 and 114 showed 17.8% dissimilarity to one another, corresponding to around 3% difference in 16S rDNA sequence. It is therefore likely that clones 73 and 114 represent two novel uncultured species of the genus *Blastomonas*. The cluster of four clones may represent a third novel species, or may be grouped in the same species as clone 114, as their *pufL* dissimilarity and predicted 16S rDNA dissimilarity to clone 114 are slightly less than the average observed among the cultured species of this group. Clones 3 and 10 are derived from another uncultured *Blastomonas* species. Clones 27 and 30 are probably derived from organisms which would be classified as members of two new genera.

Clones 20 and 111 might be expected to derive from organisms showing almost 7% difference in 16S rRNA sequence to *Rhodopseudomonas palustris* indicating that they would be classified in a new genus. Likewise, clones 51 and 87 are insufficiently similar to any known *pufL* sequence to conjecture their origin in organisms of a currently known genus. The distant phylogenetic placement of these four putative PNSB-derived clones indicates that substantial species diversity remains to be discovered even in this long-known and well-studied group. It is unlikely that known species are outnumbered by uncultured representatives by several orders of magnitude as is true of prokaryotes in general (Amann *et al.* 1995), as all described species of PNSB are capable of growth on a wide range of substrates under varying growth conditions, and so are probably more amenable to cultivation than many more fastidious bacteria. Nevertheless, as only five previously-cultivated PNSB species were detected in the OK5 mat sample by culture-based techniques, uncultured organisms comprise 45% of the total population, on the basis of this non-exhaustive search of a clone library constructed using only primer pLr4. If this sample were representative of PNSB-containing environments as a whole, it could be expected that at least another 45 species remain to be described. The diversity of aerobic phototrophic bacteria indicated by the results of the present work, and in particular the work of Beja *et al.* (2002), is even greater.

6.3.15 The dominance of sequences from aerobic phototrophic organisms

The marked dominance of sequences derived from aerobic phototrophic bacteria was perhaps the most surprising result from analysis of the clone library. Indeed, if a

clone library had successfully been constructed in initial attempts, some effort would have been directed towards isolation and characterisation of representative aerobic phototrophic organisms. Unfortunately, only minimal time was available for such work, which is presented in an appendix to this Chapter. The clone library dominance does not appear to be due to PCR primer bias. While the sequences at the primer positions of the organisms that gave rise to the clones can of course not be determined, the number of primer mismatches of the most closely related sequences from the GenBank database can be compared. This does not differ from the degree of mismatch shown by PNSB species. The ecological significance of the dominance of aerobic phototrophic bacteria among *pufL*-containing organisms in the OK5 mat is unclear, as the physiological properties of these organisms are not properly understood. They might compete directly with PNSB or fill niches that would otherwise be filled by PNSB in their absence. This is unlikely if their photosynthetic capability is limited to energy generation supplementation under conditions of carbon starvation (Suyama *et al.* 2002). If they function primarily as chemoheterotrophs, then they do not differ markedly from non-photosynthetic organisms in their interaction with PNSB.

6.4 Conclusions

- PCR amplification of *pufL* gene fragments shows promise for the culture-independent analysis of environmental samples containing photosynthetic purple bacteria
- Analysis of the OK5m library revealed the wide diversity of as yet uncultured organisms that has been found in other molecular studies of this type (Hugenholtz *et al.* 1998; Ward *et al.* 1998)
- *pufL* sequences of organisms that were known to be present in the mat sample through culture techniques were not found in the library
- No clone sequences could be attributed to currently described species
- The clone library was dominated by sequences attributable to aerobic anoxygenic phototrophic bacteria
- Four novel *pufL* sequences that can be attributed to purple nonsulfur bacteria were obtained, suggesting that many PNSB species remain to be discovered

6.5 Suggestions for further work

Attempts could be made to obtain sequences from the clones that were not successfully sequenced using pL2, either of the PCR-amplified insert using reverse primer pLr4, or directly from the plasmid using M13 sequencing primers.

Efforts could also be made to isolate the organisms which provided the novel *pufL* sequences, using the hints provided by their phylogenetic position, and perhaps employing molecular methods to monitor the success of various enrichment cultures, similarly to work published by Santegoeds *et al.* (1996), or by hybridisation of probes derived from the cloned *pufL* fragments to colony lifts. A very rapid attempt was made to enrich and isolate from the OK5 sample site aerobic phototrophic bacteria, which had not been studied in the remainder of this work. This study is reported in the appendix to this chapter which follows.

Vertical dissection of mat samples would reveal whether aerobic phototrophs are present only in the topmost layers in which cyanobacteria are actively photosynthesising, with purple nonsulfur bacteria being located deeper, or whether the two (at minimum) physiological groups of organisms are intermingled. It would be of interest to know whether aerobic phototrophs predominate in other microbial mat samples from thermal areas, or even in the more usual sites from which PNSBs have been isolated. If so, it will not be possible to use primers targeting the *puf* genes to specifically amplify all purple nonsulfur bacteria without also amplifying genes from aerobic anoxygenic phototrophs. If information is required on "true" phototrophs in particular, either a larger number of clones must be analysed to ensure that all sequences present are obtained, which should not be a great barrier to a well-funded research group, or primers specific to more narrow phylogenetic groups must be employed, such as the *Rhodobacter* and *Rhodocyclus*-specific primers that were used in the present work (though without a positive result with regard to environmental samples).

There are very considerable opportunities for further studies of the diversity of photosynthetic organisms by analysis of *puf* genes or other specific genes, including studies of varied environments and studies of temporal, spatial or physicochemical variation within single environments. The 191 bp *pufM* fragment amplified by Fode-

Vaughan *et al.* (2001) will probably be insufficient in length to allow for reliable phylogenetic placement of novel environmental sequences, but many studies of interest will no doubt be published in the future by the research groups of Achenbach, Madigan and DeLong.

Clone libraries of 16S rDNA sequences of green nonsulfur bacteria have revealed a wide range of uncultured members, many of which have no close cultured relatives (Hugenholtz *et al.* 1998). As there are non-photosynthetic members of the GNS division, it may on occasion be uncertain whether an environmental 16S rDNA sequence derives from a photosynthetic organism. This is similar to the situation that arises in the study of photosynthetic proteobacteria and, similarly, targeting of green nonsulfur reaction centre genes would provide another method of assessing the diversity of the group.

Appendix to Chapter 6

Enrichment and Isolation of Aerobic Phototrophic Organisms from Sample Site OK5

6.6 Introduction

The isolation of aerobic photosynthetic bacteria from microbial mats of hot springs has been infrequently reported. An *Erythrobacter* species was found in Bol'shaya River springs in Siberia but only at temperatures below 40°C (Yurkov *et al.* 1992); *Porphyrobacter tepidarius* was isolated from a 43°C mat sample from a brackish spring in Japan (Hanada *et al.* 1997), and "*P. cryptus*" is not yet described, but has a higher optimum temperature than *P. tepidarius* (Silva *et al.* 1998). No attempts were made to study "quasi-photosynthetic" organisms (Gest 1993) as part of the culture-based diversity studies that were reported in Chapter 4, as these organisms are physiologically distinct from purple nonsulfur bacteria, and their cultivation would have required entirely different materials and techniques from those employed for anaerobic phototrophs. However, molecular analysis of an OK5 mat sample by analysis of a *pufL* clone library revealed the presence of a large number of aerobic photosynthetic bacteria, as the dominant *pufL*-containing organisms. Consequently, it was decided to isolate and identify the dominant culturable aerobic phototrophic bacteria from the OK5 mat.

6.7 Enrichment and Isolation

No selective medium has been developed to allow the isolation of aerobic phototrophic bacteria (Yurkov and Beatty 1998). They have in general been isolated on rich organic media, containing yeast extract, peptone, TCA intermediates or sugars, on which many other heterotrophic bacteria grow well. Bacterial colonies that show colouration that might be due to carotenoids are screened for the presence of bacteriochlorophyll (Yurkov and Beatty 1998).

In initial attempts to isolate aerobic phototrophic bacteria, OK5 mat material was spread over plates of solidified LB medium, which were incubated at 37°C with a 12 hour light-dark cycle. In parallel with isolations on plates, attempts were made to

grow aerobic photosynthetic bacteria in liquid LB medium, at 37°C with shaking at 150 rpm. After 24 hours, plates were completely covered in profuse spreading growth of a white to yellow colour, and broth cultures were similarly turbid. Microscopic examination of Gram-stained samples showed Gram positive rod-shaped organisms, certainly a *Bacillus* species. LB plates amended with 100 U/mL penicillin G were then streaked with mat material. Growth of the *Bacillus* did not occur under these conditions. A wider diversity of discrete colonies was seen in its place, but no pigmented colonies could be seen, other than a few yellow to beige colonies.

Yurkov *et al.* (1996) reported resistance to the heavy-metal oxyanion tellurite in a range of aerobic photosynthetic bacteria including *Erythromicrobium ramosum*, *Roseococcus thiosulfatophilus* and *Erythrobacter litoralis* of an even greater level than the resistance exhibited by purple nonsulfur bacteria (Moore and Kaplan 1994). The majority of non-resistant organisms are inhibited in the presence of as little as 1 μg/mL (Moore and Kaplan 1992), whereas for the aerobic phototrophic bacteria tested the minimum inhibitory concentration was in general greater than 1 mg/mL, and was in some cases as high as 2.5 mg/mL. Resistance to tellurite is associated with reduction of the oxyanion to black metallic tellurium in substantial quantities, and also reduction to volatile methylated species (McCarty *et al.* 1993).

LB plates and broth were amended with 100 μg/mL potassium tellurite, a concentration sufficient to inhibit organisms that do not exhibit high-level resistance, and inoculated with mat material. Incubation was again at 37°C under a regime of 12 hours illumination and 12 hours darkness. After a week, plate cultures bore intensely black pigmented colonies and faint grey discolouration of the agar surface, while liquid cultures had turned black with colloidal tellurium, beneath which could be seen a faint pink colouration. Liquid cultures were streaked on LB/tellurite plates.

The black tellurium precipitates masked any pigmentation of resulting colonies. Colonies were transferred to LB plates containing penicillin G to inhibit any remaining *Bacillus* cells using standard velvet transfer techniques. Four colonies derived from the tellurite plate cultures showed intense orange pigmentation. Many colonies on the plates derived from the liquid cultures were pink. Absorption spectra showed the presence of bacteriochlorophyll *a*.

The orange-pigmented organism was isolated by repeated restreaking and was designated OK5APO. The pink-pigmented organism however could not be isolated as colonies were somewhat diffuse and always surrounded by non-pigmented growth.

6.8 Identification

The 16S rDNA sequence of strain OK5APO was determined according to the methods given in Sections 3.9.1 and 3.9.4. The sequence was deposited in the GenBank database under the Accession Number AY048657. BLAST search revealed that the sequence showed greater than 99% similarity to *Porphyrobacter tepidarius* and several unnamed *Porphyrobacter* strains, 98.9% similarity to *P. neustonensis*, and 98.3% similarity to *P. sanguineus*. A phylogenetic tree was constructed on the basis of their 16S rDNA sequences (Figure 6.23). As a result of this analysis, strain OK5APO is tentatively assigned to the species *P. tepidarius* (Hanada *et al.* 1997). This organism is capable of growth to a maximum of 50°C, with optimal growth at 40-48°C, which is in the range of temperatures measured at the OK5 sample site.

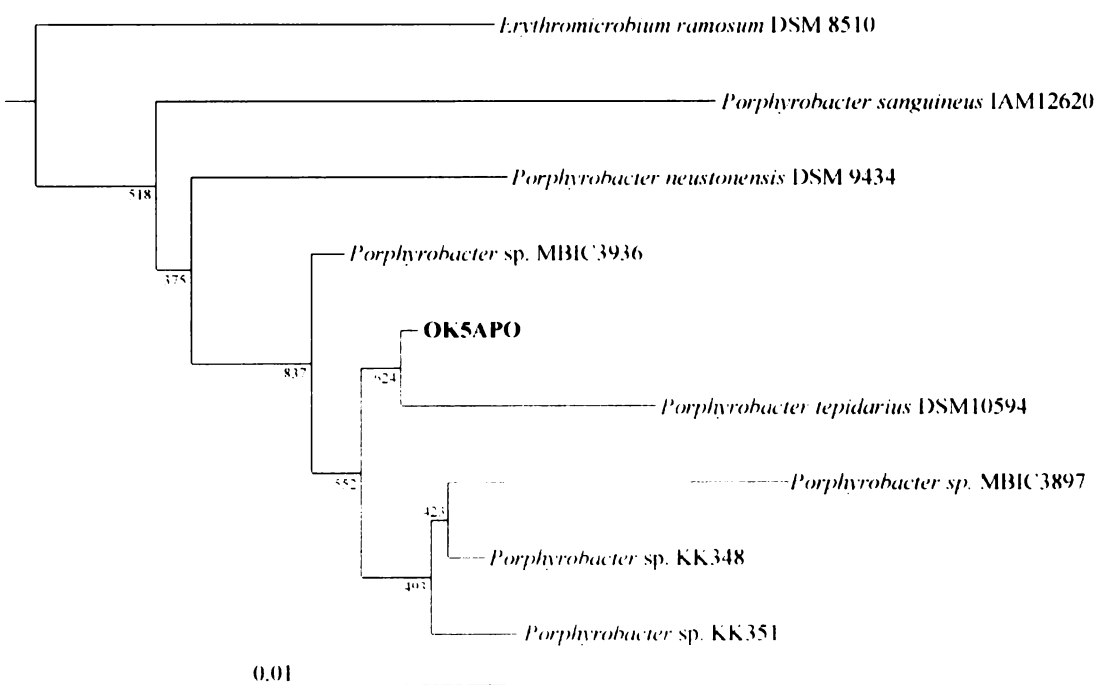


Figure 6.23 Distance matrix tree showing phylogenetic relationship of the isolate OK5APO to aerobic phototrophic bacteria of the α-4 subdivision of the Proteobacteria, based on an alignment of 1390 bases of the 16S rRNA gene. The tree was rooted using the sequence of *Erythrobacter litoralis*.

6.9 Discussion

Exploitation of the high level resistance of many aerobic phototrophic bacteria to the metal oxyanion tellurite provided a useful method for the selective growth and detection of these organisms. Two bacteriochlorophyll *a*-containing aerobic organisms were detected, but only one could be isolated. Pink-pigmented aerobic phototrophic organisms include members of *Roseobacter*, *Acidiphilium*, *Roseococcus*, *Methylobacterium* and *Roseateles*. Of these, *Roseobacter* are apparently limited to marine environments, *Acidiphilium* species are restricted to highly acidic environments, and *Roseococcus* are coccoid. The pink rod-shaped organism from OK5 may be a member of either *Methylobacterium* or *Roseateles*. The orange-pigmented organism designated OK5APO was assigned to the species *Porphyrobacter tepidarius*. *P. tepidarius* was however not represented in the clone library, so it is probable that a minor member of the mat community was cultured. However, strongly selective conditions were required in order to inhibit overgrowth by unwanted organisms, and it is possible that dominant members of the community, represented in the clone library, were unable to grow under these conditions.

Chapter 7

Degradation of Nicotinic Acid by *Rhodoplanes* species

7.1 Introduction

As described in Section 5.2.3, several *Rhodoplanes* isolates initially grew poorly in laboratory culture. Growth was enhanced by addition of 0.05% (w/v) yeast extract to the growth medium. Replacement of the yeast extract with the standard vitamin cocktail was not effective in promoting growth. A *Rhodoplanes* strain Wai3R3 vitamin test culture that had shown no growth when supplied with the standard amount of nicotinic acid was amended with 10 mg/L nicotinic acid. Rapid development of the culture followed immediately, indicating a requirement for higher than usual levels of nicotinic acid. As there seemed a slight possibility that the *Rhodoplanes* strains were exhibiting some peculiarity in nicotinic acid metabolism, growth on nicotinate as carbon source was tested, with unexpected positive results.

Other cultures were screened for growth on nicotinate. Screening of a *Blastochloris* isolate from sample site Wai3 for growth on nicotinic acid as carbon source led to development of a pink culture, revealing contamination with a bacteriochlorophyll *a*-containing organism. The culture was re-purified to yield *Blastochloris* strain Wai3G1e and the nicotinic-acid utilising strain Wai3P2 which was identified as a member of *Rhodoplanes*.

Utilisation of nicotinic acid is an exceedingly uncommon trait among purple nonsulfur bacteria. Therefore, following the discovery of the nicotinic acid-degrading abilities of members of the genus *Rhodoplanes*, further investigations were carried out, and are reported below.

7.2 Literature review

7.2.1 Metabolism of aromatic and heterocyclic compounds by the PNSB

The purple nonsulfur bacteria are regarded as being among the most metabolically versatile of all bacteria. Partly this is due to their wide range of growth modes, from the photoheterotrophy that defines the group to chemolithoautotrophy in darkness (Madigan and Gest 1979; Kompantseva 1981), but in addition, the PNSB have long

been recognised for the wide range of carbon compounds they are able to use for growth. Sasikala and Ramana (1997) provide a review of the breadth of compounds used.

Under phototrophic conditions, purple nonsulfur bacteria are freed from the need to generate energy from the catabolism of their substrates (this is indeed also true of other phototrophs, though many nonetheless assimilate only a restricted range of carbon compounds). Substrates may be almost entirely assimilated into cell carbon. Phototrophic growth, with concomitant carbon dioxide fixation, also allows PNSB to grow with high efficiency on carbon sources that under fermentative anaerobic conditions otherwise have low ATP yields, such as the short-chain fatty acids whose fermentation is possible only by a partnership between syntrophic organisms.

Several purple nonsulfur bacteria are known to possess the ability to degrade homocyclic aromatic compounds. Benzoic acid is degraded by a number of PNSB, including strains of *Blc. sulfoviridis*, *Phs. fulvum*, *Rba. blasticus*, *Rba. capsulatus*, *Rcy. purpureus*, *Rmi. vannielii*, *Rps. palustris* and *Rsp. rubrum*. The degradation pathway under phototrophic conditions, shown in Figure 7.1, has been determined in *Rps. palustris* (Perrotta and Harwood 1994) and the genes involved have been sequenced (Egland *et al.* 1997). This reductive degradation pathway is markedly different from the O_2-requiring mono- or di-oxygenase catalysed pathways employed by aerobic organisms (Harayama *et al.* 1992).

The aromatic degradation capabilities of *Rps. palustris* remain better studied than those of other PNSB. A wide range of homocyclic aromatic compounds have been found to be used as carbon sources by various PNSB, including 4-aminobenzoate, 4-aminophenol, aniline, benzyl alcohol, caffeate, chlorobenzoate isomers, cinnamate, *p*-coumarate, ferulate, hydroxybenzoate isomers, mandelate, nitrobenzene, nitrophenols, phenol, phenyl-alkanoates, phloroglucinol, protocatechuate, salicylate, syringate and vanillate (Blasco and Castillo 1992; Elder *et al.* 1992; Harwood and Gibson 1988; Kamal and Wyndham 1990; Khanna *et al.* 1992; McGrath 2000; Oda *et al.* 2001; Rahalkar *et al.* 1991, 1993; Shoreit and Shabeb 1994; Sasikala *et al.* 1994b; van der Woude *et al.* 1994; Wright and Madigan 1991). In general, it appears that aromatic compounds are funnelled to benzoate or 4-hydroxybenzoate as common degradative intermediates.

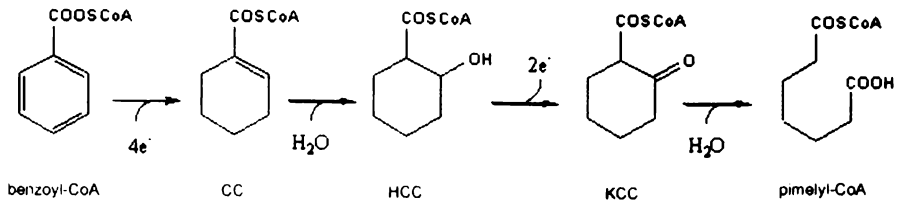


Figure 7.1 Pathway of degradation of benzoate by *Rhodopseudomonas palustris* as determined by Perrotta and Harwood (1994)
Key: CC cyclohex-1-ene carboxyl-CoA; HCC 2-hydroxycyclohexane-carboxyl-CoA; KCC 2-ketocyclohexane-carboxyl-CoA

It also appears that separate degradation pathways are used for aerobic and anaerobic metabolism of aromatic compounds (Harwood and Gibson 1988).

Blc. sulfoviridis ToP1 was found to possess the ability to degrade toluene under phototrophic conditions (Zengler *et al.* 1999). The initial step of the reaction was the addition of the methyl group of toluene to the double bond of fumaric acid to form benzylsuccinate. Further steps led to the conversion of benzylsuccinate to benzoic acid, and the release of succinate (Figure 7.2). Benzoic acid was then presumably degraded in a manner similar to the mechanism of *Rps. palustris* (Figure 7.1).

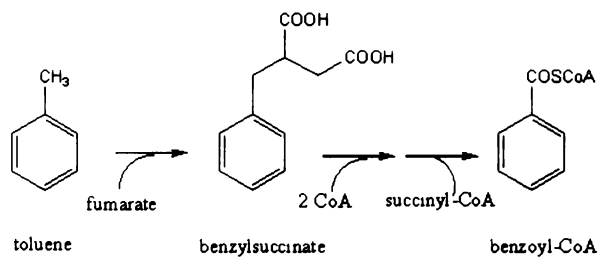


Figure 7.2 Simplified pathway of degradation of toluene by *Blastochloris sulfoviridis* ToP1 (Zengler *et al.* 1999)

Fewer studies have been carried out into metabolism of heterocyclic compounds by purple nonsulfur bacteria, despite the fact that two-thirds of known organic chemicals are heterocyclic, including many biogenic molecules such as nucleotides, alkaloids, and electron carriers, and major industrial chemicals such as herbicides and solvents (Bollag and Kaiser 1991; Kaiser *et al.* 1996).

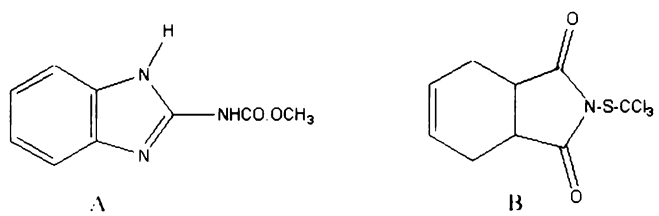
The sulfur heterocycle thiophene-2-carboxylate did not support growth of *Rps. palustris* but was transformed anaerobically to the reduced aliphatic forms tetrahydrothiophene-2-carboxylate and 3-hydroxy-tetrahydrothiophene-2-carboxylate (Tanaka *et al.* 1982). The same strain also photometabolised the oxygen-containing

analogue furan-2-carboxylate. Indole was similarly transformed by *Rhodobacter sphaeroides* (Rajasekhar *et al.* 1998).

Purines were found to be widely used as nitrogen sources in the presence of readily-used carbon sources (Aretz *et al.* 1978). Purines were degraded to uric acid as a central intermediate which was then cleaved by an oxidative mechanism to allantoin (Kaspari and Busse 1986). Pyrimidines however were degraded by a reductive pathway (Kaspari 1979). Yuan (2000) examined the metabolism of purines and pyrimidines by a range of PNSB species and determined that several PNSB were able to grow on these compounds as sole fixed carbon source, in general using mechanisms that are known in chemotrophic bacteria.

Sasikala *et al.* (1994a) reported growth of a *Rps. palustris* strain on several pyridine and pyrazine derivatives, including nicotinic acid (see Section 7.2.2) as sole carbon source.

Two of the more unusual compounds to be utilised by a purple nonsulfur bacterium are the systemic fungicides carbendazim and captan (Figure 7.3). Rajkumar and Lalithakumari (1992) reported the isolation of a strain of *Rhodopseudomonas palustris* which possessed the ability to utilise carbendazim as sole nitrogen and carbon source. Growth yield data and UV spectra of spent growth medium indicated that utilisation of the ring moiety occurred. *Rhodobacter sphaeroides* OU5 and *Rps. palustris* OU11 were reported to grow at the expense of captan, but the mode of utilisation is unclear (Rajasekhar *et al.* 2000).



A B

Figure 7.3 Structural formulae of carbendazim (A) and captan (B), unusual heterocycles catabolised by purple nonsulfur bacteria

Rhodoplanes elegans and *Rpl. roseus*, in contrast to the organisms mentioned above, are not noted for their especial nutritional diversity. Indeed, in comparison with most members of the purple nonsulfur bacteria, their range of utilisable carbon sources is narrow (see Table 5.3; Hiraishi and Ueda 1994b; Janssen and Harfoot 1991). No

ability to grow at the expense of benzoate or other aromatic compounds has been recorded in the published literature, and they are distinctive in their limited ability to use fatty acids as carbon sources.

7.2.2 Metabolism of nicotinic acid by purple nonsulfur bacteria

Harwood and Gibson (1988) reported that the type strain of *Rhodopseudomonas palustris* was unable to metabolise nicotinic acid. However, catabolism of nicotinic acid as sole carbon source has been reported in two strains of *Rhodopseudomonas palustris* - OU11, which was isolated from an industrial effluent pond, and JA1, isolated from dairy effluent. Strain OU11 was initially unable to metabolise nicotinic acid, but gained this ability after acclimation to growth on pyrazinoic acid. The doubling time for growth was 50 hours, and utilisation of the substrate was incomplete, with 40-50% degradation occurring (Sasikala *et al.* 1994a). In addition, growth on malate was markedly inhibited in the presence of nicotinate concentrations above 2.5 mM (Rajasekhar *et al.* 2000). *Rhodopseudomonas palustris* JA1 was far more resistant to high levels of nicotinate and completely degraded nicotinic acid as sole nitrogen source in the presence of malate or as sole carbon source. No attempts were made to determine the mechanism of degradation (Rajasekhar *et al.* 2000).

7.2.3 Metabolism of nicotinic acid by other bacteria

The metabolism of nicotinic acid and many other nitrogen-containing heterocyclic compounds by a wide range of bacteria are reviewed admirably by Berry *et al.* (1987), Bollag and Kaiser (1991), Fetzner (1998) and Kaiser *et al.* (1996).

Behrmann and Stanier (1957) determined the pathway of nicotinic acid degradation in a strain of *Pseudomonas fluorescens*; similar results were obtained by Gauthier and Rittenberg (1971) for *P. putida*, while Ensign and Rittenberg (1964) determined a different pathway in a *Bacillus* strain that was later described as *Bacillus niacini* (Nagel and Andreesen 1990). The initial step in both degradation pathways (Figure 7.4) was hydroxylation of nicotinate to form 6-hydroxynicotinate; the hydroxyl group was derived from water. At this point, the pathways diverged. The *Bacillus* strain carried out a second hydroxylation to form 2,6-dihydroxynicotinic acid, which was then decarboxylated forming 2,3,6-trihydroxypyridine. In the *Pseudomonas* species, 6-hydroxynicotinic acid was immediately decarboxylated to form 2,5-dihydroxypyridine. The hydroxypyridine intermediates were cleaved by a

dioxygenase to form N-formylmaleamate in both species. Thereafter the pathways were identical.

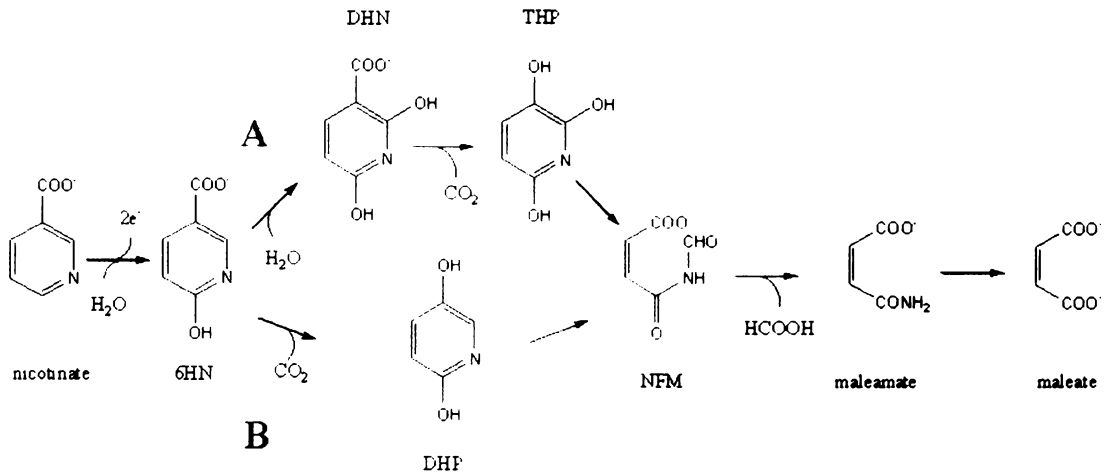


Figure 7.4 Proposed pathways for degradation of nicotinic acid in (A) *Bacillus niacini* (Ensign and Rittenburg 1964) and (B) *Pseudomonas fluorescens* (Behrman and Stanier 1957).

Key: 6HN 6-hydroxynicotinic acid; DHN 2,6-dihydroxynicotinic acid; THP 2,3,6-trihydroxypyridine; DHP 2,5-dihydroxypyridine; NFM N-formylmaleamate

The strict anaerobe *Clostridium barkeri*, now known as *Eubacterium barkeri* (Collins *et al.* 1994) was found to grow by fermentation of nicotinic acid. The pathway shown in Figure 7.5 was proposed by Tsai *et al.* (1966). As for aerobic organisms, the initial step in the pathway is hydroxylation to form 6-hydroxynicotinate.

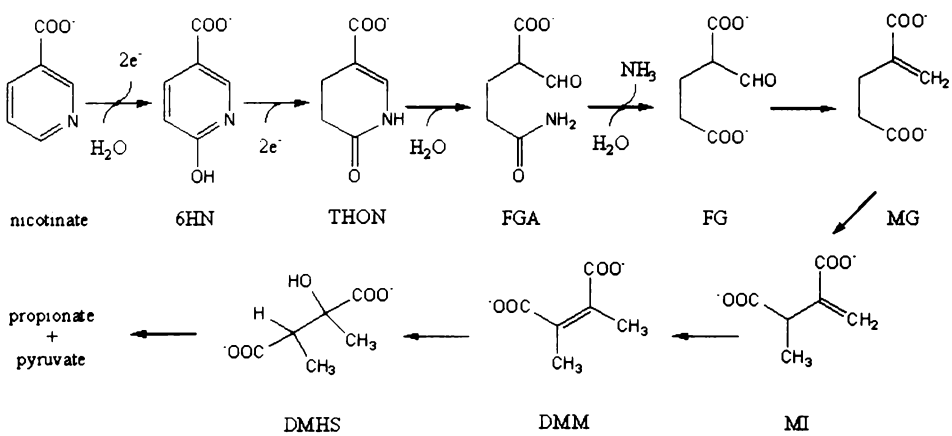


Figure 7.5 Proposed pathway for nicotinate degradation in the fermentative anaerobe *Eubacterium barkeri* (Tsai *et al.* 1966).

Key: 6HN 6-hydroxynicotinate; THON 1,4,5,6-tetrahydro-6-oxo-nicotinate; FGA 2-formyl-glutaryl-5-amide; FG 2-formyl-glutarate; MG α-methyleneglutarate; MI methylitaconate; DMM dimethylmaleate; DMHS 2,3-dimethyl-2-hydroxysuccinate

This is reduced to 1,4,5,6-tetrahydro-6-oxo-nicotinate (THON); after deamination, a complex series of carbon skeleton rearrangements lead to 2,3-dimethyl-2-hydroxysuccinate, which is cleaved to the fermentation end-products pyruvate and propionate.

Desulfococcus niacini is another anaerobe which is capable of complete nicotinate degradation coupled to the reduction of sulfate as electron acceptor (Imhoff-Stuckle and Pfennig 1983). No intermediates were detected; end products of degradation were CO_2 and NH_3. The pathway of degradation has not been investigated.

Azorhizobium caulinodans, a member of the Rhizobiaceae that grows either as a symbiont with the legume *Sesbania rostrata* or as a free-living organism, requires nicotinate as a vitamin, utilises it as a nitrogen source, and also grows poorly on nicotinic acid as sole carbon source. Kitts *et al.* (1992) proposed the degradation pathway shown in Figure 7.6. 6HN, THON and glutarate were the only detected intermediates. The identity of the ring cleavage products was inferred. The pathway is identical to that proposed for *Eubacterium barkeri* (Figure 7.5) up to the point of deamination. Thereafter, oxidative decarboxylation converts 2-formylglutarate into glutaric acid.

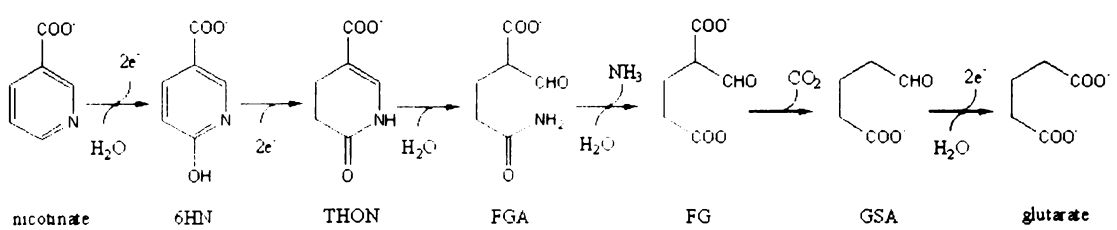


Figure 7.6 Proposed pathway for nicotinic acid degradation by *Azorhizobium caulinodans* (Kitts *et al.* 1992).
Key: 6HN 6-hydroxynicotinate; THON 1,4,5,6-tetrahydro-6-oxo-nicotinate; FGA 2-formyl-glutaryl-5-amide; FG 2-formyl-glutarate; GSA glutaryl-semialdehyde

Kitts *et al.* (1992) note that the *A. caulinodans* nicotinate catabolism pathway is a chimera containing steps found in anaerobic and aerobic organisms. They suggest that reactions catalysed by dioxygenases are avoided because cytosolic O_2 concentrations are maintained at too low a level in this aerobic but avidly N_2-fixing organism.

Strain Mena 23/3-3c, an aerobic rod-shaped organism with affiliations to the genera *Ralstonia* and *Burkholderia*, was isolated from an enrichment on 6-methylnicotinic acid, as part of a strategy designed to find organisms which do not carry out the usual hydroxylation of nicotinic acid at the 6-position (Tinschert *et al.* 1997). Mena 23/3-3c is unique amongst described bacteria in that it converts nicotinic acid to 2-hydroxynicotinate. 6-methylnicotinate is likewise converted to 2-hydroxy-6-methylnicotinate, which is then completely degraded. However, the strain is unable to grow on nicotinic acid itself, or to further metabolise 2-hydroxynicotinate. Nonetheless, this indicates the possibility of the existence of organisms that carry out metabolism of nicotinic acid by hitherto unknown pathways.

7.2.4 Enzymology of heterocycle degradation

Characterised enzymes that carry out transformations of nitrogen containing heterocyclic compounds are in general structurally-related and contain molybdenum-cofactors; these include enzymes acting on xanthine, quinoline, isoquinoline, picolinate, nicotine, and isonicotinate (Hille 1996; Kretzer *et al.* 1993). The synthesis of functioning molybdoenzymes is inhibited by the presence of tungstate, which mimics the structure of molybdate.

Several enzymes responsible for the initial steps of nicotinic acid degradation have been purified. Nicotinic acid hydroxylase was purified from *Eubacterium barkeri* (Holcenberg and Stadtman 1969) and was later characterised in detail and was found to contain molybdenum and selenium cofactors (Gladyshev *et al.* 1996).

Growth of *Bacillus niacini* on nicotinate was found to be dependent on the amount of molybdate added to the medium. Addition of tungstate inhibited growth on nicotinate and on 6-hydroxynicotinate with increasing concentration (Nagel and Andreesen 1989). The enzymes nicotinate dehydrogenase and 6-hydroxynicotinate dehydrogenase were isolated and found to be molybdoenzymes (Nagel and Andreesen 1990).

Growth of *Desulfococcus niacini* however was not dependent on addition of molybdate to the growth medium; selenite only was required (Imhoff-Stuckle and Pfennig 1983). The enzymes of nicotinate catabolism have not been characterised in this organism.

7.3 Materials and Methods

7.3.1 UV spectroscopy

Progress of nicotinate metabolism was followed by obtaining UV spectra of centrifuged culture supernatants using a Pharmacia Biotech Ultrospec 3000 spectrophotometer.

7.3.2 HPLC

HPLC was used to confirm the identity of and to quantify intermediates, using authentic compounds purchased from Sigma-Aldrich as standards. 50 µL samples of centrifuged culture media were analyzed with a reverse phase C18 column (Supelco Spheri-5 RP-18 5µ 220 x 4.6 mm) operated at 40°C using 50mM sodium phosphate, pH 5.6 as mobile phase at a flow rate of 0.5 mL/min and UV detection at 254 nm (Shimadzu), and an organic acid analysis column (BioRad Aminex HPX-87H, 300 x 7.8 mm) operated at 50°C using 0.01 N H_2SO_4 at a flow rate of 0.5 mL/min and refractive index detection (ERC 7510, Erma Optical Works, Tokyo, Japan) on a Waters Model 510 HPLC.

7.3.3 ES-MS

Intermediates of nicotinate degradation were detected using electrospray mass spectrometry (ES-MS) with a cone voltage of 20 V and water as solvent.

7.4 Results and Discussion

7.4.1 Growth on nicotinic acid and related compounds

Rhodoplanes elegans, "*Rhodopseudomonas cryptolactis*" and the thermotolerant *Rpl. elegans*-like strains were able to grow under light anaerobic conditions on nicotinic acid as sole fixed carbon source (in the presence of bicarbonate), as sole nitrogen source in the presence of mixed acids, or as both carbon and nitrogen source. During growth on nicotinate as nitrogen source, nitrogenase was derepressed, as evidenced by the extreme gas pressures generated in the tubes. Growth on nicotinic acid as carbon source was not possible in the absence of bicarbonate. *Rhodoplanes roseus* 941 and *Rhodoplanes* strain Tok2tar1 were unable to utilise nicotinic acid as carbon or nitrogen source under any conditions tested, even after 3 weeks of incubation.

The *Rhodoplanes elegans* group strains were tested for the ability to grow on a range of other aromatic compounds and derivatives of nicotinic acid. All strains were capable of growth on 6-hydroxynicotinic acid. None grew on 2-hydroxynicotinate, 6-methylnicotinate, 2-methylnicotinate, benzoate or *p*-hydroxybenzoate. The aromatic degradation repertoire of *Rpl. elegans* strains is thus limited to nicotinate and its para-hydroxylated analogue. *Rpl. roseus* and strain Tok2tar1 were unable to grow on 6-hydroxynicotinate, and were not tested on the other derivatives. Several other purple nonsulfur bacteria, including several known to possess the ability to metabolise aromatic compounds, were tested for growth on nicotinate and 6-hydroxynicotinate. *Rhodopseudomonas palustris* DSM 123, *Rps. palustris* Wai1val1, *Blastochloris sulfoviridis* Wai3G1e, *Rhodomicrobium vannielii*, *Rhodobacter capsulatus*, *Rubrivivax gelatinosus*, and *Rhodospirillum centenum* were unable to grow on these compounds.

Growth of *Rhodoplanes* strain Wai3R3 was measured at a range of nicotinate concentrations under light anaerobic conditions (Figures 7.7 and 7.8).

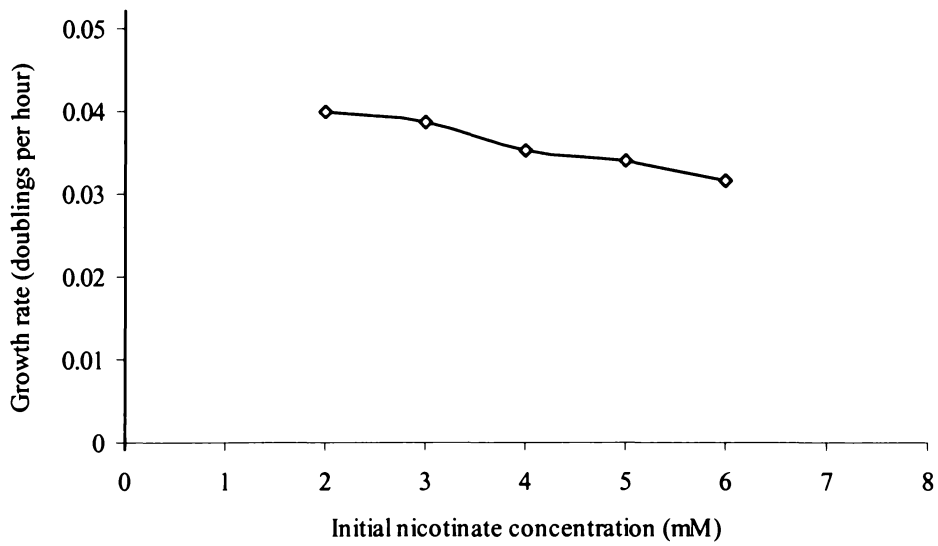


Figure 7.7 Photoheterotrophic growth rate of *Rhodoplanes* strain Wai3R3 at various initial nicotinate concentrations

Doubling time at 2 mM was approximately 25 hours; at 6 mM, doubling time was increased to almost 32 hours. For this reason, growth studies of nicotinate degradation were carried out at a concentration of 2 mM. Growth yield, however, was greatest at a concentration of 10 mM nicotinate, which was inhibitory for *Rps. palustris* OU11 and superoptimal for strain JA11 (Rajasekhar *et al.* 2000). Marginal

growth was still observed at 20 mM nicotinate. At 30 mM and above, cell growth was inhibited, as the final culture density was less than that of a control lacking any carbon source.

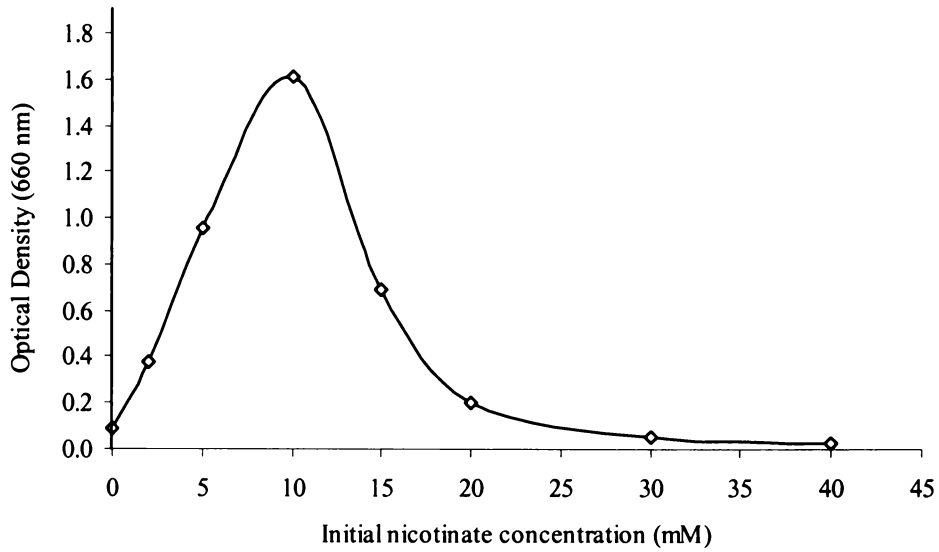


Figure 7.8 Photoheterotrophic growth yield of *Rhodoplanes* strain Wai3R3 at various nicotinate concentrations

7.4.2 Degradation of nicotinic acid

Degradation of nicotinic acid was initially followed by measuring the UV spectra of centrifuged culture supernatants taken at various time periods over the course of growth of *Rhodoplanes* strain Wai3R3 on 2 mM nicotinic acid. Sample spectra are shown in Figure 7.9. From 70 hours onwards, the characteristic absorption spectrum of nicotinic acid, with maxima at 257, 262 and 268 nm, could be seen to alter progressively (Figure 7.9 B) and by 110 hours of incubation, had been replaced by the spectrum shown in Figure 7.9 C, with absorption maxima at 252 and 296 nm. Beyond this point, no further changes to positions of the maxima were observed though peak heights decreased (Figure 7.9 D).

Comparison with a standard solution indicated that the spectrum observed in Figure 7.9 C was identical to that of 6-hydroxynicotinate. In Figure 7.9 B, therefore, both nicotinate and 6-hydroxynicotinate contribute to the overall absorption spectrum.

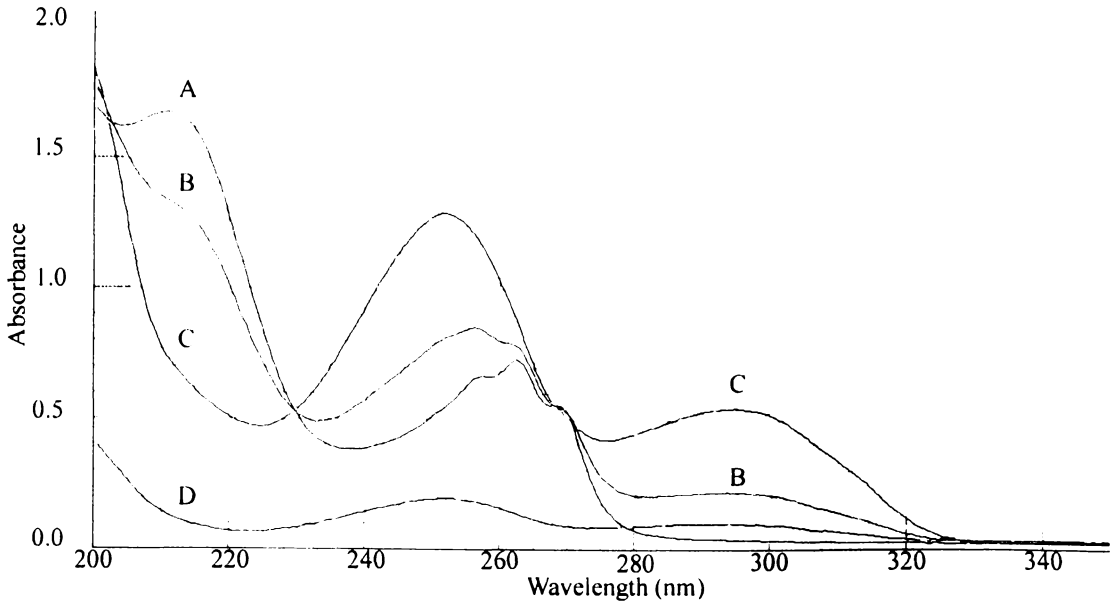


Figure 7.9 UV spectra of diluted *Rhodoplanes* strain Wai3R3 culture supernatants after (A) 15 hours, (B) 86 hours, (C) 115 hours, (D) 153 hours of growth on nicotinic acid as sole fixed carbon source

The centrifuged supernatants of cultures that had been incubated for several weeks on nicotinic acid in the absence of bicarbonate and had failed to grow were scanned to reveal the fate of the substrate. Nicotinic acid had been entirely converted to 6-hydroxynicotinate, which was not further degraded. This is surprising as both compounds are more oxidised than cell carbon.

The progress of nicotinic acid degradation by strain Wai3R3 was also examined by ESMS and HPLC analysis. Figures 7.10 through 7.14 show ESMS spectra obtained from centrifuged culture supernatant samples taken at various times during growth of strain Wai3R3 on 2 mM nicotinate. It should be noted that each spectrum is scaled to the height of the largest peak. In the 62 hour sample (Figure 7.10), a major peak at a Da/e of 122 was visible, corresponding to nicotinate. The peaks at 158 and 176 can be attributed to a species consisting of nicotinate associated with two and three water molecules respectively. At 116 and 137.5 hours, a small peak at a Da/e of 138 could be seen (arrowed in Figure 7.12), corresponding to the ionised carboxylate form of 6-hydroxynicotinic acid. By 137.5 hours (Figure 7.13), the peak at 122 had almost disappeared. By 152.5 hours (Figure 7.14), the peak at 138 was no longer visible. The ESMS spectrum of standard 6-hydroxynicotinate is shown in Figure 7.15. In this spectrum, the peaks at 156 and 174 are probably derived from hydrated forms.

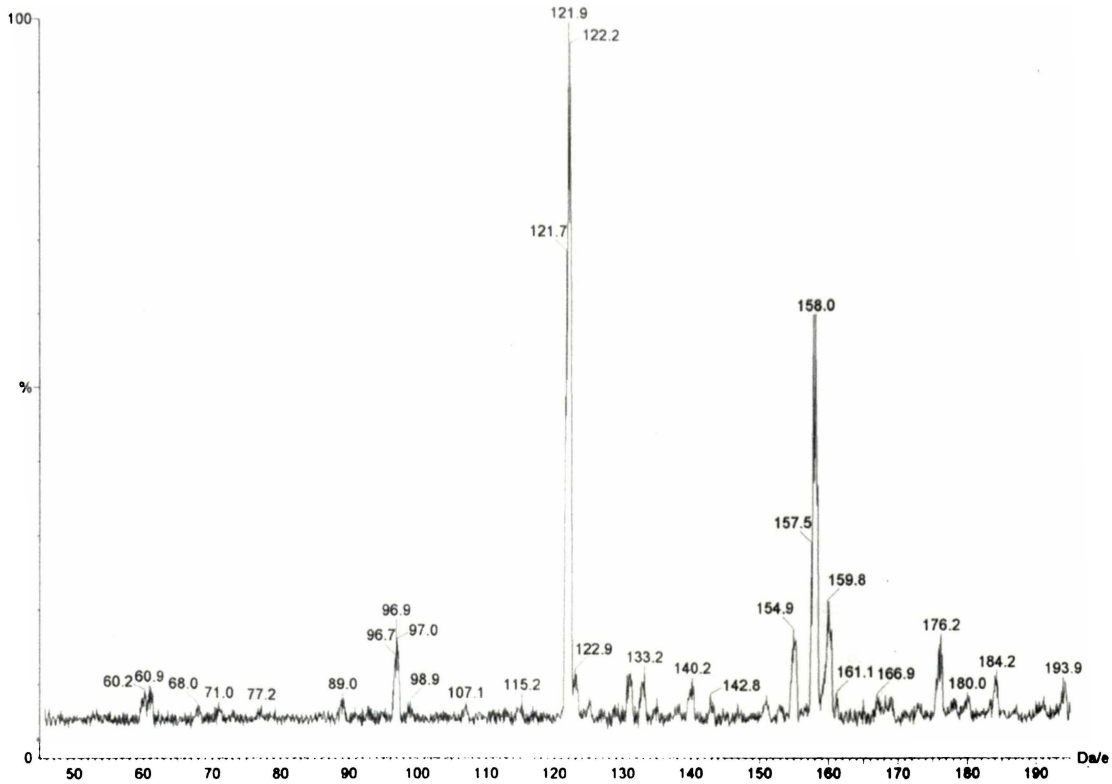


Figure 7.10 ESMS spectrum of 62 hr sample

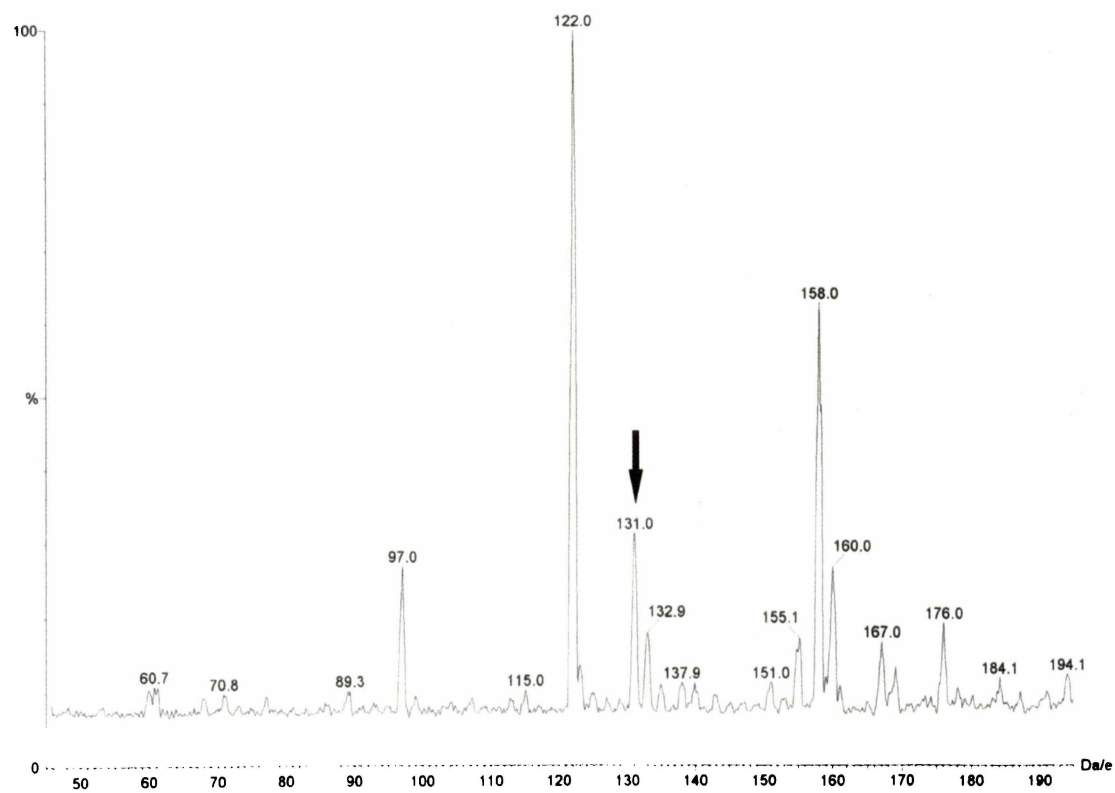


Figure 7.11 ESMS spectrum of 85.5 hr sample

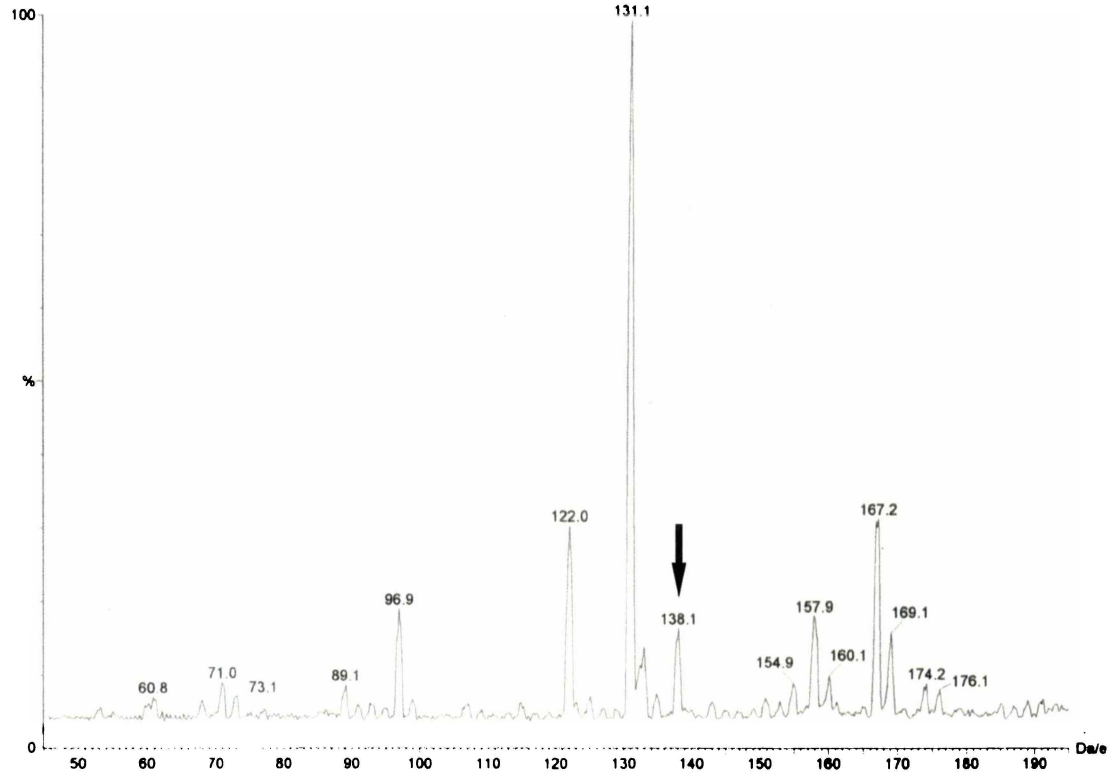


Figure 7.12 ESMS spectrum of 116 hr sample

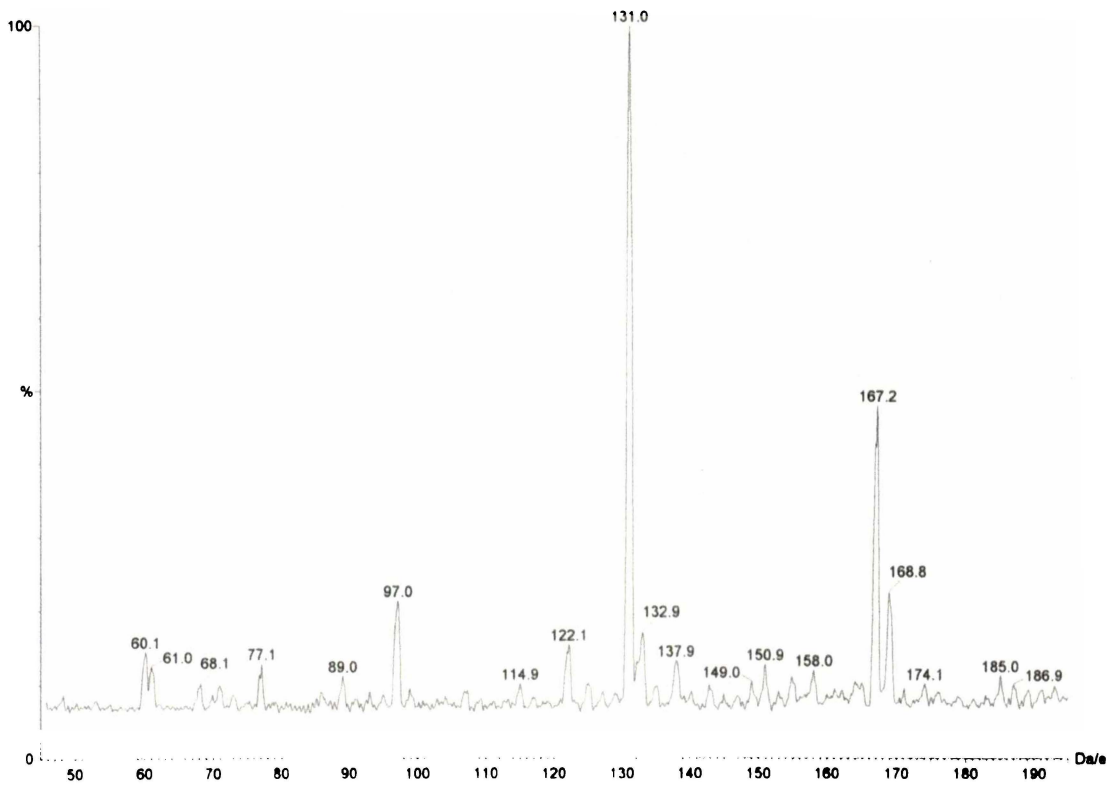


Figure 7.13 ESMS spectrum of 137.5 hr sample

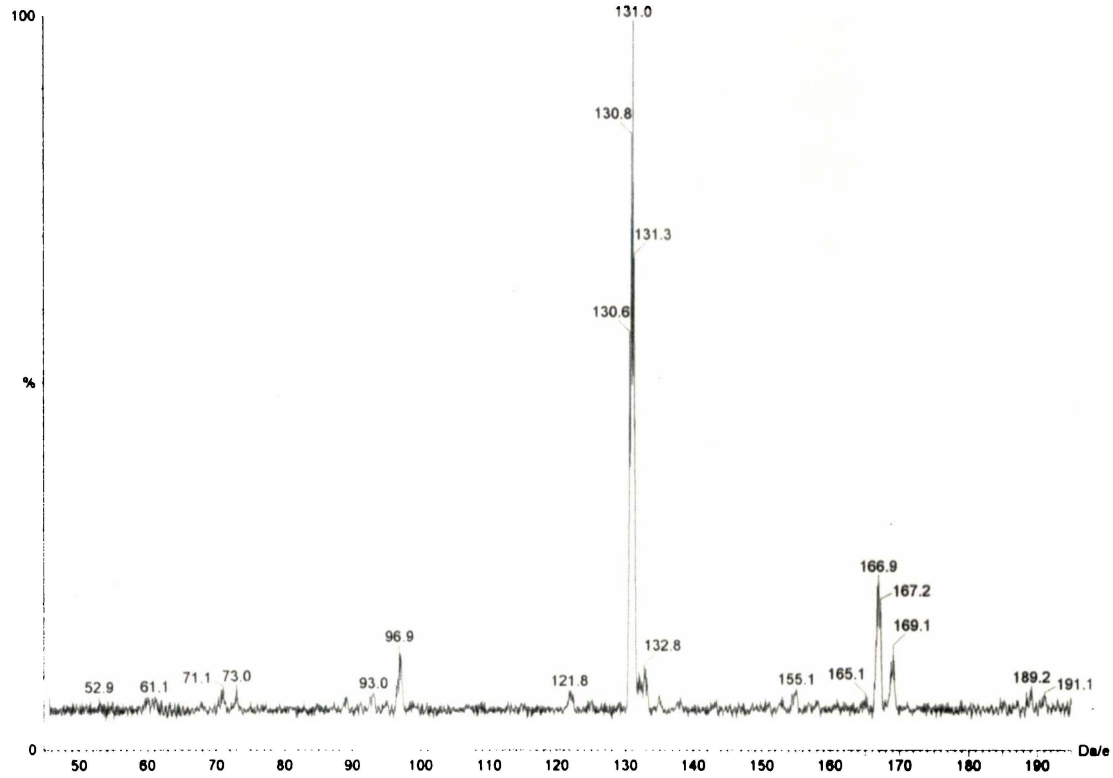


Figure 7.14 ESMS spectrum of 152.5 hr sample

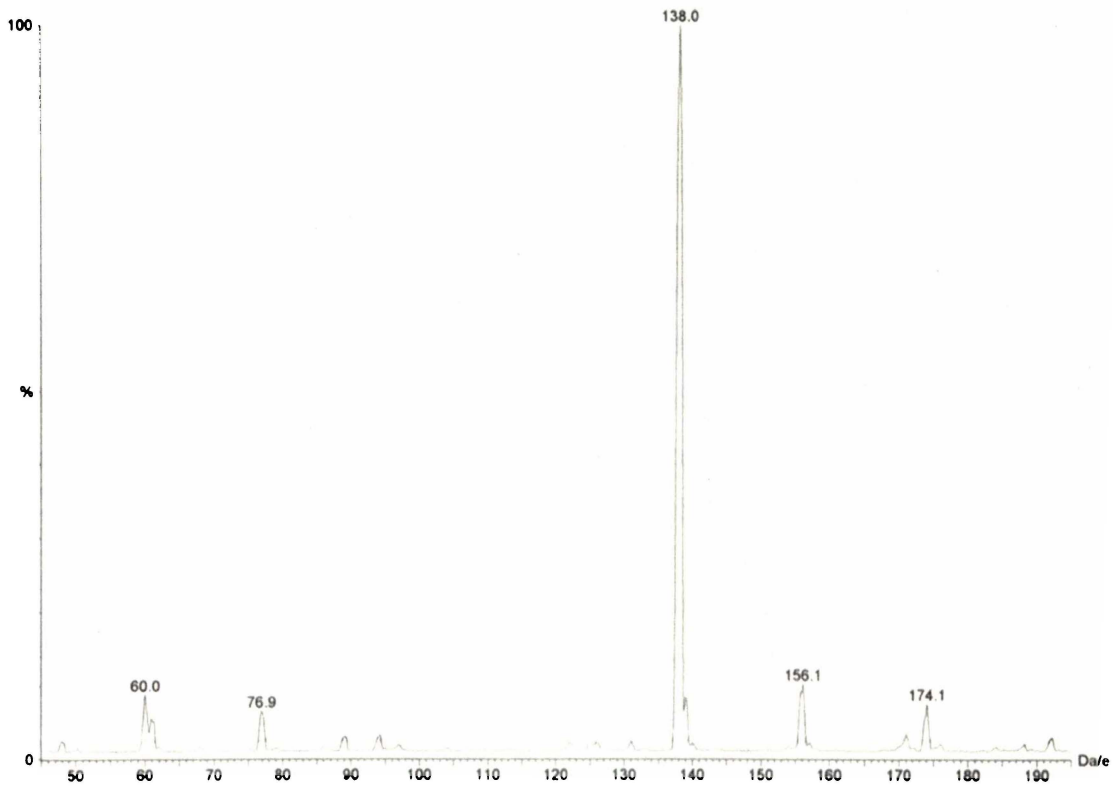


Figure 7.15 ESMS spectrum of 6-hydroxynicotinate

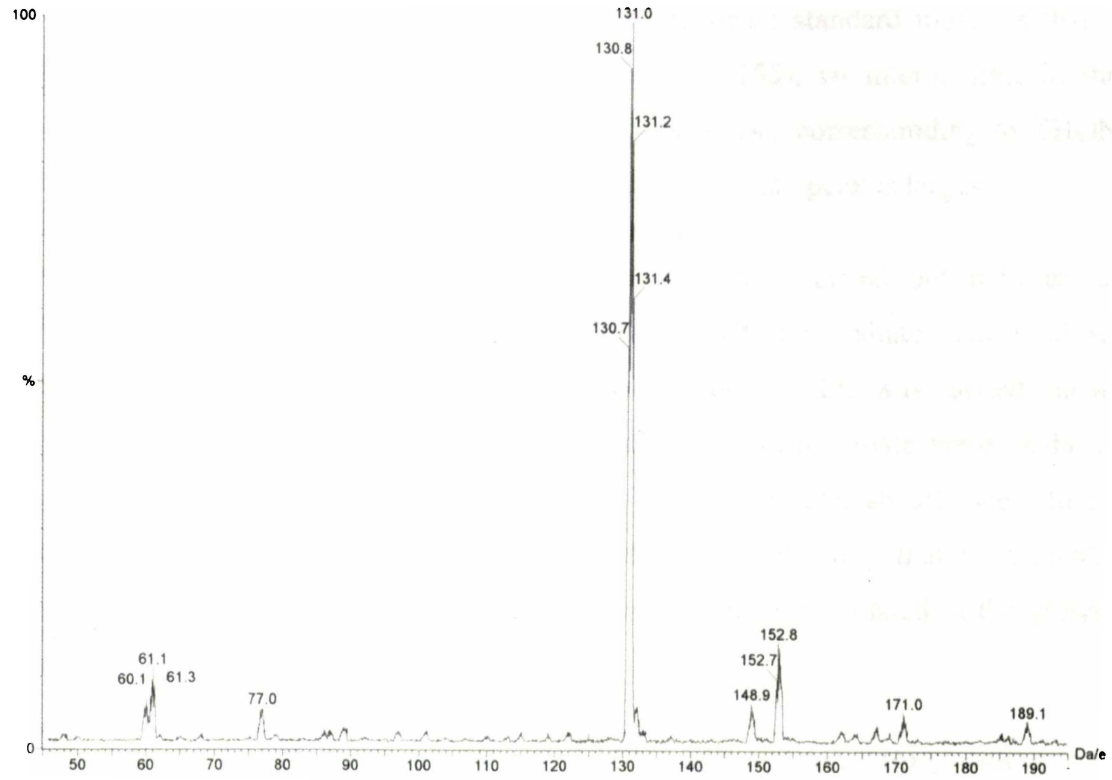


Figure 7.16 ESMS spectrum of sodium glutarate

At 85.5 hours, a peak at 131 was visible (arrowed in Figure 7.11), which thereafter was the dominant peak. Glutaric acid is the end-product of nicotinic acid metabolism in *Azorhizobium caulinodans* (Kitts *et al.* 1992), and the protonated form has a molecular weight of 132. Peaks at 149, 167 and 185 can be attributed to hydrated forms of glutarate. The ESMS spectrum of a glutarate standard is shown in Figure 7.16. The peak at 153 is perhaps derived from the species $Na^+(^-OOC(CH_2)_3COO^-)$ and the peaks at 171 and 189 to hydrated forms of this species. In the growth culture samples, a smaller proportion of the glutarate would be associated with sodium ions, due to the availability of alternative anions. The peak at 97 seen in each spectrum probably corresponds to $H_2PO_4^-$ present in the growth medium.

It was possible to detect glutarate in the 85 hour sample before a peak for 6-hydroxynicotinate was visible. Glutaric acid obviously has a higher response factor, giving a larger ESMS peak than 6-hydroxynicotinate at similar concentrations. It may be supposed that a higher proportion of glutaric acid than 6-hydroxynicotinic acid is present in the (mono)deprotonated form.

The presence of a peak at 156 in the 6-hydroxynicotinate standard indicates that it would be difficult to detect 2,6-dihydroxynicotinate (155), an intermediate in the degradation pathway of *Bacillus niacini*. No peak at 140 corresponding to THON was seen, even in Figure 7.12, where the 6-hydroxynicotinate peak is largest.

Analysis of culture supernatant samples by HPLC was carried out both as an independent confirmation of the identity of the proposed intermediates, and to allow their quantitation over the course of the growth period. HPLC was carried out as described in Section 7.3.2. Nicotinic acid and 6-hydroxynicotinate were analysed using a reverse-phase column and detected by their ultraviolet absorbance. In all culture samples tested, only two peaks were detected, indicating that no aromatic intermediates with a significantly different retention time accumulated in the growth medium.

Figure 7.17 shows the elution profiles of nicotinic acid and 6-hydroxynicotinic acid standards in comparison with the 85.5 hour sample. Elution times were identical, within the repeatability of the system. Standard curves of nicotinate and 6-hydroxynicotinate were constructed and the concentrations of these compounds in supernatants samples were measured.

Authentic glutaric acid eluted after almost 19.5 minutes; a peak with an identical elution time was present in the later samples. The elution profile of a standard and the 145 hour sample is shown in Figure 7.18. The identity of the compound eluting after 12.6 minutes could not be established. The detection limits for glutarate were relatively high and unfortunately it was not possible to accurately estimate the concentrations of glutarate in the culture samples.

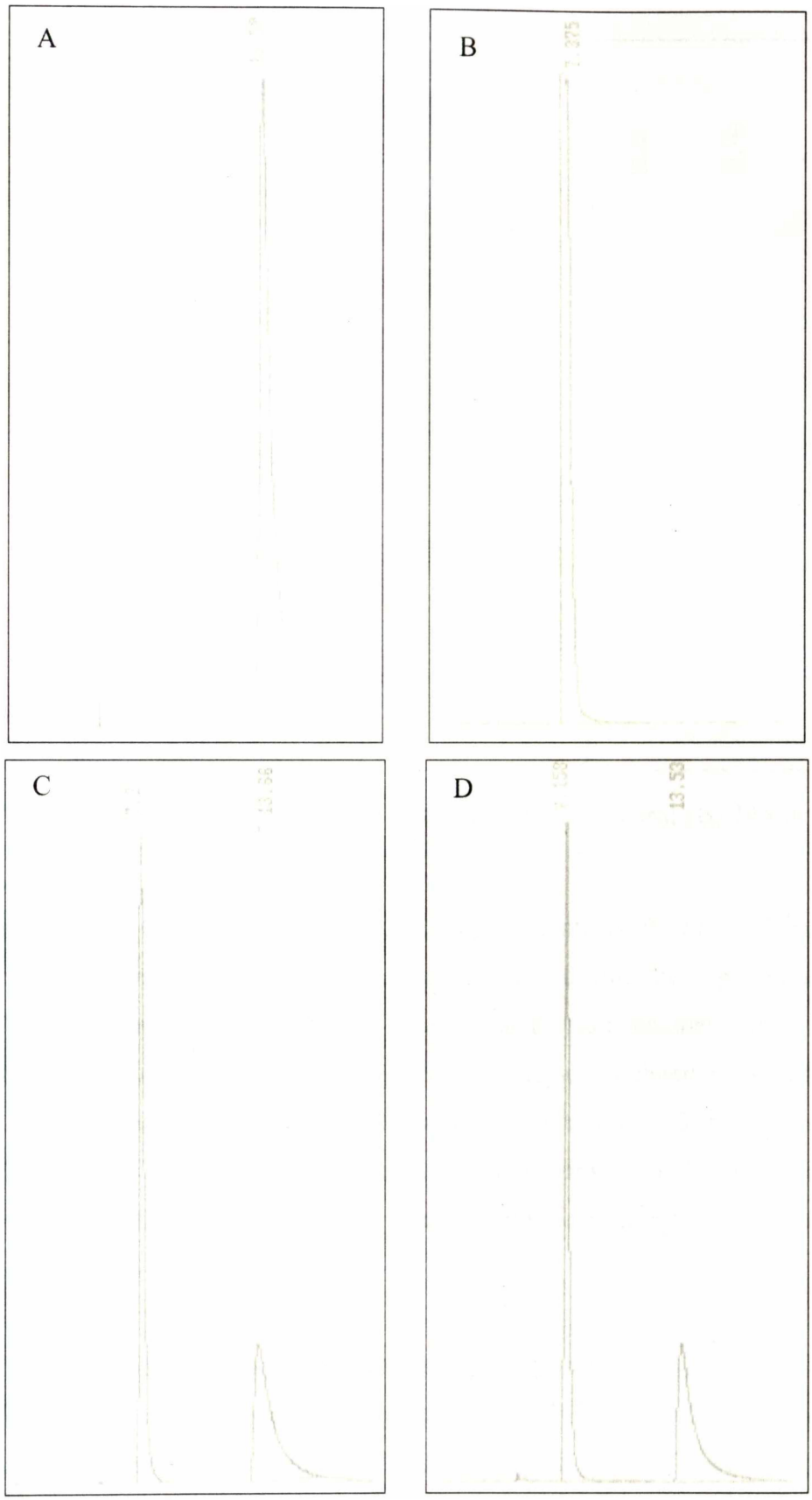


Figure 7.17 HPLC elution profiles: (A) 2mM nicotinic acid standard, eluting at 13.5 min; (B) 2mM 6-hydroxynicotinic acid standard, eluting at 7.4 min; (C) mixture of 0.3mM nicotinic acid and 0.05mM 6-hydroxynicotinic acid; (D) 85.5 hr sample of *Rhodoplanes elegans* Wai3R3 culture medium

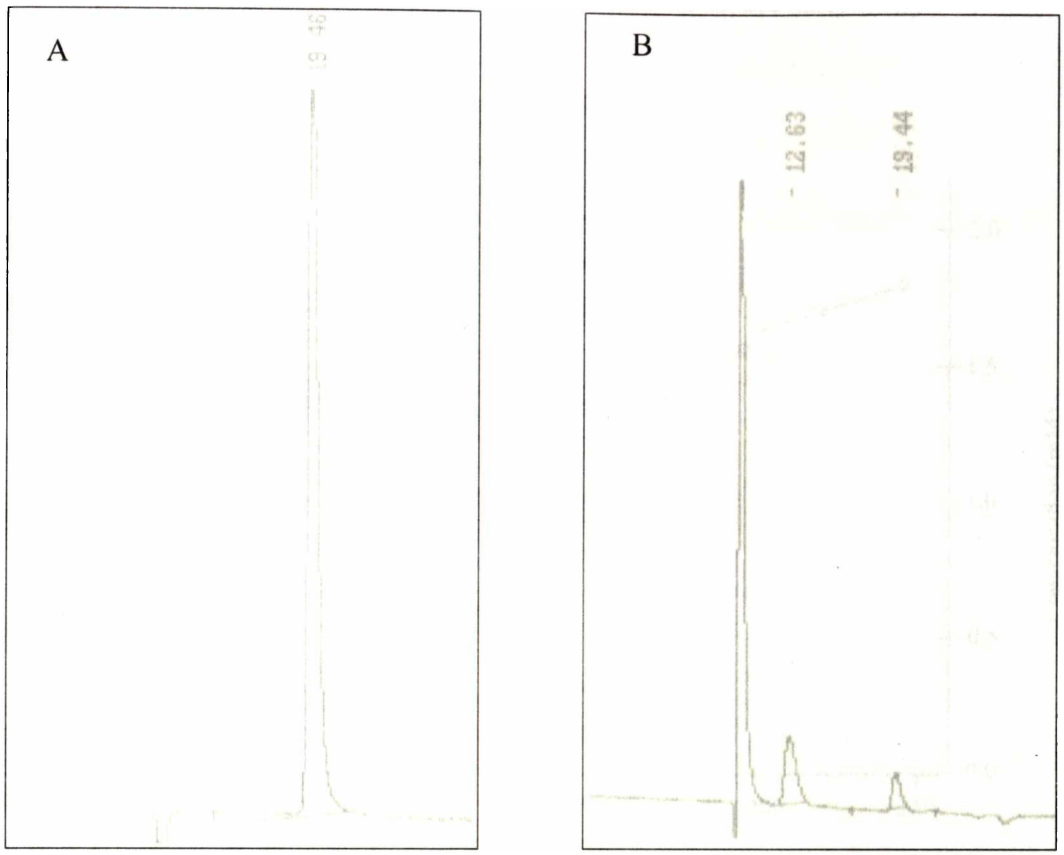


Figure 7.18 HPLC elution profiles: (A) 50mM glutarate standard; (B) 145 hr sample of *Rhodoplanes elegans* Wai3R3 culture medium

The growth curve of *Rpl. elegans* Wai3R3 on nicotinate is shown in Figure 7.19 below. The doubling time for growth on nicotinic acid during the exponential phase of growth was approximately 25 hours. Nicotinic acid was consumed entirely by the end of the sixth day of growth; 6-hydroxynicotinic acid accumulated transiently in the growth medium, reaching a maximum concentration of around 0.8 mM, 40% of the original nicotinate concentration, on the fifth day of growth, and being consumed entirely around 36 hours after this point. The maximum concentration of glutaric acid was reached around 145 hours.

7.4.3 Proposed pathway for nicotinate degradation in *Rhodoplanes elegans*

6-hydroxynicotinate and glutarate were detected as intermediates in the degradation of nicotinic acid by *Rhodoplanes elegans* Wai3R3. All nicotinic acid-degrading strains of *Rhodoplanes* were capable of growth on glutaric acid as sole carbon source. UV spectra of centrifuged culture supernatants and HPLC analysis did not reveal a third

aromatic ring compound, indicating that hydroxylation to 2,6-dihydroxynicotinate as in *Bacillus niacini* was unlikely to occur.

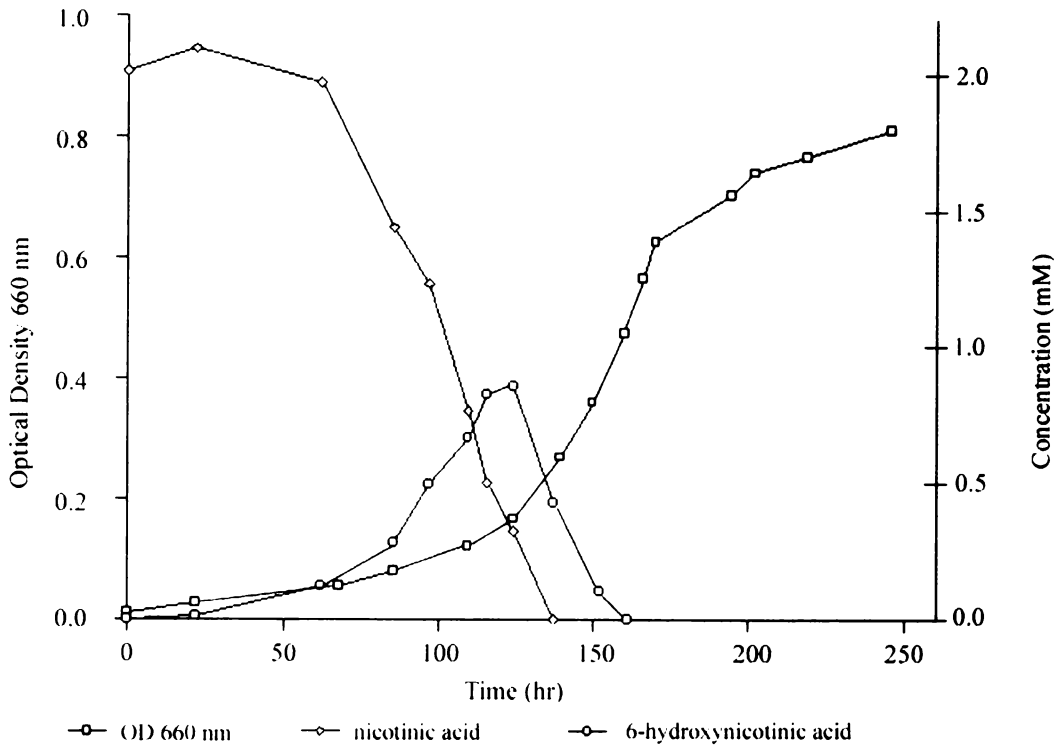


Figure 7.19 Growth of *Rhodoplanes elegans* Wai3R3 on 2 mM nicotinic acid, disappearance of nicotinic acid, and transient accumulation of the intermediate 6-hydroxynicotinic acid

However, THON could not be detected either. Thus the confirmed pathway of degradation of nicotinic acid is shown in the top line of Figure 7.19. Several possibilities for the intervening steps remain.

In A, 6-hydroxynicotinic acid is converted to THON, and the pathway is identical to that proposed for *Azorhizobium caulinodans*.

It should be borne in mind that no intermediates between THON and glutarate were detected by Kitts *et al.* (1992). Alternative series of intermediates are proposed in Figure 7.19 B and C, by analogy with the pathway of degradation of benzoate in the purple nonsulfur bacterium *Rhodopseudomonas palustris* (Figure 7.1).

In B, 6-hydroxynicotinate is converted to 2,6-dihydroxynicotinate which is then reduced to a hydroxylated nipecotic acid derivative, DKN.

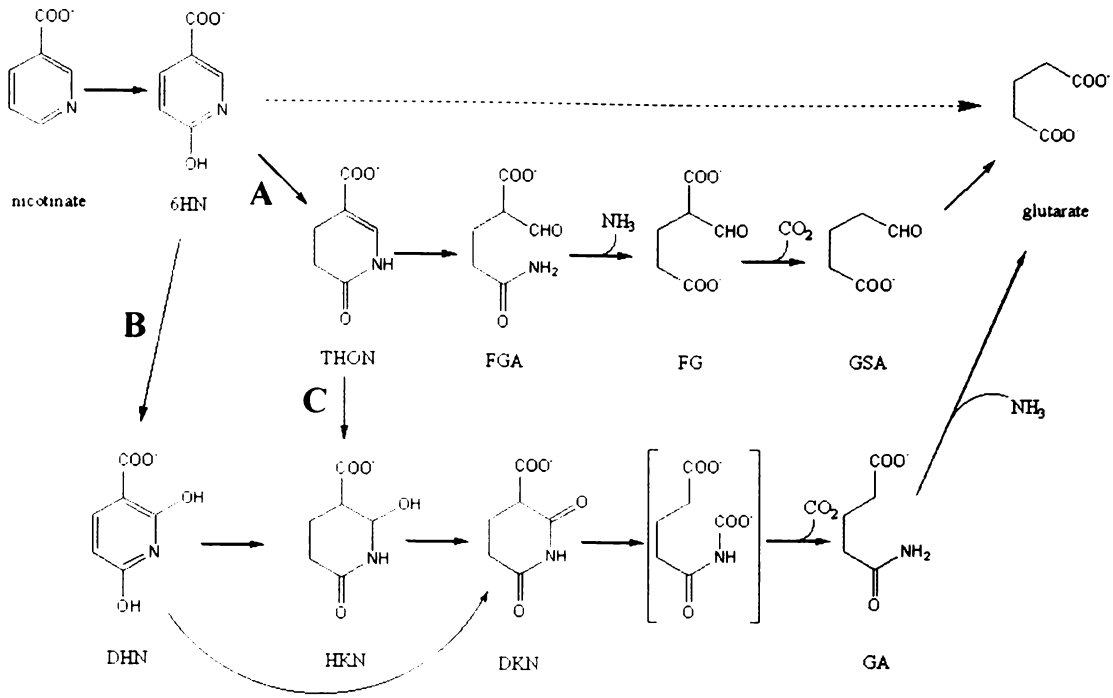


Figure 7.20 Proposed pathways for nicotinic acid degradation by *Rpl. elegans*
Key: 6HN 6-hydroxynicotinate; THON 1,4,5,6-tetrahydro-6-oxo-nicotinate; FGA 2-formyl-glutaryl-5-amide; FG 2-formyl-glutarate; GSA glutaryl-semialdehyde; DHN 2,6-dihydroxynicotinate; HKN 2-hydroxy-6-ketonipecotate; DKN 2,6-diketonipecotate; GA glutaryl-5-amide

In C, THON is converted to the same compound by addition of water across the double bond and subsequent oxidation. In this scheme, THON is analogous to cyclohexene carboxylate, and DKN to 2-ketocyclohexene carboxylate in the benzoate degradation pathway. Addition of water and decarboxylation would lead to glutaryl-5-amide, analogous to pimelate, which would be deaminated to produce glutarate. No evidence is available to determine which of the pathways is correct, and it may be that none of the intermediates accumulate during growth. Attempts were made to carry out assays on cell-free extracts to determine whether 6HN was reduced to THON or oxidised to 2,6-DHN but with no success.

7.4.4 The effect of tungstate on nicotinate metabolism

The effect of addition of sodium tungstate (Na_2WO_4) to the medium on the growth of *Rhodoplanes* strain Wai3R3 was tested (Figure 7.21 A). Growth yield was estimated by measuring the optical density at 660 nm of triplicate stationary phase cultures. Growth on acetate was unaffected by addition of 100 μM sodium tungstate, showing that there were neither general toxicity effects at this concentration nor inhibition of any enzyme required for photoheterotrophic growth. Growth on nicotinic acid in the presence of 100 μM tungstate was 90% of that achieved in the absence of tungstate,

although this decrease was well within the variation in final growth yield observed. There was also no effect of tungstate addition to growth yield on 6-hydroxynicotinate. Stationary phase was achieved in around 10 days; accordingly, no decrease in growth rate was caused by addition of tungstate.

Several strains of *Pseudomonas*, kindly provided by Dr Jackie Aislabie (Landcare-Manaaki Whenua), were screened for the ability to use nicotinic acid as sole carbon source, and one strain, *P. putida* Y5, possessed the capability. Strain Y5 achieved approximately the same culture density on 5 mM nicotinate as strain Wai3R3 achieved on one-fifth the amount. The effect of tungstate addition on growth of this strain was also tested (Figure 7.21 B). In this case, a clear inhibitory effect with increasing tungstate concentration was observed. Addition of 50 μM tungstate led to a 50% reduction in final growth yield, while 100 μM tungstate reduced the growth yield to 42%. *Bacillus niacini* was even more sensitive to tungstate – growth on both nicotinate and 6-hydroxynicotinate was almost entirely abolished at 10 μM tungstate (Nagel and Andreesen 1989). In this respect, the nicotinic acid metabolism of *P. putida* Y5 was typical of other nicotinic acid-degrading bacteria, while the response of the *Rhodoplanes* strains was anomalous.

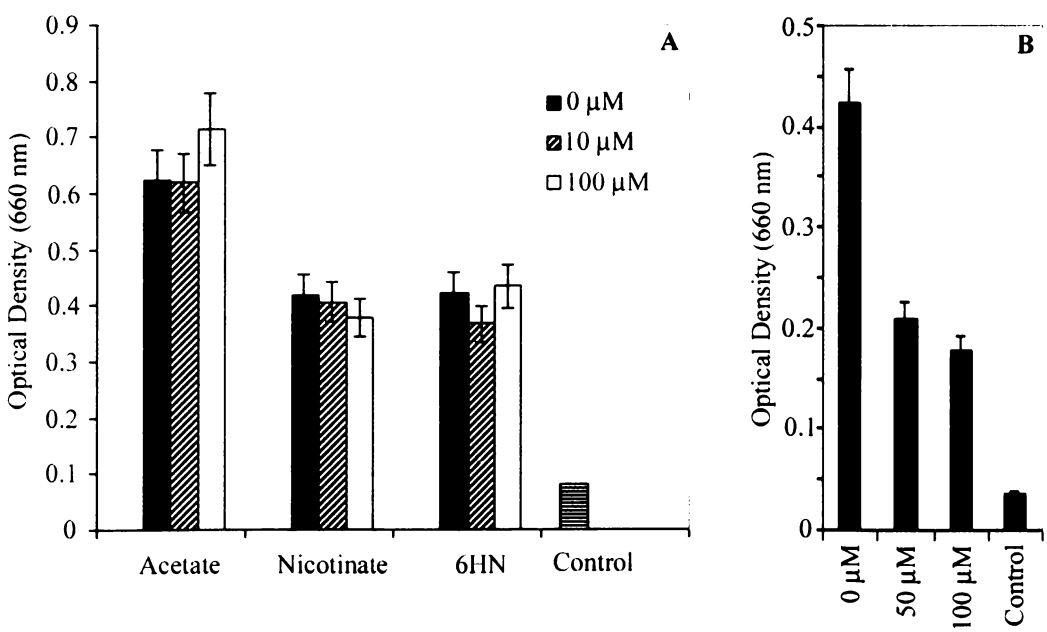


Figure 7.21 Final optical densities of (A) *Rhodoplanes* strain Wai3R3 grown on 5 mM acetate, 1 mM nicotinic acid and 1 mM 6-hydroxynicotinate (6HN) in the presence of various concentrations of sodium tungstate, and (B) *Pseudomonas putida* Y5 grown on 5 mM nicotinate in the presence of various concentrations of sodium tungstate. Control tubes are negative growth tubes lacking carbon source. Results shown are the averages of three replicates (error bars show s.d.).

PNSB are extremely resistant to the toxic effects of metal oxyanions, either by reduction of the anion, as with tellurite and selenite, or through other unknown mechanisms, as with arsenate, molybdate and tungstate (Moore and Kaplan, 1992). It was therefore thought necessary to confirm that a known molybdoenzyme was indeed inhibited in the presence of tungstate. It is unlikely that degradation of nicotinic acid by *Rhodoplanes* strains is dependent only on selenite, as was found for *Desulfococcus niacini*. This oxyanion is not included in the trace element solution added to the growth medium (see Section 3.4.1), so any requirement by *Rhodoplanes* strains must be exceedingly low.

Addition of tungstate was reported to inhibit growth of *Rhodobacter capsulatus* on xanthine (Kaspari and Busse 1986) which is dependent on the molybdoenzyme xanthine dehydrogenase (Leimkühler *et al.* 1998); Yuan (2000) observed over 90% inhibition of growth on xanthine and hypoxanthine by *Rba. azotoformans* and *Rfx. fermentans* in the presence of 100 μM tungstate. No data were available concerning tungstate inhibition in *Rhodoplanes*.

Dissimilatory (i.e. respiratory) nitrate reductase is a member of the DMSO reductase family of molybdenum-centre possessing enzymes (Hille 1996). The reduction of nitrate by *Rhodoplanes* strains Wai3R3 and OK5R2b under dark anaerobic growth conditions was checked using the dimethylnaphthylamine/sulphanilic acid method (Section 3.8.6) in the presence and absence of tungstate. Reduction of nitrate to nitrite was totally abolished by addition of 100 μM tungstate, indicating that *Rhodoplanes* strains are not inherently resistant to the inhibitory effects of tungstate. This provides circumstantial but intriguing evidence that the enzymes responsible for degradation of nicotinic acid in *Rhodoplanes* do not contain the usual molybdenum cofactors.

7.4.5 Nicotinate as a carbon source and as a vitamin

As reported in Section 5.2.1, the *Rhodoplanes elegans*-like strains have an unusually high requirement for nicotinic acid as a vitamin, some 40 times higher than the closely related *Rpl. roseus*. *Azorhizobium caulinodans* also requires high levels of nicotinate as a vitamin under N_2-fixing conditions. However, the *A. caulinodans* requirement is probably due to competitive depletion of nicotinate as the vitamin is catabolised as an alternative nitrogen source (Kitts *et al.* 1989), and is relieved by

addition of ammonium (Elmerich *et al.* 1983). By contrast, the *Rhodoplanes* strains required high levels of nicotinate when growing in medium that contained ammonium. It is possible that degradation of nicotinate is not well regulated in *Rhodoplanes*, such that nicotinate is degraded continually even in the presence of ammonium and of other carbon sources. Alternatively, it is possible that nicotinate is required at high levels for reasons entirely unrelated to the presence of a nicotinate catabolic pathway, though it is unclear what such reasons might be.

7.5 Conclusions

All *Rhodoplanes elegans*-like strains examined possessed the ability to grow on nicotinic acid as sole carbon source with a doubling time of around 25 hours. The compound was degraded via the intermediate 6-hydroxynicotinic acid, which accumulated transiently in the growth medium. Glutaric acid was also observed in the growth medium. This indicates that the pathway for degradation is similar to that observed in *Azorhizobium caulinodans* (Kitts *et al.* 1992) which lacks steps that involve use of molecular oxygen. However, degradation in *Azorhizobium* occurs only under aerobic conditions and is dependent on transfer of electrons to O_2 as terminal electron acceptor. Degradation by *Rhodoplanes* strains is truly anaerobic and non-fermentative.

Unlike all nicotinic-acid degrading organisms that have been tested, growth of *Rhodoplanes* on nicotinate is not inhibited by addition of tungstate to the growth medium, providing an indication that the early reaction steps are not catalysed by standard molybdoenzymes.

7.6 Suggestions for further work

It would be of interest to confirm the presence of THON or of 2,6-dihydroxynicotinate, either by analysis of culture supernatants or by enzyme assays of cell extracts. Although assays were unsuccessful, it is probable that more intensive efforts would allow measurement of such activity. If nicotinate or 6-hydroxynicotinate linked reduction of nitroblue tetrazolium could be achieved, it might be possible to detect and isolate the enzymes responsible for the activities on a

non-denaturing native polyacrylamide gel. Analysis of the composition of the purified enzymes would reveal whether they contain molybdenum centres.

Isolation of the genes involved in nicotinate catabolism would be of interest, but would be a difficult task in the absence of sequenced homologues and in the absence of a transformation system for *Rhodoplanes*. DNA fragments bearing the genes for nicotinic acid catabolism were isolated from *Azorhizobium caulinodans* in the course of mutant complementation studies, but no sequences were obtained (Buckmiller *et al.* 1991). This is doubly unfortunate, as *A. caulinodans* is relatively closely related to *Rhodoplanes* (see Figure 2.5). N-terminal sequences are available for the subunits of *Eubacterium barkeri* nicotinic acid hydroxylase (Gladyshev *et al.* 1996); the N-terminal sequence of the 33 kDa subunit shows promise for design of an oligonucleotide probe, but it is unlikely that the protein sequence would be conserved between *Eubacterium* and *Rhodoplanes*, especially in light of the doubt over the nature of the *Rhodoplanes* enzyme.

The requirement for higher than usual levels of a vitamin by *Rhodoplanes* strains and *Azorhizobium caulinodans* raises the possibility that previously unknown organisms could be enriched and isolated on media containing high levels of nicotinate or other vitamins. It may be of ecological significance that all *Rhodoplanes* strains with a requirement for high levels of nicotinate were isolated from microbial mat environments. Vitamins and other cell components might well be present in higher concentrations within microbial mats than in other environments, due to the high cell density and high proportion of moribund cells.

Chapter 8

Summary

8.1 Enrichment and isolation

Thermotolerant purple nonsulfur bacteria capable of growth above 40°C were found in several New Zealand thermal areas, notably Waimangu Volcanic Valley and Orakei Korako. Purple bacteria could not be enriched from most Tokaanu thermal area samples, for unknown reasons. Isolated strains were assigned to the genera *Blastochloris*, *Phaeospirillum*, *Rhodomicrobium*, *Rhodoplanes*, *Rhodopseudomonas*, *Rhodospirillum* and *Rubrivivax*.

The use of heavy metal oxyanions such as molybdate and tellurite may be of assistance in the isolation of purple nonsulfur bacteria from environmental samples that are also rich in purple sulfur bacteria or sulfate-reducing bacteria.

8.2 Unusual organisms

Several organisms observed in enrichment cultures that could not be purified were of interest. Epibionts that resembled *Vampirococcus* were seen in association with purple sulfur bacteria. Heliobacteria with a temperature range for growth lower than that of *Heliobacterium modesticaldum* were obtained in mixed culture. An organism of strange "propeller" or "bow-tie" morphology was observed in an enrichment from Warbrick Terrace (Wai5).

8.3 Characterisation of isolates

Isolated strains included *mildly thermophilic* organisms with temperature optima at or above 40°C and maxima up to 46.5°C, *thermotolerant* organisms with temperature optima much lower than their maximum growth temperatures, and organisms of limited thermotolerance.

Rhodoplanes strains were commonly found in enrichment cultures and were readily isolated. Absorption spectra of cells of New Zealand *Rhodoplanes* strains grown under low-light conditions had an unusual peak at 900 nm. Waimangu and Orakei Korako strains of *Rhodoplanes* are proposed as a novel species, *Rhodoplanes venustus* sp. nov. These strains required unusually high amounts of nicotinate as growth factor

Strain Tok2tar1 is proposed as a novel species, *Rhodoplanes segnis* sp. nov. "*Rhodopseudomonas cryptolactis*" (Stadtwald-Demchick *et al.* 1990b) was closely related to strain Tok2tar1, but differed in many phenotypic properties.

Rhodoplanes elegans and several thermotolerant *Rhodoplanes* strains had the ability to grow on nicotinic acid as sole carbon source. The pathway for degradation was similar to that proposed for *Azorhizobium caulinodans* (Kitts *et al.* 1992), with 6-hydroxynicotinate and glutarate as observed intermediates. Growth on nicotinate and 6-hydroxynicotinate was not inhibited in the presence of tungstate, suggesting that the early reactions of the pathway are not catalysed by molybdoenzymes.

Blastochloris isolates represented two novel lineages within the genus, related to *Blc. sulfoviridis*. *Blastochloris* strain GI (Resnick and Madigan 1989) was closely related to strains Wai1bG3 and Wai3G1e. A lineage comprising strains OK3G7 and Wai5bG1 showed distinctive features, but members of the other lineage could not be clearly distinguished from *Blc. sulfoviridis* on the basis of available information.

Rhodopseudomonas isolates could be classed in two groups according to 16S rDNA sequence. Members of a cluster that included the type strain *Rps. palustris* ATCC 17001 were only mildly thermotolerant and were able to use benzoate as carbon source. Members of the second cluster were more thermotolerant and were unable to use benzoate.

Two spiral-shaped organisms were isolated. One was closely related to *Rsp. photometricum*, while the other grew optimally at 40°C and is proposed as a novel species of the genus *Phaeospirillum* as *Phaeospirillum tepidarium* sp. nov.

Rubrivivax isolates were closely related to other *Rvi. gelatinosus* strains according to 16S rDNA sequence, but grew optimally at 40-44°C.

Rhodomicrobium vannielii strains from thermal areas grew faster than mesophilic strains at their maximum temperature, but differed in no other significant respects.

8.4 Temperature selection experiments

All PNSB tested, whether mesophilic with temperature maxima between 36 and 42°C, or mildly thermophilic with temperature maxima approaching 47°C, had a maximum tolerance temperature of 52°C.

Mesophilic type strains of *Rpl. elegans*, *Blc. sulfoviridis* and *Rba. capsulatus* showed rapid adaptation to growth at high temperature after serial transfer at their temperature maxima. No increase in the maximum temperature for growth occurred, but significant increases in growth rate at elevated temperatures were observed. Selected lines of *Rpl. roseus* and *Blc. viridis* did not grow better at high temperature, but showed reduced growth at low temperatures.

8.5 Molecular diversity studies

Non-culture based detection of phylogenetically-diverse purple photosynthetic bacteria was achieved using *pufL*, a gene involved in the photosynthetic process, as a specific marker. An environmental *pufL* clone library constructed from an OK5 microbial mat sample contained sequences from a wide diversity of unknown and as yet uncultured organisms. No sequences derived from cultured organisms were detected. Four novel *pufL* sequences attributable to purple nonsulfur bacteria were obtained, suggesting that many PNSB species remain to be discovered. Many of these would be representatives of novel genera. Sequences of aerobic anoxygenic phototrophic bacteria dominated the clone library, indicating that these organisms are important members of the purple photosynthetic community in this environment.

Appendix 1

GenBank Accession Numbers

1. Sequences determined in this study

16S rDNA sequences

Blastochloris [*Rhodopseudomonas*] strain GI	unsubmitted
Blastochloris strain OK3G7	AY117148
Blastochloris strain Wai1bG3	AY117149
Blastochloris strain Wai3G1e	AY117150
Phaeospirillum strain MPA1	AF487433
Porphyrobacter tepidarius OK5APO	AY048657
Rhodomicrobium vannielii OK5M2	AF487436
Rhodoplanes strain OK5R2b	AF487437
Rhodoplanes strain Tok2tar1	AF077729
Rhodoplanes strain Wai3R3	AF077730
Rhodopseudomonas strain OH3F2	AF487429
Rhodopseudomonas strain OK4bTR3	AF487430
Rhodopseudomonas strain TKT2	AF487432
Rhodopseudomonas strain Wai1S1	AF487428
Rhodopseudomonas strain Wai1val1	AF487431
Rhodospirillum strain WSSB3	AF487434
Rubrivivax gelatinosus OK303d	AF487435

pufL and *pufM* sequences

Blastochloris [*Rhodopseudomonas*] strain GI	AF161592
Blastochloris strain Wai1bG3	AF161591
Blastochloris sulfoviridis Wai3G1e	AF161590
Blastochloris strain Wai5bG1	AF161593
Rhodoplanes strain Tok2tar1	AF485346
Rhodoplanes elegans AS130	AF159440
Rhodoplanes roseus 941	AF485345
Rhodopseudomonas strain Wai1S1	AF485347
Rubrivivax gelatinosus OK303d	AF485348

Cloned *pufL* sequences from uncultured organisms

Clone OK5m03	AY048660
Clone OK5m04	AY048655
Clone OK5m10	AY048656

Clone OK5m20	AY048651
Clone OK5m27	AF484109
Clone OK5m30	AY048652
Clone OK5m36	AF484108
Clone OK5m51	AY048661
Clone OK5m69	AY048659
Clone OK5m73	AY048654
Clone OK5m77	AY048662
Clone OK5m87	AY048653
Clone OK5m111	AF484110
Clone OK5m114	AY048658

2. Sequences used in phylogenetic analyses

16S rDNA sequences of purple nonsulfur bacteria

Blastochloris sulfoviridis DSM 729	D86514
Blastochloris sulfoviridis GN1	AB033757
Blastochloris sulfoviridis ToP1	AJ012089
Blastochloris viridis ATCC 19567	D25314
Blastochloris viridis G3	AF084498
Blastochloris viridis UN	AF084496
Phaeospirillum fulvum NCIMB 11762	D14433
Phaeospirillum fulvum S3	AF508113
Phaeospirillum molischianum ATCC 14031	M59067
Rhodobaca bogoriensis LBB1	AF248638
Rhodobacter sp. SW2	X78717
Rhodobacter azotoformans KA25	D70846
Rhodobacter blasticus ATCC 33485	D16429
Rhodobacter capsulatus C5	D16427
Rhodobacter sphaeroides IL106	D16424
Rhodobacter veldkampii ATCC 35703	D16421
Rhodoblastus acidophila 7050	M34128
Rhodobium marinum ATCC 35601	D30791
Rhodobium orientis MB312	D30792
Rhodocista centenaria MT-SP-3	D12703
Rhodocyclus purpureus 6770	M34132
Rhodocyclus tenuis DSM 109	D16208
Rhodocyclus tenuis DSM 110	D16209
Rhodoferax antarcticus ANT.BR	AF084947
Rhodoferax fermentans FR2	D16211

Rhodoferax fermentans FR3	D16212
Rhodomicrobium vannielii EY33	M34127
Rhodopila globiformis DSM 161	D86513
Rhodoplanes elegans AS130	D25311
Rhodoplanes roseus 941	D25313
Rhodoplanes roseus DSM 13233	AJ289105
Rhodopseudomonas cryptolactis DSM 9987	AB087718
Rhodopseudomonas sp. B29	AB027692
"*Rhodopseudomonas faecalis*" g-c	AF123085
Rhodopseudomonas sp. IL-245	D15063
Rhodopseudomonas sp. v-1	AF095928
Rhodopseudomonas palustris ATCC 17001	D25312
Rhodopseudomonas palustris DSM 126	X87279
Rhodopseudomonas palustris F7-1	AB017261
Rhodopseudomonas palustris No.7	D89811
Rhodopseudomonas palustris RN1	AB033756
Rhodopseudomonas palustris S55	D84187
Rhodopseudomonas palustris T	AF123087
Rhodopseudomonas rhenobacensis	AB087719
Rhodospira trueperi	X99671
Rhodospirillum sp. E-12	D15064
Rhodospirillum photometricum E-11	D30777
Rhodospirillum rubrum DSM 107	X78278
Rhodospirillum rubrum DSM 467	D30778
Rhodothalassium salexigens ATCC 35888	D14431
Rhodovibrio salinarum NCIMB 2243	D14432
Rhodovulum adriaticum DSM 2781	D16418
Rhodovulum euryhalinum DSM 4868	D16426
Rhodovulum iodosum N1	Y15011
Rhodovulum robiginosum N2	Y15012
Rhodovulum strictum MB-G2	D16419
Rhodovulum sulfidophilum TW13	D16422
Roseospira mediosalina L1-66	AJ000989
Roseospirillum parvum 930I	AJ011919
Rubrivivax gelatinosus A3	D16214
Rubrivivax gelatinosus ATCC 17011	D16213
Rubrivivax gelatinosus IL144	AB016167

Other 16S sequences

Acetobacter aceti JCM 7641	D30768

Acidiphilium acidophilum ATCC 27807	D86511
Acidisphaera rubrifaciens HS AP3	D86512
Afipia felis ATCC 49715	AF338177
Agrobacterium ferrugineum IAM12616	D88522
Agrobacterium sanguineum IAM12620	AB021493
Agrobacterium tumefaciens NCPPB1650	D14506
Alcaligenes faecalis IAM12369	D88008
Amaricoccus macauensis Ben104	U88042
Antarctobacter heliothermus EL-219	Y11552
Aquaspirillum peregrinum IFO 14922	AB000479
Azoarcus denitrificans Td-15	L33688
Azorhizobium caulinodans ORS571	D11342
Azospirillum lipoferum NCIMB 11861	Z29619
Azospirillum sp. 5C	AF413109
Bartonella quintana 803	M11927
Beijerinckia indica	M59060
Blastobacter denitrificans IFAM 1005	AF338176
Blastomonas natatoria DSM 3183	Y13774
Blastomonas ursincola DSM 9006	AB024289
Bradyrhizobium elkanii NA6167	AB070561
Bradyrhizobium japonicum ATCC 10324	U69638
Bradyrhizobium lupini DSM 30140	X87273
Brucella melitensis 11-19	X13695
Burkholderia cepacia DSM 50181	X87275
Comamonas testosteroni	M11224
Craurococcus roseus NS 130	D85828
Dechlorospirillum sp. WD	AF170352
Duganella zoogloeoides IAM 12670	D14256
Erythrobacter litoralis DSM 8509	AB013354
Erythrobacter longus OCh101	L01786
Erythromicrobium ramosum DSM 8510	AB013355
Escherichia coli	J01859
Gallionella ferruginea	L07897
Gluconobacter oxydans DSM 3503	X73820
Hydrogenophaga flava DSM 619	AJ420328
Hyphomicrobium facile IFAM-B522	Y14312
Hyphomicrobium vulgare ATCC 27500	Y14302
Hyphomonas jannaschiana ATCC 33883	AJ227814
Ideonella dechloratans	X72724
Leptothrix discophora SS-1	Z18533

Leptothrix mobilis FeOx-1	X97071
Magnetospirillum gryphiswaldense	Y10109
Magnetospirillum magnetotacticum	Y10110
Magnetospirillum sp. MGT-1	D17515
Magnetospirillum sp. MSM-3	Y17389
Magnetospirillum sp. MSM-4	Y17390
Mesorhizobium loti LMG6123	Y14159
Methylobacterium organophilum JCM 2833	D32226
Natronohydrobacter thiooxidans AHO-1	AJ132383
Nitrobacter hamburgensis X14	L11663
Nitrosomonas europaea M103	AF037106
Octadecabacter antarcticus 307	U14583
Oxalobacter formigenes HC1	U49758
Paracoccus denitrificans IAM 12479	Y17512
Paracoccus versutus DSM 582	Y16931
Phenol-degrading denitrifying spirillum CC-26	AB033746
Porphyrobacter neustonensis DSM 9434	AB033327
Porphyrobacter tepidarius DSM 10594	AB033328
Propionivibrio decarboxylicus DSM 5885	Y17601
Rhodobacter apigmentum	AF035433
Rickettsia prowazekii	M21789
Roseobacter denitrificans OCh114	M96746
Roseobacter litoralis ATCC 49566	X78312
Roseovarius tolerans Ekho Lake-172	Y11551
Rubrimonas cliftonensis OCh317	D85834
Sandaracinobacter sibiricus RB16-17	Y10678
Simonsiella muelleri ATCC 29453	AF328147
Sphaerotilus natans 565	Z18534
Sphingobium chlorophenolicum	X87161
Sphingomonas paucimobilis EPA 505	X94100
Sulfitobacter pontiacus ChLG10	Y13155
Tetracoccus cechii Italian	Y09609
Xanthobacter flavus 301	X94199
Zymomonas mobilis	AF281033

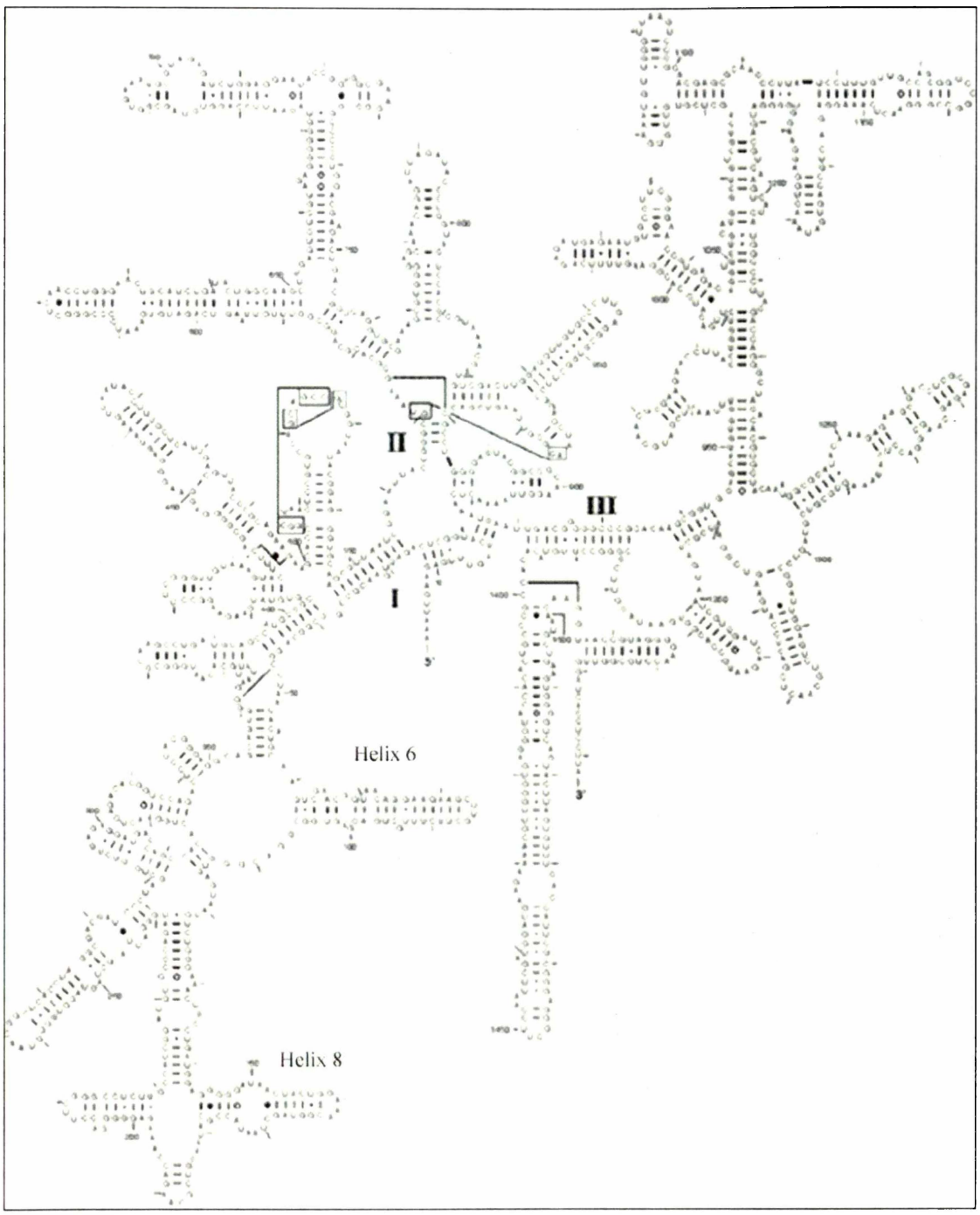
pufL sequences

Acidiphilium acidophilum	ABO13379
Acidiphilium angustum	AB005219
Acidiphilium cryptum	AB005220
Acidiphilium multivorum	AB00521

Acidiphilium organovorum	AB005222
Acidiphilium rubrum	AB005218
Allochromatium vinosum D	AB011811
Blastochloris sulfoviridis DSM 729	AB017041
Blastochloris viridis	X03915
Blastomonas natatoria IFO15649	AB012060
Blastomonas sp. NT12	AB026182
Blastomonas ursincola DSM 3183	AB031015
Ectothiorhodospira shaposhnikovii	AF018955
Erythrobacter litoralis IAM 14332	AB010981
Erythrobacter longus MBIC 2294	AB035570
Erythromicrobium ramosum IAM 14333	AB010873
Phaeospirillum molischianum	D50654
Porphyrobacter neustonensis DSM 9434	AB011073
Porphyrobacter sanguineus IAM 12620	AB011074
Porphyrobacter tepidarius MBIC 3363	AB020599
Rhodobacter blasticus	D50649
Rhodobacter capsulatus	Z11165
Rhodobacter sphaeroides	AJ010302
Rhodocyclus tenuis	D50651
Rhodoferax fermentans	D50650
Rhodomicrobium vannielii	AB010830
Rhodoplanes elegans	AF159440
Rhodoplanes roseus	AF485345
Rhodopseudomonas palustris	AB015977
Rhodospirillum centenum	L81173
Rhodospirillum photometricum	D50681
Rhodospirillum rubrum	J03731
Rhodovulum sulfidophilum	AB020784
Roseateles depolymerans DSM 11813	AB028938
Roseobacter denitrificans MBIC 2684	AB016989
Rubrivivax gelatinosus IL144	D16822
Thiocystis gelatinosa	D50653

Appendix 2

Secondary structure of *Escherichia coli* 16S rRNA



Appendix 3

Deduced *pufL* amino acid sequence alignment

```
Rsp. rubrum                 GTALIVWGAALGP-----SWTFWQISINPPDVSYGLAMAPMAKGGLWQII
Rps. palustris              GTALIIWNTALGP-----TWNLWQISVNPPDAKYGLGFAPLAEGGIWQWV
Bradyrhizobium ORS278       GVALILYATALGP-----TWNLWQISINPPDAKYGLSFAPLTEGGFWQLI
OK5m111                     GVAMIMYGALLGP-----TWNIWLISINPPPPEYGLAFAPLKEGGIWQIV
OK5m20                      GVAMIMYGAALGP-----TWNIWLISINPPPPEYGLAFAPLKEGGIWQIV
Rmi. vannielii              GTALIVWGAAQGH-----TLNIWKINIAPPDLSYGLGVAPLAKGGLWQLI
Blc. sulfoviridis           GICFIGYAASQGP-----TWDPFAISINPPDLKYGFAAAPLLEGGFWQAI
Blc. viridis                GVSLIGYAASQGP-----TWDPFAISINPPDLKYGLGAAPLLEGGFWQAI
Rpl. roseus                 GTALIIWGAALGP-----TWNLWQINIAPPDLSYGLALAPLREGGLWQLI
Rpl. elegans                GTALIIWGAALGP-----TWNIWQINIAPPDLSYGLTLAPLKEGGLWQII
OK5m51                      GTALIIWGAALGP-----TWNIWQISIAPPDLKYGLALAPLKEGGLWQLI
Rsp. centenum               GTALIIYGAALGP-----TWNIWQINIAPPDIKYGLALAPLKEGGLWQLI
OK5m87                      GTALIVYGAALGP-----TWNLWQINIAPPDLSYGLRLAPLAEGGLWQLI
R. depolymerans             GTLLIVWGASQGG-----TWNLWRISIAPPDLKYGLGMAPLMEGGLWQLI
Rfx. fermentans             GTFMILWGASQGP-----TWNLWQISIAPPDLSYGLGIAPLMEGGLWQLV
Rcy. tenuis                 GTLLIVLGASWGP-----TWNLWQISIAPPDLKYGLGVAPLREGGLWQAI
Rvi. gelatinosus            GTALIIWGASQGP-----TWNLWQISIAPPDLKYGLGVAPLMEGGLWQII
Phs. molischianum           GVALIVLAASHGP-----TWNPWEISIAPPDLSYGLGFAPLNEGGFWQFI
Rsp. photometricum          GIALILLGTALGP-----TWNPLRINIAPPDLSYGLGFAPLMKGGLWQLI
BAC-65D09                   GTALILVGASMGD-----TWNIWRINIAPPDLSYGLGVAPFAEGGAWQLI
A. vinosum                  GVLLIVWGATIGPNAELQTYNIWQISIAPPDLSYGLGMAPMTEGGLWQII
T. gelatinosa               GTLLIVWGATLGPNAELQTFNIWQISIAPPDLSYGLGMAPMTEGGLWQII
E. shaposhnikovii           GTLLIVWGAAMGP-----TWNIWQISINPPDLSYGLGFAPLTEGGLWQMI
OK5m04                      GTALIFYGAAIGP-----TWNPWLISVEPPDLSYGLGLAPLKEGGLWQII
OK5m69                      GTALIFYGAAIGP-----TWNPWLISIEPPDLSYGLGLAPLKEGGLWQII
OK5m77                      GTALIFYGAAIGP-----TWNPWLISIEPPDLSYGLGLAPLKEGGLWQII
OK5m114                     GTALIFWGASQGP-----TWNPWLISITPPDIKYGLGLAPLMEGGLWQII
OK5m73                      GTLLILYGAAIGP-----TWNPWLISIEPPDLKYGLGLAPLAEGGLWQII
B. natatoria                GTALIFYGASQGG-----TWNPWLINIAPPDLKYGLGLAPLKEGGLWQII
OK5m10                      GTALILYAASQGP-----TWNPWLISIEPPDLKYGLGLAPLSEGGFWQVI
Blastomonas NT12            GTALILYGASLGP-----TWNPWLISIAPPDISYGLGFAPLKAGGLWQII
OK5m30                      GTALIFYGASIGP-----TWNPWQINIAPPDLKYGLGLAPLAEGGLWQVI
A. sanguineum               GTALIFAAAAQGP-----TLNPWLISINPPSIEAGLAFAPLSEGGYWQVI
P. tepidarius               GTALIFAAATQGP-----TLNPWLISINPPPVEYGLAFAPLKEGGYWQVI
P. neustonensis             GTMLIFAAATQGP-----TLNPWLISINPPPVEYGLSLAPLAQGGYWQII
E. ramosum                  GTMLIFAAATQGP-----TLNPWLISINPPPVEYGLSLAPMAQGGYWQII
E. longus                   GTMLIFAAAAAGP-----TLNPWLIDIQPPAIEAGLAFAPLNEGGYHQII
E. litoralis                GTALIFAAAIEGP-----TLNPWLIDIQPPPIEAGLALAPLSEGGYWQII
OK5m27                      GTALIFLGASMEN-----TWNPWLISIDPPSIDYGLQLAPMAEGGIWQMV
A. acidophilum              GTALIIWGAAIGP-----TWNIWEINIAPPNLSYGLAFAPLAHGGLWQII
A. rubrum                   GTALIIWGAAIGP-----TWNIWQINIAPPDLSYGLGFAPLAHGGLWQII
A. cryptum                  GTALIIWGAALGP-----TWNIWQISIAPPDLSYGLGLAPLAKGGLWQII
R. denitrificans            GTILIFWGASQQG-----TFNPWLINIAPPDLSYGLGLAPLLEGGLWQII
Rba. sphaeroides            GIILIAWSAVLQG-----TWNPQLISVYPPALEYGLGGAPLAKGGLWQII
Rba. capsulatus             GFLLILWGAAMQG-----TWNPQLISIFPPPVENGLNVAALDKGGLWQVI
Rba. blasticus              GTLLILYGTAMEG-----VWNPQLISIEPPSVENGLAFAPLAEGGLWQLI
Rov. sulfidophilum          GTAMIFWGAALQG-----TFNPLKIHIAPPDLSYGLSIAPLAEGGLWQFI
                            *  :*   :              * : **  . *:  *.:  **  * :
```

```
Rsp. rubrum                 TFSAIGAFVSWALREVEICRKLGIGYHIPFAFGFAILAYVSLVVIRPVMM
Rps. palustris              TIFATGAFCSWALREVEICRKLGIGYHVPFAFSFAIFAYLTLVVIRPVLM
Bradyrhizobium ORS278       TVCAHGAFVSWALREVEICRKLAIGYHVPFAFGFAIFAYFTLEVIRPVLM
OK5m111                     TVCALGAFGSWALREVEICRKLGIGYHVPFAFSIAIFAYFTLVVIRPVLM
OK5m20                      TVCALGAFGSWALREVEICRKLGIGYHVPFAFSIAIFAYFTLVVIRPVLM
Rmi. vannielii              TICATGAFISWALREVEICRKLGIGYHVPIVFSVAIFAYLTLVVIRPVLL
Blc. sulfoviridis           TVCALGAFFSWMLREVEISRKLGMGWHVPLAFCVPIFMFCVLQVFRPILM
Blc. viridis                TVCALGAFISWMLREVEISRKLGIGWHVPLAFCVPIFMFCVLQVFRPLLL
Rpl. roseus                 TVCALGAFCSWALRQAEIARKLGMGYHIPVAFSFAIFAYIALVVIRPLLM
Rpl. elegans                TVCALGAFCSWALRQAEIARKLGMGLHIPVAFGFAIFAYVALVVIRPLLM
OK5m51                      TICALGAFGSWALRQAEISRKLGMGYHIPVAYSVAILAYFTLVVVRPVLL
Rsp. centenum               SICALGAFVSWALRQVEISRKLGMGLHVPVAYSVAVFAYFTLVVIRPVLM
OK5m87                      TICAIGAFVSWALREVEICRKLGIGYHVPVAFGFAILAYVTLVVIRPLLM
R. depolymerans             TVCAIGAFVSWALREVEICRKLGMGYHVPFAFAVAIFAYVTLVVVRPVLM
Rfx. fermentans             TVCAIGAFVSWMLREVEICRKLGMGLHVPLAFGVAIFAYVTLVVIRPVLM
Rcy. tenuis                 TVCAIGVFVSWALREVEICRKLGIGFYIPFAFSFAILAYITLVVIRPVLM
Rvi. gelatinosus            TVCAIGAFVSWALREVEICRKLGMQYHVPIAFSFAILAYVTLVVIRPILM
Phs. molischianum           TICATGAFVSWALRQVEIARKLGMGLHVPFAFSFAILAYVTLVVIRPVLM
Rsp. photometricum          TICAIGRFCCWALRQVEIARKLGMGLHIPFAFSFAIFAYLALVVIRPLLL
BAC-65D09                   TICAIGAFVSWILRQVEIARKLGMGLHIPFAFSYAVLAYVTLVVIRPMLL
A. vinosum                  TICAIGAFVSWALREVEICRKLGIGFHIPFAFAFAIGAYLVLVVVRPILM
T. gelatinosa               TFCAIGAFVSWALRQVEISRKLGIGFHVPFAFSFAIGAYITLVVIRPILL
E. shaposhnikovii           TICALGAFVSWALRQAEIARKLGMGLHLPIAFSVAVFAYFTLVVIRPVLL
OK5m04                      TICAIGAFVSWALREVEICRKLGMGYHVPFAFGVAIFAYVTLVVFRPFLL
OK5m69                      TICAIGAFVSWALREVEICRKLGMGYHVPFAFGVAIFAYVTLVVFRPFLL
OK5m77                      TICAIGAFVSWALREVEICRKLGMGYHVPFAFGVAIFAYVTLVVFRPFLL
OK5m114                     TICAVGAFVSWALREVEICRKLGMGYHVPVAFGVAIFAYMTLVIFRPFLL
OK5m73                      TICAIGAFVSWALREVEICRKLGMGYHVPFAFGVAIFAYVTLVVFRPLLL
B. natatoria                TVCAIGAFVSWALREVEICRKLGMGYHVPVAFGVAIFAYVTLVVFRPFLL
OK5m10                      TVCALVSFVSWALREVEICRKLGMGYHVPVAFGFAIFAYFSLVVIRPVLL
Blastomonas NT12            TICAVGAFCSWALREVEICRKLGMGYHVPIAFGFAIFAYVSLVVIRPILL
OK5m30                      TVCAIGAFVSWALREVEICRKLGMGYHVPVAFGFAIFAYVTLVVIRPVLM
A. sanguineum               TACAVVAFSSWVLRQAEISRKLGMSYHVPIAFGVAVFAYVTLNVIRPLWM
P. tepidarius               TICAVIAFGSWVMRQAEISRKLGMSYHVPIAFSVAVFAYVTLNVIRPVWM
P. neustonensis             TCCAVIAFSSWVLRQAEISRKLGMSYHVPIAFAVAVFAYVSLNVIRPLWM
E. ramosum                  TCCAVVAFVSWVLRQAEISRKLGMSYHVPIAFSVAVFAYVSLNVIRPLWM
E. longus                   TLCAVIAFGSWVLRQAEISRKLGMGYHVPIAFSVAVFAFVTLNIIRPFWM
E. litoralis                TACAVVAFYSWVFRQAEISRKLGMGYHVPVAFSFAVFAYVTLNVIRPVWM
OK5m27                      TICAIGAFVSWALREVEISRKLGMGLHVPIAFSVAIFAYVTLVVIRPVLM
A. acidophilum              TICAVGSFGSWALREVEISRKLGIGLHVPAAFSVAIFAYVSLEVIRPLLM
A. rubrum                   TICAVGSFSSWALREVEISRKLGIGLHVPAAFSVAIFAYVTLEVIRPLLM
A. cryptum                  TVCAIGAFGSWALREVEISRKLGIGLHVPAAFSVAIFAYVTLEVIRPLLM
R. denitrificans            TICATGAFISWALREVEICRKLGMGYHVPFGFAAAIIAYMTLVIFRPLLM
Rba. sphaeroides            TICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAYLTLVLFRPVMM
Rba. capsulatus             TVCATGAFCSWALREVEICRKLGIGFHIPVAFSMAIFAYLTLVVIRPMMM
Rba. blasticus              TICALGXFISXALREVEICRKLGIGLHIPFAFSFAILAYAVLVVFRLLLM
Rov. sulfidophilum          TLCAIGAFVSWALREVEICRKLGIGYHIPIAFGMAIFAYLTLEVFRPMLM
                            :  *   * .  :*:.**.***.:  ::*  :  .:  :  * :.* . :
```

```
Rsp. rubrum                 GAWGYGFPYGFMTHLDWVSNTGYQYANFHYNPAHMLGITLFFTTCLALAL
Rps. palustris              GSWSYGFPYGIFTHLDWVSNTGYQYGQFHWNPGHMIAITFFFTTCLALAL
Bradyrhizobium ORS278       GSWSYAFPYGIITHLDWVSNTGYQFGNFHYNPAHMIAITFFFTTCLALAL
OK5m111                     GSWSYGFSYGIMSHLDWVSNTGYQYGNFHYNPAHMIAITFFFTTCLALAL
OK5m20                      GSWSYGFPYGIMSHLDWVSNTGYQYGNFHYNPAHMIAITFFFTTVLAPGL
Rmi. vannielii              GAWGFGFPYGILSHLDWVSNTGYQYLHFHYNPAHMLAISFFFLTALLLAM
Blc. sulfoviridis           GGWGFAFPYGILSHLDWVNNFGFQYLNWHYNPGHMSSVSFLFANAAALGL
Blc. viridis                GSWGHAFPYGILSHLDWVNNFGYQYLNWHYNPGHMSSVSFLFVNAMALGL
Rpl. roseus                 GAWGHGFPYGIMSHLDWVSNVGYQYLHFHYNPAHMIAITFFFTNALALAM
Rpl. elegans                GAWGHGFPYGIMSHLDWVSNVGYQNLHFHYNPAHCIAITFFFTNALALAM
OK5m51                      GAWGHGFPYGIISHLDWVSDVGYQYLHFHYNPAHMIAVSFFFTTTFALAL
Rsp. centenum               GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMIAVSFFFATTFALSL
OK5m87                      GAWGHGFPYGIISHLDWVSNVGYQYLHFHYNPAHMIAVSFFFTTALALSL
R. depolymerans             GAWGHGFPYGIISHLDWVSNTGYQYLHFHYNPAHMLAVSFFFATTFALSL
Rfx. fermentans             GAWGHGFPYGIYSHLDWGSNVGYQYLHFHYNPAHMLAISFSFGTTFALSL
Rcy. tenuis                 GAWGHGFPYGIMSHLDWVSNTGYQYLHFHYNPGHMLGIAFFFTTTLALAL
Rvi. gelatinosus            GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMLAITFFFTTTLAMSM
Phs. molischianum           GAWGYGFPYGIFSHLDWVSNVGYQFLHFHYNPAHMIAISFFFTNALALAM
Rsp. photometricum          GAWGHGXPYGILSHLDWVSNIGYQFLHFHYNPAHMIGITFFFTNCMAFGM
BAC-65D09                   GAWGHGFPYGILSHLDWVSNVGYQFLHFHYNPAHMIAITFFFTTAMALSM
A. vinosum                  GAWGHGFPYGILSHLDWVSNVGYQFLHFHYNPAHMLAITFFFTNCLALSM
T. gelatinosa               GAWGHGFPYGIMSHLDWVSNVGYQFLHFHYNPAHMLAITFFFTNALALAM
E. shaposhnikovii           GAWGHGFPYGIMSHLDWVSNTGYQFLHFHYNPAHMLAIAFFFTTALALSL
OK5m04                      GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMIAVSFFFTTTLALSL
OK5m69                      GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMIAVSFFFTTTLALSL
OK5m77                      GAWGHGFPYGTFSHLDWVSNVGYQYLHFHYNPAHMIAVSFFFTTTLALSL
OK5m114                     GAWGHGFPYGIFSHLDWVSNVGYQFLHFHYNPAHMIAVSFFFTTTLALSL
OK5m73                      GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMLAVTFFFTTTLALSL
B. natatoria                GAWGHGFPYGIFSHLDWVSNVGYQYLHFHYNPAHMLAVTFFFTTTLALSM
OK5m10                      GGWHFGFPYGIFSHLDWVSNTGYQYLHFHYNPAHMLAVSFFFTTTFALAL
Blastomonas NT12            GAWGFGFPYGIFSHLDWVSNTGYQYLHFHYNPAHMIAVSFFFTTTLALAL
OK5m30                      GAWGHGFPYGIWSHLDWVSNVGYQYLHFHYNPAHMLAVTFFFTTTLALAL
A. sanguineum               GAWGNGFPYGIWTHLDWVSNVGYAFGNFHYNPVHMLAITFFFTNCLALAL
P. tepidarius               GAWANGFPYGIWTHLVWVSNVGYAFGNFHYNPVHMLAITFFFTNCLALAL
P. neustonensis             GAWGNGFPYGIWTHLEWVSNVGYAFGNFHYNPVHMLAITFFFTNCLALAL
E. ramosum                  GAWGNGFPYGIWTHLEWVSNVGYAFGNFHYNPVHMLAISFFFTNALALAL
E. longus                   GAWGHAFPYGIWSHLDWVSYTGYNYVNFHYNPVHMLAITFFFTNCLALAL
E. litoralis                GAWGHAFPYGIWSHLDWVSNTGYMYVNFHYNPVHMLAITFFFTNCLALAL
OK5m27                      GAWGHGFPYGIMSHLDWVSNTGYAYGNFHYNPAHMIAITFFFATVLALSL
A. acidophilum              GAWGVGFPYGIMSHLDWASNTGYNYINFEYNPMHMVAVTLFFTTTFALAL
A. rubrum                   GAWGVGFPYGIMSHLDWVSNTGYNYINFEYNPMHMVAVTLFFTTTFALAL
A. cryptum                  GAWGNGFPYGIMSHLDWVSNTGYAYLNFEYNPMHMVAVTLFFTTTLALAL
R. denitrificans            GAWGHGFPYGIFSHLDWVSNVGYAYLHFHYNPAHMLAVTLFFTTTLALAL
Rba. sphaeroides            GAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALAL
Rba. capsulatus             GSWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPFHMLGISLFFTTAWALAM
Rba. blasticus              GSWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMLGISFFFTTALALAL
Rov. sulfidophilum          GAWGHAFPYGIFTHLDWVSNTGYHYLHFHYNPFHMLGVSLFFATCLALAL
                            *.*  . .**  :** * .  *:   ::.:** *  .::: * .    .:
```

```
Rsp. rubrum                    HGSLILSAANPGKG-EVVKGPEHENTYFQDTIGY
Rps. palustris                 HGGLVLSAINPDRG-EPVKSPEHENTVFRDLVGY
Bradyrhizobium ORS278          HGSLILSAANPGKG-QEMKSPEHENTMFRDLIGY
OK5m111                        HGSLILSATNPKPG-ETVRTAEHENTFFRDTIGW
OK5m20                         HGGLVLSAANPPKG-QVMKTAEHENTYFRDLIGY
Rmi. vannielii                 HGGLILSALNPGDR-DVVKTPEHENAFTRDLLGY
Blc. sulfoviridis              HGGLILSVTNPGDG-DRVKTAEHENAYFRDVVGY
Blc. viridis                   HGGLILSVANPGDG-DKVKTAEHENQYFRDVVGY
Rpl. roseus                    HGSLIVSAANPQAG-EVVKGAEHENTIFRDTIGY
Rpl. elegans                   HGSLIVSAANPRKG-EIVKGAEHENTIFRDTVGY
OK5m51                         HGSLILSATNPAPG-EAVKTAEHENTFFRDTLGY
Rsp. centenum                  HGALVLSAVNVGPDEDVIKTSEHENTFYRDTIGY
OK5m87                         HGALVLSATNPKPG-ERMKTAEHENTMFRDTIGY
R. depolymerans                HGALVLSATNPAPG-EVVKTAEHENTFFRDAIGY
Rfx. fermentans                HSSLIVSASNPKKG-EKVCAPEAEDTFFRDIIGY
Rcy. tenuis                    HGSLILSATNPGKX-QVVKTAEHENTFFRDVVGY
Rvi. gelatinosus               HGGLILSAANPKKG-EPMKTTDHEDTFFRDAVGY
Phs. molischianum              HGGLILSASNPGKG-EVVKAAEHEDTFFRDVVGY
Rsp. photometricum             HGSIILSVLNPGKG-EKVKGSEHENTFFRDVVGY
BAC-65D09                      HGSLILMATDPRDG-EKVKTGEHENTFFRDDIGY
A. vinosum                     HGSLILSVTNPQKG-EEVKTSEHENTFFRDIVGY
T. gelatinosa                  HGSLNLSVTNPQEG-EPVKTSEHENTFFRDIVGY
E. shaposhnikovii              HGGLILSATNPKKG-EPVKTPEYEDTFFRDVVGY
OK5m04                         HGSLILSAANPAKG-ETVKSSEHEDMFFQDIIGY
OK5m69                         HGSLILSAANPAKG-ETVKSSEHEDMFFQDIIGY
OK5m77                         HGSLILSAANPAKG-ETVKSSEHEDMFFQDIIGY
OK5m114                        HGSLILSAANPAKG-ETVKSSEHEDMFFQDIIGY
OK5m73                         HGSLILSAANPGKG-EKVKGSEHEDMFFHDMIGY
B. natatoria                   HGALVLSATNPAKG-EEVKSPEYEDTFFRDLIGY
OK5m10                         HGSLVLSAVNPAPG-EEVKSPEYEDTFFRDLIGY
Blastomonas NT12               HGGLVLSAVNPQKG-EDVKSPEYEDAFFRDTIGY
OK5m30                         HGGLVLSATNPPEG-EVVKTPEHENIFFRDFIGY
A. sanguineum                  HGGLVLSAVNPTGG-TDVKTPEYEDTYFRDFIGY
P. tepidarius                  HGGLVLSAVNPTGG-TDVKSPEYEDTYFRDFIGY
P. neustonensis                HGGLVLSAVNPTRG-TDVKTPEYEDTYFRDFIGY
E. ramosum                     HGGLVLSAVNPSGG-TDVKSPEYEDTYFRDFIGY
E. longus                      HGGLVLSAVNVGKG-NEVRSPEYEDTYFRDFIGY
E. litoralis                   HGGLVLSAVNPSNG-TNVKTPEYEDTYFRDFIGY
OK5m27                         HGALVLSALNPPKG-EPVKTPEHENTFFRDLIGY
A. acidophilum                 HGSLVLGAVNPAKG-ETAKFTEHEDTFFRDFIGY
A. rubrum                      HGSLVLAAINPAKG-EVVKFTEHEDTFFRDFIGY
A. cryptum                     HGSLVLAAINPPAG-ETVKFAEHEDTFFRDFIGY
R. denitrificans               HGGLILSACNPEKG-EEAKTPDHEDTFFRDFIGY
Rba. sphaeroides               HGALVLSAANPEKG-KEMRTPDHEDTFFRDLVGY
Rba. capsulatus                HGALVLSAANPVKG-KTMRTPDHEDTYFRDLMGY
Rba. blasticus                 HGALVLSAANPEKG-QEMKTADHEDTFFRDLVGY
Rov. sulfidophilum             HGGLILSACNPEKG-EEAKTPDHEDSFFRDLIGY
                               *..: : . :           : *:   :* :*:
```

References

Achenbach, L.A., Carey, J. and Madigan, M.T. (2001). Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Applied and Environmental Microbiology* **67**: 2922-2926.

Adkins, J.P., Madigan, M.T., Mandelco, L., Woese, C.R. and Tanner, R.S. (1993). *Arhodomonas aquaeolei* gen. nov. and sp. nov., an aerobic, halophilic bacterium isolated from a subterranean brine. *International Journal of Systematic Bacteriology* **43**: 514-520.

Akiba, T., Usami, R. and Horikoshi, K. (1983). *Rhodopseudomonas rutila*, a new species of nonsulfur purple photosynthetic bacteria. *International Journal of Systematic Bacteriology* **33**: 551-556.

Althoff, K., Schutt, C., Steffen, R., Batel, R. and Müller, W.E.G. (1998). Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondra panicea* - harbor also for putatively toxic bacteria. *Marine Biology* **130**: 529-536.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**: 143-169.

Ambler, R.P. (1996). The distance between bacterial species in sequence space. *Journal of Molecular Evolution* **42**: 617-630.

Aminov, R.I., Chee-Sanford, J.C., Garrigues, N., Terefedegne, B., Krapac, I.J., White, B.A. and Mackie, R.I. (2002). Development, validation, and application of PCR primers for detection of tetracycline efflux genes of Gram-negative bacteria. *Applied and Environmental Microbiology* **68**: 1786-1793.

Aretz, W., Kaspari, H. and Klemme, J.-H. (1978). Utilization of purines as nitrogen source by facultative phototrophic bacteria. *FEMS Microbiology Letters* **4**: 249-253.

Beer-Romero, P. and Gest, H. (1987). *Heliobacillus mobilis*, a peritrichously flagellated anoxyphototroph containing bacteriochlorophyll *g*. *FEMS Microbiology Letters* **41**: 109-114.

Behrman, E.J. and Stanier, R.Y. (1957). The bacterial oxidation of nicotinic acid. *Journal of Biological Chemistry* **12**: 923-945.

Beja, O., Aravind, L., Koonin, E.V., Suzuki, M.T., Hadd, A., Nguyen, L.P., Jovanovich, S.B., Gates, C.M., Feldman, R.A., Spudich, J.L., Spudich, E.N. and DeLong, E.F. (2000). Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**: 1902-1906.

Beja, O., Spudich, E.N., Spudich, J.L., Leclerc, M. and DeLong, E.F. (2001). Proteorhodopsin phototrophy in the ocean. *Nature* **411**: 786-789.

Beja, O., Suzuki, M.T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., Eisen, J.A., Fraser, C.M. and DeLong, E.F. (2002). Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* **415**: 630-633.

Bennett, A.F. and Lenski, R.E. (1993). Evolutionary adaptation to temperature. II. Thermal niches of experimental lines of *Escherichia coli*. *Evolution* **47**: 1-12.

Berry, D.F., Francis, A.J. and Bollag, J.-M. (1987). Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiological Reviews* **51**: 43-59.

Blasco, R. and Castillo, F. (1992). Light dependent degradation of nitrophenols by phototrophic bacterium *Rhodobacter capsulatus* E1F1. *Applied and Environmental Microbiology* **57**: 2067-2073.

Bollag, J.-M. and Kaiser, J.-P. (1991). The transformation of heterocyclic aromatic compounds and their derivatives under anaerobic conditions. *Critical Reviews in Environmental Control* **21**: 297-329.

Boomer, S.M., Lodge, D.P., Dutton, B.E. and Pierson, B. (2002). Molecular characterization of novel red green nonsulfur bacteria from five distinct hot spring communities in Yellowstone National Park. *Applied and Environmental Microbiology* **68**: 346-355.

Boomer, S.M., Pierson, B.K., Austinhirst, R. and Castenholz, R.W. (2000). Characterization of novel bacteriochlorophyll-a-containing red filaments from alkaline hot springs in Yellowstone National Park. *Archives of Microbiology* **174**: 152-161.

Braker, G., Fesefeldt, A. and Witzel, K.P. (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology* **64**: 3769-3775.

Brock, T.D. and Brock, M.L. (1971). Microbiological studies of thermal habitats of the central volcanic region, North Island, New Zealand. *New Zealand Journal of Marine and Freshwater Research* **5**: 233-258.

Brockman, H., Jr. and Lipinski, A. (1983). Bacteriochlorophyll *g*, a new bacteriochlorophyll from *Heliobacterium chlorum*. *Archives of Microbiology* **136**: 17-19.

Brocks, J.J., Logan, G.A., Buick, R. and Summons, R.E. (1999). Archean molecular fossils and the early rise of eukaryotes. *Science* **285**: 1033-1036.

Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I., Achenbach, L.A. and Madigan, M.T. (1999). *Heliorestis daurensis*, gen. nov., sp. nov., an alkaliphilic rod-to-coiled-shaped phototrophic heliobacterium from a Siberian soda lake. *Archives of Microbiology* **172**: 167-174.

Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I. and Imhoff, J.F. (2000). *Thioalkalicoccus limnaeus* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium with bacteriochlorophyll *b*. *International Journal of Systematic and Evolutionary Microbiology* **50 Pt 6**: 2157-2163.

Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I., Tourova, T.P., Kuznetsov, B.B. and Osipov, G.A. (2000). Alkaliphilic heliobacterium *Heliorestis baculata* sp. nov. and emended description of the genus *Heliorestis*. *Archives of Microbiology* **174**: 283-291.

Buckmiller, L.M., LaPointe, J.P. and Ludwig, R.A. (1991). Cloning of *Azorhizobium caulinodans* nicotinate catabolism genes and characterization of their importance in N_2 fixation. *Journal of Bacteriology* **173**: 2017-2025.

Burgess, J.G., Kawaguchi, R., Yamada, A. and Matsunaga, T. (1994). *Rhodobacter marinus* sp. nov.: a new marine hydrogen producing photosynthetic bacterium which is sensitive to oxygen and sulfide. *Microbiology* **140**: 965-970.

Burke, M.E., Gorham, E. and Pratt, D.C. (1974). Distribution of purple photosynthetic bacteria in woodland and woodland habitats of central and northern Minnesota. *Journal of Bacteriology* **117**: 826-833.

Castenholz, R.W., Bauld, J. and Jørgenson, B.B. (1990). Anoxygenic microbial mats of hot springs: thermophilic *Chlorobium* sp. *FEMS Microbiology Ecology* **74**: 325-336.

Chaintreuil, C., Giraud, E., Prin, Y., Lorquin, J., Ba, A., Gillis, M., de Lajudie, P. and Dreyfus, B. (2000). Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. *Applied and Environmental Microbiology* **66**: 5437-47.

Charlton, P.J. (1997) Aspects of the carbon metabolism of the purple nonsulfur bacteria. M.Sc. Thesis, Department of Biological Sciences, University of Waikato.

Clarke, K.J., Finlay, B.J., Vicente, E., Lloréns, H. and Miracle, M.R. (1993). The complex life-cycle of a polymorphic prokaryote epibiont of the photosynthetic bacterium *Chromatium weissei*. *Archives of Microbiology* **59**: 498-505.

Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitts, C.L. (1998). Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *Journal of Microbiological Methods* **31**: 135-142.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A.E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *International Journal of Systematic Bacteriology* **44**: 812-826.

Compeau, G.C. and Bartha, R. (1985). Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. *Applied and Environmental Microbiology* **50**: 498-502.

Corson, G.E., Nagashima, K.V.P., Matsuura, K., Sakuragi, Y., Wettasinghe, R., Qin, H., Allen, R. and Knaff, D.B. (1999). Genes encoding light-harvesting and reaction center proteins from *Chromatium vinosum*. *Photosynthesis Research* **59**: 39-52.

Cotton, F.A. and Wilkinson, G. (1988). Molybdenum and tungsten: Group VIB(6). Advanced Inorganic Chemistry. New York, Chichester, Brisbane, Toronto, Singapore, John Wiley and Sons: 804-847.

Dilling, W., Liesack, W. and Pfennig, N. (1995). *Rhabdochromatium marinum* gen. nom. rev., sp. nov., a purple sulfur bacterium from a salt marsh microbial mat. *Archives of Microbiology* **164**: 125-131.

Dobbin, P.S., Warren, L.H., Cook, N.J., McEwan, A.G., Powell, A.K. and Richardson, D.J. (1996). Dissimilatory iron (III) reduction by *Rhodobacter capsulatus*. *Microbiology* **142**: 765-774.

Drews, G. (1981). *Rhodospirillum salexigens*, spec. nov., an obligatory halophilic phototrophic bacterium. *Archives of Microbiology* **130**: 325-327.

Drews, G. and Giesbrecht, P. (1966). *Rhodopseudomonas viridis*, nov. spec., ein neu isoliertes, obligat phototrophes Bakterium. *Archiv für Mikrobiologie* **53**: 255-262.

Duchow, E. and Douglas, H.C. (1949). *Rhodomicrobium vannielii*, a new phototrophic bacterium. *Journal of Bacteriology* **58**: 409-416.

Eckersley, K. and Dow, C.S. (1980). *Rhodopseudomonas blastica* sp. nov.: a member of the Rhodospirillaceae. *Journal of General Microbiology* **119**: 465-473.

Edwards, U., Rogall, T., Blocker, H., Emde, M. and Bottger, E.C. (1989). Isolation and direct nucleotide determination of entire genes. Characterisation of a gene coding for 16S ribosomal RNA. *Nucleic Acid Research* **17**: 7843-7853.

Egland, P.G., Pelletier, D.A., Dispensa, M., Gibson, J. and Harwood, C.S. (1997). A cluster of bacterial genes for anaerobic benzoate ring degradation. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 6484-6489.

Ehrenreich, A. and Widdel, F. (1994). Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Applied and Environmental Microbiology* **60**: 4517-4526.

Elder, D.J.E., Morgan, P. and Kelly, D.J. (1992). Anaerobic degradation of trans-cinnamate and ω-phenylalkane carboxylic acids by the photosynthetic bacterium *Rhodopseudomonas palustris*: evidence for a β-oxidation mechanism. *Archives of Microbiology* **157**: 148-154.

Elmerich, C., Dreyfus, B. and Aubert, J.-P. (1983). Nicotinic acid requirement and degradation by *Sesbania rhizobium* [sic] strain ORS571. *FEMS Microbiology Letters* **19**: 281-284.

Elsaied, H. and Naganuma, T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Applied and Environmental Microbiology* **67**: 1751-1765.

Ensign, J.C. and Rittenberg, S.C. (1964). The pathway of nicotinic acid oxidation by a *Bacillus* species. *Journal of Biological Chemistry* **239**: 2285-2291.

Esmarch, E. (1887). Über die Reincultur eines Spirillum. *Zentralblat für Bakteriologie und Parasitenkunde* **1**: 225-230.

Esteve, I., Gaju, N., Mir, J. and Guerrero, R. (1992). Comparison of techniques to determine the abundance of predatory bacteria attacking Chromatiaceae. *FEMS Microbiology Ecology* **86**: 205-211.

Esteve, I., Guerrero, R., Montesinos, E. and Abellà, C. (1983). Electron microscopy study of the interaction of epibiontic bacteria with *Chromatium minus* in natural habitats. *Microbial Ecology* **9**: 57-64.

Fathir, I., Ashikaga, M., Tanaka, K., Katano, T., Nirasawa, T., Kobayashi, M., Wang, Z.-Y. and Nozawa, T. (1998). Biochemical and spectral characterization of the core light harvesting complex 1 (LH1) from the thermophilic purple sulfur bacterium *Chromatium tepidum*. *Photosynthesis Research* **58**: 193-202.

Fathir, I., Tanaka, K., Yoza, K., Kojima, A., Kobayashi, M., Wang, Z.Y., Lottspeich, F. and Nozawa, T. (1997). The genes coding for the L, M and cytochrome subunits of the photosynthetic reaction center from the thermophilic purple sulfur bacterium *Chromatium tepidum*. *Photosynthesis Research* **51**: 71-82.

Favinger, J., Stadtwald, R. and Gest, H. (1989). *Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. *Antonie van Leeuwenhoek* **55**: 291-296.

Fenchel, T. (1998a). Artificial cyanobacterial mats: structure and composition of the biota. *Aquatic Microbial Ecology* **14**: 241-251.

Fenchel, T. (1998b). Formation of laminated cyanobacterial mats in the absence of benthic fauna. *Aquatic Microbial Ecology* **14**: 235-240.

Ferguson, S.J., Jackson, J.B. and McEwan, A.G. (1987). Anaerobic respiration in the Rhodospirillaceae: characterisation of pathways and evaluation of roles in redox balancing during photosynthesis. *FEMS Microbiology Reviews* **46**: 117-143.

Fesefeldt, A. and Gliesche, C.G. (1997). Identification of *Hyphomicrobium* spp. using PCR-amplified fragments of the *mxaF* gene as a molecular marker. *Systematic and Applied Microbiology* **20**: 387-396.

Fetzner, S. (1998). Bacterial degradation of pyridine, indole, quinoline, and their derivatives under different redox conditions. *Applied Microbiology and Biotechnology* **49**: 237-250.

Fleischman, D. and Kramer, D. (1998). Photosynthetic rhizobia. *Biochimica et Biophysica Acta - Bioenergetics* **1364**: 17-36.

Fode-Vaughan, K.A., Wimpee, C.F., Remsen, C.C. and Collins, M.L. (2001). Detection of bacteria in environmental samples by direct PCR without DNA extraction. *BioTechniques* **31**: 598-604.

Fox, G.E., Wisotzkey, J.D. and Jurtshuk, P., Jr. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic Bacteriology* **42**: 166-170.

Fuerst, J.A., Hawkins, J.A., Holmes, A., Sly, L.I., Moore, C.I. and Stackebrandt, E. (1993). *Porphyrobacter neustonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-synthesizing budding bacterium from freshwater. *International Journal of Systematic Bacteriology* **43**: 125-134.

Gaju, N., Esteve, I. and Guerrero, R. (1992). Distribution of predatory bacteria that attack Chromatiaceae in a sulfurous lake. *Microbial Ecology* **24**: 171-179.

Gauthier, J.J. and Rittenberg, S.C. (1971). The metabolism of nicotinic acid I. Purification and properties of 2,5-dihydroxypyridine oxygenase from *Pseudomonas putida* N9. *Journal of Biological Chemistry* **246**: 3737-3742.

Gest, H. (1993). Photosynthetic and quasi-photosynthetic bacteria. *FEMS Microbiology Letters* **112**: 1-6.

Gest, H. and Favinger, J. (1998). Taxonomic problems, exemplified with anoxygenic phototrophic bacteria. *ASM News* **64**: 434-435.

Gest, H. and Favinger, J. (2001). Taxonomic ambiguities: a case history. *International Journal of Systematic and Evolutionary Microbiology* **51**: 707-710.

Gest, H. and Favinger, J.L. (1983). *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a "new" form of bacteriochlorophyll. *Archives of Microbiology* **136**: 11-16.

Gest, H., Favinger, J.L. and Madigan, M.T. (1985). Exploitation of N_2-fixation capacity for enrichment of anoxygenic photosynthetic bacteria in ecological studies. *FEMS Microbiology Ecology* **31**: 317-322.

Gich, F., Garcia-Gil, J. and Overmann, J. (2001). Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. *Archives of Microbiology* **177**: 1-10.

Giesberger, G. (1947). Some observations on the culture, physiology and morphology of some brown-red *Rhodospirillum* species. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **13**: 135-148.

Gladyshev, V.N., Khangulov, S.V. and Stadtman, T.C. (1996). Properties of the selenium- and molybdenum-containing nicotinic acid hydroxylase from *Clostridium barkeri*. *Biochemistry* **35**: 212-223.

Glaeser, J. and Overmann, J. (1999). Selective enrichment and characterization of *Roseospirillum parvum*, gen. nov. and sp. nov., a new purple nonsulfur bacterium with unusual light absorption properties. *Archives of Microbiology* **171**: 405-416.

Gorham, H. and Dow, C.S. (1996). A bacteriophage of *Rhodopseudomonas blastica*. *Microbiology* **142**: 979-983.

Gorlenko, V.M. (1969). Spore formation in a budding phototrophic bacterium. *Microbiology* **38**: 106-111.

Gorlenko, V.M., Bonch-Osmolovskaya, E.A., Kompantseva, E.I. and Starynin, D.A. (1987). Differentiation of microbial communities in connection with a change in the physicochemical conditions in thermophile spring. *Microbiology* **56**: 250-257.

Gorlenko, V.M., Kompantseva, E.I. and Puchkova, N.N. (1985). Influence of temperature on the prevalence of phototrophic bacteria in hot springs. *Microbiology* **54**: 848-853.

Gossling, J. (1989). Genus *Gemmiger* Gossling and Moore 1975, 206^{AL}. Bergey's Manual of Systematic Bacteriology Volume 3. J.T. Staley, M.P. Bryant, N. Pfennig and J.G. Holt. Baltimore, Hong Kong, London, Sydney, Williams & Wilkins: 1971-1973.

Guerrero, R., Pedrós-Alió, C., Esteve, I., Mas, J., Chase, D. and Margulis, L. (1986). Predatory prokaryotes: Predation and primary consumption evolved in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 2138-2142.

Guyoneaud, R., Matheron, R., Baulaigue, R., Podeur, K., Hirschler, A. and Caumette, P. (1996). Anoxygenic phototrophic bacteria in eutrophic coastal lagoons of the French Mediterranean and Atlantic Coasts (Prévost Lagoon, Arcachon Bay, Certes fishponds). *Hydrobiologia* **329**: 33-43.

Guyoneaud, R., Matheron, R., Liesack, W., Imhoff, J.F. and Caumette, P. (1997). *Thiorhodococcus minus*, gen. nov., sp. nov., a new purple sulfur bacterium isolated from coastal lagoon sediments. *Archives of Microbiology* **168**: 16-23.

Halloren, E., McDermott, G., Lindsay, J.G., Miller, C., Freer, A.A., Isaacs, N.W. and Cogdell, R.J. (1995). Studies on the light-harvesting complexes from the thermotolerant purple bacterium *Rhodopseudomonas cryptolactis*. *Photosynthesis Research* **44**: 149-155.

Hanada, S., Hiraishi, A., Shimada, K. and Matsuura, K. (1995). *Chloroflexus aggregans* sp. nov., a filamentous phototrophic bacterium which forms dense cell aggregates by active gliding movement. *International Journal of Systematic Bacteriology* **45**: 676-681.

Hanada, S., Kawase, Y., Hiraishi, A., Takaichi, S., Matsuura, K., Shimada, K. and Nagashima, K.V.P. (1997). *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. *International Journal of Systematic Bacteriology* **47**: 408-413.

Hanada, S., Takaichi, S., Matsuura, K. and Nakamura, K. (2002). *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *International Journal of Systematic and Evolutionary Microbiology* **52**: 187-193.

Hansen, T.A. and Imhoff, J.F. (1985). *Rhodobacter veldkampii*, a new species of phototrophic purple nonsulfur bacteria. *International Journal of Systematic Bacteriology* **35**: 115-116.

Hansen, T.A. and Veldkamp, H. (1973). *Rhodopseudomonas sulfidophila*, nov. spec., a new species of purple nonsulfur bacteria. *Archives of Microbiology* **92**: 45-58.

Harayama, S., Kok, M. and Neidle, E.L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annual Reviews of Microbiology* **46**: 565-601.

Harfoot, C.G. and Janssen, P.H. (1985). Growth of *Rhodocyclus purpureus* on short-chain fatty acids. *FEMS Microbiology Letters* **29**: 177-179.

Harwood, C.S. and Gibson, J. (1988). Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. *Applied and Environmental Microbiology* **54**: 712-717.

Head, I.M., Gray, N.D., Clarke, K.J., Pickup, R.W. and Jones, J.G. (1996). The phylogenetic position and ultrastructure of the uncultured bacterium *Achromatium oxaliferum*. *Microbiology* **142**: 2341-2354.

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998). Microbial evolution, diversity and ecology - a decade of ribosomal RNA analysis of uncultivated organisms. *Microbial Ecology* **35**: 1-21.

Heising, S., Dilling, W., Schnell, S. and Schink, B. (1996). Complete assimilation of cysteine by a newly isolated non-sulfur purple bacterium resembling *Rhodovulum sulfidophilum* (*Rhodobacter sulfidophilus*). *Archives of Microbiology* **165**: 397-401.

Heising, S. and Schink, B. (1998). Phototrophic oxidation of ferrous iron by a *Rhodomicrobium vannielii* strain. *Microbiology* **144**: 2263-2269.

Herbert, R.A. (1976). Isolation and identification of the photosynthetic bacteria *Rhodopseudomonas* from Antarctic marine and freshwater sediments. *Journal of Applied Bacteriology* **41**: 75-80.

Hille, R. (1996). The mononuclear molybdoenzymes. *Chemical Reviews* **96**: 2757-2816.

Hiraishi, A. (1994). Phylogenetic affiliations of *Rhodoferax fermentans* and related species of phototrophic bacteria as determined by automated 16S rDNA sequencing. *Current Microbiology* **28**: 25-29.

Hiraishi, A. (1997). Transfer of the bacteriochlorophyll b-containing phototrophic bacteria *Rhodopseudomonas viridis* and *Rhodopseudomonas sulfoviridis* to the genus *Blastochloris* gen. nov. *International Journal of Systematic Bacteriology* **47**: 217-219.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K.A., Fukuyama, M. and Tabuchi, K. (1995a). Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. *Applied and Environmental Microbiology* **61**: 2099-2107.

Hiraishi, A. and Hoshino, Y. (1984). Distribution of rhodoquinone in *Rhodospirillaceae* and its taxonomic implications. *Journal of General and Applied Microbiology* **30**: 435-448.

Hiraishi, A., Hoshino, Y. and Satoh, T. (1991). *Rhodoferax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the "*Rhodocyclus gelatinosus*-like" group. *Archives of Microbiology* **155**: 330-336.

Hiraishi, A. and Kitamura, H. (1984). Distribution of phototrophic purple nonsulfur bacteria in activated sludge systems and other aquatic environments. *Bulletin of the Japanese Society of Scientific Fisheries* **50**: 1929-1937.

Hiraishi, A., Kuraishi, H. and Kawahara, K. (2000b). Emendation of the description of *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997 as an aerobic photosynthetic bacterium and reclassification of *Erythromonas ursincola* Yurkov et al. 1997 as *Blastomonas ursincola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **50**: 1113-1118.

Hiraishi, A., Matsuzawa, Y., Kanbe, T. and Wakao, N. (2000a). *Acidisphaera rubrifaciens* gen. nov., sp nov., an aerobic bacteriochlorophyll-containing bacterium isolated from acidic environments. *International Journal of Systematic and Evolutionary Microbiology* **50**: 1539-1546.

Hiraishi, A., Muramatsu, K. and Ueda, Y. (1996). Molecular genetic analyses of *Rhodobacter azotoformans* sp. nov. and related species of phototrophic bacteria. *Systematic and Applied Microbiology* **19**: 168-177.

Hiraishi, A., Muramatsu, K. and Urata, K. (1995b). Characterisation of new denitrifying *Rhodobacter* strains isolated from photosynthetic sludge for wastewater treatment. *Journal of Fermentation and Bioengineering* **79**: 39-44.

Hiraishi, A., Santos, T.S., Sugiyama, J. and Komagata, K. (1992). *Rhodopseudomonas rutila* is a later subjective synonym of *Rhodopseudomonas palustris*. *International Journal of Systematic Bacteriology* **42**: 186-188.

Hiraishi, A. and Shimada, K. (2001). Aerobic anoxygenic photosynthetic bacteria with zinc-bacteriochlorophyll. *Journal of General and Applied Microbiology* **47**: 161-180.

Hiraishi, A. and Ueda, Y. (1994a). Intrageneric structure of the genus *Rhodobacter*. Transfer of *Rhodobacter sulfidophilus* and related marine species to the genus *Rhodovulum* gen. nov. *International Journal of Systematic Bacteriology* **44**: 15-23.

Hiraishi, A. and Ueda, Y. (1994b). *Rhodoplanes* gen. nov., a new genus of phototrophic bacteria including *Rhodopseudomonas rosea* as *Rhodoplanes roseus* comb. nov. and *Rhodoplanes elegans* sp. nov. *International Journal of Systematic Bacteriology* **44**: 665-673.

Hiraishi, A. and Ueda, Y. (1995). Isolation and characterization of *Rhodovulum strictum* sp. nov. and some other purple nonsulfur bacteria from colored blooms in tidal and seawater pools. *International Journal of Systematic Bacteriology* **45**: 319-326.

Hiraishi, A., Umezawa, T., Yamamoto, H., Kato, K. and Maki, Y. (1999). Changes in quinone profiles of hot spring microbial mats with a thermal gradient. *Applied and Environmental Microbiology* **65**: 198-205.

Hiraishi, A., Urata, K. and Satoh, T. (1995c). A new genus of marine budding phototrophic bacteria, *Rhodobium* gen. nov., which includes *Rhodobium orientis* sp. nov. and *Rhodobium marinum* comb. nov. *International Journal of Systematic Bacteriology* **45**: 226-234.

Hiraishi, A., Yonemitsu, Y., Matsushita, M., Shin, Y.K., Kuraishi, H. and Kawahara, K. (2002). Characterization of *Porphyrobacter sanguineus* sp. nov., an aerobic bacteriochlorophyll-containing bacterium capable of degrading biphenyl and dibenzofuran. *Archives of Microbiology* **178**: 45-52.

Hirner, A.V., Feldmann, J., Krupp, E., Grümping, R., Goguel, R. and Cullen, W.R. (1998). Metal(loid) compounds in geothermal gases and waters. *Organic Geochemistry* **29**: 1765-1778.

Holcenberg, J.S. and Stadtman, E.R. (1969). Nicotinic acid metabolism. III. Purification and properties of a nicotinic acid hydroxylase. *Journal of Biological Chemistry* **244**: 1194-1203.

Holding, A.J. and Collee, J.G. (1971). Routine biochemical tests. Methods in Microbiology. J.R. Norris and D.W. Ribbons. London, New York, Academic Press Inc.: 2-32.

Holland, S. (2001). Analytic Rarefaction 1.3 for Windows, web address http://www.uga.edu/~strata/software/index.html.

Horne, I.M., Pemberton, J.M. and McEwan, A.G. (1998). Manganous ions suppress photosynthesis gene expression in *Rhodobacter sphaeroides*. *Microbiology* **144**: 2255-2261.

Horz, H.P., Yimga, M.T. and Liesack, W. (2001). Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. *Applied and Environmental Microbiology* **67**: 4177-4185.

Hougardy, A. and Klemme, J.-H. (1995). Nitrate reduction in a new strain of *Rhodoferax fermentans*. *Archives of Microbiology* **164**: 358-362.

Hougardy, A., Tindall, B.J. and Klemme, J.-H. (2000). *Rhodopseudomonas rhenobacensis* sp. nov., a new nitrate-reducing purple non-sulfur bacterium. *International Journal of Systematic and Evolutionary Microbiology* **50**: 985-992.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacteria diversity. *Journal of Bacteriology* **180**: 4765-4774.

Imhoff, J.F. (1983). *Rhodopseudomonas marina* sp. nov., a marine photosynthetic bacterium. *Systematic and Applied Microbiology* **4**: 512-521.

Imhoff, J.F. (2001). Transfer of *Rhodopseudomonas acidophila* to the new genus *Rhodoblastus* as *Rhodoblastus acidophilus* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1863-1866.

Imhoff, J.F. (2001). True marine and halophilic anoxygenic phototrophic bacteria. *Archives of Microbiology* **176**: 243-254.

Imhoff, J.F., Petri, R. and Süling, J. (1998a). Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the α-Proteobacteria: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. *International Journal of Systematic Bacteriology* **48**: 793-798.

Imhoff, J.F. and Pfennig, N. (2001). *Thioflavicoccus mobilis* gen. nov., sp. nov., a novel purple sulfur bacterium with bacteriochlorophyll *b*. *International Journal of Systematic and Evolutionary Microbiology* **51**: 105-110.

Imhoff, J.F. and Süling, J. (1996). The phylogenetic relationship among Ectothiorhodospiraceae: a reevaluation of this taxonomy on the basis of 16S rDNA analyses. *Archives of Microbiology* **165**: 106-113.

Imhoff, J.F., Süling, K. and Petri, R. (1998b). Phylogenetic relationships among the *Chromatiaceae*, their taxonomic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. *International Journal of Systematic Bacteriology* **48**: 1129-1141.

Imhoff, J.F., Then, J., Hashwa, F. and Trüper, H.G. (1981). Sulfate assimilation in *Rhodopseudomonas globiformis*. *Archives of Microbiology* **130**: 234-237.

Imhoff, J.F. and Trüper, H.G. (1989). Purple nonsulfur bacteria (*Rhodospirillaceae* Pfennig and Trüper 1971, 17^{AL}). Bergey's Manual of Systematic Bacteriology Volume 3. J.T. Staley, M.P. Bryant, N. Pfennig and J.G. Holt. Baltimore, Hong Kong, London, Sydney, Williams & Wilkins: 1658-1682.

Imhoff, J.F. and Trüper, H.G. (1992). The genus *Rhodospirillum* and related genera. The Prokaryotes. A. Balows, H.G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag: 2141-2155.

Imhoff, J.F., Trüper, H.G. and Pfennig, N. (1984). Rearrangement of the species and genera of the phototrophic "purple nonsulfur bacteria". *International Journal of Systematic Bacteriology* **34**: 340-343.

Imhoff-Stuckle, D. and Pfennig, N. (1983). Isolation and characterization of a nicotinic acid-degrading sulfate-reducing bacterium, *Desulfococcus niacini* sp. nov. *Archives of Microbiology* **136**: 194-198.

Janssen, P.H. (1985) Photoassimilation of fatty acids by members of the purple nonsulfur bacteria. M.Sc. Thesis, Biological Sciences, University of Waikato.

Janssen, P.H. and Harfoot, C.G. (1991). *Rhodopseudomonas rosea* sp. nov., a new purple nonsulfur bacterium. *International Journal of Systematic Bacteriology* **41**: 26-30.

Kaiser, J.-P., Feng, Y. and Bollag, J.-M. (1996). Microbial metabolism of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions. *Microbiological Reviews* **60**: 483-498.

Kamal, V.S. and Wyndham, R.C. (1990). Anaerobic phototrophic metabolism of 3-chlorobenzoate by *Rhodopseudomonas palustris* WS17. *Applied and Environmental Microbiology* **56**: 3871-3873.

Kaplan, I.R. (1956). Evidence of microbiological activity in some of the geothermal regions of New Zealand. *New Zealand Journal of Science and Technology* **37**.

Kashefi, K., Holmes, D.E., Reysenbach, A.L. and Lovley, D. (2002). Use of Fe(III) as an electron acceptor to recover previously uncultured hyperthermophiles: isolation and characterization of *Geothermobacterium ferrireducens* gen. nov., sp. nov. *Applied and Environmental Microbiology* **68**: 1735-1742.

Kaspari, H. and Busse, W. (1986). Oxidative degradation of purines by the facultative phototrophic bacterium *Rhodopseudomonas capsulata*. *Archives of Microbiology* **144**: 67-70.

Kato, S.-I., Urakami, T. and Komagata, K. (1985). Quinone systems and cellular fatty acid composition in species of *Rhodospirillaceae* genera. *Journal of General and Applied Microbiology* **31**: 381-398.

Kawasaki, H., Hoshino, Y., Hirata, A. and Yamasoto, K. (1993a). Is intracytoplasmic membrane structure a genetic criterion? It does not coincide with phylogenetic interrelationships among phototrophic purple nonsulfur bacteria. *Archives of Microbiology* **160**: 358-362.

Kawasaki, H., Hoshino, Y., Kuraishi, Y. and Yamasoto, Y. (1992). *Rhodocista centenaria* gen. nov., sp. nov., a cyst-forming anoxygenic photosynthetic bacterium and its phylogenetic position in the Proteobacteria alpha group. *Journal of General and Applied Microbiology* **38**: 541-551.

Kawasaki, H., Hoshino, Y. and Yamasoto, K. (1993b). Phylogenetic diversity of phototrophic purple non-sulfur bacteria in the *Proteobacteria* α group. *FEMS Microbiology Letters* **112**: 61-66.

Kawasaki, H., Yamasoto, K. and Sugiyama, J. (1997). Phylogenetic relationships of the helical-shaped bacteria in the α-proteobacteria inferred from 16S rDNA sequences. *Journal of General and Applied Microbiology* **43**: 89-95.

Keppen, O.I., Baulina, O.I. and Kondratieva, E.N. (1994). *Oscillochloris trichoides* neotype strain DG-6. *Photosynthesis Research* **41**.

Keppen, O.I. and Gorlenko, V.M. (1975). A new species of purple budding bacteria containing bacteriochlorophyll *b*. *Microbiology* **44**: 224-229.

Khanna, P., Rajkumar, B. and Jothikumar, N. (1992). Anoxygenic degradation of aromatic substances by *Rhodopseudomonas palustris*. *Current Microbiology* **25**: 63-67.

Kimble, L.K. and Madigan, M.T. (1992). Nitrogen fixation and nitrogen metabolism in heliobacteria. *Archives of Microbiology* **158**: 155-161.

Kimble, L.K., Mandelco, L., Woese, C.R. and Madigan, M.T. (1995). *Heliobacterium modesticaldum*, sp. nov., a thermophilic heliobacterium of hot springs and volcanic soils. *Archives of Microbiology* **163**: 259-267.

Kimble, L.K., Stevenson, A.K. and Madigan, M.T. (1994). Chemotrophic growth of heliobacteria in darkness. *FEMS Microbiology Letters* **115**: 51-56.

Kitts, C.L., Lapointe, J.P., Lam, V.T. and Ludwig, R.A. (1992). Elucidation of the complete *Azorhizobium* nicotinate catabolism pathway. *Journal of Bacteriology* **174**: 7791-7797.

Kitts, C.L., Schaecter, L.E., Rabin, R.S. and Ludwig, R.A. (1989). Identification of cyclic intermediates in *Azorhizobium caulinodans* nicotinate metabolism. *Journal of Bacteriology* **171**: 3406-3411.

Klemme, J.-H., Chyla, I. and Preuss, M. (1980). Dissimilatory nitrate reduction by strains of the facultatively phototrophic bacterium *Rhodopseudomonas palustris*. *FEMS Microbiology Letters* **9**: 137-140.

Kolber, Z.S., Plumley, F.G., Lang, A.S., Beatty, J.T., Blankenship, R.E., Van Dover, C.L., Vetriani, C., Koblizek, M., Rathgeber, C. and P.G., F. (2001). Contribution of aerobic photoheterotrophic bacteria to the carbon cycle in the ocean. *Science* **292**: 2492-2495.

Kolber, Z.S., Van Dover, C.L., Niederman, R.A. and Falkowski, P.G. (2000). Bacterial photosynthesis in surface waters of the open ocean. *Nature* **407**: 177-179.

Kompantseva, E.I. (1981). Utilization of sulfide by nonsulfur purple bacteria *Rhodopseudomonas capsulata*. *Mikrobiologiya* **50**: 429-436.

Kompantseva, E.I. (1985). New halophilic purple bacteria *Rhodobacter euryhalinus* sp. nov. *Mikrobiologiya* **54**: 974-982 (in Russian).

Kompantseva, E.I. (1989). A new species of budding purple bacterium: *Rhodopseudomonas julia* sp. nov. *Microbiology* **58**: 254-259.

Kompantseva, E.I. and Gorlenko, V.M. (1985). A new species of moderately halophilic purple bacterium *Rhodospirillum mediosalinum* sp. nov. *Microbiology* **53**: 775-781.

Kompantseva, E.I. and Panteleeva, E.E. (1998). Strains of the halophilic species *Rhodobium marinum* that inhabit freshwater hot springs. *Microbiology* **67**: 98-104.

Kompantseva, E.I., Panteleeva, E.E., Ariskina, E.V., Lysenko, A.M., Imhoff, J.F. and Gorlenko, V.M. (1996). Phylogenetic relationships among budding purple bacteria of the genus *Rhodopseudomonas*. *Microbiology* **65**: 344-351.

Kompantseva, E.I., Panteleeva, E.E., Lysenko, A.M., Imhoff, J.F., Thiemann, B. and Mityushina, L.L. (1998). Taxonomic analysis of a group of strains of bacteriochlorophyll *b*-containing purple bacteria of the genus *Blastochloris*. *Microbiology* **67**: 323-329.

Kretzer, A., Frunzke, K. and Andreesen, J. (1993). Catabolism of isonicotinate by *Mycobacterium* sp. INA1: extended description of the pathway and purification of the molybdoenzyme isonicotinate dehydrogenase. *Journal of General Microbiology* **139**: 2763-2772.

Kuntikov, E.I. and Gorlenko, V.M. (1998). Interrelation between halo- and thermotolerance in anoxygenic phototrophic bacteria. *Microbiology* **67**: 245-250.

Labrenz, M., Collins, M.D., Lawson, P.A., Tindall, B.J., Schumann, P. and Hirsch, P. (1999). *Roseovarius tolerans* gen. nov., sp. nov., a budding bacterium with variable bacteriochlorophyll *a* production from hypersaline Ekho Lake. *International Journal of Systematic Bacteriology* **49**: 137-147.

Lee, C.L.H. (1992) Studies on the utilization of aliphatic alcohols by the Purple Nonsulfur Bacteria. D.Phil. Thesis, Department of Biological Sciences, University of Waikato.

Leimkühler, S., Kern, M., Solomon, P.S., McEwan, A.G., Schwarz, G., Mendel, R.R. and Klipp, W. (1998). Xanthine dehydrogenase from the phototrophic purple bacterium *Rhodobacter capsulatus* is more similar to its eukaryotic counterparts than to prokaryotic molybdenum enzymes. *Molecular Microbiology* **27**: 853-869.

Liu, W.-T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rDNA. *Applied and Environmental Microbiology* **63**: 4516-4522.

Mack, E.E., Mandelco, L., Woese, C.R. and Madigan, M.T. (1993). *Rhodospirillum sodomense*, sp. nov., a Dead Sea *Rhodospirillum* species. *Archives of Microbiology* **160**: 363-371.

Madigan, M.T. (1984). A novel photosynthetic purple sulfur bacterium isolated from a Yellowstone hot spring. *Science* **225**: 313-315.

Madigan, M.T. (1986). *Chromatium tepidum* sp. nov., a thermophilic photosynthetic bacterium of the Family Chromatiaceae. *International Journal of Systematic Bacteriology* **36**: 222-227.

Madigan, M.T. (2001). Thermophilic anoxygenic phototrophs Diversity and ecology. Thermophiles: Biodiversity, Ecology, and Evolution. A.L. Reysenbach, M. Voytek and R. Mancinelli. New York, Kluwer Academic / Plenum Publishers: 103-123.

Madigan, M.T. and Gest, H. (1979). Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with hydrogen as the energy source. *Journal of Bacteriology* **137**: 524-530.

Madigan, M.T., Jung, D.O., Woese, C.R. and Achenbach, L.A. (2000). *Rhodoferax antarcticus* sp nov., a moderately psychrophilic purple nonsulfur bacterium isolated from an Antarctic microbial mat. *Archives of Microbiology* **173**: 269-277.

Manchester, L.C., Poeggeler, B., Alvares, F.L., Ogden, G.B. and Reiter, R.J. (1995). Melatonin immunoreactivity in the photosynthetic prokaryote *Rhodospirillum rubrum* - implications for an ancient antioxidant system. *Cellular and Molecular Biology Research* **41**: 391-395.

McCarty, S., Chasteen, T., Marshall, M., Fall, R. and Bachofen, R. (1993). Phototrophic bacteria produce volatile, methylated sulfur and selenium compounds. *FEMS Microbiology Letters* **112**: 93-98.

McGrath, J.E. (2000) Anaerobic transformation of halocarboxylic acids and phenolic compounds by photosynthetic bacteria. Ph.D. Thesis, Biological Sciences, University of Waikato.

McGrath, J.E. and Harfoot, C.G. (1997). Reductive dehalogenation of halocarboxylic acids by the phototrophic genera *Rhodospirillum* and *Rhodopseudomonas*. *Applied and Environmental Microbiology* **63**: 3333-3335.

Milford, A.D., Achenbach, L.A., Jung, D.O. and Madigan, M.T. (2000). *Rhodobaca bogoriensis* gen. nov and sp nov., an alkaliphilic purple nonsulfur bacterium from African Rift Valley soda lakes. *Archives of Microbiology* **174**: 18-27.

Miller, S.R. and Castenholz, R.W. (2000). Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus*. *Applied and Environmental Microbiology* **66**: 4222-4229.

Minz, D., Flax, J.L., Green, S.J., Muyzer, G., Cohen, Y., Wagner, M., Rittman, B.E. and Stahl, D.A. (1999). Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Applied and Environmental Microbiology* **65**: 4666-4671.

Molisch, H. (1907). Die Purpurbakterien nach neueren Untersuchungen. Jena, Gustav Fischer Verlag.

Mongold, J.A., Bennett, A.F. and Lenski, R.E. (1999). Evolutionary adaptation to temperature. VII. Extension of the upper thermal limit of *Escherichia coli*. *Evolution* **53**: 386-394.

Moore, M.D. and Kaplan, S. (1992). Identification of intrinsic high-level resistance to rare-earth oxides and oxyanions in members of the Class Proteobacteria: Characterization of tellurite, selenite and rhodium sesquioxide reduction in *Rhodobacter sphaeroides*. *Journal of Bacteriology* **174**: 1505-1514.

Moore, M.D. and Kaplan, S. (1994). Members of the family *Rhodospirillaceae* reduce heavy-metal oxyanions to maintain redox poise during photosynthetic growth. *ASM News* **60**: 17-23.

Moreira, D. (1998). Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Research* **26**: 3309-3310.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Nagashima, K.V.P., Hanada, S., Hiraishi, A., Shimada, K. and Matsuura, K. (1995). Phylogenetic analysis of photosynthetic reaction centers of purple bacteria and green filamentous bacteria. Proceedings of the 10th International Congress of Photosynthesis.

Nagashima, K.V.P., Hiraishi, A., Shimada, K. and Matsuura, K. (1997a). Horizontal transfer of genes coding for the photosynthetic reaction centers of purple bacteria. *Journal of Molecular Evolution* **45**: 131-136.

Nagashima, K.V.P., Matsuura, K., Ohyama, S. and Shimada, K. (1994). Primary structure and transcription of genes encoding B870 and photosynthetic reaction center apoproteins from *Rubrivivax gelatinosus*. *Journal of Biological Chemistry* **269**: 2477-2484.

Nagashima, K.V.P., Matsuura, K., Wakao, N., Hiraishi, A. and Shimada, K. (1997b). Nucleotide sequences of genes coding for photosynthetic reaction centers and light-harvesting proteins of *Acidiphilium rubrum* and related aerobic acidophilic bacteria. *Plant and Cell Physiology*.

Nagel, M. and Andreesen, J.R. (1989). Molybdenum-dependent degradation of nicotinic acid by *Bacillus* sp. DSM2923. *FEMS Microbiology Letters* **59**: 147-152.

Nagel, M. and Andreesen, J.R. (1990). Purification and characterization of the molybdoenzymes nicotinate dehydrogenase and 6-hydroxynicotinate dehydrogenase from *Bacillus niacini*. *Archives of Microbiology* **154**: 605-613.

Naylor, G.W., Addlesee, H.A., Gibson, L.C.D. and Hunter, C.N. (1999). The photosynthesis gene cluster of *Rhodobacter sphaeroides*. *Photosynthesis Research* **62**: 121-139.

Nepple, B.B. and Bachofen, R. (1997). Induction of stress proteins in the phototrophic bacterium *Rhodobacter sphaeroides*. *FEMS Microbiology Letters* **153**: 173-180.

Nepple, B.B., Flynn, I. and Bachofen, R. (1999). Morphological changes in phototrophic bacteria induced by metalloid oxyanions. *Microbiological Research* **154**: 191-198.

Neutzling, O., Imhoff, J.F. and Trüper, H.G. (1984). *Rhodopseudomonas adriatica* sp. nov., a new species of the Rhodospirillaceae, dependent on reduced sulfur compounds. *Archives of Microbiology* **137**: 256-261.

Neutzling, O. and Trüper, H.G. (1982). Assimilatory sulfur metabolism in *Rhodopseudomonas sulfoviridis*. *Archives of Microbiology* **133**: 145-148.

Nickens, D., Fry, C.J., Ragatz, L., Bauer, C.E. and Gest, H. (1996). Biotype of the purple nonsulfur photosynthetic bacterium, *Rhodospirillum centenum*. *Archives of Microbiology* **165**: 91-96.

Nissen, H. and Dundas, I.D. (1984). *Rhodospirillum salinarum* sp. nov., a halophilic photosynthetic bacterium isolated from a Portuguese saltern. *Archives of Microbiology* **138**: 251-256.

Nübel, U., Bateson, M.M., Madigan, M.T., Kuhl, M. and Ward, D.M. (2001). Diversity and distribution in hypersaline microbial mats of bacteria related to *Chloroflexus* spp. *Applied and Environmental Microbiology* **67**: 4365-4371.

Nübel, U., Garcia-Pichel, F. and Muyzer, G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* **63**: 3327-3332.

Oda, Y., de Vries, Y.P., Forney, L.J. and Gottschal, J.C. (2001). Acquisition of the ability for *Rhodopseudomonas palustris* to degrade chlorinated benzoic acids as the sole carbon source. *FEMS Microbiology Ecology* **38**: 133-139.

Ormerod, J.G., Kimble, L.K., Nesbakken, T., Torgersen, Y.A., Woese, C.R. and Madigan, M.T. (1996). *Heliophilum fasciatum* gen. nov., sp. nov. and *Heliobacterium gestii* sp. nov.: endospore-forming heliobacteria from rice field soils. *Archives of Microbiology* **165**: 226-234.

Orso, S., Gouy, M., Navarro, E. and Normand, P. (1994). Molecular phylogenetic analysis of *Nitrobacter* spp. *International Journal of Systematic Bacteriology* **44**: 83-86.

Page, R.D.M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357-358.

Pantazopoulous, P.E. and Madigan, M.T. (2001). Primary alcohols and di-alcohols as growth substrates for the purple nonsulfur bacterium *Rhodobacter capsulatus*. *Canadian Journal of Microbiology* **46**: 1166-1170.

Patel, B.K., Morgan, H.W. and Daniel, R.M. (1985). A simple and efficient method for preparing and dispensing anaerobic media. *Biotechnology Letters* **7**: 277-278.

Pellerin, N.B. and Gest, H. (1983). Diagnostic features of the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. *Current Microbiology* **9**: 339-344.

Perrotta, J.A. and Harwood, C.S. (1994). Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation, by *Rhodopseudomonas palustris*. *Applied and Environmental Microbiology* **60**: 1775-1782.

Pfennig, N. (1969a). *Rhodospirillum tenue* sp. n., a new species of the purple nonsulfur bacteria. *Journal of Bacteriology* **99**: 619-620.

Pfennig, N. (1969b). *Rhodopseudomonas acidophila*, sp. n., a new species of the budding purple nonsulfur bacterium. *Journal of Bacteriology* **99**: 597-602.

Pfennig, N. (1974). *Rhodopseudomonas globiformis*, sp. n., a new species of the Rhodospirillaceae. *Archives of Microbiology* **100**: 197-206.

Pfennig, N. (1978). *Rhodocyclus purpureus* gen. nov. a ring-shaped, vitamin B_{12}-requiring member of the family Rhodospirillaceae. *International Journal of Systematic Bacteriology* **28**: 283-288.

Pfennig, N., Lünsdorf, H., Süling, J. and Imhoff, J.F. (1997). *Rhodospira trueperi* gen. nov., spec. nov., a new phototrophic Proteobacterium of the alpha group. *Archives of Microbiology* **168**: 39-45.

Pfennig, N. and Trüper, H.G. (1989). I. Purple Bacteria. Family I. Chromatiaceae Bavendamm 1924, 125, ^{AL} Emended description Imhoff 1984, 339. Bergey's Manual of Systematic Bacteriology Volume 3. J.T. Staley, M.P. Bryant, N. Pfennig and J.G. Holt. Baltimore, Hong Kong, London, Sydney, Williams & Wilkins: 1637-1653.

Pierson, B.K. and Castenholz, R.W. (1974). A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. *Archives of Microbiology* **100**: 5-24.

Pierson, B.K., Giovannoni, S.J., Stahl, D.A. and Castenholz, R.W. (1985). *Heliothrix oregonensis*, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll *a*. *Archives of Microbiology* **142**: 164-167.

Pierson, B.K., Valdez, D., Larsen, M., Morgan, E. and Mack, E.E. (1994). *Chloroflexus*-like organisms from marine and hypersaline environments: Distribution and diversity. *Photosynthesis Research* **41**: 35-52.

Pond, F.R., Gibson, I., Lalucat, J. and Quackenbush, R.L. (1989). R-body producing bacteria. *Microbiological Reviews* **53**.

Priemé, A., Braker, G. and Tiedje, J.M. (2002). Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology* **68**: 1893-1900.

Puchkova, N.N., Imhoff, J.F. and Gorlenko, V.M. (2000). *Thiocapsa litoralis* sp. nov., a new purple sulfur bacterium from microbial mats from the White Sea. *International Journal of Systematic and Evolutionary Microbiology* **50 Pt 4**: 1441-1447.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.-P. and Wagner, M. (2000). Phylogeny of all recognised species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity studies. *Applied and Environmental Microbiology* **66**: 5368-5382.

Quayle, J.R. and Pfennig, N. (1975). Utilization of methanol by the Rhodospirillaceae. *Archives of Microbiology* **102**: 193.

Ragatz, L., Jiang, Z.-Y., Bauer, C.E. and Gest, H. (1995). Macroscopic phototactic behavior of the purple photosynthetic bacterium *Rhodospirillum centenum*. *Archives of Microbiology* **163**: 1-6.

Rahalkar, S.B., Joshi, S.R. and Shivaraman, N. (1991). Biodegradation of aromatic compounds by *Rhodopseudomonas gelatinosa*. *Current Microbiology* **22**: 155-158.
Rahalkar, S.B., Joshi, S.R. and Shivaraman, N. (1993). Photometabolism of aromatic compounds by *Rhodopseudomonas palustris*. *Current Microbiology* **26**: 1-9.

Rajasekhar, N., Sasikala, C. and Ramana, C.V. (1998). Photobiotransformation of indole to its value-added derivatives by *Rhodobacter sphaeroides* OU5. *Journal of Industrial Microbiology and Biotechnology* **20**: 177-179.

Rajasekhar, N., Sasikala, C. and Ramana, C.V. (2000). Toxicity of N-containing heterocyclic aromatic compounds and their utilization for growth by a few purple non-sulfur bacteria. *Bulletin of Environmental Contamination and Toxicology* **65**: 375-382.

Rajkumar, B. and Lalithakumari, D. (1992). Carbendazim as the sole carbon and nitrogen source for a phototrophic bacterium. *Biomedical Letters* **47**: 337-346.

Redburn, A.C. and Patel, B.K.C. (1993). Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiology Letters* **113**: 81-86.

Resnick, S.M. and Madigan, M.T. (1989). Isolation and characterization of a mildly thermophilic nonsulfur purple bacterium containing bacteriochlorophyll *b*. *FEMS Microbiology Letters* **65**: 165-170.

Rijkenberg, M.J.A., Kort, R. and Hellingwerf, K.J. (2001). *Alkalispirillum mobile* gen. nov., spec. nov., an alkaliphilic non-phototrophic member of the Ectothiorhodospiraceae. *Archives of Microbiology* **175**: 369-375.

Roldan, M.D., Reyes, F., Moreno-Vivian, C. and Castillo, F. (1994). Chlorate and nitrate reduction in the phototrophic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. *Current Microbiology* **29**: 241-245.

Rosado, A.S., Duarte, G.F., Seldin, L. and van Elsas, J.D. (1997). Molecular microbial ecology - a minireview. *Revista de Microbiologia* **28**: 135-147.

Rotthauwe, J.H., Witzel, K.P. and Liesack, W. (1997). The ammonia monooxygenase structural gene *amoA* as a functional marker - molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology* **63**: 4704-4712.

Rubin, C.A. and Madigan, M.T. (1986). Amino acid and ammonia metabolism by the halophilic nonsulfur purple bacterium *Rhodospirillum salexigens*. *FEMS Microbiology Letters* **34**: 73-77.

Rüger, H.J. and Höfle, M.G. (1992). Marine star-shaped-aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom. rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev. *International Journal of Systematic Bacteriology* **42**: 133-143.

Sackett, M.J., Armitage, J.P., Sherwood, E.E. and Pitta, T.P. (1997). Photoresponses of the purple nonsulfur bacteria *Rhodospirillum centenum* and *Rhodobacter sphaeroides*. *Journal of Bacteriology* **179**: 6764-6768.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Santegoeds, C.M., Nold, S.C. and Ward, D.M. (1996). Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. *Applied and Environmental Microbiology* **62**: 3922-3928.

Sasikala, C. and Ramana, C.V. (1997). Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. *Advances in Microbial Physiology* **39**: 339-377.

Sasikala, C., Ramana, C.V. and Raghuveer Rao, P. (1994b). Photometabolism of heterocyclic aromatic compounds by *Rhodopseudomonas palustris* OU11. *Applied and Environmental Microbiology* **60**: 2187-2190.

Sasikala, C., Ramana, C.V. and Rao, P. (1994a). Nitrogen fixation by *Rhodopseudomonas palustris* OU 11 with aromatic compounds as carbon source / electron donors. *FEMS Microbiology Letters* **122**: 75-78.

Satoh, T., Hoshino, Y. and Kitamura, H. (1976). *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. *Archives of Microbiology* **108**.

Sawayama, S., Hanada, S. and Kamagata, Y. (2000a). Isolation and characterization of phototrophic bacteria growing in lighted upflow anaerobic sludge blanket reactor. *Journal of Bioscience and Bioengineering* **89**: 396-399.

Sawayama, S., Tsukuhara, K. and Yagishita, T. (2000b). Organic acid consumption of phototrophic bacteria in a lighted upflow anaerobic sludge blanket reactor. *Journal of Bioscience and Bioengineering* **90**: 241-246.

Scala, D.J. and Kerkhof, L.J. (1999). Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. *Applied and Environmental Microbiology* **65**: 1681-1687.

Schwieger, F. and Tebbe, C.C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology* **64**: 4870-4876.

Seewaldt, E., Schleifer, K.-H., Bock, E. and Stackebrandt, E. (1982). The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. *Archives of Microbiology* **131**: 287-290.

Shiba, T. (1991). *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. nov., aerobic pink-pigmented bacteria which contain bacteriochlorophyll *a*. *Systematic and Applied Microbiology* **14**: 140-145.

Shiea, J., Brassell, S.C. and Ward, D.M. (1991). Comparative analysis of extractable lipids in hot spring microbial mats and their component photosynthetic bacteria. *Organic Geochemistry* **17**: 309-319.

Shoreit, A.A.M. and Shabeb, M.S.A. (1994). Utilization of aromatic compounds by phototrophic purple nonsulfur bacteria. *Biodegradation* **5**: 71-76.

Siefert, E. and Pfennig, N. (1979). Chemoautotrophic growth of *Rhodopseudomonas* species with hydrogen and chemotrophic utilization of methanol and formate. *Archives of Microbiology* **122**: 177-182.

Sievers, M., Ludwig, W. and Teuber, M. (1994). Phylogenetic positioning of *Acetobacter*, *Gluconobacter*, *Rhodopila* and *Acidiphilium* species as a branch of acidophilic bacteria in the α-subclass of *Proteobacteria* based on 16S ribosomal DNA sequences. *Systematic and Applied Microbiology* **17**: 189-196.

Silva, J., Nobre, M.F., Rainey, F. and da Costa, M.S. (1998). *Porphyrobacter cryptus* sp. nov., has a higher optimum growth temperature, but is otherwise very similar to *Porphyrobacter tepidarius*. Thermophiles '98, Le Quant-Brest, France.

Sly, L.I. and Cahill, M.M. (1997). Transfer of *Blastobacter natatorius* (Sly 1985) to the genus *Blastomonas* gen. nov. as *Blastomonas natatoria* comb. nov. *International Journal of Systematic Bacteriology* **47**: 566-568.

Sommer, R. (1996). Yeast extracts: production, properties and components. 9th International Symposium on Yeasts, Sydney.

Sorokin, D.I., Turova, T.P., Kuznetsov, B.B., Bryantseva, I.A. and Gorlenko, V.M. (2000). *Roseinatronobacter thiooxidans* gen. nov., sp. nov., a new alkaliphilic aerobic bacteriochlorophyll-*a*-containing bacteria from a soda lake. *Mikrobiologiya* **69**: 89-97.

Stackebrandt, E., Fischer, A., Roggentin, T., Wehmeyer, U., Bomar, D. and Smida, J. (1988). A phylogenetic survey of budding, and/or prosthecate, non-phototrophic eubacteria: membership of *Hyphomicrobium*, *Hyphomonas*, *Pedomicrobium*, *Filomicrobium*, *Caulobacter* and "*Dichotomicrobium*" to the alpha-subdivision of purple non-sulfur bacteria. *Archives of Microbiology* **149**: 547-556.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kampfer, P., Maiden, M.C., Nesme, X., Rossello-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C. and Whitman, W.B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**: 1043-1047.

Stackebrandt, E. and Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* **44**: 846-849.

Stackebrandt, E., Rainey, F.A. and Ward-Rainey, N. (1996). Anoxygenic phototrophy across the phylogenetic spectrum: current understanding and future perspectives. *Archives of Microbiology* **166**: 211-223.

Stadtwald-Demchick, R., Turner, F.R. and Gest, H. (1990a). Physiological properties of the thermotolerant photosynthetic bacterium, *Rhodospirillum centenum*. *FEMS Microbiology Letters* **67**: 139-144.

Stadtwald-Demchick, R., Turner, F.R. and Gest, H. (1990b). *Rhodopseudomonas cryptolactis*, sp. nov., a new thermotolerant species of budding phototrophic purple bacteria. *FEMS Microbiology Letters* **71**: 117-122.

Stahl, D.A., Lane, D.J., Olson, G.J. and Pace, N.R. (1985). Characterization of a Yellowstone hot spring community by 5S rRNA sequences. *Applied and Environmental Microbiology* **49**: 1379-1384.

Staley, J.T. and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Reviews of Microbiology* **39**: 321-46.

Steffan, R.J., Goksøyr, J., Bej, A.K. and Atlas, A.M. (1988). Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology* **54**: 2908-2915.

Stenklo, K., Thorell, H.D., Bergius, H., Aasa, R. and Nilsson, T. (2001). Chlorite dismutase from Ideonella dechloratans. *Journal of Biological Inorganic Chemistry* **6**: 601-607.

Stevenson, A.K., Kimble, L.K., Woese, C.R. and Madigan, M.T. (1997). Characterization of new phototrophic heliobacteria and their habitats. *Photosynthesis Research* **53**: 1-12.

Straub, K.L., Rainey, F.A. and Widdel, F. (1999). *Rhodovulum iodosum* sp. nov., and *Rhodovulum robiginosum* sp. nov., two new marine phototrophic ferrous-iron-oxidizing purple bacteria. *International Journal of Systematic Bacteriology* **49**: 729-735.

Strimmer, K. and von Haeseler, A. (1996). Quartet puzzling: a quartet-maximum likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution* **13**: 964-969.

Suwanto, A. and Kaplan, S. (1989). Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: presence of two unique circular chromosomes. *Journal of Bacteriology* **171**: 5850-5859.

Suyama, T., Shigematsu, T., Suzuki, T., Tokiwa, Y., Kanagawa, T., Nagashima, K.V.P. and Hanada, S. (2002). Photosynthetic apparatus in *Roseateles depolymerans* 61A is transcriptionally induced by carbon limitation. *Applied and Environmental Microbiology* **68**: 1665-1673.

Suyama, T., Shigematsu, T., Takaichi, S., Nodasaka, Y., Fujikawa, S., Hosoya, H., Tokiwa, Y., Kanagawa, T. and Hanada, S. (1999). *Roseateles depolymerans* gen. nov., sp. nov., a new bacteriochlorophyll *a*-containing obligate aerobe belonging to the β-subclass of the *Proteobacteria*. *International Journal of Systematic Bacteriology* **49**: 449-457.

Suzuki, T., Muroga, Y., Takahama, M. and Nishimura, Y. (1999). *Roseovivax halodurans* gen. nov., sp. nov. and *Roseovivax halotolerans* sp. nov., aerobic bacteriochlorophyll-containing bacteria isolated from a saline lake. *International Journal of Systematic Bacteriology* **49**: 629-634.

Suzuki, T., Muroga, Y., Takahama, M. and Nishimura, Y. (2000). *Roseibium denhamense* gen. nov., sp. nov. and *Roseibium hamelinense* sp. nov., aerobic bacteriochlorophyll-containing bacteria isolated from the east and west coasts of Australia. *International Journal of Systematic and Evolutionary Microbiology* **50**: 2151-2156.

Suzuki, T., Muroga, Y., Takahama, M., Shiba, T. and Nishimura, Y. (1999). *Rubrimonas cliftonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium isolated from a saline lake. *International Journal of Systematic and Evolutionary Microbiology* **49**: 201-205.

Tanaka, H., Maeda, H., Suzuki, H., Kamibayashi, A. and Tonomura, K. (1982). Metabolism of thiophene-2-carboxylate by a photosynthetic bacterium. *Agricultural Biological Chemistry* **46**: 1429-1438.

Teske, A., Wawer, C., Muyzer, G. and Ramsing, N.B. (1996). Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology* **62**: 1405-1415.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876-4882.

Tindall, B.J. (2001). *Rhodocista centenaria* vs *Rhodospirillum centenum*: a reply to Gest and Favinger. *International Journal of Systematic and Evolutionary Microbiology* **51**: 711-713.

Tinschert, A., Kiener, A., Heinzmann, K. and Tschech, A. (1997). Isolation of new 6-methylnicotinic acid-degrading bacteria, one of which catalyses the regioselective hydroxylation of nicotinic acid at position C2. *Archives of Microbiology* **168**: 355-361.

Tsai, L., Pastan, I. and Stadtman, E.R. (1966). Nicotinic acid metabolism: the isolation and characterization of intermediates in the fermentation of nicotinic acid. *Journal of Biological Chemistry* **241**: 1807-1813.

Uffen, R.L. and Wolfe, R.S. (1970). Anaerobic growth of purple nonsulfur bacteria under dark conditions. *Journal of Bacteriology* **104**: 462.

van Berkum, P. and Eardly, B.D. (2002). The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen-fixing symbiont of *Aeschynomene indica*. *Applied and Environmental Microbiology* **68**: 1132-1136.

van der Woude, B.J., de Boer, M., van der Put, N.M.J., van der Geld, F.M., Prins, R.A. and Gottschal, J.C. (1994). Anaerobic degradation of halogenated benzoic acids by photoheterotrophic bacteria. *FEMS Microbiology Letters* **119**: 199-208.

van Niel, C.B. (1944). The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. *Bacteriological Reviews* **8**: 1-118.

Wahlund, T.M., Woese, C.R., Castenholz, R.W. and Madigan, M.T. (1991). A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. *Archives of Microbiology* **156**: 81-90.

Wakao, N., Shiba, T., Hiraishi, A., Ito, M. and Sakurai, Y. (1993). Distribution of bacteriochlorophyll *a* in species of the Genus *Acidiphilium*. *Current Microbiology* **27**: 277-279.

Wakao, N., Yokoi, N., Isoyama, A., Hiraishi, A., Shimada, K., Kobayashi, M., Kise, H., Iwaki, M., Itoh, S., Takaichi, S. and Sakurai, Y. (1996). Discovery of natural photosynthesis using Zn-containing bacteriochlorophyll in an aerobic bacterium *Acidiphilium rubrum*. *Plant and Cell Physiology* **37**: 889-893.

Ward, D.M., Ferris, M.J., Nold, S.C. and Bateson, M.M. (1998). A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiology and Molecular Biology Reviews* **62**: 1353-1370.

Ward, D.M., Weller, R. and Bateson, M.M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**: 63-65.

Watanabe, K., Teramoto, M., Futumata, H. and Harayama, S. (1998). Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Applied and Environmental Microbiology* **64**: 4396-4402.

Wawer, C., Jetten, M.S. and Muyzer, G. (1997). Genetic diversity and expression of the [NiFe] hydrogenase large-subunit gene of *Desulfovibrio* spp. in environmental samples. *Applied and Environmental Microbiology* **63**: 4360-4369.

Weaver, P.F., Wall, J.D. and Gest, H. (1975). Characterization of *Rhodopseudomonas capsulata*. *Archives of Microbiology* **105**: 207-216.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B. and Schink, B. (1993). Ferrous ion oxidation by anoxygenic phototrophic bacteria. *Nature* **362**: 834-836.

Widjojoatmodjo, M.N., Fluit, A.C. and Verhoef, J. (1995). Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *Journal of Clinical Microbiology* **33**: 2601-2606.

Wiggli, M., Schenk, A., Horath, T., Stettler, R., Lüthy, L., Grüter, D., Buchs, U., Smallcombe, A. and Bachofen, R. (1998). Chemical, physiological and molecular-biological investigations in the microbial mats West of Cadagno in the Piora Valley. *Documenta Istituto italiano di Idrobiologia* **63**: 127-136.

Wimpenny, J.W.T., Gest, H. and Favinger, J.L. (1986). The use of two dimensional gradient plates in determining the responses of non-sulphur purple bacteria to pH and NaCl concentration. *FEMS Microbiology Letters* **37**: 367-371.

Woese, C.R. (2000). Interpreting the universal phylogenetic tree. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 8392-8396.

Wong, F.Y.K., Stackebrandt, E., Ladha, J.K., Fleischmann, D.E., Date, R.A. and Fuerst, J.A. (1994). Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aeschynomene* species grown in separated geographical regions. *Applied and Environmental Microbiology* **60**: 940-946.

Wright, G.E. and Madigan, M.T. (1991). Photocatabolism of aromatic compounds by the phototrophic purple bacterium *Rhodomicrobium vannielii*. *Applied and Environmental Microbiology* **57**: 2069-2073.

Xiong, J., Fischer, W.M., Inoue, K., Nakahara, M. and Bauer, C.E. (2000). Molecular evidence for the early evolution of photosynthesis. *Science* **289**: 1724-1730.

Yamamoto, H., Hiraishi, A., Kato, K., Chiura, H.X., Maki, Y. and Shimizu, A. (1998). Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. *Applied and Environmental Microbiology* **64**: 1680-1687.

Yildiz, F.H., Gest, H. and Bauer, C.E. (1991a). Attenuated effect of oxygen on photopigment synthesis in *Rhodospirillum centenum*. *Journal of Bacteriology* **173**: 5502-5506.

Yildiz, F.H., Gest, H. and Bauer, C.E. (1991b). Genetic analysis of photosynthesis in *Rhodospirillum centenum*. *Journal of Bacteriology* **173**: 4163-4170.

Yuan, J. (2000) Growth of purple nonsulfur bacteria on purines and pyrimidines. M.Sc. Thesis, Biological Sciences, University of Waikato.

Yurkov, V., Stackebrandt, E., Buss, O., Vermeglio, A., Gorlenko, V. and Beatty, J.T. (1997). Reorganization of the genus *Erythromicrobium*: description of "*Erythromicrobium sibiricum*" as *Sandaracinobacter sibiricus* gen. nov., sp. nov., and of "*Erythromicrobium ursincola*" as *Erythromonas ursincola* gen. nov., sp. nov. *International Journal of Systematic Bacteriology* **47**: 1172-1178.

Yurkov, V., Stackebrandt, E., Holmes, A., Fuerst, J.A., Hugenholtz, P., Golecki, J., Gad'on, N., Gorlenko, V.M., Kompantseva, E.I. and Drews, G. (1994). Phylogenetic positions of novel aerobic, bacteriochlorophyll a-containing bacteria and description of *Roseococcus thiosulfatophilus* gen. nov., sp. nov., *Erythromicrobium ramosum* gen. nov., sp. nov., and *Erythrobacter litoralis* sp. nov. *International Journal of Systematic Bacteriology* **44**: 427-434.

Yurkov, V.V. and Beatty, J.T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiology and Molecular Biology Reviews* **62**: 695-724.

Yurkov, V.V. and Gorlenko, V.M. (1992a). A new strain RB-5 of the purple nonsulfur bacterium *Rhodopseudomonas blastica*, isolated from a sulfide alkaline spring. *Mikrobiologiya* **61**: 76-80.

Yurkov, V.V. and Gorlenko, V.M. (1992b). Ecophysiological characteristics of phototrophic microorganisms of Bol'sherechenskii thermal springs microbial communities. *Mikrobiologiya* **61**: 86-92.

Yurkov, V.V., Gorlenko, V.M., Mityushina, L.L. and Starynin, D.A. (1992). Effect of limiting factors on the structure of phototrophic associations in thermal springs. *Microbiology* **60**: 129-138.

Yurkov, V.V., Jappé, J. and Verméglio, A. (1996). Tellurite resistance and reduction by obligately aerobic photosynthetic bacteria. *Applied and Environmental Microbiology* **62**: 4195-4198.

Yurkov, V.V., Krieger, S., Stackebrandt, E. and Beatty, J.T. (1999). *Citromicrobium bathymarinum*, a novel aerobic bacterium isolated from deep-sea hydrothermal vent plume waters that contains photosynthetic pigment-protein complexes. *Journal of Bacteriology* **181**: 4517-4525.

Zehnder, A.J.B. and Wuhrmann, K. (1976). Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* **194**: 1165-1166.

Zengler, K., Heider, J., Rossello-Mora, R. and Widdel, F. (1999). Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*. *Archives of Microbiology* **172**: 204-212.

Zhang, D., Yang, H., Huang, Z., Zhang, W. and Liu, S.-J. (in press). *Rhodopseudomonas faecalis* sp. nov., a phototrophic bacterium isolated from an anaerobic reactor that digests chicken faeces. *International Journal of Systematic and Evolutionary Microbiology*.