Studies on Sulfate-reducing Bacteria which Degrade Fatty Acids and on an Obligately Anaerobic Agarolytic Bacterium

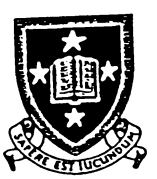
A thesis submitted in partial fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Biological Sciences
by

Gavin Nigel Rees



University of Waikato

1989

ABSTRACT

Five methods were used to isolate sulfate-reducing bacteria using palmitate as the enrichment substrate. (1) Standard enrichment procedures gave rise to pure cultures of *Desulfovibrio sapovorans*. (2) In enrichments which involved the continual transfer of sediment throughout the isolation, two rod-shaped strains were isolated in pure culture and designated strains HoPa1 and AmPa1. These latter strains had a number of characteristics similar to *D. sapovorans*, but also varied from the general description by a number of characteristics. Determination of their DNA base composition is required for confirmation of their identification. (3) When pasteurized sediment was used in the enrichments with palmitate as the electron donor, a strain of *Desulfotomaculum sapomendens* was isolated. (4) When enrichments were carried out at 13°C, a sulfate-reducing bacterium was enriched and purified. This bacterium was identified as as *Desulfobacterium* species. (5) A series of enrichments were carried out whereby an increasing dilution of mud was used as the source material. Tubes with mud from the lower dilutions gave rise to typical *Desulfovibrio sapovorans*. However, mud used at higher dilutions gave rise to *Desulfobacterium* species, suggesting that these organisms may be present in higher numbers.

Enrichments were carried out using branched-chain fatty acids as the electron donor. Freshwater enrichments with isobutyrate and 2-methyl butyrate gave rise to *Desulfotomaculum acetoxidans* and *Desulfovibrio sapovorans*, designated Amib and Am2mb respectively. Estuarine enrichments with isobutyrate and 2-methylbutyrate gave rise to

Desulfobacterium vacuolatum, designated Okib and a *Desulfosarcina* species designated Ok2mb. Enrichments from both freshwater and estuarine sources using isovalerate grew very slowly and were not purified in this study. Studies on the physiology of strain Okib showed it was able to oxidize a range of amino acids and keto acids including proline, branched-chain amino acids, 5-aminovalerate and keto isocaproate. It neither fermented amino acids nor carried out Stickland reactions with pairs of amino acids.

During the course of isolating the sulfate-reducing bacteria in this study, an obligately anaerobic agarolytic bacterium was enriched in association with a *Desulfovibrio* species. The agarolytic bacterium was obtained in pure culture and designated 16AV. This is the first account of the degradation of agar by an obligate anaerobe. Strain 16AV grew only on agar, agarose, galactose and cellobiose producing ethanol, acetate, H_2 and CO_2. Strain 16AV probably represents a new species of the genus *Acetivibrio*.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr CG Harfoot for his guidance throughout this project and also for his constructive criticisms during the preparation of this thesis. I would also like to thank Professor HW Morgan for his assistance as joint supervisor, particularly during the absence of Dr Harfoot.

Special thanks are due to Peter Janssen for many a stimulating discussion of things microbial and otherwise. I would also like to acknowledge Louis, Gordon, John Fam, Andrew, Keith, Ron, Greg, Shona and Roger for their contributions over the last couple of years, who along with the Welsh Rugby team, supplied some of the lighter moments. Special mention should be made of Tim Coolbear and Mark Patchett for answering those often trivial biochemistry questions, Bharat Patel for assistance with electron microscopy, Daniel Stangl for instruction of amino analysis, Colin Monk for help with HPLC operation and Andrew Hudson for proof reading.

I would like to give a special vote of thanks to my typing pool, Beverly, Sarah and Andrea whose efforts have been invaluable.

I would like to thank MIRINZ for financial support. I must also thank DAAD for funding a short study at the University of Konstanz, Federal Republic of Germany. I would like to thank Professor Norbert Pfennig, Dr Friedhelm Bak and Jürgen Seitz for the stimulating discussions while in Germany.

Finally, most important thanks are due to my wife Sarah for her support and encouragement throughout this project. I would also like to thank my family for their encouragement while I have carried out my university studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Plates bear no legends or page numbers. The legends and page numbers appear on the facing pages.

CHAPTER ONE

INTRODUCTION

Dissimilatory sulfate-reducing bacteria are an eco-physiological group of obligately anaerobic bacteria which are able to use inorganic sulfate as a terminal electron acceptor for oxidative degradation of their substrate. Dissimilatory sulfate reduction results in production of hydrogen sulfide. It is this compound that gives recognition of these organisms in nature, as hydrogen sulfide reacts with metal ions giving rise to black muds where sulfate is present. In addition, hydrogen sulfide has a particularly offensive smell. The black odorous sediments produced by the action of sulfate-reducing bacteria has contributed a degree of infamy on this group of organisms.

From the first observation of sulfate-reducing bacteria by Beijerink (1895) up until the mid 1970's, the group of organisms included a limited number of species comprising 2 genera. Their metabolic patterns were very restricted, being able to oxidize lactate, pyruvate, some alcohols and small organic acids such as malate. Under modified conditions, Widdel and Pfennig (1977) and Widdel (1980) described a variety of hitherto unknown sulfate-reducing bacteria able to use fatty acids and aromatic compounds. Fatty acids from acetate to octadecanoate were oxidized by some species, often completely to CO_2. Autotrophic growth on H_2/CO_2, and formate were also shown. Following the work of Widdel (1980) further sulfate-reducing bacteria have been isolated which can use an even wider range of compounds.

The central aim of this thesis was to study organisms that could degrade fatty acids. First, a number of enrichment techniques were used in order to isolate sulfate-reducing bacteria able to use palmitate (hexadecanoate) as an electron donor. The use of palmitate by sulfate-reducing bacteria was first observed by Widdel (1980) and has since been used as a compound for the selective enrichment of *Desulfovibrio sapovorans*. Second, attempts were made to isolate organisms from estuarine and freshwater sediments which could oxidize branched-chain fatty acids. Sulfate-reducing bacteria have been described in the literature which can use branched-chain fatty acids. However, the use of these fatty acids as enrichment substrates has not been comprehensively studied. The organisms that were isolated using isobutyrate, 2-methylbutyrate and isovalerate were characterized and aspects of their physiology studied.

These two central studies gave rise to two further studies. Attempts were made to isolate bacteria able to degrade palmitate in syntrophic association. It was hoped that such cultures could be characterized and then used for comparative studies of palmitate degradation with sulfate-reducing bacteria. In the final part of this thesis, a study was made of an agarolytic bacterium that was isolated from an agar shake dilution tube designed to isolate sulfate-reducing bacteria. Though "dissimilatory sulfate-reducing bacteria" is the correct term to use for the main group of organisms described in this thesis, the terms "sulfate-reducing bacteria" and "sulfate-reducers" are used by author for convenience sake. While recognizing the correct term, the two shortened terms are used throughout this thesis. The term "syntrophic bacterium" is also used for convenience throughout this thesis as a shortened term for the "organism which <u>produces</u> hydrogen in syntrophic associations" when oxidizing its substrate.

CHAPTER TWO

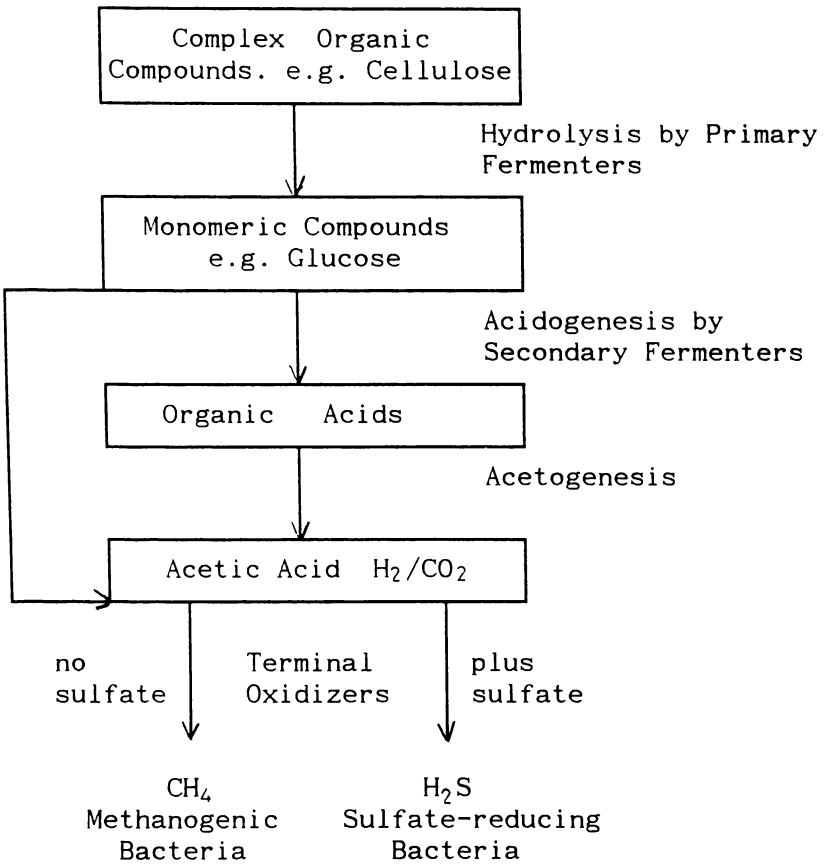
SULFATE-REDUCING BACTERIA:- A LITERATURE REVIEW

2.1 PROCESSES OF ANAEROBIC DEGRADATION OF ORGANIC MATERIAL.

The oxidation of organic material in aerobic environments is a relatively simple process as most compounds, be they complex polymers or monomeric compounds, are oxidized completely to CO_2. In anaerobic environments the degradation of organic material is a more complex process. Organic material is degraded incompletely by one group of bacteria, yielding compounds which are further degraded by different bacteria until the final oxidation state of the organic substrate is reached. Anaerobic degradation can be regarded as a food chain, varying in complexity in different environments.

The simplest schematic diagram to represent the major metabolic groups is shown in figure 2.1. This situation results from the restricted metabolism of the anaerobes in each of the groups. The groups involved are: (i) the primary fermenters, responsible for the breakdown of organic polymers and for producing monomeric compounds, acetate, H_2 and CO_2, small organic acids and alcohols, (ii) the secondary fermenters, bacteria using monomers, acids and alcohols and producing smaller organic acids, acetate, H_2 and CO_2, (iii) the bacteria which bring about acetogenic fermentation of organic acids, (iv) the terminal oxidizers, methanogenic bacteria or sulfate-reducing bacteria depending on the environmental conditions. Acetate, H_2 and CO_2 feature on this diagram and while methanogens have been described which produce methane from formate (Ferry *et al.* 1974),

Figure 2.1 Generalized Scheme For Anaerobic Mineralization of Carbon Compounds in Nature



methanol (Bryant and Boone 1987) and methylamines (Mah and Kuhn 1984), acetate and H_2/CO_2 are generally regarded as the major substrates for methanogesis. In the rumen, acetate has been shown not to be a significant methanogenic substrate (Opperman *et al.* 1961).

In environments which have very low levels of sulfate, such as man-made anaerobic digesters, rumens and freshwater sediments, the terminal oxidation is carried out by methanogens. In marine and estuarine sediments, sulfate reduction is the principol terminal oxidation process. Sulfate-reducing bacteria which oxidize acetate and H_2 are able to outcompete methanogens for those substrates, a phenomenon studied by a number of workers (e.g Cappenberg 1974, Winfrey and Zeikus 1977). The reason for the dominance of sulfate-reducing bacteria over methanogens was studied by Kristjansson *et al.* (1982) and Schöhneit *et al.* (1982) who showed that *Desulfovibrio vulgaris* (Marburg) had K_s values for hydrogen and acetate of 1 μM and 0.2 mM respectively, whereas *Methanobrevibacter arborphilicus* (AZ) had a K_s value of 6μM for H_2 and *Methanosarcina* had a K_s value for acetate of 3mM. The lower K_s values shown by the sulfate-reducing bacteria for these substrates confers competitive advantage of the sulfate-reducing bacteria over methanogens.

However, sulfate-reducing bacteria do not completely displace methanogens in sulfate-containing sediments, nor do methanogens completely displace sulfate-reducing bacteria from freshwater environments. Winfrey and Ward (1983) studied the activity of methanogenic bacteria and sulfate-reducing bacteria in marsh, estuarine and beach intertidal sediments and demonstrated slow rates of methane generation in each of the sediments studied. Sulfate

reduction was dominant with rates 100 to 1000 times higher than rates for methanogenesis. Mountfort and Asher (1981) also showed that methanogens could grow in environments where sulfate reduction was occurring. Further, a number of methanogens have been isolated from estuarine and marine environments, many having a requirement for sodium chloride. Examples include *Methanococcus halophilus* (Zhilina 1983) and *Methanogenium cariaci* (Romesser *et al.* 1979).

In general, methanogenesis is the dominant terminal process in fresh water sediments where sulfate concentrations are low, (typically 0.01-0.2 mM) compared to seawater (typically 20-30 mM). This is particularly the case in sediments of eutrophic lakes (Winfrey and Zeikus 1977). Despite this, sulfate reduction has been observed in eutrophic lakes (Ingvorsen and Brock 1982). The uptake kinetics of sulfate were studied by Ingvorsen and Jorgensen (1984) who showed that *Desulfovibrio vulgaris* strain Marburg and *D. vulgaris* strain Hildenborough, both fresh water isolates, had K_s values for sulfate of 5 μM and 32 μM respectively. These values can be considered to be very low; freshwater sediments have sulfate levels ranging from a few micromolar to 200 micromolar. The very low K_s values explain why sulfate-reducing bacteria can occur in freshwater sediment. K_s values for sulfate have also been calculated for marine strains of sulfate-reducing bacteria. Despite the concentration of sulfate in seawater, the K_s values found were quite low; about 200 μM for *Desulfobacter postgatei* and 77μM in *Desulfovibrio salexigens* (Ingvorsen *et al.* 1984, Ingvorsen and Jorgensen 1984). The latter approaches the values generally associated with high affinity uptake systems for a substrate.

2.2 ROLE OF SULFATE-REDUCING BACTERIA IN DEGRADATION OF ORGANIC MATERIAL.

2.2.1 GENERAL MICROBIOLOGY OF SULFATE-REDUCING BACTERIA.

Mention has already been made of sulfate-reducing bacteria and some of their activities. These bacteria will be discussed here in more detail.

Heterotrophy and Sulfate Respiration.

Sulfate-reducing bacteria are a group of obligate anaerobes which carry out respiratory metabolism using sulfate as the terminal electron acceptor, reducing it to hydrogen sulfide. This is the key characteristic which assigns bacteria to this group. Up until 1980, the sulfate-reducing bacteria were considered to be a group of metabolically restricted organisms, capable of degrading lactate, pyruvate and some further small organic acids, as well as some alcohols. In 1980 Widdel described a number of species able to degrade both short and long chain fatty acids, aromatic compounds and some which could grow autotrophically on H_2 and CO_2 (Widdel 1980). Since this work appeared there have been a number of descriptions of sulfate-reducing bacteria able to degrade an even wider range of substrates. Some examples include nicotinic acid (Imhoff-Stuckle and Pfennig 1983), indole (Bak and Widdel 1986a), phenol (Bak and Widdel 1986b), catechol (Szewzyk and Pfennig 1987) and fructose (Ollivier 1988).

Autotrophic Growth of Sulfate-reducing Bacteria.

Studies which set out to isolate autotrophic sulfate-reducing bacteria have shown members of the genus *Desulfobacterium* to be widely distributed in marine sediments. They carry out the reaction:

$$4H_2 + SO_4^{2-} + 2H^+ \longrightarrow H_2S + 4H_2O$$

thus they play a major role in the removal of H_2 from the environment. The removal of hydrogen is also the likely role in nature of the H_2-utilizing *Desulfovibrio* species. Members of this genus are typically isolated from sediment using lactate as the enrichment electron donor. Many strains, though isolated with lactate, are also able to use hydrogen as an electron donor for sulfate reduction provided a carbon source such as acetate is available and so nature hydrogen is more likely to be their major electron donor.

Sulfate-reducing Bacteria as Fermenters.

Sulfate-reducing bacteria have also been shown to be able to grow using means other than by sulfate reduction. The ability of some species to ferment pyruvate and fumarate in the absence of sulfate is well documented (e.g Miller and Wakerley 1966, Postgate 1984). Laanbroek *et al.* (1982) showed that *Desulfobulbus propionicus* was able to ferment lactate and ethanol to propionate and acetate in the absence of sulfate.

A newly-observed metabolic process has been described whereby *Desulfovibrio sulfodismutans* was able to ferment thiosulfate and sulfite according to the equations 1 and 2 below, obtaining energy for growth (Bak and Pfennig 1987, Bak and Cypionka 1987). A carbon source was required in the form of acetate.

Equation 1) $S_2O_3^{2-} + H_2O \longrightarrow SO_4^{2-} + HS^- + H^+$

Equation 2) $4SO_3^{2-} + H^+ \longrightarrow 3SO_4^{2-} + HS^-$

Of the other sulfate-reducing bacteria tested, *Desulfobacter curvatus* was also able to disproportionate thiosulfate and sulfite. A further unnamed strain was able to use only thiosulfate as an energy source.

Other Metabolic Activities.

It was shown that *Desulfotomaculum orientis* could carry out homoacetogenic growth using H_2/CO_2, formate and methanol (Klemps *et al.* 1985). This indicates a degree of similarity with some clostridia and *Acetobacterium* species.

The ability of sulfate-reducing bacteria to use nitrate as electron acceptor has been observed in a number of species (Widdel and Pfennig 1982, Keith and Herbert 1983, Seitz and Cypionka 1986, Szewzyk and Pfennig 1987). Nitrogen fixation has been shown to be carried out by some species of sulfate-reducing bacteria (Postgate and Kent 1985).

It is evident that sulfate-reducing bacteria comprise a complex assemblage of organisms, able to participate in a great variety of

metabolic processes. Sulfate-reducing bacteria have a very important role in the oxidation of carbon compounds. There are several reports of sulfate-reducing bacteria able to grow autotrophically, some that use acetate and those that use more complex compounds. With the knowledge that there are sulfate-reducing bacteria able to use compounds like dicarboxylic acids, catechol and indole, sulfate-reducing bacteria are also potential competitors with fermentative bacteria for such compounds. They are also able to compete with syntrophic organisms for compounds such as fatty acids and benzoate.

2.2.2 HABITATS OF SULFATE-REDUCING BACTERIA.

Sulfate-reducing bacteria can be isolated from a wide range of aquatic and terrestrial anaerobic environments. Whereas sulfate-reducing bacteria are predominantly found in environments with high sulfate availability, such as estuaries, marine sediment and salt marsh sediments, the occurrence of sulfate-reducing bacteria has also been reported in nonsaline environments (Trüper *et al.* 1969, Pfennig *et al.* 1981, Keith *et al.* 1982). Sulfate-reducing bacteria have been isolated from environments as diverse as the rumen (Coleman 1960, Huising *et al.* 1974), piggery wastes (Widdel and Pfennig 1977), human feces (Beerens and Romond 1977), geothermal water pools (Zeikus *et al.* 1983), spoiled foods (Werkman and Weaver 1927) and soil (Campbell *et al.* 1957). There has been one report of sulfate-reducing bacteria isolated from a clinical blood specimen (Porschen and Chan 1977). It was suggested by the authors that the patient had probably acquired the organism from some soil or water source and carried it in the gastrointestinal tract, as opposed to it growing freely in their blood as blood could be regarded as an aerobic environment.

2.2.3 IDENTIFICATION AND TAXONOMIC STATUS OF SULFATE-REDUCING BACTERIA.

Sulfate-reducing bacteria are an eco-physiological group; many species bearing little apparent taxonomic similarity with one another. Examples of the group include *Desulfonema magnum*, a large (6-8 μm x 9-13 μm) filamentous gliding organism, *Desulfobacter* a gram-negative rod and *Desulfotomaculum*, a sporulating organism that stains gram negatively but possesses a gram-positive type wall (Sleytr *et al.* 1969).

The presently-used system for identification of sulfate-reducing bacteria is based on that proposed by Pfennig *et al.* (1981) which took into account those organisms isolated by Widdel (1980). Previous to this, the schemes proposed by Campbell and Postgate (1965) and Postgate and Campbell (1966) were used which described two genera, *Desulfotomaculum* and *Desulfovibrio*. *Desulfotomaculum* and *Desulfovibrio* were shown to be nutritionally limited, growing typically on lactate, pyruvate and some alcohols. Sporulating species were assigned to the genus *Desulfotomaculum* whereas non-sporulating species assigned to the genus *Desulfovibrio*. Widdel (1980) isolated a great variety of sulfate-reducing bacteria able to degrade fatty acids and aromatic compounds. Since this publication, there have been a number of descriptions of sulfate-reducing bacteria able to degrade an even wider range of substrates. Table 2.1 lists those sulfate-reducing bacteria presently described in the literature, the substrate on which they were enriched and important characteristics.

Table 2.1 Sulfate-reducing bacteria Described in the Literature

Organism	Enrichment Substrate	Oxidation	Important Characteristic, Compound used	Reference
Desulfotomaculum				
nigrificans	La	i	Growth at 55°C	(1)
orientis	La	i	Methanol	(1)
ruminis	La	i	Alanine	(1)
antarcticum	La	i	Glucose	(2)
acetoxidans	Ac	c	Butyrate	(3)
guttoideum	La	i	Morphology	(4)
sapomendens	La	c	Very versatile	(5)
geothermicum	H_2	c	Fatty acids at 55°C	(6)
Desulfovibrio				
desulfuricans	La	i	Choline	(7)
vulgaris	La	i	High DNA composition	(7)
gigas	La	i	Morphology	(8)
africanus	La	i	Long thin vibrio	(9)
salexigens	La	i	Requires salt	(7)
baculatus	La	i	Rod-shaped	(10)
sulfodismutans	La	i	Dismutates sulfite	(11)
thermophilus	La	i	Growth at 55°C	(12)
sapovorans	Pa	i	Fatty acids	(13)
baarsii	St	c	Fatty acids	(13)
fructosovorans	H_2	i	Fructose	(14)
giganteus	La	i	Size	(15)
carbinolicus	La	i	Ferments Glycerol	(16)
Desulfomonas				
pigra	La	i	Large rod, High G+C content	(17)
Thermodesulfobacterium commune	La	i	Growth at 70°C	(18)
Desulfobulbus				
propionicus	Pr	i	Oval-shaped	(19)
elongatus	Pr	i	Long rod	(20)
Desulfobacter				
postgatei	Ac	c	Small rod, uses acetate	(21)
hydrogenophilus	Ac	c	H_2, can fix N_2	(22)
latus	Ac	c	Large cells	(22)
curvatus	Ac	c	Vibrio-shaped	(22)

Table 2.1 continued

Desulfococcus				
multivorans	Bz	c	Aromatic Compounds	(14)
*niacini**	Nic	c	Nicotinic acid	(23)
Desulfosarcina				
variabilis	Bz	c	Variety of aromatic compounds	(14)
Desulfobacterium				
autotrophicum	H_2	c	Autotrophic	(24)
indolicum	In	c	Indole	(25)
phenolicum	Ph	c	Phenol	(26)
vacuolatum	Ib	c	Forms vacuoles	(27)
catecholicum	Ca	c	Catechol	(28)
Desulfonema				
magnum	Bz	c	Large filaments	(29)
limicola	Ac	c	Filaments Fatty acids	(29)

Table 2.1 is after Widdel (1988)

* Considered to be reclassified as *Desulfobacterium niacini* (Widdel 1988)

Abbreviations used:
La, Lactate; Ac, Acetate; Pa, Palmitate; St, Stearate; Pr, propionate; Bz, Benzoate; Ni, Nicotinate; In, Indole; Ib, Isobutyrate; Ph, Phenol; Ca, catechol; i = incomplete oxidation; c = complete oxidation

References are as follows:
(1), Campbell and Postgate (1965); (2), Iizuka *et al.* (1969); (3) Widdel and Pfennig (1977); (4) Gogotova and Vainshtein (1983); (5) Cord-Ruwisch and Garcia (1985); (6) Daumas *et al.* (1988); (7) Postgate and Campbell (1966); (8) LeGall (1963); (9) Campbell *et al.* (1966); (10) Rozonova and Nazina (1976); (11) Bak and Pfennig (1987); (12) Rozonova and Khudyakova (1974); (13) Widdel (1980); (14) Ollivier *et al.* (1988); (15) Esnault *et al.* (1988); (16) Nanninga and Gottschal (1987); (17) Moore *et al.* (1976); (18) Zeikus *et al.* (1983); (19) Widdel and Pfennig (1982); (20) Samain *et al.* (1984); (21) Widdel and Pfennig (1981a); (22) Widdel (1987); (23) Imhoff-Stuckle and Pfennig (1983); (24) Brysch *et al.* (1987); (25) Bak and Widdel (1986a); (26) Bak and Widdel (1986b); (27) Widdel (1980); (28) Szewzyk and Pfennig (1987); (29) Widdel *et al.* (1983).

In the classification scheme proposed by Pfennig *et al.*(1981), the first major division is whether or not the organism had the ability to oxidize its substrate completely. Incomplete oxidizers belonged to the 'classical' *Desulfovibrio* species (those sulfate-reducers that were first to be obtained in pure culture and studied in detail), *Desulfomonas pigra* and most of the early discovered *Desulfotomaculum* strains. In this group, *Desulfotomaculum sapovorans* degrades long-chain fatty acids having an even number of carbon atoms incompletely to acetate and degrades long-chain fatty acids having an odd number of carbon atoms to acetate and propionate. *Desulfobulbus propionicus* degrades propionate to acetate and *Thermodesulfobacterium commune* also oxidizes substrates such as lactate, incompletely to acetate. The second group, oxidize their substrate completely to CO_2 and comprise the genera *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfonema and Desulfobacterium.* Complete oxidation is also carried out by one species of *Desulfovibrio*, namely *D. baarsii.* Other physiological studies would suggest this organism may in fact not be closely related to other desulfovibrios. Isolation of *Desulfobacterium* species gave rise to further difficulties in the identification of sulfate-reducing bacteria as isolates were obtained which were either specialist degraders of aromatic compounds and not able to degrade fatty acids or vice versa. The scheme of Pfennig *et al.* (1981) is still used as the basis of identification of sulfate-reducing bacteria, but additional characteristics often have to be considered when identifying some strains. Difficulties can arise when identifying newly isolated strains of sulfate-reducing bacteria as there are so few chatacteristics that one can routinely determine. Consequently, a difference of 2 of 3 characteristics can place an

organism "in limbo" between the benchmark descriptions of accepted species.

With one exception, all the sulfate-reducing bacteria described in the literature have been eubacteria. In 1987, Stetter described the isolation of novel, extremely thermophilic, archaebacterial sulfate reducers (Stetter *et al.* 1987). *Archaelobus fulgidis* was named and described more fully at a later date (Stetter 1988). This group represents a new taxon of archaebacteria. Growth of *A. fulgidis* occurs between 60°C and 95°C with sulfate, sulfite and thiosulfate, but not sulfur, as electron acceptors which are reduced to sulfide. Belonging to the Kingdom Archaebacteria, this organism clearly has very distant affinities to either the mesophilic sulfate-reducing bacteria or the thermophilic eubacterial sulfate-reducing bacteria such as *T.commune*, *Desulfovibrio thermophilus* and *Desulfotomaculum nigrificans*. It has been suggested that *A. fulgidis* may represent a biochemical "missing link" between methanogenic and sulfur-metabolizing archaebacteria.

In a study of their phylogenetic relationship, nine species of seven genera of sulfate-reducing bacteria were analyzed by comparative oligonucleotide cataloguing of their 16S ribosomal RNA (Fowler *et al.* 1986). The results have both *Desulfotomaculum acetoxidans* and *Desulfotomaculum nigrificans* clustered with the *Clostridium* branch of the Gram-positive eubacteria. All the other genera cluster together, though in not so coherent a fashion. The 16S RNA analyses of the sulfate-reducers used in this study were in good agreement with the classification system of genera based on physiological characteristics. Of interest in this study was that morphological features, other than sporulation, appeared to be of no significance

in determining phylogenetic relationships compared with nutritional features. One such example was *Desulfosarcina* and *Desulfonema*. These two organisms are morphologically very different, *Desulfonema* is a large filamentous bacterium while *Desulfosarcina* forms typical sarcina packets. These two genera however, possess both similar nutritional characteristics and S_{AB} values for their 16sRNA similarities (Fowler *et al.* 1986).

2.3 SYNTROPHY AND INTERSPECIES HYDROGEN TRANSFER.

2.3.1 THE CONCEPT OF SYNTROPHY.

Early concepts of anaerobic degradation led to the belief that a complex of fermentative bacteria degrade the primary substrates, carbohydrates, protein and lipid to a variety of acidic and products. These were then thought to be fermented by methanogenic bacteria. One of the earliest such organisms described in the literature was *Methanobacillus omelianskii* (Barker 1940). This "organism" was able to use ethanol as the growth substrate and produced methane as an end product according to the equation:

$$2CH_3CH_2OH + HCO_3^- \longrightarrow CH_3COO^- + H^+ + CH_4 + H_2O$$

$$\Delta G^{\circ\prime} = -116.4 \text{ kJ/reaction}$$

The overall equation for the fermentation of ethanol as carried out by *M. omelianskii* has a negative $\Delta G^{\circ\prime}$. A negative value indicates such a reaction is thermodynamically feasible. Subsequent studies showed *M. omelianskii* to be a mixed culture of two bacteria. The reason for such an association became obvious when the thermodynamics of the reaction were considered in greater detail. Fermentation of ethanol at standard conditions proceeds according to the equation:

$$2CH_3CH_2OH + 2H_2O \longrightarrow 2CH_3COO^- + 4H_2 + 2H^+$$

$$\Delta G^{\circ\prime} = +19.2 \text{ kJ/reaction}$$

At standard conditions of one atmosphere, this reaction is thermodynamically unfavourable, indicated by the $\Delta G^{\circ\prime}$ = +19.2 KJ/reaction. If however, the hydrogen is removed, then the $\Delta G^{\circ\prime}$ becomes negative and so the reaction can proceed to the right.

$$2CH_3CH_2OH + 2H_2O \longrightarrow 2CH_3COO^- + 4H_2 + 2H^+ \ (10^{-4} \text{ atmos.})$$

$$\Delta G^{\circ\prime} = -71.8 \text{ kJ/reaction}$$

The methanogen was also able to grow; it obtained energy for growth by reduction of CO_2 by H_2, producing methane according to the equation below:

$$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O \quad \Delta G^{\circ\prime} = -135.6 \text{ kJ/reaction}$$

The methanogen has subsequently been isolated in pure culture using H_2 as electron donor and is now known as *Methanobacterium bryantii* strain MoH. *M. omelianskii* was rationalized as consisting of a non-methanogenic species, designated the "s-organism", which fermented ethanol, but only if a H_2-utilizing strain, in this case a methanogen, kept the partial pressure of H_2 low.

2.3.2 DEGRADATION BY SYNTROPHIC CULTURES.

It was recognized that fermentation of fatty acids other than formate and acetate would also be unfavourable at standard conditions as the reactions have positive $\Delta G^{\circ\prime}$ values. These compounds could in theory be degraded in syntrophic association with a H_2 scavenging organism.

McInerney *et al.* (1979) reported the isolation of an anaerobe that degraded butyrate, caproate and caprylate to acetate and H_2 while valerate and heptanoate were degraded to propionate, acetate and H_2. In order to grow, this bacterium required the presence of a hydrogen-utilizing organism, either a methanogen or H_2-utilizing sulfate reducing bacterium. No growth took place if a H_2-utilizer was not present. This organism was later described as a new species of the new genus *Syntrophomonas wolfei* (McInerney *et al.* 1981). A fatty-acid degrading anaerobe was described (Boone and Bryant 1980) which was only able to use propionate and in the presence of a H_2-utilizing *Desulfovibrio*. Isolation of this bacterium was not successful with a methanogen.

Other bacteria have been isolated in defined mixed cultures that showed obligate syntrophy when degrading fatty acids. Stieb and Schink (1985) isolated and described *Clostridium bryantii* using caproate as the growth substrate for isolation with either a methanogen or *Desulfovibrio* species as the H_2-utilizing partner. *C. bryantii* degrades even numbered fatty acids up to 10 carbon atoms to acetate and H_2 while odd-numbered fatty acids up to 11 carbon atoms, including the branched fatty acid 2-methylbutyrate, to propionate, acetate and H_2. Roy *et al.* (1986) isolated a defined mixed culture able to degrade long-chain fatty acids. In this culture, palmitate was fermented by *Syntrophomonas sapovorans* if hydrogen was removed by either a methanogen or sulfate-reducer.

Other descriptions of syntrophic cultures in the literature include: anaerobic degradation of isovalerate by a defined methanogenic co-culture (Stieb and Schink 1986), defined co-cultures consisting of (a), a Gram-negative, non sporeforming bacterium, plus a methanogen

and (b), a Gram-positive spore-forming bacterium plus methanogen, both these co-cultures were isolated on butyrate from a 3-chlorobenzoate degrading consortium (Shelton and Tiedge 1984). The syntrophic sporeformer was also reported to degrade isobutyrate.

Syntrophic degradation of aromatic compounds has been studied in some detail (Mountfort and Bryant 1982, Mountfort *et al.* 1984). These reports contained observations and descriptions of bacteria degrading benzoate in syntrophic associations.

Syntrophic degradation of alcohols received some attention from Eichler and Schink (1985). They isolated ethanol oxidizers similar to the "s-organism" of *M. omelianskii* and found that their strains were not obligate syntrophs but could be grown in pure culture on 1,2-propandiol. This led to the view that other syntrophs could be isolated in pure culture on fermentable substrates, that is, free of any H_2-utilizing strain if those organisms were grown on 1,2-diol compounds. This led Lee and Zinder (1988) to use ethylene glycol as a substrate in order to isolate a thermophilic acetate degrading co-culture. Growth of *Syntrophomonas wolfei* in pure culture on crotonate has also been reported (Beaty and McInerney 1987).

Interspecies electron transfer via formate.

Thiele *et al.* (1988) and Thiele and Zeikus (1988) examined purified bacterial flocs from an anaerobic whey-processing chemostat which were shown to account for 87% of the total methanogenic activity of digester and 76% of the total ethanol consuming acetogenic activity. Within the flocs, ethanol was converted to methane by interspecies electron transfer. They examined the role of formate in this system

as compared to hydrogen metabolism and concluded that in this system 79% of the syntrophic ethanol conversion to CH_4 was mediated by interspecies formate transfer and that less than 10% was mediated via H_2 transfer. *Methanobacterium formicum* was the probable methanogenic species involved as it is known to be prevalent in methanogenic anaerobic digestions (Chartrain and Zeikus 1986). Interspecies formate transfer may exist in nature though to what extent it may take place in other methanogenic systems such as freshwater sediment and with other substrates, such as fatty acids, is unknown.

2.4 PALMITIC ACID AND ITS DEGRADATION.

2.4.1 ORIGINS OF PALMITIC ACID.

Most studies on anaerobic lipid metabolism have been confined to the rumen. The most abundant long-chain fatty acids in nature have 14-22 carbon atoms, with C_{16} and C_{18} predominating (Harfoot 1981). Palmitic acid is the long-chain fatty acid with 16 carbon atoms ~~and~~ possessing no double bonds. Palmitate occurs in anaerobic environments as a result of the action on the acylglycerol linkages of triglycerides by lipolytic bacteria such as the rumen bacterium *Anaerovibrio lypolytica* (Hobson and Mann 1961, Henderson 1971). Palmitate can also be produced as a consequence of hydrogenation of free unsaturated C_{16} fatty acids, such as palmitoleic acid, released by lipolysis. Bio-hydrogenation has been studied extensively in the rumen. Here, unsaturated fatty acids can be partially or completely hydrogenated by the action of carbohydrate- fermenting bacteria (Harfoot 1981).

2.4.2 DEGRADATION OF PALMITIC ACID.

The degradation of palmitate has been mentioned in the sections on sulfate-reducing bacteria and syntrophy above. The fate of palmitate depends on the environmental conditions in which it is formed. In sulfate-containing sediments, palmitate is oxidized by sulfate-reducing bacteria. Enrichment using palmitate as electron donor yields large vibrio-shaped cells (1.5 x 3.5-5.5 μm), identified as *Desulfovibrio sapovorans* (Widdel 1980). That *D. sapovorans*-like sulfate-reducing bacteria are not the only ones to use palmitate has been shown by Brysch *et al.* (1987) who isolated a number of sulfate-reducing bacteria using H_2/CO_2 and subsequently showed these bacteria were also able to use palmitate. There have been no systematic studies on the use of palmitate as as the enrichment compound for the isolation of sulfate-reducing bacteria other than *D. sapovorans*. Thus, we have little knowledge of the possible diversity of palmitate-utilizing sulfate-reducing bacteria; at the present time, it is assumed that *D. sapovorans* is largely responsible.

In sulfate-limited environments, degradation of palmitate is presumably carried out by syntrophic organisms. As described above, a hydrogen-utilizing species is required to keep the hydrogen concentration low in order to enable oxidation to proceed thermodynamically.

Oxidation of palmitate in sulfate-reducing bacteria occurs by β-oxidation. Incomplete oxidation of palmitate results in production of acetate. Bacteria incompletely oxidizing fatty acids with an odd number of carbon atoms produce acetate and propionate.

2.5 BRANCHED-CHAIN FATTY ACIDS AND THEIR DEGRADATION.

2.5.1 INTRODUCTION.

A large number of branched-chain fatty acids occur in association with fermentation processes, but in this study, the term is restricted to include the isomers of butyric and valeric acids.

The study of the fate of branched-chain volatile fatty acids is relatively poorly understood. In anaerobic systems, isovalerate has been used as an enrichment substrate (Stieb and Schink 1986) as has isobutyrate (Tholozan *et al.* 1988). Study of the degradation of branched-chain fatty acids has to date consisted of testing pure cultures of bacteria obtained by enrichment on fatty acids such as acetate, propionate, butyrate, palmitate and on aromatic compounds such as benzoate, cresol and indole for their ability to use branched-chain fatty acids.

2.5.2 ORIGINS OF BRANCHED-CHAIN FATTY ACIDS.

Branched-chain fatty acids are produced largely by the degradation of the branched amino acids valine, isoleucine and leucine. In aerobic systems, bacteria such as *Pseudomonas putida* completely degrade branched-chain amino acids, producing branched-chain fatty acids as intermediates which in turn, are oxidized completely to CO_2 (Massey *et al.* 1976). In anaerobic systems, branched-chain amino acids are fermented, with the corresponding branched-chain fatty acids produced as the fermentation end-products. The end-products are then excreted by bacteria. Some of the bacteria known to produce branched-chain fatty acids from amino acids described above include:

a number of *Clostridium* species (Barker 1981), *Spirocheaeta isovalerica* (Harwood and Canale-Parola 1983), *Bacteroides ruminocola* and *Megasphaera elsdenii* (Allison 1978).

2.5.3 FATE OF BRANCHED-CHAIN FATTY ACIDS.

Having been formed as end-products of fermentation, branched-chain fatty acids can enter a number of metabolic pathways.

Cycling of Branched-chain Fatty Acids.

Branched-chain fatty acids are used by some bacteria for the synthesis of branched-chain amino acids. This is particularly important in the rumen, where several organisms are known to require the pre-formed branched chain for synthesis of such amino acids. The branched chain is also used for synthesis of long-chain branched-chain fatty acids (Allison 1969). When such bacteria are grown in pure culture, rumen fluid or artificially prepared growth factors, consisting of a range of branched-chain fatty acids, are included in the growth medium.

Degradation of Branched-Chain Fatty Acids.

The pathways of degradation of branched-chain fatty acids depend on the environmental conditions. The fermentation of branched-chain fatty acids results in the formation of H_2 which at standard conditions is thermodynamically unfavourable. If H_2 is removed then the fermentation becomes feasible, similar to the process described above with the fermentation of ethanol (section 2.3.1). In sulfate-limited environments methanogens would be largely responsible for H_2

removal. In sulfate-containing sediments, two different pathways are theoretically possible. Fermentation by syntrophic bacteria, such as those described by Stieb and Schink (1985, 1986), could take place, but in this case, the H_2 would be removed by H_2-utilizing sulfate-reducing bacteria. The second possibility is for the branched-chain fatty acid to be oxidized directly by a sulfate-reducing bacterium.

Stieb and Schink (1985) described *Clostridium bryantii* a sporeforming, obligately syntrophic bacterium, isolated using caproate as substrate, and able to degrade fatty acids with up to 11 carbon atoms including 2-methyl butyrate. In the presence of *Methanospirillum hungatei*, *C. bryantii* degraded 2-methyl butyrate to acetate, propionate and methane. Anaerobic degradation of isovalerate by a defined methanogenic coculture was described by Stieb and Schink (1986). The organism degrading the isovalerate, a Gram-positive thick rod with rounded ends, was not named though some aspects of its physiology were investigated. Isovalerate was the only substrate utilized and was fermented to 3 mol acetate and 1 mol hydrogen (scavenged by a methanogen or H_2-utilizing sulfate-reducing bacterium) per mol substrate.

Isobutyrate was fermented in the presence of a methanogen by a rod-shaped bacterium, strain SF-1, isolated from an anaerobic consortium that mineralized 3-chlorobenzoic acid. The same consortium contained a sporeforming rod that grew on 2-methylbutyrate (Shelton and Tiedge 1984). Tholozan *et al.* (1988) reported an undefined mixed culture that grew on isobutyrate and produced methane. Microscopic examination of this culture revealed the presence of several bacterial morphologies, including *Methanothrix*-like filaments.

However, a dominant morphology to which the substrate utilization could be attributed could not be ascertained.

Pathways of degradation of Branched-chain fatty acids and Related Amino Acids.

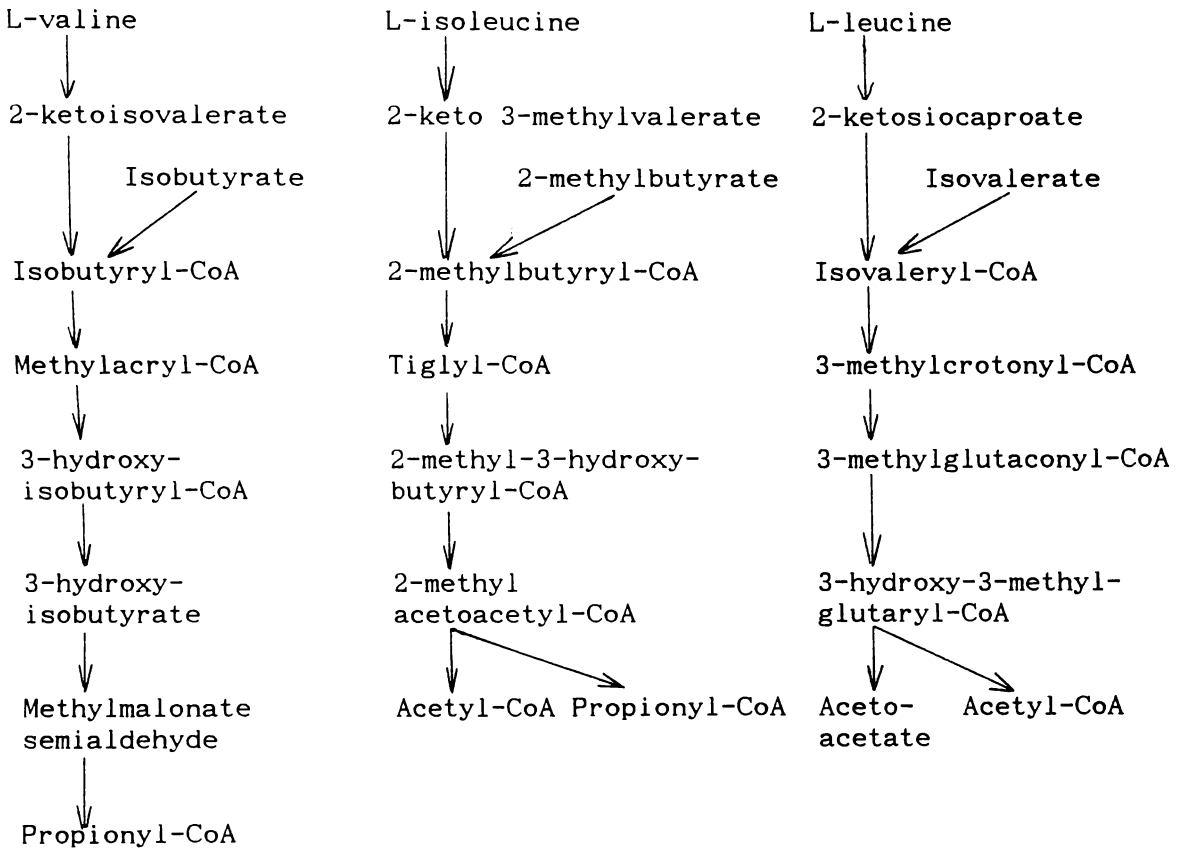
Comprehensive investigations into the enzymatic conversion of branched-chain fatty acids have been made in both microbial and mammalian systems. *Pseudomonas putida*, as mentioned above, degrades branched-chain amino acids completely to CO_2, producing branched-chain fatty acids as intermediates. *Pseudomonas putida* has provided much of the presently known information on bacterial degradation of amino acids and provides a basis for possible pathways of branched-chain fatty acids. These are seen in figure 2.2 from the respective amino acid.

2.6 AMINO ACIDS AND THEIR DEGRADATION.

2.6.1 ORIGINS OF AMINO ACIDS.

Amino acids in nature are produced by hydrolysis of proteins. A large number of anaerobic proteolytic microorganisms are known to exist in nature, examples of which include *Clostridium sporogenes*, *Clostridium sticklandii* (Smith and Hobbs 1974), *Bacteroides nodosus* (Holdeman and Moore 1974), *Bacteroides ruminocola* (Hazelwood and Edwards 1981) and *Eubacterium ruminantium* (Blackburn and Hobson 1962).

Figure 2.2 Pathway for Catabolism of Branched-chain Amino Acids and Related Branched-chain Fatty acid



2.6.2 DEGRADATION OF AMINO ACIDS.

Fermentation of Single Amino Acids.

Amino acids are fermented by a number of *Clostridium* species. The products of fermentation differ depending on the particular amino acid and also the bacterium carrying out the fermentation. An example of fermentation of a single amino acid is lysine fermentation by *Clostridium* species. The ultimate yield per mol of lysine fermented is 1 mol each of acetate and butyrate and 2 mol ammonia (Barker 1981). The difference in products from different bacteria can be seen in the metabolism of threonine. Cardon and Barker (1942) showed that *Clostridium propionicum* degrades threonine to propionate plus butyrate, *Clostridium sordelii* produced aminobutyrate from threonine, whereas *C. tetani* deaminated the aminobutyrate intermediate and therefore produced butyrate as the endproduct (Barker 1981).

In a recent publication, Zindel *et al.* (1988), described *Eubacterium acidaminophilum*, a versatile amino acid- degrading anaerobe which in pure culture, had the ability to ferment glycine to acetate, ammonia and CO_2 and serine to acetate, ethanol, CO_2, H_2 and ammonia. Whereas growth of *E. acidaminophilum* was poor on alanine in pure culture, *Desulfovibrio*, *Methanospirillum hungatei* and *Acetobacterium* could serve as H_2 scavengers and greatly improved the yield. The substrate range was extended when co-cultures were used. In addition to growth on amino acids, *E. acidaminophilum* could also use sarcosine and creatine as substrates for growth provided that H_2 or formate were

added as electron donors. *E. acidaminophilum* thus shows the ability either to produce or utilize H_2 depending on the growth conditions.

Fermentation of Amino Acid Pairs.

An important mechanism of amino acid metabolism among many of the *Clostridium* species, particularly the proteolytic organisms is the fermentation of pairs of amino acids, one of which acts as an electron donor, undergoing oxidation, while the other acts as an electron acceptor so becoming reduced. This type of metabolism was first shown to exist by Stickland in 1934 hence the naming of the fermentation as "The Stickland Reaction". Stickland (1935a, 1935b) showed that alanine and glycine were metabolized as a pair according to the formulae:

$$\underset{\text{(alanine)}}{CH_3CH(NH_2)COOH} + 2H_2O \longrightarrow CH_3COOH + CO_2 + NH_3 + 4H$$

$$\underset{\text{(glycine)}}{2NH_2CHCOOH} + 4H \longrightarrow 2CH_3COOH + NH_3$$

Total Reaction:

$$\text{alanine} + 2[\text{glycine}] \longrightarrow 3[\text{acetate}] + CO_2 + 3NH_3$$

In the alanine/glycine pair, alanine is oxidatively deaminated to pyruvate which undergoes reaction with CoA-SH to form acetyl-CoA. This is then cleaved yielding ATP. The electrons are accepted by NAD, forming NADH, which can be used by the cell for the reductive deamination of glycine to acetate. The net yield of the reaction is 1 mol ATP per mole of alanine oxidized.

It was also shown by Stickland that oxidative deamination of alanine could be coupled to the reduction of proline to 5-aminovaleric acid.

Proline reductase is specific for the D- isomer of proline and organisms carrying out proline reduction have been shown to possess an active racemase converting L-proline to D-proline.

Further investigations have shown a number of amino acids can potentially act as hydrogen donors, namely leucine, isoleucine, norleucine, valine, histidine, phenylalanine and tryptophan, while others can act as electron acceptors namely, hydroxyproline, ornithine, arginine, tyrosine and tryptophan. Tryptophan, as can be seen, can act both in the role of electron acceptor and electron donor.

The Involvement of Sulfate-reducing Bacteria in Amino Acid Degradation.

In so far that some sulfate-reducing bacteria have been demonstrated to oxidize branched-chain and small mono- and dicarboxylic acids, they are implicated in the overall anaerobic degradation of amino acids. However, accounts of oxidation of amino acids by sulfate-reducing bacteria in the literature are limited.

Though Coleman described the use of L- and D- alanine by *Desulfotomaculum* sp. as early as 1960, relatively little is known about the potential use of amino acids by sulfate-reducing bacteria. Smith and Klug (1981) carried out studies on Wintergreen Lake (Michigan, U.S.A.) with molybdate, a specific inhibitor of sulfate reduction, and concluded that a major proportion of amino acid degradation involved sulfate-reducing bacteria.

Two marine strains of *Desulfovibrio* were described by Stams *et al.* (1985) which were able to use alanine, serine and glycine as substrates; aspartate, cysteine, threonine and branched-chain amino acids supported only slow growth. Oxidation products of alanine, serine, glycine, aspartate and cysteine were acetate and ammonium ions. Threonine oxidation also led to production of propionate as well as acetate. Growth experiments with the branched-chain amino acids were carried out in the presence of 5mM acetate as an additional carbon source was required. These amino acids were degraded incompletely to their respective branched-chain fatty acid.

The other reports that have been made in the literature of sulfate-reducing bacteria using amino acids as substrates for growth include the use of glutamate by *Desulfococcus niacini* (Imhoff-Stuckle and Pfennig 1983) and some strains of *Desulfobacterium autotrophicum* (Brysch *et al.* 1987)

A second process by which sulfate-reducing bacteria play a role in amino acid degradation is to act in syntrophic association as the H_2-utilizing partner with an amino acid-fermenting organism. Stams and Hansen (1984) described the fermentation of glutamate and other amino acids by *Acidaminobacter hydrogenoformans* in the presence of *Desulfovibrio* sp. strain HL21. Pure cultures of *A. hydrogenoformans* grew slowly on glutamate and formed acetate, CO_2, formate and H_2. More rapid fermentation was obtained in mixed culture with *Desulfovibrio* sp. HL21. Mixed cultures could also utilize glutamine, arginine, ornithine, theronine, lysine, alanine, valine, leucine and isoleucine.

In summary: a number of sulfate-reducing bacteria have been isolated using a range of compounds and subsequently shown to use other compounds. There have been no systematic studies on the the use of palmitate as the enrichment compound for the isolation of sulfate-reducing bacteria other than *Desulfovibrio sapovorans*. That *D. sapovorans* are not the only sulfate-reducing bacteria known to oxidize palmitate indicates the possible diversity of palmitate-oxidizing bacteria in nature is not known.

Descriptions of sulfate-reducing bacteria described in the literature that can oxidize branched-chain fatty acids, is restricted to those bacteria isolated in pure culture using other compounds and subsequent testing for their ability to oxidize isobutyrate, isovalerate and 2-methylbutyrate.

Knowing this information, attempts were made to increase the knowledge of sulfate-reducing bacteria which can degrade fatty acids.

CHAPTER THREE

ISOLATION AND CHARACTERIZATION OF SULFATE-REDUCING BACTERIA:- MATERIALS AND METHODS

3.1 SOURCES OF ORGANISMS.

A variety of organisms was used in this study. The following paragraphs below indicate the sources of gift and purchased cultures. Details of the organisms isolated during the course of this study appear in the chapter four.

3.1.1 MUDS.

A variety of environments were used to obtain mud for isolation of bacteria. Anaerobic zones were sampled, the mud placed in sterile bottles and stored at 4°C. Each site was designated a code which follows in table 3.1, along with a brief description of the site material.

Table 3.1 Description of Sample Materials Used for the Isolation of Bacteria

Site Abbreviation	Site Description
Am:	Anaerobic sediment from an abattoir waste-water settling pond
Ho:	Anaerobic material from an anaerobic digester, MIRINZ pollution control lab., Horotiu, New Zealand.
Ok:	Estuarine mud from a tidal mangrove creek, Oakleigh Harbour, Northland, New Zealand.
FJ:	Estuarine sediment from Suva Bay, Suva, Fiji Islands.
VE:	Marine sediment, silty in texture, from Rio Marin, Venice.

The AM and FJ mud were collected by the author of this thesis (GNR), OK was kindly provided by Dr C G Harfoot, University of Waikato. VE mud was procured by GNR when visiting Professor Norbert Pfennig, University of Konstanz, Federal Republic of Germany.

3.1.2 GIFT / PURCHASED CULTURES.

The following is a list of additional organisms used in this study, some pertinent features or reason for the use in this thesis and the source:

Escherichia coli strain W. University of Waikato Collection. Gram stain control.

Bacillus circulans. University of Waikato Collection. Gram stain control.

Desulfovibrio sapovorans. DSM 2055. Sulfate-reducing bacterium incompletely oxidizing long-chain fatty acids.

Desulfovibrio sp. strain HL.21. DSM 2555. Sulfate-reducing bacterium which oxidizes H_2.

Desulfovibrio vuglaris strain marburg. University of Konstanz Collection. Sulfate-reducing bacterium which oxidizes H_2.

Methanobacterium bryantii strain MoH. University of Konstanz Collection. Methanogen which uses H_2/CO_2.

Methanospirillum hungatei. DSM 864. Methanogen which uses H_2/CO_2.

Desulfobacterium autotrophicum strain HRM2. Dr Widdel, University of Marburg, Federal Republic of Germany. Autotrophic sulfate-reducing bacterium.

Desulfobacterium vacuolatum. DSM 3385. Sulfate-reducing bacterium which degrades isobutyrate.

DSM = Deutsche Sammlung von Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG.

3.2 MEDIA PREPARATION.

3.2.1 INITIAL PREPARATION.

The culturing of anaerobes, once thought to require a great many skills and specialized equipment not associated with typical aerobic microbiology, are now practised by many laboratories throughout the world. Anaerobic microbiology does have some specialized techniques all based on the principle of degassing the culture medium, reduction of the medium with an appropriate reducing agent and dispensing into culture vessels which are air-tight, typically sealed with butyl rubber seals. The method of Patel *et al.* (1985) with some modifications was followed in this study. The basal mineral salts were prepared and degassed by boiling for 2-3 minutes. The hot medium was placed in an Oxford Dispenser Model S-A (Oxford Laboratories, Foster City, CA, USA) to which a gas line was attached. The medium was cooled under a constant stream of oxygen-free nitrogen (New Zealand Industrial Gases) until it reached a temperature in the vicinity of 40°C. At this temperature the medium could be handled with ease.

3.2.2 DISPENSING OF MEDIUM.

After boiling and cooling, the medium was dispensed into culturing vessels which were simultaneously gassed with oxygen-free nitrogen. The headspace was further flushed with nitrogen, vessels were capped and autoclaved. For all routine culturing, carbon source study and maintenance culturing, Hungate-tubes (Bellco Glass Inc., Vineland, NJ, USA) were used as the culturing vessels. When larger volumes of cell material were required eg., for dry weight analysis, 125ml serum

bottles (Wheaton Scientific, Millville, NJ, USA) were used. All anaerobic media were left at least 12 hours after addition of the reducing agent before using to ensure the media were sufficiently reduced.

3.2.3 PREPARATION OF MEDIUM FOR AGAR SHAKE DILUTION TUBES.

Basal medium was autoclaved in medical flat bottles and sealed immediately after sterilizing was complete. When the medium had cooled to 30-40°C, the remaining components that were required for completion of the medium were added and the medium was aseptically distributed between the bottles and so stored completely full thereby preventing oxidation of the medium on storing.

3.2.4 MEDIUM FOR CULTURE OF LARGE VOLUMES OF CELL MATERIAL.

When one litre volumes of cultures were required, the medium was prepared in one litre Schott bottles and autoclaved. After autoclaving, the headspace was flushed with O_2-free nitrogen. Additions were made aseptically while gassing with O_2-free nitrogen.

3.3 MEDIA COMPOSITION.

The basal salts used were those of Widdel (1980) modified by increasing the calcium concentration to 2mM. Long-chain fatty acids have been shown to have inhibitory effects on anaerobic digesters (Hanaki *et al.* 1981). These authors showed addition of calcium chloride reduced the inhibitory effect. Roy *et al.* (1985) used equimolar calcium and long-chain fatty acid for the isolation of syntrophic fatty acid-degrading cultures. Calcium forms insoluble

calcium salts with the long-chain fatty acid, thereby removing the inhibitory effect. The compositions of the media used are seen in table 3.2. For growth of sulfate-reducing bacteria, sulfate was included at 20 mM. When used for growing methanogenic or other non-sulfate reducing bacteria no sulfate was added. Resazurin, 0.001 g/l was used as a redox indicator in media.

Table 3.2 Basal Medium Components (mM)

	Freshwater	Estuarine	Marine
KH_2PO_4	1.5	1.5	1.5
KCl	4.0	4.0	4.0
NH_4Cl	5.0	5.0	5.0
NaCl	20	120	340
$MgCl_2.6H_2O$	2.0	6.0	15.0
$CaCl_2.2H_2O$	2.0	2.0	2.0
pH= 7.2-7.4			

3.4 MAINTAINING CULTURE COLLECTION ORGANISMS.

The gift and purchased organisms were maintained on the following media with electron donor/c-source etc. noted as required:

Clostridium sporogenes. Cooked Meat Medium (BBL, Cockeysville, USA).
Desulfovibrio vulgaris strains Marburg and HL/21. Freshwater + SO_4 + lactate (10mM).
Desulfovibrio sapovorans. Freshwater +SO_4+ palmitate(1mM).
Desulfobacterium autotrophicum. Marine + SO_4 + Butyrate (10mM).
Methanospirillum hungatei. FWM - SO_4. H_2/CO_2.
Methanobacterium bryantii. FWM - SO_4. H_2/CO_2.
Bacillus circulans. Nutrient agar (BBL, Cockeysville, USA)
Escherichia coli. Nutrient agar (BBL, Cockeysville, USA)

3.5 VITAMIN SOLUTIONS.

It was felt that if potentially novel organisms were to be isolated then perhaps a wider vitamin requirement than previously described for sulfate-reducing bacteria might exist and so the vitamin 8 (below) was used. In addition, for isolation of sulfate-reducing bacteria and syntrophic cultures the medium included riboflavin and vitamin B_{12}. Details of these three vitamin solutions are in table 3.3. Soon after obtaining an organism in pure culture, tests were made to see if the organism required vitamin B_{12}, riboflavin or the vitamin 8 mix.

Table 3.3 Compositions of vitamin solutions.

Vitamin 8 Solution	
4 amino benzoate	40mg
Biotin	10mg
Folic acid	30mg
Lipoic acid	10mg
Nicotinic acid	100mg
Pantothenate	50mg
Pyradoxamine.HCl	100mg
Thiamine.HCl	100mg
Prepared in 1000 ml	
50 mM Phosphate Buffer pH=7.0	

Vitamin B_{12} Solution	
Vitamin B_{12}	50mg
Water	1000ml

Riboflavin Solution	
Riboflavin	50mg
0.3 mM acetic acid	20ml
Water	1000ml

Each solution was filter-sterilized using 0.2 μm pore-sized membrane filters and stored at 4°C. The riboflavin solution was also protected from light. All the solutions were added at 1ml per litre.

3.6 TRACE ELEMENTS.

Trace element solution SL-10 of Imhoff-Stuckle and Pfennig (1983) was initially used in this study and proved successful for the isolation

of strain 16AV (Chapter ten). Later in the study, a change was made to SL-10a, and was used for isolation of all other strains described in this study. Though very similar in composition, SL-10a had been used with a good degree of success when culturing methanogens as well as some of the recent isolates of sulfate reducing bacteria (Bak personal communication). The compositions of the trace element solutions are seen in table 3.4.

Table 3.4 Composition of Trace Element Solutions

	SL-10	SL-10a
H_2O	990ml	990ml
HCl (25% v/v)	10ml	10ml
$FeCl_2.4H_2O$	1.5g	1.0g
$ZnCl_2$	70mg	70mg
$MnCl_2.4H_2$	100mg	100mg
H_3BO_3	6mg	6mg
$CoCl_2.6H_2O$	190mg	130mg
$CuCl_2.5H_2O$	2mg	2mg
$NiCl_2.6H_2O$	24mg	29mg
$NaMoO_4.2H_2O$	36mg	36mg

SL-10 is added at 1 ml per litre while SL-10a is added at 2ml per litre.

When organisms were grown on aromatic compounds, selenite and tungstate were also added to the growth medium at final concentrations of 10 μmoles/litre and 12 μmoles/litre respectively. The stock solution of selenite and tungstate contained 0.5g/l sodium hydroxide.

3.7 BICARBONATE BUFFER.

Important properties of Widdel's medium are thought to be the presence of phosphate at 4 mM (considered low compared to many other microbiological media) and use of a bicarbonate buffer. Bicarbonate was prepared as follows: a 1M sodium bicarbonate solution was prepared in CO_2 saturated distilled water. CO_2 saturation was obtained by gassing water with CO_2, contained in an Erlenmeyer flask, stoppering, then shaking. The flask was regassed, stoppered and shaken until no further CO_2 would dissolve in the water. This could easily be determined as a slight vacuum was produced in the flask as the CO_2 dissolved in the water. The bicarbonate solution was placed in 125 ml Wheaton bottles, the headspace flushed with CO_2 and stoppered with a butyl rubber seals and finally autoclaved. The bicarbonate solution was added at 30ml per litre.

3.8 SULFIDE REDUCING AGENT.

Sulfide was routinely used as the reducing agent. A stock solution of neutralized sodium sulfide was prepared at a concentration so that after addition to medium, the final concentration was 1-1.5mM. The sodium sulfide solution was prepared in degassed distilled water and added to 50 ml serum bottles, the headspace flushed with O_2-free nitrogen and autoclaved. Media were reduced at least one day prior to use as described above.

3.9 DITHIONITE AS AN ADDITIONAL REDUCING AGENT.

Some sulfate-reducing bacteria are known to require small amounts of dithionite in the growth medium as an additional reducing agent while

with some other sulfate-reducing bacteria the lag phase can be significantly reduced by the addition of 50 μM dithionite. Dithionite has also been used in methanogenic cultures to improve growth rate (Seitz. personal communication).

Dithionite solutions are very unstable and so can neither be autoclaved nor stored. Consequently, solutions are always made fresh, immediately prior to use, then discarded soon after. It has been observed that sodium dithionite obtained from chemical suppliers is infact sterile. Numerous tests were carried out on the sodium dithionite obtained for this study (Purified Sodium Dithionite, J.T. Baker Chemical Co., NJ, USA) and they showed it also to be free of bacterial contamination. Nevertheless, when dithionite solutions were added to media, strict microscopic examination, along with tests for contamination were used to keep a check on the purity of the reducing agent.

Dithionite was prepared as follows. Distilled water was degassed by attaching the bottle containing the water to a vacuum line. Bijou bottles were filled (7 ml) and the bottles were autoclaved. When required, dithionite was aseptically weighed on a sterile aluminum tray, added to the bottle and capped. Dithionite was added to medium contained in Hungate-tubes using a hypodermic needle and syringe so as to give a final concentration in the order of 100 μM.

3.10 COMPOUNDS USED AS ELECTRON DONORS/CARBON-SOURCES.

A wide range of compounds were used throughout this study so only a brief description will be given here. Most compounds were prepared as sterile stock solutions at 100 times final concentration,

generally autoclaved, although some were filter sterilized using 0.2 μm pore-sized membrane filters. Unless otherwise stated, L-isomers of organic acids and D-isomer of carbohydrates were used. Organic acids were prepared as sodium salts. With the long-chain fatty acids, heating of the stock solution was required to melt them prior to addition to the medium. Important substrates and concentrations used are shown in table 3.5, along with appropriate comments. Unless specified substrates were autoclaved. Further details of the electron donors appears in the appropriate tables as they were used (see later).

Table 3.5 Preparation of Electron Donors/carbon-sources.

Compound(s)	Final Concentration (mM)	Comments
Alcohols	10	
Formate	5, 10	
Acetate	10	
Propionate, Butyrate	10	
C_5-C_8 Fatty acids	5.0	
C_{10}-C_{14} fatty acid	0.5	
C_{16}-C_{18} fatty acid	1	
Amino acids	10, 20	Filter-sterilized
Dicarboxylic Acids	3	glutarate
	10	succinate
	5	all others
Carbohydrates	2 - 10	Filter-sterilized
Sugar alcohols	10	Filter-sterilized
Pyruvate	10	Filter-sterilized
Benzoate	3	
Keto-acids	5, 10	Filter-sterilized
Nicotinic compounds	5, 10	Filter-sterilized

The ability to use hydrogen as an electron donor was tested under two conditions. The first was to test for autotrophic growth on H_2/CO_2, the second was H_2 as electron donor with acetate (1 mM final concentration) added as the carbon source. When hydrogen was used as

an electron donor, 20 ml of the appropriate medium was dispensed into 50 ml serum bottles and completed in the usual manner. After inoculation, H_2/CO_2 (80:20) was added to the bottles via a hypodermic needle to give one atmosphere over pressure. On some occasions further pressurization with H_2/CO_2 was carried out to replace the hydrogen used by the growing organism. The bottles with hydrogen were incubated at 28°C in a shaking incubator.

3.11 COMPOUNDS USED AS ELECTRON ACCEPTORS.

Sulfate-reducing bacteria were tested for their ability to use electron acceptors other than sulfate. Stock solutions of sodium thiosulfate (1M), sodium sulfite (1M) and potassium nitrate (1M) were prepared at pH=7. The sulfur compounds were filter-sterilized using 0.2 μm pore-sized membrane filters. The nitrate solution was autoclaved. Electron acceptors were added to sulfate-free medium to give final concentrations of 10 mM. When elemental sulfur was used as the terminal electron acceptor, a small spatula of sulfur was added to the culture vessel prior to addition of medium.

3.12 ISOLATION OF BACTERIA.

3.12.1 ENRICHMENT OF SULFATE-REDUCING BACTERIA.

Standard Methods of Enrichment.

Medium prepared in medical flat bottles (section 3.2.3) was completed by addition of the three vitamin solutions and an appropriate substrate. Mud was aseptically added to a sterile culture vessel, (approximately 5% inoculum). In this study Hungate-tubes and 50 ml

glass bottles were both used for enrichments. The vessels were completely filled with the appropriate medium and a few crystals of sodium dithionite added to ensure the medium was sufficiently reduced. The vessels were sealed and incubated at 28°C. A control enrichment was also initiated which contained the source material, medium, but no substrate. As growth takes place within the sediment at early stages of enrichment, sulfide production was used as the method for estimating growth of the cultures. Sulfide was determined using the rapid method (section 3.18.5). Sulfide determinations were also carried out on the control enrichment so as to determine the sulfide production attributable to growth on compounds in the mud. When high sulfide concentrations were observed, (10-15 mM), the enrichment vessel was shaken and the culture transferred to fresh medium using a 5% inoculum. This procedure was repeated, i.e., measuring sulfide in the enrichment culture and control, and transferring to fresh medium when high sulfide levels were detected. After some 4-5 transfers, the culture contained no mud. At this point in the purification, bacteria were growing freely in the medium and not just associated with the sediment. Microscopic examination of these cultures showed they often contained only two or three cell types. Most of the organisms present in the initial enrichment had been eliminated by the continual transfer into fresh medium. Cultures were finally purified by application of the agar shake dilution procedure (below).

Mud Dilution Method.

A series of enrichments were initiated using mud in which increasing dilutions of mud were used as the source material. Mud samples were diluted in the liquid medium without substrate to give dilutions to

10^{-4}. These dilutions were then inoculated into medium containing a carbon-source as is usual.

3.12.2 ENRICHMENT OF SYNTROPHIC CULTURES.

The same basic procedures were used with some modifications. The medium contained no sulfate so eliminating the prospect of sulfate-reducing bacteria out-competing the syntrophic bacteria. Initializing the enrichments and transfer was the same as described above. Culture were transferred after significant gas production had taken place as compared to that in the control enrichment.

3.12.3 AGAR-SHAKE DILUTION TUBES.

Sulfate-reducing bacteria.

Agar shake dilutions were carried out according to the method of Pfennig and Trüper (1981). Purified agar was prepared as a 2.2 % (w/v) solution in distilled water. 3 ml aliquots were added to test tubes, cotton wool plugs were fitted and the tubes autoclaved. For dilution series, the agar of six tubes was melted and the tubes transferred to a 50°C water bath. Approximately 7 ml of pre-warmed medium was added to the molten agar and serial dilutions were carried out using material from the enrichment. The tubes were further reduced with 10µl of a 2 % (w/v) solution of sodium dithionite solution and sealed with wax plugs (100 g paraffin wax + 200 ml liquid paraffin). Tubes were incubated at 28°C.

When colonies had developed, a tube containing well-dispersed colonies was broken open, a colony drawn into a fine Pasteur pipette,

transferred to fresh liquid medium and incubated. If a colony was to be transferred to medium contained in a Hungate-tube, a small volume of medium was first drawn into a syringe via a hypodermic needle and the colony drawn into the syringe. The colony plus medium was transferred to a Hungate-tube. Agar shake dilutions were carried out until pure cultures were obtained.

Syntrophic cultures.

Agar shakes for syntrophic bacteria were carried out exactly the same as for the sulfate-reducing bacteria but prior to inoculating the tubes, 1ml of a well grown culture of a hydrogen utilizing species was added to each tube. Two possibilities were available; the first was to use medium with no sulfate and include a methanogen as the H_2-utilizing species and the second was to use a sulfate containing medium and add a hydrogen utilizing sulfate-reducing bacterium. The H_2-utilizing organisms available are described above (section 3.1.2).

3.13 ESTIMATION OF GROWTH.

3.13.1 OPTICAL DENSITY.

Routine estimation of growth was carried out by measuring optical density of cultures. Optical density was measured directly in Hungate-tubes using a Spectronic 20 spectrophotometer (Bausch and Lomb, NY, USA) fitted with an adapter designed to hold long tubes. Absorbance was measured at 660 nm.

3.13.2 TURBIDIMETRIC SCORING.

A system was devised to give a simple qualitative method for estimating growth. Visual observations were made of the turbidity in each tube, compared against positive and negative controls and used for scoring very good growth as "+++", modest growth as "++", poor growth as "+" and no growth as "-". These scores approximately correspond to optical density values of 0.4, 0.3, 0.1 and 0. Qualitative estimation of growth was possible with the long-chain fatty acids as growth on the could be seen by the disappearance of the white "fluffy" precipitate. Turbidimetric scoring was used to estimate growth in carbon and energy source tests etc. where only qualitative estimates were required.

3.14 PURITY CHECKS.

All cultures were routinely checked for purity, examining those cultures both for contaminant aerobes as well as anaerobes. Nutrient agar was used to test for the presence of contaminant aerobes. Thioglycollate medium (Oxoid) was used to test for the presence of contaminant anaerobes. Tests were also made for contaminant sulfate-reducing bacteria by growing cultures on medium containing fumarate plus pyruvate. Cultures grown on different carbon sources were also examined microscopically. This latter test was also useful to detect changes in morphology that might occur when a strain was grown on different substrates.

3.15 MICROSCOPY.

3.15.1 GRAM STAIN.

A modified method of Hucker as described by Doetsch (1981) was used to determine the Gram reaction. 95% ethanol (v/v) was used as the decolorizing agent and counter-staining was carried out using carbol fuchsin. *Escherichia coli* and *Bacillus circulans* were used as the respective Gram-negative and Gram-positive controls.

3.15.2 SUDAN BLACK STAIN.

The method described by Doetsch (1981) has been used to observe the presence of poly-β-hydroxy butyrate (PHB). Janssen (1989) showed however that some granules which stained with sudan black were not infact PHB. The staining was carried out recognizing the non-specific nature of this staining procedure

3.15.3 PHASE CONTRAST MICROSCOPY.

Wet mounts prepared for phase contrast microscopy were used to check purity of cultures and also to determine the general morphological characteristics of size, shape and motility. Photomicrographs were taken using a Reichert-Jung Polyvar microscope (Reichert AG, Vienna, Austria) after pretreating slides with the agar-coating method of Pfennig and Wagener (1986). For the preparation of slides for microscopy, cleaned slide were coated with 2ml of a 1% (w/v) agar solution which was left to dry. Young cultures were placed on the slide and examined. Where growth was poor 1 ml of culture was

centrifuged and resuspended in 0.1 ml of medium. This more dense cell suspension was then used for examinations.

3.15.4 ELECTRON MICROSCOPY.

Transmission electron micrographs were taken of negatively-stained cells using a modified method of Patel (1984).

Butvar was obtained from Probing and Structure (Aitkenville, Queensland, Australia). Butvar films were cast using the method of Handley and Olsen (1979) with the modifications of Patel (1984). Microscope slides (Blue Star Microslides, Chance and Proper, England) were cleaned, made dust free and then used as a base for casting the films. The film was removed dipping the coated slide into a beaker of water. Copper grids (3.05 mm diameter) (Polaron Equipment Ltd., Watford, England) were dropped shiny side down onto the prepared film. The film (plus grid) was picked up using a piece of parafilm (American Can Company, Greenwich, CT, USA).

A 1% (w/v) uranyl acetate (BDH, Poole, England) solution was prepared in distilled water and negative staining was carried out as follows. A piece of parafilm was placed on a bench. One drop of the specimen was placed on the parafilm along with a drop of distilled water that had been filtered through a 0.2 μm pore-sized membrane filter. In addition, 2 separate drops of stain, similarly filtered, plus a further drop of filtered water were also placed on the film. A grid was placed dark side down onto the specimen for 5 minutes. The grid was then run through the first drop of water, the first drop of stain and finally placed on the second drop of stain. The exact time required for clear staining varied depending on the individual cells

though most stained well after being in contact with the stain for 30 seconds. The grid was finally run through the last drop of water and dried using filter paper, touching only the edge of the grid.

The grids were examined using a Philips EM 400 electron microscope (NV Philips, Eindhoven, The Netherlands)

3.16 PRESENCE OF ENDOSPORES.

Cultures were examined for the presence of spores. Cultures grown on different carbon sources were left for 4 weeks and examined for the presence of refractile bodies using phase contrast microscopy. The importance of examining an organism grown on more than one substrate was demonstrated by Widdel and Pfennig (1981b) who showed that *Desulfotomaculum acetoxidans* produces spores when grown on butyrate but not acetate. Cultures showing refractile bodies were centrifuged for 10 minutes at 10000 x *g* and re-examined using phase contrast microscopy. Centrifugation collapses gas vacuoles which also appear as refractile bodies. Sporulation is not always a predictable event. However, even where no spores are visible using microscope, they can be detected by their heat resistance. Cultures to be examined were incubated at 80°C for ten minutes, allowing 1-2 minutes for the culture to heat up. One ml of the heat treated culture was transferred to fresh medium, incubated at the appropriate temperature and examined at regular intervals fo up to 4 weeks.

3.17 MOST PROBABLE NUMBER ESTIMATION.

Estimation of cell numbers in sediments were carried out using the five tube most probable number method (MPN). Tubes were incubated

for 6 weeks and checked for growth. Growth was measured using optical density, substrate disappearance, product formation and sulfide production. For counts of syntrophic organisms, MPN tubes were prepared with sulfate-free medium,inoculated and incubated as usual. Production of methane as well as optical density increase indicated positive growth. Disappearance of insoluble substrates such as palmitate could be monitored visually. The most probable numbers were taken from statistical tables (American Public Health Association 1971)

3.18 CHEMICAL DETERMINATIONS.

3.18.1 ORGANIC ACIDS.

Organic acids, with the exception of isobutyrate, were determined using HPLC. The pump used was a Waters Model 510 (Millipore Corporation, Bedford, USA) fitted to a Bio-Rad HPX-87H organic acid column and Bio-Rad Cation H^+ guard cartridge. (Bio-Rad, Richmond, CA, USA). The column was heated to 50°C. The solvent, 0.1N H_2SO_4 was run at a flow rate of 0.5 ml/min. Compounds were detected using a refractive index detector (Model ERC-7510, ERMA Optical Works Ltd., Tokyo, Japan). Samples were centrifuged to remove cells and approximately 40 μl was loaded into the injection loop which inturn put 20 μl onto the column. All likely fermentation intermediates could be detected using this system with run time of 30 minutes.

Because isobutyrate eluted at the same time as CO_2 using the HPLC conditions above, isobutyrate was measured using a PYE GCD Gas Chromatograph equipped with a flame ionization detector. The column

was packed with Chromosorb 101, 100-120 mesh (Supelco Inc., Bellefonte, PA, USA). The oven was heated to 130°C, the detector 225°C, and the injecter 215°C. Oxygen-free nitrogen was used as the carrier gas at a flow rate of 60 ml/min. Propionate was used as the internal standard at a concentration of 10 mM. Peak size was integrated using a Spectra Physics Model SP4100 integrater.

3.18.2 AMINO ACIDS.

Amino acids were measured using an HPLC amino acid analyzer. Detection was carried out after a precolumn derivatizing procedure with phenylisothiocyanate (PITC) (Sigma Chemical Co., St Louis, USA).

Reagents: A) Coupling Buffer, Acetonitrile/Ethanol/Trimethylamine. (2:1:1).

B) Coupling Reagent. 5% (v/v) Phenylisothiocyanate in acetonitrile

C) Injection Buffer. 0.1M phosphate buffer, pH=15 % (v/v) acetonitrile

D) Solvent Buffer 1. Potassium acetate 130 mM, pH=5.6

E) Solvent Buffer 2. 84 % (v/v) acetonitrile

Sample Preparation: 20 μl samples were dried in Eppendorf tubes using a vacuum line. 40 μl of coupling buffer A) were added, the samples mixed well using a vortex mixer and redried. A further 25 μl of coupling buffer A), plus 50 μl coupling reagent B), were added to the tubes and the contents mixed. The samples were incubated at room temperature for 20 minutes then dried. The PITC-amino acid derivatives were usually used immediately. If this was not the case they were stored dry at -20°C, for no longer than 48 hours. Samples were suspended in 50 μl injection buffer C) and injected onto the HPLC.

The HPLC system used consisted of Waters Pumps, a Water Novapak Reverse Phase column (Millipore Corporation, Bedford, MD, USA) and a Shimadzu SPD-6A Detector (Shimadzu, Kyoto, Japan). The solvent buffers were run in proportions according to the gradient table below (table 3.6). This achieved good separation of the derivatized amino acids. The column temperature was 40°C and the solvent flow rate 1 ml/min.

Table 3.6 Gradient Table of Buffers used for Amino Acid Analysis

Time (min)	Flow Rate	% Buffer A	% Buffer B
Initial	1.0	91	9
.10	1.0	88	12
4.0	1.0	87	13
6.0	1.0	87	13
10	1.0	75	25
12	1.0	64	36
16	1.0	10	90
17	1.0	95	5
20	1.0	95	5
20.1	1.0	91	9

3.18.3 GAS ANALYSIS.

Methane, H_2 and CO_2 were detected using a PYE PU4500 Gas Chromatograph. Details of the apparatus as well as the running conditions are given in chapter nine.

3.18.4 AMMONIA ANALYSIS.

Ammonia concentration was measured using an enzymatic kit (Boehringer Mannheim, GmbH, FGR). In the presence of glutamate dehydrogenase (GlDH) and NADH ammonia reacts with 2-oxo-glutarate to give

glutamine, whereby NADH is oxidized to NADH. The amount of NADH oxidized is directly proportional to the amount of ammonia present. NADH concentration was measured at 340 nm; the extinction coefficient of NADH is 6.3 ($mol^{-1}.cm^{-1}$).

3.18.5 SULFIDE DETERMINATION.

Rapid Method: For the rapid determination of sulfide the method of Cord-Ruwisch (1984) was used. This method is particularly useful for following enrichment studies to obtain reasonably accurate total sulfide concentrations. The reagent used was 5mM $CuSO_4$ in 50mM HCl For qualitative measurements 0.5 ml of sample was added to 2ml of reagent and immediately mixed. Copper sulfide (dark brown precipitate) was compared to control tubes to gauge intensity. Quantitative measurements were done carried out by adding 100 μl of sample to 4ml of reagent and mixing immediately. Optical density was recorded at 480 nm.

Methylene Blue Method: When highest accuracy was required, eg. for stoichiometric balancing, the more commonly employed methylene blue method of Trüper and Schlegel (1964) was used. A sample (0.5 ml) of unknown sulfide concentration was added to a 100 ml volumetric flask containing 20 ml of a zinc acetate solution (20 $g.l^{-1}$). The flask was filled to about 80ml with distilled water to which 10 ml of a NN-dimethyl-*p*-phenylenediamine sulfate solution (2 $g.l^{-1}$ in 20% v/v H_2SO_4) was added while gently shaking. Finally 0.5ml of an ammonium ferric sulfate solution (100 $g.l^{-1}$ in 2% v/v H_2SO_4) was added. Flasks were immediately shaken, made up to 100 ml left to stand for 10 minutes to allow the full development of the colour. Optical density was determined against a distilled water blank at 670 nm

using a Shimadzu UV-250 spectrophotometer. Sulfide concentration was estimated by use of a standard curve.

3.19 PREPARATION OF CELL-FREE EXTRACTS.

The presence of different types of cytochromes and sulfite reductases are used as taxonomic tests for identification of sulfate-reducing bacteria. In order to analyze for the different types of cytochromes that may be present and to demonstrate conclusively the presence of desulfoviridin, a type of sulfite reductase, cell-free extracts must be prepared.

100 ml of a well-grown culture of the particular sulfate-reducing bacterium was used, grown on its enrichment substrate. Cells were collected by centrifugation at 13000 x *g* for 20 minutes. Cell material was washed in 1 ml of phosphate buffer (50 mM, pH=7.0) and recentrifuged at 12800 x *g* for 5 minutes using a microcentrifuge (Runne RS 85-1, Heidelberg, FRG). The pellet was suspended in 0.1 ml of buffer and sonicated for 4x30 second intervals with cooling on ice between sonication steps. Microscopic examination revealed complete lysis after this treatment. The volume was made up to 1 ml and the preparation was centrifuged for 5 minutes at 12800 x*g* to remove cell debris. The supernatant was removed for analysis. In most cases this volume could be diluted by half and still provide enough material for analyses.

3.20 DESULFOVIRIDIN ANALYSIS.

Two methods were used to test for the presence of desulfoviridin.

3.20.1 FLUORESCENCE TEST ON WHOLE CELLS.

The fluorescence test of Postgate (1959) was used. Cell material from 10 ml of a log phase culture was collected by centrifugation, washed and recentrifuged. The pellet was resuspended in 1 ml of medium and placed under a UV lamp at 365 nm immediately after the addition of 2 drops of 2.0 M NaOH. Any red fluorescence observed was due to the release of the chromophore of the pigment desulfoviridin and was taken as evidence for the presence of desulfoviridin in whole cells.

3.20.2 SPECTROPHOTOMETRIC ANALYSIS OF CELL-FREE EXTRACT.

The cell-free extract (section 3.19) was scanned from 420 nm to 700 nm using a Shimadzu UV-250 double-beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Desulfoviridin is known to have an absorption maximum at 630 nm (Postgate 1956); any peak detected at this wavelength was taken as evidence for the presence of desulfoviridin.

3.21 CYTOCHROME ANALYSIS.

The cell-free extracts, prepared as described above (section 3.19) were examined. Difference spectra (oxidized minus reduced), were obtained using a 1 cm light path with a Shimadzu UV-250 double-beam spectrophotometer. Cell-free extract was placed in both the reference and sample cells. A small amount of sodium dithionite was added to the sample cuvette giving a reduced sample while the reference cuvette was shaken in air to give the oxidized reference. A very sensitive absorbancy scale was necessary to enable detection

of the peaks. This was typically a 0-0.02 scale though this did vary according to the quantity of material present.

3.22 DNA BASE COMPOSITION.

The DNA base composition was determined by thermal denaturation using DNA isolated using the modified Marmur method of Owen and Lapage (1976). Log phase cells were harvested by centrifugation at 13000 x *g* for 20 minutes. The volume of culture required varied with different organisms so long as a pellet of at least 0.75-1 g wet weight was obtained. In most cases this could be obtained from 2 litres of medium. The harvested cells were resuspended in 25 ml 0.15M NaCl/0.1M EDTA, pH=8. The high pH and EDTA inhibit DNAse activity. Cell lysis was effected by addition of 10 mg lysozyme (Sigma Chemical Co., St. Louis, MO, USA), incubated at 37°C for 30 minutes, followed by addition of 2ml of 25 % (w/v) sodium lauryl sulfate. The preparation was then incubated at 60°C for 10-15 minutes after which lysis could be seen by the preparation becoming viscous and showing a good degree of clearing. Sodium hypochlorite was added to give a final concentration of 1M and the preparation deproteinized with the addition of an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The preparation was shaken gently in a ground glass-stoppered flask at room temperature for 30 minutes. The resulting emulsion was separated into three layers by centrifugation for 15 minutes using an MSE Centaur 2 benchtop centrifuge (MSE Scientific Instruments, Crawley, Sussex, England). The aqueous layer, containing the DNA, was removed and over this was added two volumes of 95 % (v/v) ethanol. DNA was precipitated onto a glass rod by stirring at the interface of the two liquids. The precipitated DNA was then dissolved in 0.015M NaCl/0.0015M tri-sodium citrate, pH=7.0 (0.1 SSC Buffer). This DNA

was then diluted with further 0.1 SSC to obtain a working concentration of DNA (OD_{260} approximately equal to 0.5) and then thermally denatured.

The thermal denaturation of the DNA was carried out by measuring the change in absorbance with increasing temperature of the DNA solution using a PYE SP8-400 spectrophotometer fitted with a heating block. The temperature of the DNA solution was measured directly in the cuvette with a thermister probe.

Having determined the denaturation temperature (T_m) the base composition was calculated using the formula of Owen *et al.* (1969) below:

$$\%\text{mol G+C} = (2.08 \times T_m) - 106.4$$

CHAPTER FOUR

ENRICHMENT AND ISOLATION:- RESULTS AND DISCUSSION

4.1 INTRODUCTION.

This chapter discusses the results obtained from a number of procedures that were carried out to isolate sulfate-reducing bacteria. The organisms that were isolated are described and discussed in detail in the following chapter. The first section here describes the enrichment of sulfate-reducing bacteria able to oxidize palmitic acid. The second section describes the isolation of syntrophic cultures able to degrade fatty acids. The third and final section describes the isolation of sulfate-reducing bacteria able to degrade branched-chain fatty acids.

4.2 ISOLATION OF PALMITATE-OXIDIZING SULFATE-REDUCING BACTERIA.

4.2.1 ENRICHMENT CULTURES.

A number of enrichment techniques were used to isolate palmitate-oxidizing sulfate-reducing bacteria from different sediments. This was carried out in the hope of gaining more understanding of the types of organisms that might be responsible for degradation of long-chain fatty acids in sediments. A summary of the results of enrichments for sulfate-reducing bacteria oxidizing palmitate is seen in table 4.1.

Table 4.1 Summary of Enrichments For Sulfate-reducing Bacteria Using Palmitate.

Method of enrichment	Site*	Dominant organism(s) in enrichment	Organism obtained in pure culture	Strain designation
Standard	Am	Large vibrios	As in enrichment	AmPa2
	FJ	Large vibrios	As in enrichment	FJPa1
	Ok	Large vibrios	As in enrichment	OkPa1
Sediment transfer	Ho	Square rods	As in enrichment	HoPa1
	Am	Square rods	As in enrichment	AmPa1
Pasteurized sediment	Am	Many cell types	Sporulating rod seen prior to pasteurization	AmPa3
13°C Enrichment	Am	Enrichment was abandoned.	NA	
	Ok	Oval-shaped cells	As in enrichment	OkPa13
Diluted mud enrichment	Ok	Low dilutions:large vibrio-shaped cells	As in enrichment	ND
		High dilutions:oval-shaped cells		ND

*Site abbreviations: Am, abattoir waste water pond mud; Ho, abattoir anaerobic digester material; FJ, estuarine sediment; Ok, estuarine sediment.
ND, not determined; NA, not applicable.

Standard Enrichment Procedures.

Enrichments were carried out as described in section 3.12.1 with Oakleigh harbour (Ok), Fiji (FJ) and waste water pond (Am) sediments (section 3.1.1). In all cases, large vibrio-shaped, (1-1.5 x 2-3.5 μm), sulfate-reducing bacteria were isolated from these sediments. Organisms from FJ, Ok and Am sediments were purified and designated FJPa1, OKPa1 and AmPa2.

Sediment Transfer.

Enrichments were initiated using anaerobic digester material (Ho) and Am sediment (section 3.1.1) and incubated at 28°C. These cultures showed extensive sulfide production, (approximately 10-15 mM), after about 3 weeks and were transferred to fresh medium. Instead of the usual practice of shaking the tube and transferring a homogeneous mixture of mud and culture supernatant, the transfer was made by transferring a good proportion of sediment. In all subsequent transfers, mud was also always transferred. By carrying out enrichments in this manner, mud was carried through a greater number of transfers than would normally be the case. In addition to measuring the sulfide produced, growth of the organisms in the enrichment was also followed by visual observation. Instead of most growth gradually becoming associated with the culture supernatant, as is the usual practice when isolating anaerobes, it took place attached to the sediment and insoluble palmitate and remained in clumps. The clumps were disrupted by vigorous shaking, transferred to agar-shake tubes and the organisms making up the clumps subsequently purified. When colonies were picked and transferred to

liquid medium they no longer grew as clumps but instead grew dispersed throughout the culture tube.

Using the sediment transfer method, two strains were isolated. Microscopic examination showed both strains to be round-ended rods and not the typical 'sapovorans-type' vibrios usually isolated from palmitate enrichments. The two strains were designated AmPa1 and HoPa1. As sulfate-reducing bacteria with this morphology had never been isolated using palmitate in the past, these strains were studied further.

Pasteurized Sediment Enrichment.

An enrichment was initiated and transferred through similar passages as described for the sediment transfer above. After 4 transfers, the culture was subjected to pasteurization at 80°C for 10 minutes and inoculated into fresh medium. When the pasteurized culture had grown, it was transferred to agar shake dilution tubes. A pure culture was subsequently obtained and designated AmPa3.

13°C Enrichment.

Enrichments were initiated and incubated at 13°C using Am and Ok muds with palmitate as the substrate. After 5 weeks incubation of the Ok series, the enrichment showed considerably more sulfide production compared to that in the control (10-15 mM in the enrichment compared to 1-1.5 mM in the control) and was transferred to fresh medium, whereas the Am enrichment showed no more sulfide present than was in the control enrichment. After a 3 week incubation, the Ok subculture had again produced large amounts of sulfide compared to the control

enrichment. Sulfide production was again measured as an indicator of growth in the Am culture and it was decided to abandon further purification as the culture still had not produced sulfide to a greater extent than had the control. Microscopic examination of the Ok culture showed that it was dominated by a large oval-shaped organism. No large vibrios were observed. The Ok culture was purified using agar shakes and the purified strain designated OkPa13. The growth rate of OkPa13 decreased once in pure culture and cultures incubated at 13°C could only be transferred after 4-5 weeks. Characterization of this organism was carried out in order to allow identification to the level of genus.

Enrichment Using a Dilution Series of Mud.

A series of enrichments were initiated using estuarine mud in which increasing dilutions of mud were used as the source material. Mud samples were diluted in the liquid medium without substrate to give dilutions to 10^{-4}. These dilutions were then inoculated into medium containing palmitate plus sulfate. The organisms isolated were examined microscopically. The mud added from the 10^{-1} to 10^{-3} dilutions gave rise to cultures that were typically vibrio-shaped, very similar to *Desulfovibrio sapovorans*. The mud added from both the 10^{-4} and 10^{-5} dilutions gave rise to oval-shaped organisms that appeared very similar to strain OkPa13. These organisms were purified late in this study so allowing no time to carry out further experiments.

4.2.2 DISCUSSION OF ENRICHMENTS USING PALMITATE.

Standard enrichment techniques using palmitate as the carbon-source in the past have given rise to *Desulfovibrio sapovorans* strains. In this study, five enrichment techniques were used with palmitate as the carbon source in order to see whether novel sulfate-reducing bacteria could be isolated. Results using these different approaches are discussed below.

Standard Enrichments.

It has been suggested that palmitate can be used for specific enrichment of *Desulfovibrio sapovorans* (Widdel 1980, Pfennig *et al.* 1981, Widdel and Pfennig 1984, Widdel 1988). Cord-Ruwisch and Garcia (1985) reported other workers' observations that spore-forming sulfate-reducing bacteria had never been observed in enrichment cultures using palmitate and that *Desulfotomaculum sapomendens* could only be isolated if the sample material was first pasteurized. *Desulfovibrio sapovorans* uses fatty acids with 4 to 18 carbon atoms, and as had already been described (Widdel 1980), oxidizes these compounds incompletely. Enrichments with butyrate also yield cultures of *Desulfovibrio sapovorans*. As well as isolating 5 strains using palmitate, Widdel (1980) also described a further 3 strains isolated with butyrate as the substrate. These too have been classified in the 'Sapovorans-group'. Nanninga and Gottschal (1987) also isolated *D. sapovorans* using butyrate as the enrichment substrate. It is of interest that other sulfate-reducing bacteria described in the literature, while not isolated with palmitate, have subsequently shown to use palmitate as an electron donor (e.g

Desulfobacterium autotrophicum, Brysch *et al.* (1987); *Desulfovibrio baarsii* Widdel (1980).

In this study, all those strains isolated using standard techniques were vibrio-shaped and showed incomplete oxidation of their substrate. These characteristics identified these strains as *Desulfovibrio sapovorans* confirming reports "that palmitate is used for the selective isolation of *Desulfovibrio sapovorans*".

Sediment Transfer.

It was hoped that as sediment was carried through a greater number of transfers, organisms might be selected which had an association or even a requirement for sediment or a surface. Standard enrichment techniques select for organisms able to grow without a surface. The sediment enrichment culture showed clumps of bacteria attached to the sediment and substrate; palmitate being insoluble. The insoluble nature of palmitate implies that all organisms growing at its expense would become attached. However, bacteria growing on palmitate are easily dispersed throughout the medium and when growing on soluble fatty acids like butyrate, show no tendency to form clumps.

The organisms isolated using the sediment transfer method showed an association for sediment during the enrichment stages of their isolation; after purification using agar shakes, no requirement for a sediment was observed. When pure culture were grown on palmitate, growth was associated with the insoluble substrate but the cells could easily be distributed throughout the medium. Butyrate-grown cells did not display any tendency to form clumps.

The reason why these two strains developed as opposed to typical *Desulfovibrio sapovorans* cells is difficult to explain and one can only speculate. These enrichments were carried out with the aim of allowing isolation of surface-requiring sulfate-reducing bacteria by ensuring that a suitable surface was present, in this case, the actual source mud. One would have expected to see a), conventional *Desulfovibrio sapovorans*-type of cells growing after 2-3 transfers and b), possibly other sulfate-reducing bacteria, dependent to some extent, on the solid surfaces supplied by the mud particles. Infact, only the latter persisted through the 6-7 transfers, after which time, mud sediment was still obviously present. It was anticipated that after colonies were picked and transferred to fresh medium, either no growth would occur in the liquid medium, or it would only take place attached to the palmitate as no other surface material was added. Growth may also have been possible at the medium/tube interface. During the normal course of isolating sulfate-reducing bacteria, the cells are invariably associated with the enrichment mud and it is not until 2-3 transfers that microbial activity is associated with the culture supernatant, after which growth always takes place in the liquid. With standard enrichments, sediment is only present for 4-5 transfers as compared to the sediment transfer method here when 6-7 transfers were carried out, at which point sediment was still obviously present. Under standard conditions, strains HoPa1 and AmPa1 would probably not be isolated.

The two strains had morphologies unlike those described in the literature and so these two strains were studied in greater detail, details appearing in the following chapter.

Having shown that morphologically interesting organisms could be isolated using the sediment transfer technique, an extension to the method was made by taking the culture through several transfers then pasteurizing for 10 minutes at 80°C.

Pasteurized Sediment Enrichment.

Sulfate-reducing bacteria have been isolated with pasteurized sample materials using palmitate as the substrate (Cord-Ruwisch and Garcia 1985, Klemps *et al.* 1985). Results here showed that organisms could be isolated using this method. As pasteurization selects for sporulating organisms, it was likely then that the sulfate-reducing bacteria that would be isolated using this method would be *Desulfotomaculum* species. Strain AmPa3 was purified from the pasteurized sediment enrichment. Strain AmPa3 was characterized so as to allow comparison with the organisms described above.

It is of interest that during the early enrichment stage, although many bacterial morphologies were present, there was no obvious presence of spore-forming organisms and it was not until pasteurization of the sediment was carried out and cells obtained in pure culture that spores were seen. Observation made with pure culture studies have shown sporulation in *Desulfotomaculum* is an unpredictable event. The role of sporulating sulfate-reducing bacteria and the production of spores in nature has not been studied. It is however, difficult to make microscopic examinations of sediments for spores as many refractile bodies are visible when viewed with phase contrast microscopy.

Enrichment at 13°C.

Enrichment of sulfate-reducing bacteria is usually carried out at temperatures from 20-28°C. 13°C is a more representative temperature of the sediment and so it was thought of interest to investigate which organism(s) might be isolated if all the procedures were carried out at 13°C.

Standard enrichments at 28°C using the Ok sediment gave rise to typical *Desulfovibrio sapovorans*. However, when enrichments and purification were carried using the same sample incubated at 13°C, vibrio-shaped organisms were not seen. Instead, a large oval-shaped organism was isolated (designated OkPa13). Details of this strain follow in chapter five.

Enrichment Using a Dilution Series of Mud.

The enrichment carried out using mud taken through a dilution series gave interesting results. Tubes inoculated with the most mud (least dilute) all gave rise to typical *Desulfovibrio sapovorans* while mud added from the 10^{-4} and 10^{-5} dilutions all gave rise to oval-shaped bacteria similar to OkPa13 and strain Okib (discussed later). These latter strains were tentatively identified as *Desulfobacterium* spp.. This would seem to suggest that these oval-shaped organisms may in fact be present in higher numbers than the *Desulfovibrio* species. If their specific growth rates were greater than those of the *Desulfovibrio sapovorans* strains present, then one might expect them also to dominate the lower dilution tubes, which clearly was not the case. One might therefore speculate that *Desulfovibrio sapovorans* is present in lower numbers but its higher growth rate allows it to

dominate enrichments when large inocula are used, whereas the oval-shaped organisms are present at higher numbers, but due to their slower growth rate, are rapidly outnumbered by *D. sapovorans* and can only be isolated, as was this case, when inocula of dilutions greater than 10^{-3} of sample were used. The phenomenon of isolating different bacteria from the same source using different inoculum sizes has been noted by other workers (Bak, personal communication). Using very small inocula for isolating bacteria may prove to be a very useful technique both to isolate novel organisms and to investigate which organisms might play an important role in metabolism in nature due to their numerical superiority compensating for their low growth rate.

4.3 ISOLATION OF SYNTROPHIC CULTURES.

4.3.1 ENRICHMENT CULTURES.

The reader is reminded that the term "syntrophic bacterium" is used in this section for convenience sake when referring to the organism growing in a syntrophic association which oxidizes the substrate and produces hydrogen.

Enrichment cultures of syntrophic bacteria were obtained from a variety of sample sites using palmitate as substrate. Enrichments were initiated using freshwater (Ho and Am), estuarine (Ok) and marine (Ve) sources with no sulfate in the medium. These cultures all evolved methane and could be maintained by transfer to fresh medium.

Enrichment cultures of syntrophic organisms were obtained which produced methane at different rates. In the primary enrichment with

material from the anaerobic waste pond, gas production took place in 1 week. This compared with 5-6 weeks for production of gas to be observed in the estuarine and marine sources. As cultures were enriched and transferred to fresh medium, the growth rate of the freshwater culture decreased, whereas that of the estuarine and marine cultures increased. A point was reached where both freshwater and marine cultures were transferred at monthly intervals.

As pure cultures could not be obtained simply by continued liquid transfer, agar shake dilutions were carried out as described in section 3.12.3. Several attempts at isolating defined syntrophic cultures were made using both methanogens and sulfate-reducing bacteria as H_2-utilizing species in agar shake tubes. Agar shake tubes gave rise to colonies that were very slow growing. After 6-8 weeks incubation, colonies were picked and transferred to liquid medium. On all occasions, these picked colonies failed to grow. Either growth conditions were not adequate or the colonies picked were not 'syntrophic colonies'. Table 4.2 describes each of the syntrophic cultures and the dominant organisms in each of the cultures.

All the freshwater cultures that were isolated possessed morphological characteristics similar to *Syntrophomonas sapovorans*. *S. sapovorans* is a vibrio-shaped organism that was isolated from an anaerobic digester using palmitate as the enrichment substrate (Roy *et al.* 1985). The primary enrichment period for isolation of *S. sapovorans* was two months and subsequent agar-shake dilution series incubated for periods of six weeks. Similar time periods were used to enrich the organisms of this study while agar shakes were generally incubated for six to eight weeks. The time permissible for

Table 4.2 Description of Syntrophic Cultures Obtained From Different Sites.

Source	Salinity	Substrate	Designated Name	Dominant Organisms	Suggested role
Am	FW	Palmitate	AmPaM	Vibrio	Palmitate-oxidizer
				Long spiral	H_2-utilizer (methanogen)
		Caproate	AmCaM	Vibrio	Caproate-oxidizer
				Long spiral	H_2-utilizer (methanogen)
Ho	FW	Palmitate	HoPaM	Vibrio	Palmitate-oxidizer
				Long spiral	H_2-utilizer (methanogen)
		Caproate	HoCaM	Vibrio	Caproate-oxidizer
				Long spiral	H_2-utilizer (methanogen)
Ok	Est	Palmitate	OkPaM	Vibrio	Palmitate-oxidizer
				Long spiral	H_2-utilizer (methanogen)
Ve	Mar	Palmitate	VePaM	Square-ended rods	Unknown+
				Vibrio	Unknown+
				Flat disc-shaped cells	Unknown+

Abbreviations: FW Freshwater, Est Estuarine, Mar Marine
+, see text for details.

completion of this thesis did not make it possible to continue attempts to obtain defined pure cultures.

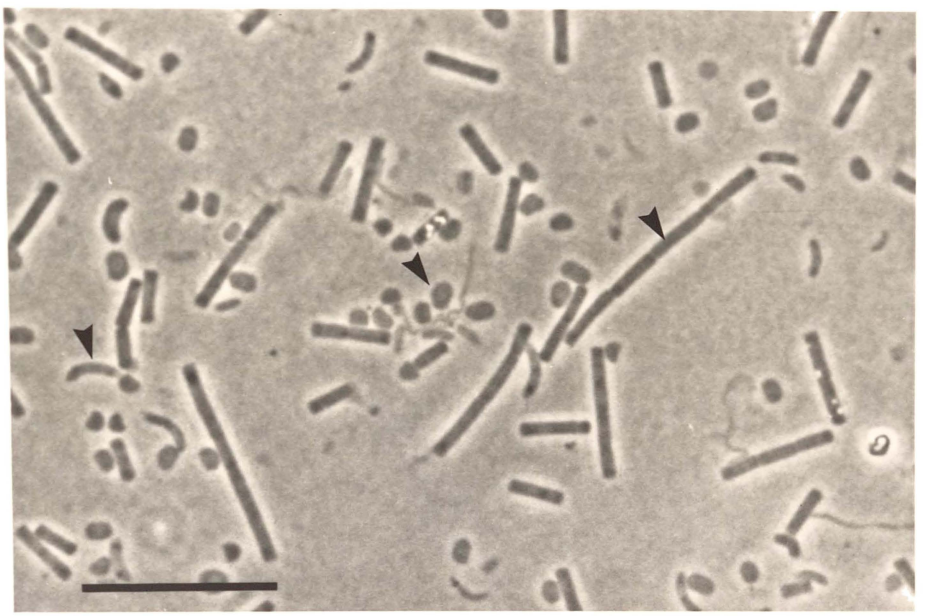
Caproate has been suggested as useful for specific enrichment of *Clostridium bryantii*, a sporulating organism which degrades fatty acids with up to 10 carbon atoms in syntrophic associations (Stieb and Schink 1985). Sporulating organisms were not observed in any of the caproate cultures isolated in this study. HoCaM and AmCaM were also subjected to pasteurization followed by inoculation into separate media with no sulfate plus *Methanospirillum hungatei* and sulfate-containing media plus *Desulfovibrio vulgaris*. No growth took place on these media and it was concluded that these two cultures did not form spores.

OkPaM was isolated from an estuarine source. This culture had very similar appearance to the freshwater culture, one of the dominant organisms having the vibrio-shaped *Syntrophomonas sapovorans*-like cells described above. The methanogenic, or probable H_2-utilizing species was tentatively identified as *Methanospirillum* sp. in both the AmPaM and OkPaM cultures. The long spirillum showed weak fluorescence when excited with light in the 395-446 nm range of wavelengths. Methanogens are known to fluoresce when excited with light at 420 nm due to excitation of the co-enzyme F420.

The Venice culture (marine) was unlike the two cultures described above. There were 3 morphologies that dominated this culture (plate 1); palmitate degradation could not be attributed specifically to any one of these morphologies. The dominant organisms were: a large square-ended rod, a vibriod species and a flat disc-shaped organism. The flat nature of the cells of the latter organism was clearly

Plate 1.
Syntrophic culture degrading palmitate

1.1 Phase contrast micrograph of VenPaM. Arrows indicate the three dominant organisms. For details, see text. (Bar = 10μm)



apparent when the cells tumbled during streaming of the liquid on the slide. No *Methanospirillum*-like cells were observed.

4.3.2 ADDITIONAL ATTEMPTS TO PURIFY SYNTROPHIC CULTURES.

Attempts were made to isolate syntrophic species from enrichment cultures in pure culture using fermentable substrates. This was carried out using agar shakes, as in addition to a syntrophic organism being able to carry out fermentation of the substrates that were chosen, there would most likely be contaminant organisms also present in the enrichment cultures which would be able to ferment these substrates. As the enrichment cultures had been taken through several passages, it was felt likely that these contaminants would only be present in only low numbers. Consequently, the syntrophic bacterium alone should be present in the higher dilutions. The compounds used and the cultures tested are shown in table 4.3.

Table 4.3 Substrates Used for Isolation of Syntrophs in Pure Culture.

Culture(s) tested	Compound(s) used
AmCaM	Acetoin
HoCaM	2,3 butanediol
AmPaM	2,3 butanediol
HoPaM	acetoin ethylene glycol
OkPaM	2,3 butanediol acetoin

All cultures gave rise to colonies in the 4th and 5th tubes in the dilution series. These colonies were picked and transferred to liquid medium. All the freshwater strains were vibrio-shaped,

0.5-0.8 μm x 1.5-2.5 μm. The organism first obtained in pure culture was from the HoCaM enrichment culture, designated HoCaM-2 and studied in greatest detail.

An experiment was carried out using medium containing caproate plus sulfate to test whether HoCaM-2 was capable of growing syntrophically. The medium was inoculated with the pure culture HoCaM-2 and *Desulfovibrio vulgaris* (Marburg). This assumes that HoCaM-2 produces H_2 from caproate. If HoCaM-2 was capable of syntrophic growth, then sulfide should be detected in the medium as *D. vulgaris* (Marburg) is unable to use caproate as an electron donor, but can grow using H_2 as an electron donor. Tubes were incubated for 3 weeks and observed for any turbidity and sulfide production. No increase on optical density or production ~~production~~ of sulfide occurred and it was concluded that HoCaM-2 was not capable of syntrophic growth on caproate with *D. vulgaris*. Similar experiments were carried out on the organisms purified from the remaining agar shakes. These too proved not to be syntrophic organisms.

4.3.3 ENUMERATION OF SYNTROPHIC PALMITATE-DEGRADING BACTERIA.

The number of organisms present in the waste water settling pond sediment(Am) and an estuarine sediment(Ok) capable of degrading palmitate in syntrophic association were estimated using the five tube MPN method (section 3.17). The results are seen in table 4.4. (over page).

Table 4.4 Most Probable Number Enumeration of Syntrophic Palmitate-Degrading Organisms in Estuarine Sediment and Anaerobic Waste Pond (cells/ml). Values given are within 95% confidence limits.

Site	Cell Numbers
Anaerobic Waste Pond (Am)	9×10^7
Estuarine Sediment (Ok)	3.5×10^2

The most probable number estimates of palmitate-degrading syntrophic bacteria in the sediments showed a large difference between the estuarine sediment and the anaerobic waste pond. These values suggest that syntrophy is of less importance in the mineralization of carbon compounds in estuarine environments than in the anaerobic waste pond, where syntrophic degradation of palmitate would seem to be a more important process.

4.3.4 DISCUSSION OF SYNTROPHIC CULTURES.

Although cultures were obtained which degraded palmitate in association with a methanogenic bacterium, they were not able to be isolated in defined culture. Attempts to isolate the syntrophic organism in pure culture using fermentable substrates also failed as the organisms isolated were subsequently shown not to oxidize fatty acids.

Enumeration of palmitate-oxidizing syntrophs were carried out using sulfate-free medium and most probable numbers were estimated from statistical tables after scoring positive growth by observing substrate disappearance and gas formation. The $\Delta G^{\circ\prime}$ for oxidation of palmitate to acetate plus H_2 = +391 kJ/mol at standard conditions

(calculated from tables in Thauer *et al.* 1977). In sulfate-free medium, the disappearance of palmitate can therefore only take place if it is oxidized syntrophically; one organism oxidizing the palmitate, the other bacterium removing the hydrogen generated. The numbers of syntrophic bacteria obtained were 9×10^7 and 3.5×10^2 cells per ml in the respective freshwater and estuarine samples. The medium that was used contains no sulfate. As syntrophic degradation is dependent on consumption of H_2, this medium relied on the activity of methanogens to keep the H_2 partial pressure low and not sulfate-reducing bacteria. Hence, it is worth noting that these values are probably minimum-number estimates as the method used assumed sufficient numbers of H_2-utilizing bacteria were present in the sediment. Reports of numbers of fatty acid-oxidizing syntrophic bacteria are seen in table 4.5.

Table 4.5 Most Probable Number of Fatty Acid-oxidizing Syntrophic Bacteria In Different Source Materials.
Values given are within 95% confidence limits.

Source	Substrate	Number (cells/ml)	Reference
Laboratory Scale Anaerobic Digester	Butyrate	5×10^5	Roy et al. (1986)
	Palmitate	2×10^6	Roy et al. (1986)
Commercial Anaerobic Digester	Butyrate	4.5×10^5	McInerney et al. (1979)
Waste Water Pond	Palmitate	9×10^7	This study
Estuarine Sediment	Palmitate	3.5×10^2	This study

The numbers obtained from the waste water sample of this study is consistent with other reports of fatty acid-oxidizing syntrophs. No reports of enumerations from estuarine samples have been made.

The organism likely to be responsible for palmitate oxidation in both the freshwater and estuarine cultures is the vibrio-shaped organism. The organism observed in each of these cultures resembles *Syntrophomonas* sp. Spores were never observed in either the caproate or palmitate cultures. This is interesting as caproate has been described as being suitable for the specific enrichment of *Clostridium bryantii* (Stieb and Schink 1985). *C. bryantii* oxidizes even-numbered fatty acids with up to 10 carbon atoms in obligately syntrophic association with H_2-utilizing bacteria. It has been isolated from marine and freshwater mud samples as well as anaerobic digesters.

Methanospirillum spp. appeared to be the dominant methanogenic bacteria. A possible explanation for this may be that *Methanospirillum* sp. grows well in autotrophic media; the only organic compounds added here were the carbon source and usually simple vitamin mixes. Methanogens like *Methanobrevibacter ruminantium* would not grow as they have a requirement for Coenzyme M (Boone and Whitman 1988).

Three morphologies were present in the Venice (marine) sample. It could not be said with any certainty which organism(s) may have been responsible for oxidation of palmitate and which organism(s) may have been methanogenic. The dominant organisms were a square-ended rod and an irregular-shaped disc. The rod does not resemble any organism described in the literature which is likely to be present in a palmitate-oxidizing syntrophic culture. The flat disc-shaped organism is however, similar in appearance to *Methanoplanus* sp. (Wildgruber *et al.* 1982), a marine methanogenic bacterium.

Examination with fluorescence microscopy did not reveal any fluorescent bacteria.

4.4 ISOLATION OF BRANCHED-CHAIN FATTY ACID-OXIDIZING SULFATE-REDUCING BACTERIA.

4.4.1 ENRICHMENT CULTURES.

Table 4.6 summarizes observations made on the enrichments and the cultures isolated in pure culture using branched-chain fatty acids. enrichments from both freshwater and estuarine sediments using isobutyrate and 2-methylbutyrate yielded cultures able to degrade their enrichment substrates with concomitant reduction of sulfate. When enrichments with isovalerate were carried out with the Am sediment, methanogenesis occurred in the culture tubes after 4 enrichment transfers, that is, even when very little sediment was left. Sulfide production was very low, (1-1.5 mM), in both the enrichment and the control without added substrate. Sulfide production did not match the levels that were produced in the other enrichments using branched-chain fatty acids (typically 10-15 mM). The freshwater strain isolated using isovalerate was not studied further as its growth rate was too slow for the confines of this study. Enrichments with estuarine samples and isovalerate gave rise to a culture dominated by a large oval organism similar to that isolated on isobutyrate. Microscopic examination of this culture suggested this organisms accounted for an estimated 90% of the biomass. It was decided not to continue purification of this isolate as it appeared very similar to the isobutyrate strain. Strain Ok2mb was purified late in this study and so only a limited number of characteristics could be determined.

Table 4.6 Summary of Enrichments For Sulfate-reducing Bacteria Using Branched-chain Fatty Acids.

Site	Carbon Source	Dominant organism in enrichment	Organism obtained in pure Culture	Strain Designation
Am	isobutyrate	Pointed rod Twisting motility	Same organism as seen in enrichment	Amib
	2 methyl butyrate	1.Large vibrio 2.Small ovals	Both cell types seen in the enrichment	1. Am2mb 2. Am2mb/Pr
	isovalerate	Many cell types seen	Not obtained in pure culture	NA
Ok	isobutyrate	Oval cells	Same organism as seen in enrichment	Okib
	2 methyl butyrate	Sarcina	Same organism as seen in enrichment	Ok2mb
	isovalerate	Oval cells similar to Okib	Not obtained in pure culture	NA

4.4.2 ENUMERATION OF BRANCHED-CHAIN FATTY ACID-OXIDIZING SULFATE-REDUCING BACTERIA.

Enumeration of sulfate-reducing bacteria capable of oxidizing the three branched-chain fatty acids and their straight chain isomers from estuarine and freshwater sites was carried out using the five-tube most probable number method (section 3.17). Results are seen in table 4.7A and B.

Table 4.7 Most Probable Numbers of Sulfate-reducing Bacteria Able to Oxidize Branched-Chain Fatty Acids and Their Straight-Chain Isomers. Values given are within 95% confidence limits.

A. Am Sediment (freshwater)

Compound	Number (cells/ml)
Butyrate	1.7×10^7
Isobutyrate	7.9×10^5
Valerate	9.2×10^6
2 methyl-butyrate	5.4×10^6
Isovalerate	2.4×10^5

B. Ok Sediment (estuarine)

Compound	Number (cells/ml)
Butyrate	2.4×10^7
Isobutyrate	1.3×10^7
Valerate	3.5×10^7
2 methyl-butyrate	2.4×10^7
Isovalerate	5.4×10^7

The numbers of cells able to degrade isomers of butyrate and valerate in the freshwater sample differed up to 100 fold (table 4.7A). This was most clearly seen with 1.7×10^7 cells per ml able to degrade butyrate but only 7.9×10^5 able to degrade isobutyrate. A similar trend was also observed with valerate. Roughly the same number of cells were present that could degrade valerate and 2 methyl butyrate but 10 fold less able to degrade isovalerate.

Sulfate-reducing bacteria able to degrade isomers of butyrate and valerate in the estuarine sediment (table b) occurred at reasonably high numbers ranging from 1.3×10^7 cells/ml able to oxidize isobutyrate, to 5.4×10^7 cells/ml able to oxidize isovalerate.

4.4.3 DISCUSSION OF BRANCHED-CHAIN FATTY ACID-DEGRADING ENRICHMENT CULTURES.

Several strains of sulfate-reducing bacteria were isolated which were capable of using branched-chain fatty acids. A freshwater isolate

from the isovalerate enrichment was not obtained in pure culture as this enrichment was considerably-slower growing than were the enrichments using other fatty acids. Pure cultures of sulfate-reducing bacteria growing on isovalerate have been observed by others to be slow growing (Widdel 1988).

MPN estimation of these bacteria showed organisms able to use isovalerate were also present in the lowest number. That numbers in the range of $10^{7}/ml$ were obtained, implies that the oxidation of branched-chain fatty acids and their straight chain isomers by sulfate-reducing bacteria is a relatively important process in both freshwater and estuarine environments. Numbers of organisms present on each of the branched-chain isomers from the 0k sample do not differ significantly from one another at the 95% confidence levels. The values obtained from the waste-water pond show significant differences at the 95% confidence levels. The number of organisms present degrading isobutyrate was significantly less than the number able to degrade butyrate. Likewise, the population of isovalerate-oxidizing sulfate-reducing bacteria is less than those able to degrade 2-methylbutyrate. No comments can be made as to what proportion of a population degrading a particular isomer is able to degrade other isomers.

The primary enrichment from the freshwater source with 2-methylbutyrate demonstrated co-isolation of 2 sulfate-reducing bacteria, with both organisms present in roughly equal proportions. Agar shake tubes gave rise to colonies that either consisted of a large spiral-shaped organism or mixed colonies of the spiral plus small oval. Because the small oval cells never appeared in pure colonies on 2-methylbutyrate, it was thought that there may have been

some dependence of the small oval on the spiral-shaped organism. Further studies on this culture, transferred into agar shakes with propionate as the carbon-source, produced a pure culture of the small oval organism. Detailed discussion of the organisms isolated using branched-chain fatty acids follows in the next chapter.

CHAPTER FIVE

CHARACTERIZATION OF ISOLATES:- RESULTS AND DISCUSSION

5.1 INTRODUCTION.

This chapter describes the cytological and metabolic characteristics of the organisms that were isolated in pure culture during the course of this study. Discussions of the results are included in this chapter when they relate to the results that were obtained. Organization of the material in this form allows the reader to follow the results through a logical progression. The reader is reminded that unless otherwise specified, growth in substrate tests etc. were scored as follows: +++, ++, +, -, which correspond to optical density values of 0.4, 0.3, 0.1 and 0 respectively.

5.2 CHARACTERIZATION OF SULFATE-REDUCING BACTERIA CAPABLE OF OXIDIZING PALMITATE.

The following section describes the characterization and identification of some of those sulfate-reducing bacteria isolated using palmitate as the enrichment substrate. Each organism is considered separately as the number of characteristics determined for each of the strains differed.

5.2.1 STANDARD ENRICHMENT PROCEDURES.

Standard enrichments from both freshwater and estuarine sample sites gave rise to vibrio-shaped sulfate-reducing bacteria, typically 1-1.5 x 3-5 μm. All strains used fatty acids as substrates; in all cases,

the fatty acid substrates were oxidized incompletely. Extensive study was not carried out on these strains as they were all tentatively identified as being typical *Desulfovibrio sapovorans*.

5.2.2 SEDIMENT TRANSFER METHOD.

Characterization of Strains HoPa1 and AmPa1.

Using the sediment transfer method, two rod-shaped strains of sulfate-reducing bacteria were isolated using palmitate as the enrichment electron donor. Organisms with this morphology have not been previously isolated using palmitate as the enrichment substrate. The two strains were designated AmPa1 and HoPa1 and their characteristics are described comprehensively below.

General Characteristics.

Both strains were rod-shaped with rounded ends (1 x 2-3 μm) (plate 2.1 and 2.2). Cells were occasionally seen in pairs, or in chains of up to 6-7 cells in old cultures. Both organisms stained Gram-negative. Motility was never observed. In stationary-phase cultures observed using negative phase contrast microscopy, cells were shown to contain 3-5 intracellular bodies that appeared as typical PHB granules. Cells possessing these inclusions were subjected to Sudan black staining (section 3.15.2) and were observed to take up the stain. Janssen (1989) observed the presence of small intracellular granules which stained with Sudan black but were subsequently shown not to be PHB. The granules observed by Janssen were considerably smaller than those seen in AmPa1 and HoPa1 and so it was concluded the intracellular inclusions observed in these strains were probably PHB.

Enrichment and isolation of these two strains was carried out in the presence of vitamin 8 solution and Vitamin B_{12} (section 3.5). Once in pure culture, both strains were transferred through several passages in medium which contained no added vitamins. Strains AmPa1 and HoPa1 showed no vitamin requirement; vitamins were therefore not included in further media.

Electron Donors.

The compounds that were tested for use as electron donors by strains HoPa1 and AmPa1 are seen in table 5.1. Numbers in brackets are the concentrations used in mM. AmPa1 and HoPa1 grew only on fatty acids from C_4 chain length to C_{18}. No growth took place on propionate, acetate, or formate, or on formate plus acetate. No growth was also obtained on any of the dicarboxylic acids that were tested. No growth was obtained on aromatic compounds or on H_2/CO_2.

Electron Acceptors Used By Strains AmPa1 *and* HoPa1.

Both strains were able to use sulfate and sulfite as terminal electron acceptors when growing using palmitate as the electron donor. Thiosulfate, nitrate and oxygen could not be used.

Growth in the Absence of Sulfate.

Both strains were inoculated into separate media containing lactate, pyruvate, fumarate, malate and glucose as fermentable substrates; each in the absence of a terminal electron acceptor. Neither strain grew on any of the substrates indicating their inability to ferment those substrates.

Table 5.1 Compounds Tested for use as Electron Donors by Strains AmPa1 and HoPa1.

Electron Donor	Organism	
	AmPa1	HoPa1
H_2/CO_2	-	-
Formate(10)	-	-
Formate plus Acetate(10/1)	-	-
Acetate(10)	-	-
Propionate(10)	-	-
Butyrate(10)	+++	+++
Isobutyrate(5)	-	-
Valerate(5)	+++	+++
2-methylbutyrate(5)	-	-
Isovalerate(5)	-	-
Hexanoate(5)	+++	+++
Octanoate(0.5)	+++	+++
Decanoate(0.5)	+++	+++
Dodecanoate(0.5)	+++	+++
Tetradecanoate(0.5)	+++	+++
Hexadecanoate(1)	+++	+++
Octadecanoate(1)	+++	+++
Lactate(10)	-	-
Pyruvate(10)	-	-
Succinate(10)	-	-
Malate(10)	-	-
Fumarate(10)	-	-
Maleate(10)	-	-
Benzoate(3)	-	-
Ethanol(10)	-	-
Propanol(10)	-	-
Butanol(10)	-	-

Cell Pigments of Strains AmPa1 *and* HoPa1.

Both the fluorescence test and spectral scans of cell-free extracts were used to determine the presence of desulfoviridin. No fluorescence was seen after addition of sodium hydroxide to the cell pellets of either HoPa1 or AmPa1. A cell pellet of *Desulfovibrio vulgaris* served as the positive control and gave red fluorescence. No characteristic absorbance peak was detected at 630 nm in the cell-free extracts, whereas *D. vulgaris* showed an absorption maximum, confirming the absence of desulfoviridin in these strains (figure 5.1).

Difference spectra (oxidized minus reduced) of cell preparations showed absorption maxima at a number of wavelengths, details of which can be seen in table 5.2. *Desulfovibrio vulgaris* served as a control for spectral scans.

Table 5.2 Absorption Maxima of Difference Spectra (Oxidized minus Reduced) From Cell-free Extracts.

	Absorption Maxima		
	α	β	γ
HoPa1	558	524	424
AmPa1	556	523	423
Desulfovibrio vulgaris	553	523	421
Cytochrome c_3 *	553	522	419
Cytochrome b *	629	558	527

*, Jones (1972)

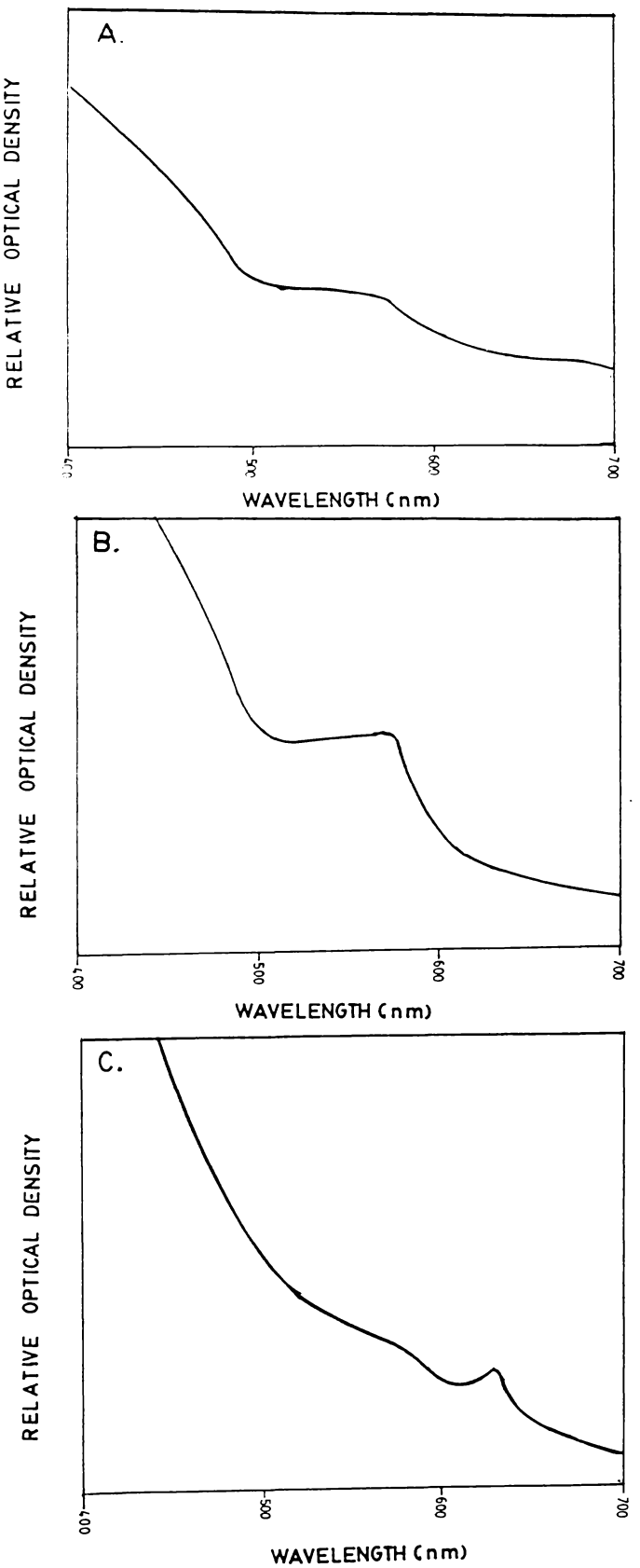
Strains AmPa1 and HoPa1 appear to possess both b- and c-type cytochromes though an absorption maximum at λ 629 nm was not observed.

Analyses of End Products.

Cells grown on palmitate, heptadecanoate and hexanoate were analyzed for end product formation. Results are seen in table 5.3. Both AmPa1

Figure 5.1 Spectral scans for desulfoviridin.

A. HoPal: B. AmPal: C. *Desulfovibrio vulgaris* strain Marburg



and HoPal were incomplete oxidizers, producing acetate alone from even-numbered chain length fatty acids and propionate and acetate from fatty acids with an odd number of carbon atoms.

Table 5.3 Endproduct Formation of Strains AmPal and HoPal Grown on Fatty Acids.

Organism	Substrate (Concentration)	Product(s) Formed (concentration)
AmPal	Palmitate (1mM)	Acetate (7mM)
	Hexanoate (5mM)	Acetate (14mM)
	Heptadecanoate (1mM)	Acetate (7mM)
		Propionate (1mM)
HoPal	Palmitate (1mM)	Acetate (8mM)
	Hexanoate (5mM)	Acetate (14mM)
	Heptadecanoate (1mM)	Acetate (7.5mM)
		Propionate (1mM)

The amount of substrate oxidized and products formed are in good agreement with the equation of Widdel (1980), 1 and 2 below, indicating incomplete oxidation:

1. $CH_3(CH_2)_{2n}COO^- + n/2\ SO_4^{2-} \longrightarrow (n+1)CH_3COO^- + n/2HS^- + H^+$

2. $CH_3(CH_2)_{2n+1}COO^- + n/2\ SO_4^{2-} \longrightarrow CH_3CH_2COO^- + nCH_3COO^- + n/2\ HS^- + n/2\ H^+$

Comparison of Strains AmPal and HoPal with *Desulfovibrio sapovorans*.

Sediment transfer enrichments from a freshwater source gave rise to strains AmPal and HoPal. Both AmPal and HoPal oxidized fatty acids with an even number of carbon atoms to acetate and fatty acids with an odd number of carbon atoms to acetate plus propionate. Thus both AmPal and HoPal resembled *D. sapovorans* metabolically suggesting

that both AmPal and HoPal were strains of *Desulfovibrio sapovorans*. However, *D. sapovorans* is a vibrio- to spirillum-shaped organism whereas AmPal and HoPal were both rods. Further characteristics were determined in order to see how these two organisms resembled *D. sapovorans*. Details are seen in table 5.4.

Table 5.4. Characteristics of *Desulfovibrio sapovorans* and Strains AmPal and HoPal.

Characteristic	*Desulfovibrio sapovorans*	AmPal	HoPal
Morphology			
Width (μm)	1.5	1	1
Length (μm)	3-3.5*	2-3	2-3
Motility	present	absent	absent
Substrates Used with sulfate			
H_2/CO_2	-	-	-
Formate	-	-	-
C_2-C_3	-	-	-
C_4-C_{16}	+	+	+
C_{18}	-	+	+
2-methylbutyrate	+	-	-
Lactate	+	-	-
Pyruvate	+	-	-
Dicarboxylic acids	-	-	-
Alcohols	-	-	-
Pyruvate-SO_4^{2-}	+	-	-
Cytochromes	b,c	b,c	b,c
Desulfoviridin	absent	absent	absent

Abbreviations used: +, good growth; -, no growth
*, Some strains are smaller oval to vibrio-shaped, 0.5-0.7 x 1-1.5 μm.

Strains HoPal and AmPal differed from *D. sapovorans* in morphology, motility, inability to use 2-methylbutyrate, lactate, pyruvate and inability to ferment pyruvate in the absence of sulfate. A number of attempts were made to extract DNA from both AmPal and HoPal.

However, these attempts failed and consequently, the DNA composition could not be determined.

Relatively few distinguishing characteristics can be easily determined for sulfate-reducing bacteria and so difficulties can arise with identification of newly isolated strains. Strains AmPa1 and HoPa1 are clearly very similar; so similar that they can be regarded as different strains of the same species. However, they differ in a number of ways from *D. sapovorans* and the differences might be regarded as sufficient to place them in a different species. The DNA composition can be important in describing a new species of sulfate-reducing bacteria and is clearly required for the identification of these two strains. However, three attempts to isolate the DNA from these two strains failed. In this case here, should the DNA composition of strains AmPa1 and HoPa1 be shown to be significantly different from that of *D. sapovorans*, then the description of a new species would be required to accommodate these two strains. Until such information is available, no such taxon should be created.

5.2.3 PASTEURIZED SEDIMENT ENRICHMENT.

Characterization of Strain AmPa3.

Enrichment cultures using the sediment transfer technique gave rise to atypical sulfate-reducing bacteria and inturn prompted the use of an isolation procedure involving pasteurization of each sediment. Spores were never apparent in these enrichment cultures when viewed with the microscope. However, pasteurization of the sediment allowed

isolation of a sporulating sulfate-reducing bacterium. The isolate was designated AmPa3.

General Characteristics.

Strain AmPa3 was a pointed rod (0.8 x 2.5-3 μm) (Plate 3.1). Examination of strain AmPa3 using phase contrast microscopy showed the presence of refractile intracellular bodies. Cultures of this organism retained viability after pasteurization at 80°C for 10 minutes, demonstrating the presence of endospores. Vacuoles were never observed.

Cell pigments.

Both the fluorescence test and scans of cell-free extracts, (figure 5.2) demonstrated the absence of desulfoviridin from AmPa3. Difference spectra (oxidized minus reduced), showed absorption maxima at 526, 560 and 570 nm. The absorption maxima at 560 nm and 526 nm are characteristic of b-type cytochromes. The absorption maximum at 629, also characteristic of cytochrome b, was however, not detected.

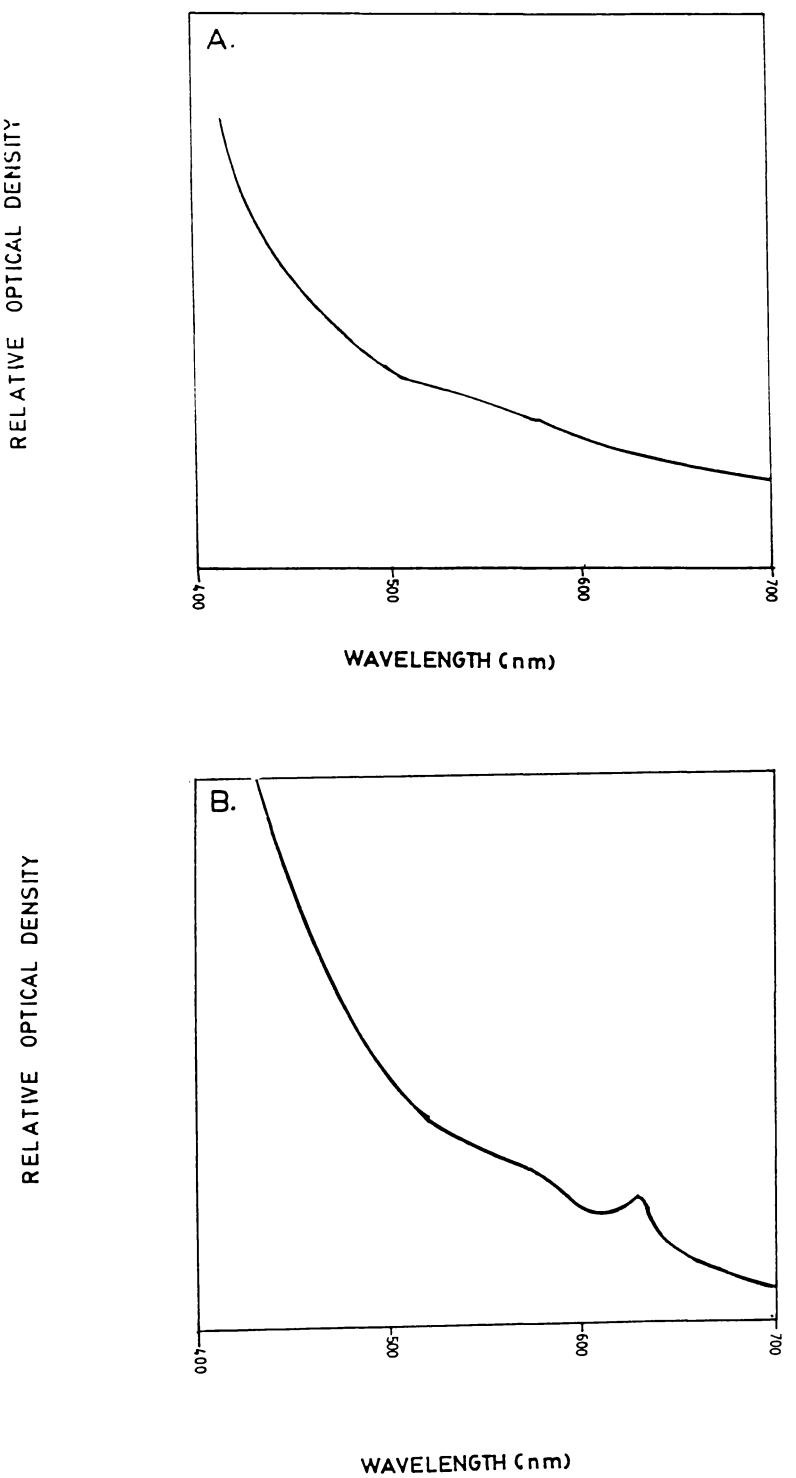
End Products Analysis.

Qualitative measurements of butyrate oxidation by AmPa3 showed the presence of acetate as an end product, but at concentrations intermediate between that expected from incomplete oxidation, (i.e. 2 acetate per butyrate oxidized) and complete oxidation, (i.e. no acetate produced). Quantitative analysis of butyrate oxidation (mean of three experiments) is seen in table 5.5.

The amount of acetate produced compared with the amount of butyrate used is inconsistent with true incomplete oxidation where 500 μmol butyrate would lead to production of 1 mmol acetate. When these

Figure 5.2 Spectral scans for desulfoviridin

A. AmPa3: B. *Desulfovibrio vulgaris* Marburg



observation were made, such a phenomenon had not been reported in *Desulfotomaculum* spp.. However, late in this study, a report was made by Daumas *et al.* (1988) who showed *Desulfotomaculum geothermicum* produced acetate, but at a concentration inconsistent with either complete or incomplete oxidation when growing on butyrate. Strain AmPa3 was able to grow, albeit very slowly, on acetate.

Table 5.5 Stoichiometry of Butyrate Oxidation by Strain AmPa3.

Butyrate Added	670 μmol
Cells Formed (Dry Weight)	4.2 mg
Butyrate Consumed	550 μmol
Butyrate assimilated*	43 μmol
Butyrate oxidized	507 μmol
Acetate Formed	200 μmol
Sulfide Formed	850 μmol

*Substrate assimilated was calculated using the emperical formula for a cell from Widdel (1980): $<C_4H_7O_3>$.

Approximately three weeks incubation on 10 mM acetate at 28°C was required to reach a final optical density of 0.05. In the experiment summarized in table 5.5, 5 mM butyrate was used as the substrate in the presence of 20 mM sulfate as electron acceptor. Oxidation of butyrate could theoretically take place in a manner akin to that in *Desulfobacterium* without the sulfate becoming limiting. The oxidation of butyrate in *Desulfobacterium* takes place according to the formula 1 below (Schauder *et al.* 1986):

1. $1\ \text{butyrate} + 1.5\ SO_4^{2-} \longrightarrow 1\ \text{acetate} + 1.5HS^-$.

Using this formula, 507 μmol butyrate would result in production of 760 μmol sulfide and 507 μmol acetate. As AmPa3 was shown to use acetate, subsequent oxidation of acetate would lead to further production of sulfide, acetate oxidation taking place according to the formula 2 below (Schauder *et al.* 1986):

2. $CH_3COO^- + SO_4^{2-} + 3H^+ \longrightarrow 2CO_2 + H_2S + 2H_2O$

The overall oxidation of butyrate by AmPa3 could be summarized by combining equations 1 and 2 above:

$$\begin{array}{ll} & 507 \text{ butyrate} + 760\ SO_4^{2-} \longrightarrow 507 \text{ acetate} + 760\ H_2S \\ & 307 \text{ acetate} + 307\ SO_4^{2-} \longrightarrow 307 \text{ acetate} + 307\ H_2S \\ \hline 3. & 507 \text{ butyrate} + 1067\ SO_4^{2-} \longrightarrow 200 \text{ acetate} + 1067\ H_2S \end{array}$$

The amount of sulfide produced as shown in table 5.5 is 20 % less than the theoretical sulfide production given by equation three.

Identification of Strain AmPa3.

Isolation of strain AmPa3 involved the pasteurization of the enrichment culture. Pasteurization selects for sporulating organism and so it was assumed that any sulfate-reducing bacteria isolated using this procedure would probably be members of the genus *Desulfotomaculum*. The presence of spores was subsequently demonstrated in AmPa3 confirming its identification as a *Desulfotomaculum* species. End-product analysis of a culture grown on butyrate showed the present of acetate, but at a concentration inconsistent with incomplete oxidation. The use of fatty acids by *Desulfotomaculum* spp. has been reported in *D. sapomendens* (Cord-Ruwisch and Garcia 1985) and *Desulfotomaculum acetoxidans* (Widdel and Pfennig 1977, Widdel and Pfennig 1982b). *D. acetoxidans* is able to

grow on only a very limited range of compounds whereas *D. sapomendens* is also able to grow on long-chain fatty acids and carboxylic acids. Strain AmPa3 clearly did not resemble *D. acetoxidans* and so was compared to *D. sapomendens*. A comparison of the characteristics of *D. sapomendens* and AmPa3 is seen in table 5.6.

Table 5.6. Comparison of Characteristics of *Desulfotomaculum sapomendens* and Strain AmPa3.

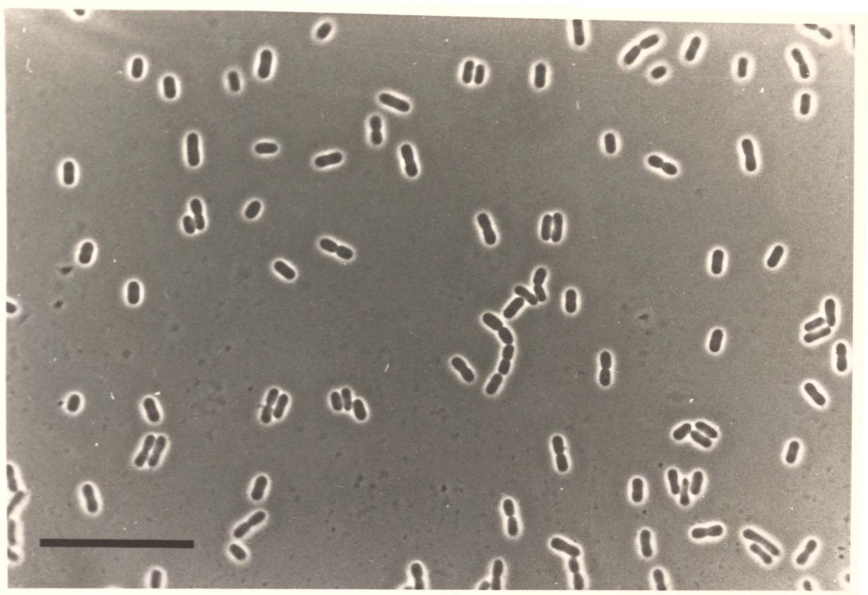
	*Desulfotomaculum sapomendens**	AmPa3
Morphology		
Width	1.2-2.0 μm	0.8 μm
Length	5-7 μm	2.5-3 μm
Gas Vacuoles	present	absent
Substrates used		
Formate	+	+
Acetate	+	+
Propionate	+	-
C_4-C_{16} fatty acids	+	+
Isovalerate	+	+
2 methylbutyrate	-	-
Isobutyrate	+	+
Ethanol	+	+
Dicarboxylic acids	+	-

Strain AmPa3 differs from *D. sapomendens* in its ability to use acetate, propionate and carboxylic acids and in the absence of vacuoles. Though there are these differences, they are not significant enough to warrant the description of a new species and so strain AmPa3 is a strain of *Desulfotomaculum sapomendens*.

Plate 2.
Sulfate-reducing bacteria isolated which oxidize palmitate

2.1 Phase contrast micrograph of strain HoPa1. (Bar = 15μm)

2.2 Phase contrast micrograph of strain AmPa1. (Bar = 15μm).



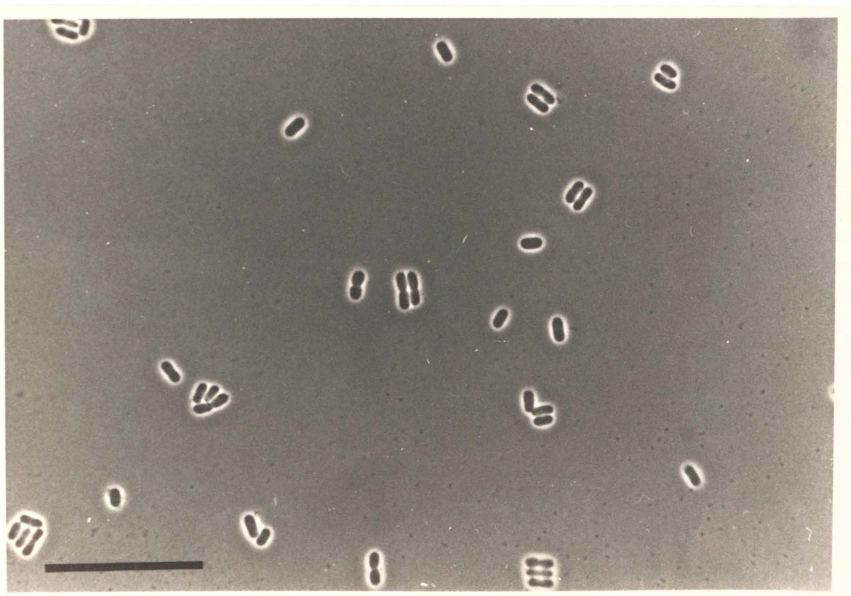
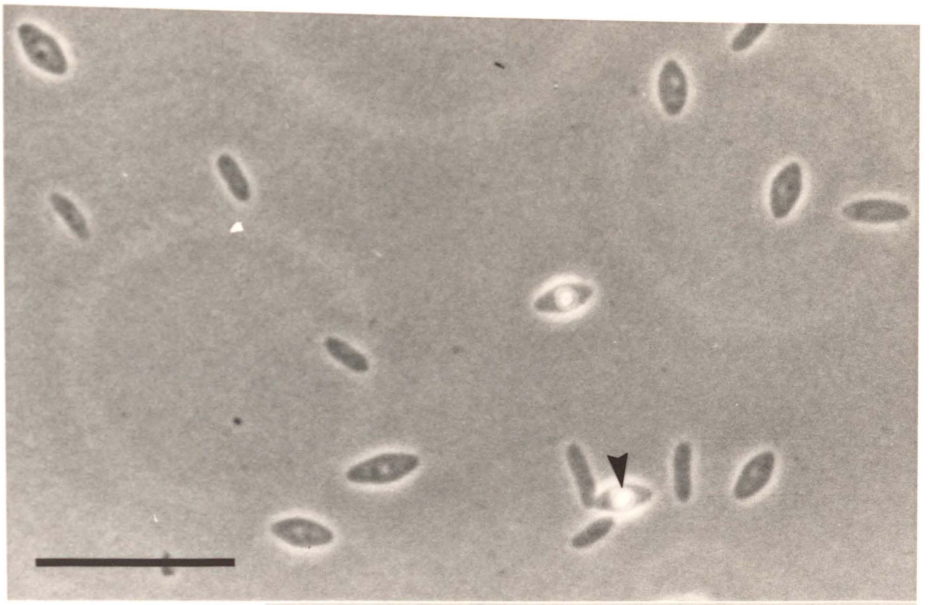


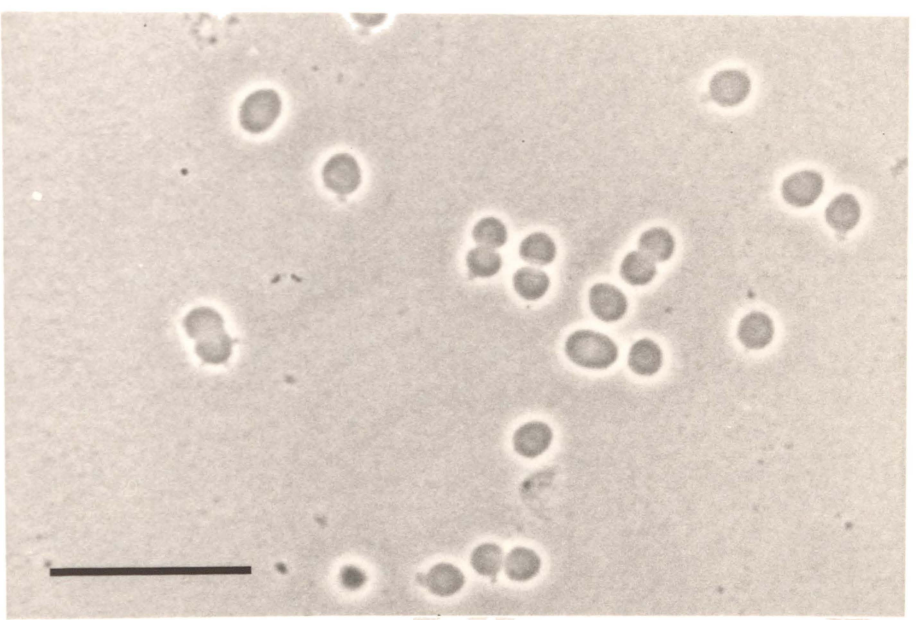
Plate 3.
Sulfate-reducing bacteria isolated which oxidize palmitate (continued)

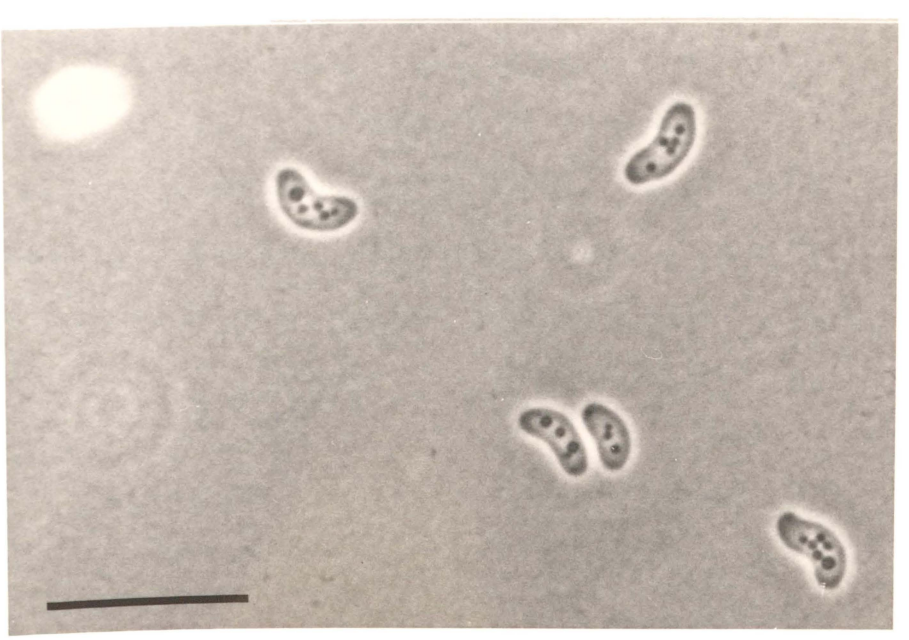
3.1 Phase contrast micrograph of strain AmPa3. Arrow indicates spore. (Bar = 10μm)

3.2 Phase contrast micrograph of strain OkPa13. (Bar = 10μm).

3.3 Phase contrast micrograph of *Desulfovibrio sapovorans* strain DSM 2055. (Type strain). This shows the typical organism isolated when using palmitate as the enrichment carbon source. (Bar = 10μm)







5.2.4 ENRICHMENT AT 13°C.

Characterization of Strain OkPa13.

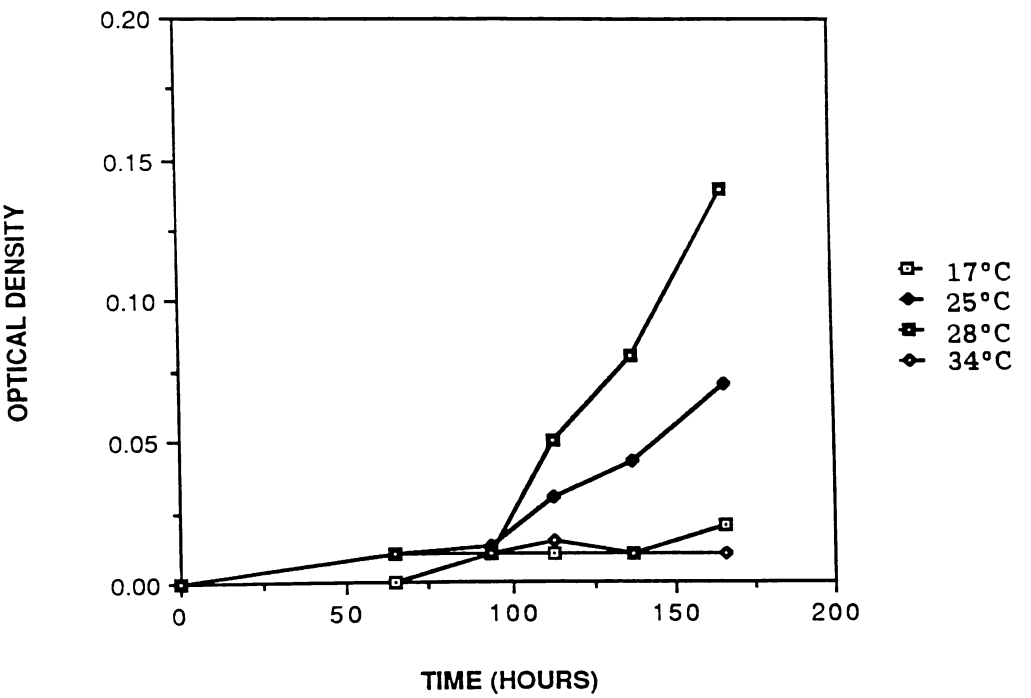
Only those characteristics were determined for strain OkPa13 which allowed identification to the level of genus. Those characteristics determined are summarized in the following paragraphs.

General Characteristics.

Strain OkPa13 was an oval-shaped organism (1-1.5 μm x 2-2.5 μm), often occurring in pairs (Plate 3.2). Motility was never observed at any stage of growth in any cultures. OkPa13 was shown to grow well on fatty acids with chain lengths from C_4 to C_{18}, while only very slow growth took place on propionate. No growth was recorded in the incubation time (three weeks) on formate or acetate. Strain OkPa13 grew well on carboxylic acids, proline and casamino acids.

As OkPa13 was enriched at 13°C, its growth response to temperature was investigated in order to determine its optimum growth temperature. Growth response is shown in figure 5.3. Although enrichment and isolation was carried out at 13°C, optimum growth rate was measured at 28°C. Growth was slower at 25°C than at 28°C. At 17°C, growth was very slow, showing as increase in optical density of 0.04 over the 116 hours incubation period. Strain OkPa13 grew faster than did strain Okib (see below) when incubated at 17°C, which failed to show any growth after 390 hour incubation (see figure 5.4).

Figure 5.3 Growth response of strain OkPa13 to temperature.



End Product Analysis.

Measurements were made of substrate disappearance and product formation, including sulfide, in order to determine general metabolic patterns. The results are seen in table 5.7.

Table 5.7 Stoichiometry of Fatty Acid Metabolism by Strain OkPa13.

Substrate	Substrate Added (μmol)	Substrate Remaining (μmol)	Acetate Produced (μmol)	Sulfide Produced (μmol)
Butyrate	1000	0	1000	1580
Valerate	500	0	160	1320
Palmitate	100	0*	0	1460

* Visual observation showed OkPa13 had used all the palmitate at the end of the incubation period.

The data in table 5.7 shows that OkPa13 was capable of complete oxidation of those substrates tested. Incomplete oxidation of these substrate would occur according to the following generalized equations (from Widdel 1980):

1. $10\ \text{Butyrate} + 5\ SO_4^{2-} \longrightarrow 20\ \text{Acetate} + 5\ HS^-$
2. $5\ \text{Valerate} + 2.5\ SO_4^{2-} \longrightarrow 5\ \text{Propionate} + 5\ \text{Acetate} + 2.5\ HS^-$
3. $1\ \text{Palmitate} + 3.5\ SO_4^{2-} \longrightarrow 8\ \text{Acetate} + 3.5\ HS^-$

Instead, oxidation of butyrate took place according to the formula:

4. $\text{Butyrate} + 1.5SO_4^{2-} \longrightarrow 1\ \text{Acetate} + 1.5HS^-$

Oxidation according to this formula is typical of *Desulfobacterium* sp.. The substrate oxidation balance for OkPa13 was carried out

using 1000 μmol butyrate which if oxidized according to the formula of Schauder *et al.* (1986), would have a theoretical sulfide production of 1500 μmol. The amount of sulfide produced measured was 105% that of the theoretical value indicating this is the likely pathway for butyrate oxidation. No growth had been demonstrated on acetate which explains the good agreement with the theoretical values.

Although OkPa13 was enriched and isolated at 13°C, it was shown to have an optimum growth rate at 28°C. Ingvorsen *et al.* (1981) measured the effect of temperature on rates of sulfate reduction in sediments from a freshwater lake. The temperature of the sediment sampled ranged from 10°C in winter to 13°C in summer. Despite these values, the optimum temperature for sulfate reduction was 36-37°C, considerably higher than the *in situ* temperatures. Whereas sulfate reduction can take place at temperatures in the vicinity of 10°C, the organisms that can be isolated at those temperatures do not necessarily have optima at those temperatures, accounting for the measurements obtained by Ingvorsen *et al.* (1981). A number of sulfate-reducing bacteria have also been isolated, using an incubation temperature of 25°C, from Antarctic lake sediments which typically have *in situ* temperatures of around 4°C (Rees 1985, Rees *et al.* 1986). These strains were subsequently shown to have optimum growth temperatures ranging from 25-37°C.

5.3 CHARACTERIZATION OF SULFATE-REDUCING BACTERIA CAPABLE OF OXIDIZING BRANCHED-CHAIN FATTY ACIDS.

The following section discusses the characterization of the organisms that were isolated using branched-chain fatty acids as enrichment

substrates. The characteristics that are necessary for the identification of sulfate-reducing bacteria are considered individually for each of the strains. The most detailed study was that carried out on strain Okib whereas strain Amib was studied to a lesser degree. Strain Ok2mb was purified late in this study and so is only briefly mentioned as time allowed determination of only a few characteristics. A sufficient number of details of this latter organism were established so as to allow tentative identification to the level of genus. The description and discussion of the strains that were isolated are considered separately.

5.3.1 DESCRIPTION AND DISCUSSION OF THE Am2mb CULTURE.

Agar shake dilution tubes from the Am2mb culture gave rise to two colony types. In the first, spirillum-shaped cells were observed and in the second, the spirillum, along with a small oval were observed (Plate 4.3). The spirillum was purified and shown to oxidize fatty acids incompletely, therefore was identified as belonging to the *Desulfovibrio sapovorans* group. *D. sapovorans* has been shown to oxidize 2-methylbutyrate producing acetate and propionate (Widdel 1980). For this reason, it was thought the small oval was either a *Desulfobacter* sp. growing on acetate, or a *Desulfobulbus* sp. growing on propionate. Agar shakes with propionate as the electron donor gave rise to pure cultures of the oval organism which was designated Am2mb/Pr. Substrate tests showed that Am2mb/Pr used propionate incompletely according to Widdel and Pfennig (1982) and that 2-methylbutyrate was not used. This confirmed its identification as a strain of *Desulfobulbus*. Am2mb/Pr presumably required the presence of the *Desulfovibrio sapovorans* strain for the production of propionate hence its occurrence only in mixed colonies.

5.3.2 DESCRIPTION AND DISCUSSION OF STRAIN Amib.

Characterization of Amib.

General Characteristics.

Strains Amib was a large pointed rod, (1.2-1.5 x 4-5.5 μm) (Plate 4.1). Wet mount preparations demonstrated that motility was present. When examined using negative phase contrast microscopy, intracellular granules characteristic of PHB were not observed. Cells were therefore not subjected to the Sudan black staining procedure. However, cells of Amib showed internal refractile bodies when grown on butyrate and isobutyrate, but not when grown on acetate. Amib cultures grown on butyrate showed heat resistance (80°C for 10 minutes), confirming that the refractile bodies were endospores.

In addition to the spherical refractile endospores, Amib also produced a second, more irregularly-shaped refractile body. High speed centrifugation (10000 x *g*) of cultures which had cells possessing this second refractile body, caused the latter refractile body to disappear. This second refractile body was therefore, probably a gas vacuole; it is known that one of the properties of gas vacuoles is their disruption following centrifugation (Widdel 1988).

Growth Response to Temperature.

Detailed growth studies of Amib's response to temperature were not carried out. However, qualitative tests showed that strain Amib grew fastest at 34°C.

Electron Donors Used By Strain Amib.

Strain Amib was able to grow using acetate, butyrate, isobutyrate and butanol. No other alcohols, straight chain organic acids or aromatic compounds could support growth. The concentrations of electron donor tested were the same as those described in table 5.1 above unless otherwise indicated (in brackets). Results are seen in table 5.8.

Table 5.8 Compounds Tested for use as Electron Donors by Strain Amib.

Electron Donor	Growth	Electron Donor	Growth
H_2/CO_2	-	Methanol	-
Formate	-	Ethanol	-
Acetate	++	Propanol	-
Propionate	-	Butanol	-
Butyrate	+++	Fumarate	-
C_5-C_{16} fatty acids	-	Malate	-
C_{18} fatty acid	-	Succinate	-
Isobutyrate	+++	Pimelate(5)	-
2-methylbutyrate	-	Glutarate(3)	-
Isovalerate	-	Lactate	-
		Pyruvate	-

Identification of Strain Amib.

Strain Amib was shown to produce endospores although the isolation procedures did not involve pasteurization. Sporulating sulfate-reducing bacteria have been assigned to the genus *Desulfotomaculum* (Campbell and Singleton 1986). Two species have been described in the literature which are able to oxidize fatty acids, namely *Desulfotomaculum acetoxidans* (Widdel and Pfennig 1977) and *Desulfotomaculum sapomendens* (Cord-Ruwisch and Garcia 1985). *D. acetoxidans* was isolated from piggery waste using acetate as the electron donor and is only able to use acetate, butyrate, valerate,

isobutyrate and butanol. *D. sapomendens* was isolated from pasteurized soil with palmitate as the electron donor and is able to use both short and long-chain fatty acids, organic acids, alcohols and aromatic compounds. Strain Amib was able to use only acetate, butyrate and isobutyrate. The characteristics determined identify strain Amib as *Desulfotomaculum acetoxidans*.

Stain Amib was isolated from abattoir waste water, a similar environment to that of *D. acetoxidans*. It was suggested that *D. acetoxidans* may in fact be a gut organism as it had a temperature optimum for growth of 35°C. A similar optimum temperature was observed in Amib.

5.3.3 DESCRIPTION AND DISCUSSION OF STRAIN Ok2mb.

Characterization of Strain Ok2mb.

Strain Ok2mb was purified during the last stages of this project allowing only determination of the general morphological features and certain electron donor tests to be carried out (Plate 4.2).

General Characteristics.

Colonies of strain Ok2mb were found to be difficult to disrupt when picked with a pasteur pipette. As a consequence, purification of this strain took longer than was usual as contaminant organisms within the colony were also often transferred. In liquid culture, strain Ok2mb grew in sarcinae packets which retained their structure after transfer through several subcultures. Cell size was difficult to determine due to the clumping of the cells. Neither motile cells nor spores were ever observed.

Compounds Used as Electron Donors by Strain Ok2mb.

Strain Ok2mb was tested for the range of compounds it could use as electron donor and shown to grow on all fatty acids tested from C_2 to C_{16}, as well as some organic acids. Formate did not support growth. The aromatic compounds tested were not used. Results are seen in table 5.9. The concentrations of electron donor tested were the same as those in table 5.1 unless otherwise specified (in brackets).

Table 5.9. Compounds Tested For Use as Electron Donors By Strain Ok2mb.

Electron Donor	Growth	Electron Donor	Growth
H_2/CO_2	++	Lactate	+++
Formate	-	Pyruvate	+++
Acetate	++	Fumarate	+++
Propionate	++	Malate	+++
Butyrate	+++	Succinate	+++
Isobutyrate	+++	Pimelate	+++
2-methylbutyrate	+++	Glutarate	+++
Isovalerate	+++	Benzoate	-
Methanol	-	3-hydroxy benzoate(3)	-
Ethanol	+++	4-hydroxy benzoate(3)	-
Propanol	+++		

Identification of Strain Ok2mb.

Irregularly-shaped cells occurring in large sarcina-like packets in liquid media have been assigned to the genus *Desulfosarcina* (Widdel and Pfennig 1984). One species, *D. variabilis*, has been isolated from marine sediment and will not grow on media containing less than 1% NaCl and 0.2% $MgCl_2.6H_2O$. As the name indicates, *D. variabilis* can use a wide range of organic compounds as electron donors for sulfate reduction. It is also able to grow autotrophically on H_2/CO_2

or formate. In addition, growth is possible on lower and fatty acids, alcohols, other organic acids and aromatic compounds.

The morphology of strain Ok2mb would tentatively place this strain in the genus *Desulfosarcina*. The range of compounds that strain Ok2mb was able to use supports its indicated identification. Strain Ok2mb was however unable to use benzoate. The use of aromatic compounds is a characteristic of the genus *Desulfosarcina*. Further study of this strain is required to allow complete identification.

5.3.4 DESCRIPTION AND DISCUSSION OF STRAIN Okib.

Characterization of Strain Okib.

General Characteristics.

Strain Okib was an oval-shaped organism (1.5-2 x 2.5-3.5 μm). The sizes of the cells occasionally varied in a culture (Plate 5.1, 5.2). Slow motility was observed in young cultures when observing wet-mount preparations. Cultures examined with negative phase contrast microscopy did not contain granules typical of PHB and so were not subjected to Sudan black staining procedures. Refractile granules indicative of endospores were never observed.

The response of Okib to different temperature is shown in figure 5.4. Fastest growth took place at 28°C. Slower growth took place at 25°C and only very slight growth at 17°C after 390 hours incubation. No growth took place at 34°C indicating the steep decline in growth at temperatures greater than 28°C.

Figure 5.4 Growth response of strain Okib to temperature

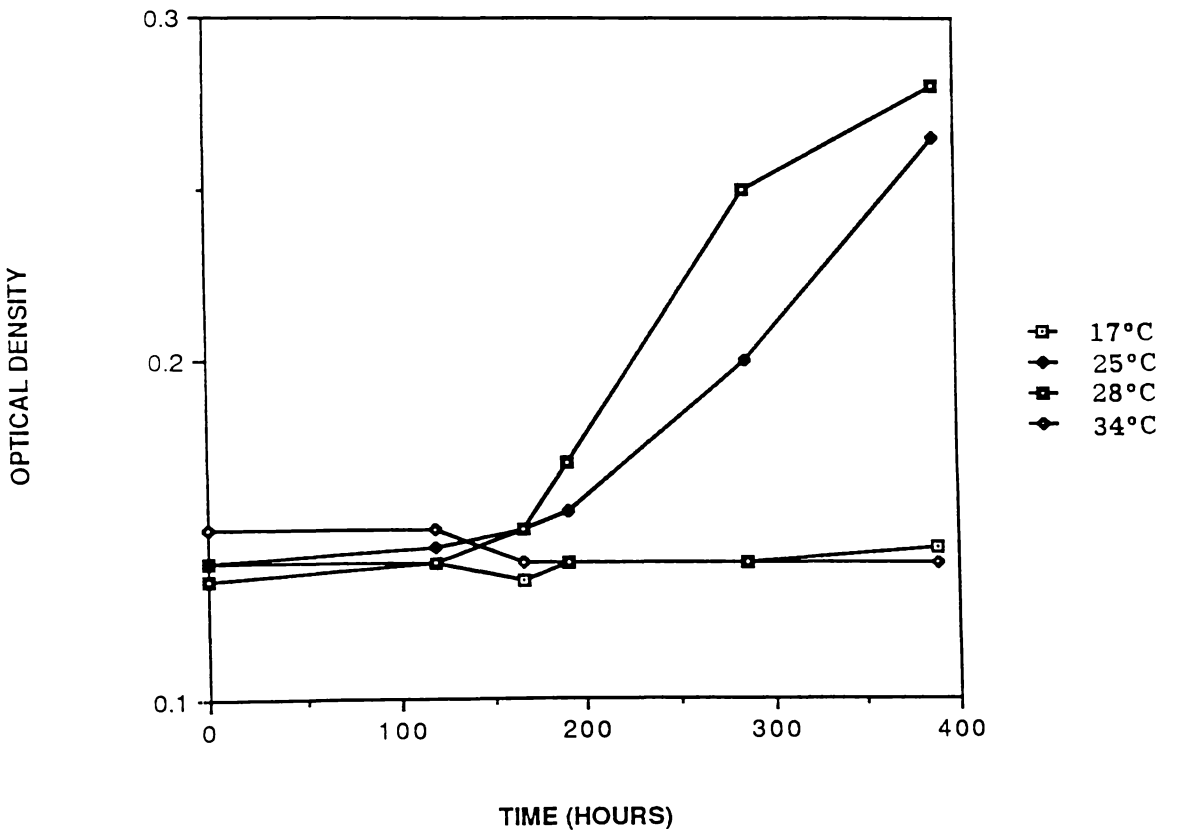
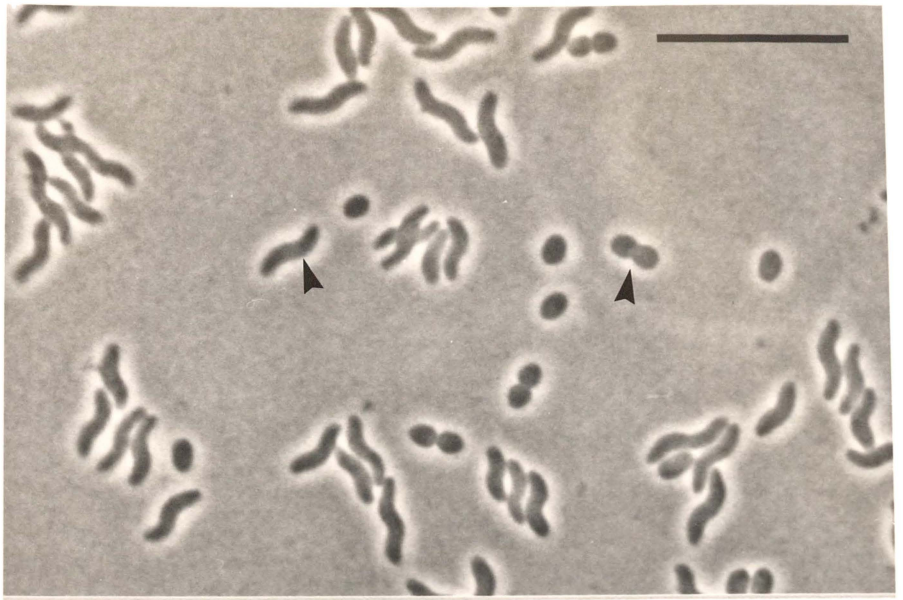


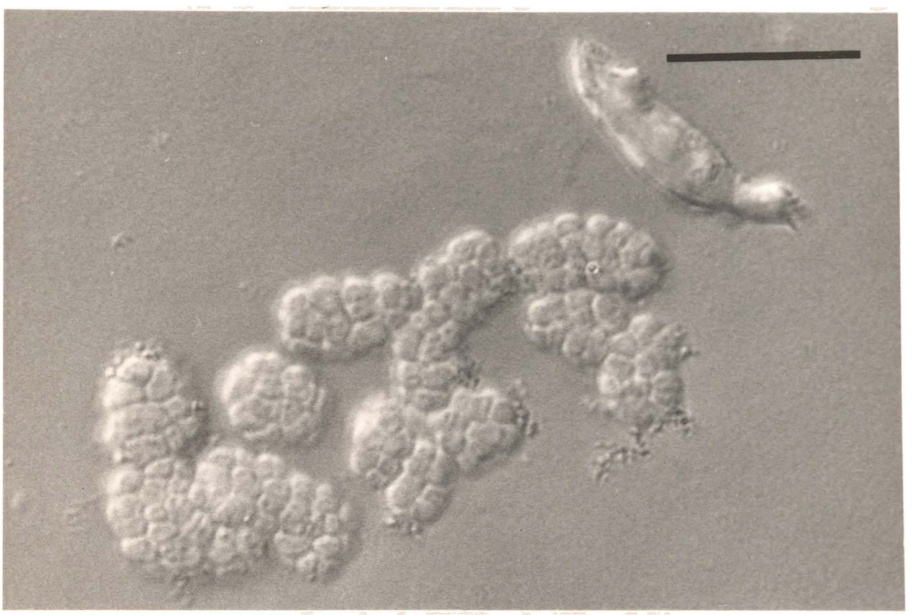
Plate 4.
Sulfate-reducing bacteria isolated which oxidize branched-chain fatty acids

4.1 Phase contrast micrograph of strain Amib. Arrow indicates a refractile spore. (Bar = 10μm).

4.2 Interference contrast of strain Ok2mb. (Bar = 10μm)

4.3 Phase contrast micrograph of Am2mb culture. Arrows indicate the two morphologies present. See text for details. (Bar = 10μm)





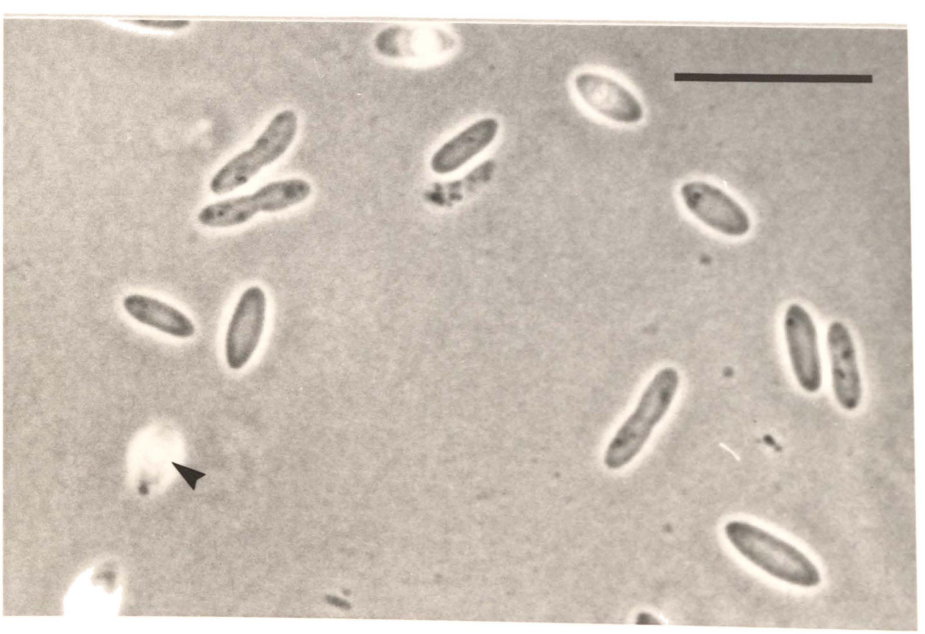
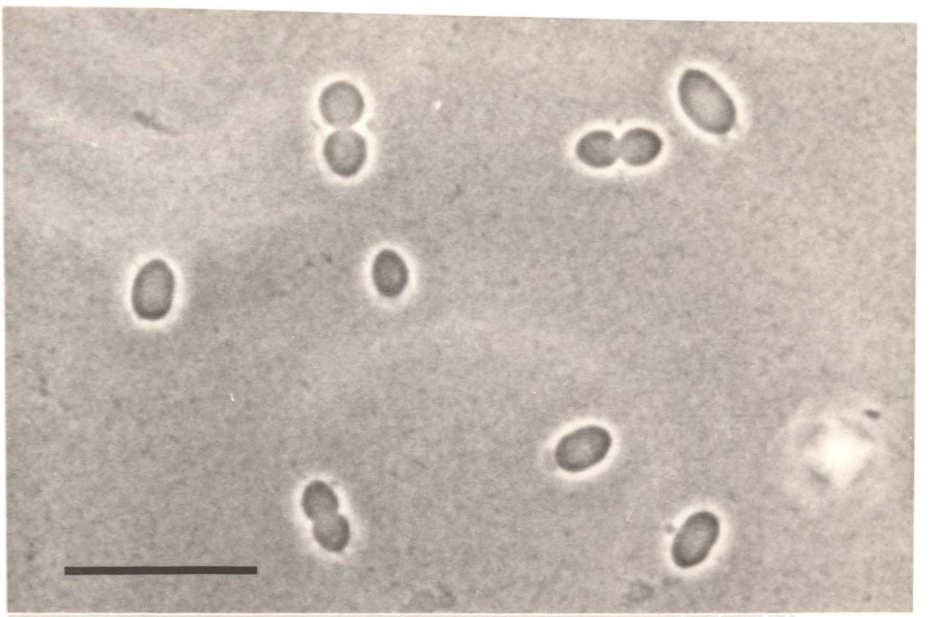
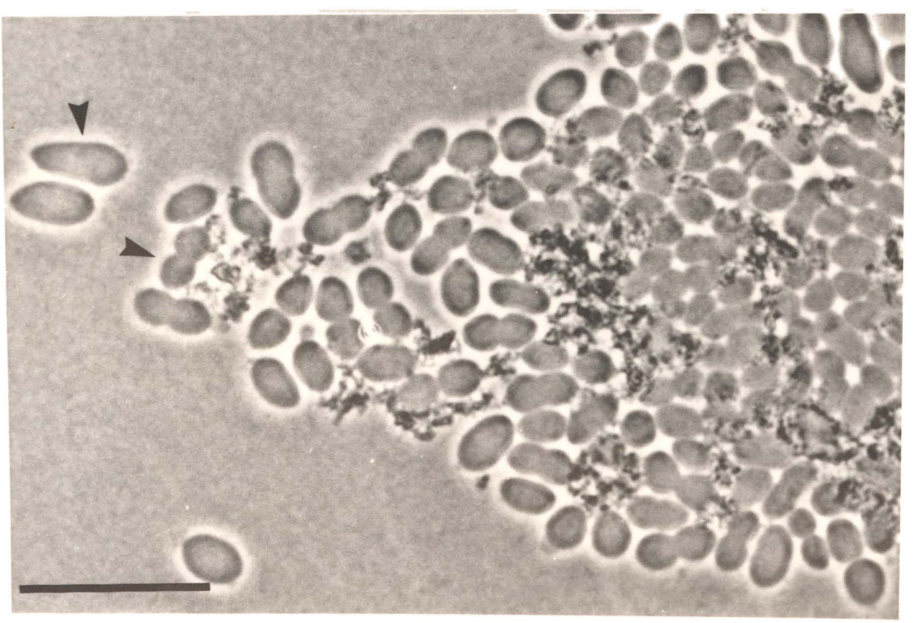


Plate 5.
Sulfate-reducing bacteria isolated which oxidize branched-chain fatty acids (continued)

5.1 Phase contrast micrograph of strain Okib. (Bar = 10μm)

5.2 Phase contrast micrograph of strain Okib. Arrows indicate the possible variation in morphology. (Bar = 10 μm)





Compounds Used as Election Donors by Strain Okib.

Strain Okib was able to grow on an extensive range of carbon compounds. The complete list of compounds tested and growth response can be seen in table 5.10. Numbers in brackets refer to concentration used (mM).

Table 5.10 Compounds Tested for use as Electron Donors by Strain Okib.

Electron Donor	Growth	Electron Donor	Growth
H_2/CO_2	+++	Methanol(10)	-
Formate(10)	-	Ethanol(10)	+++
Acetate(10)	+	Propanol(10)	+++
Propionate(10)	+	Butanol(10)	+++
Butyrate(10)	+++	Glycerol(10)	++
Isobutyrate(5)	+++	2,3 Butanediol(10)	-
Valerate(5)	+++	α-keto glutarate(10)	+++
2methyl butyrate(5)	+++	α-ketoisocaproate(10)	+++
Isovalerate(5)	+++	α-Ketoisovalerate(10)	-
Hexanoate(5)	+++	Citrate(10)	+++
Heptanoate(0.5)	+++	Isocitrate(10)	-
Octanoate(0.5)	+++	Phenyl acetate(3)	-
Nonanoate(0.5)	+++	3-hydroxy benzoate(3)	-
Decanoate(0.5)	+++	4-hydroxy benzoate(3)	-
Dodecanoate(0.5)	+++	Benzoate(3)	-
Tetradecanoate(1)	+++	Resorcinol(0.5)	-
Hexadecanoate(1)	+++	Phenol(1)	-
Octadecanoate(1)	+	Catechol(0.5)	-
Fumarate(5)	+++	Hydroxy quinoline(0.5)	-
Malate(5)	+++	Pyrogallol(0.5)	-
Tiglate(10)	+++	Cinnamate(2)	-
Crotonate(10)	+++	Imidazole(10)	-
Hydroxy butyrate	+++	Nicotinate(10)	-
Tartrate(10)	-	Nicotinamide(10)	-
Oxalate(10)	-	Indole (1)	-
Malonate(10)	-	Lactate(10)	+++
Succinate(10)	+++	Pyruvate(10)	+++
Pimelate(5)	+++		
Glutarate(3)	+++		

Growth took place on all those fatty acids tested though growth was poor on octadecanoate. Growth on acetate was very slow; cultures took

about 3 weeks to reach an optical density of 0.1 on 10mM acetate. No growth was observed on formate. Strain Okib was taken through 5 transfers (1% inoculum) in medium containing H_2/CO_2 as the only energy and carbon source. On the 5th transfer, the optical density of a 2 week old culture grown according to the method described (section 3.10) without replacement of H_2/CO_2 was 0.04 and had produced approximately 3mM sulfide showing that strain Okib was capable of autotrophic growth. Okib was able to grow on the dicarboxylic acids tested except for oxalate and malonate. Growth was also possible on a number of other small organic acids such as malate, fumarate, crotonate and tiglate. A number of amino acids and amino compounds were shown to support growth and are discussed in greater detail below. Of the alcohols tested, methanol was the only one that could not support growth. None of the aromatic compounds tested (including phenylalanine and tyrosine, details below) supported growth.

Alternative electron acceptors.

Okib was shown to use thiosulfate, sulfite and sulfate as terminal electron acceptors. Nitrate, sulfur and oxygen were not used. It was shown that Okib could grow on fumarate in the absence of sulfate (below), making it difficult to test for its possible use as a terminal electron acceptor as media containing butyrate and fumarate with no sulfate could still be supplying a fermentable substrate. Substrate metabolism patterns were monitored using HPLC of a culture in medium containing butyrate plus fumarate with no sulfate. Growth occurred, but only at the expense of fumarate; fumarate did not act serve as an electron acceptor for butyrate oxidation.

Growth in the Absence of Sulfate.

Strain Okib was able to ferment fumarate, pyruvate and malate in the absence of sulfate. Lactate, succinate, glucose and glutarate were not fermented.

Cell Pigments.

The fluorescence test and spectral scans of cell-free extracts was used to investigate the presence of desulfoviridin in strain Okib. *Desulfovibrio vulgaris* was used as the positive control. No fluorescence was seen after addition of the sodium hydroxide to Okib cell pellets. In addition, no characteristic peak at 630 nm was observed as compared with *Desulfovibrio vulgaris* confirming the absence of desulfoviridin in Okib.

Difference spectra (oxidized minus reduced) were obtained for strain Okib in order to determine the presence and type of cytochromes. The absorption maxima are seen in Table 5.11.

Table 5.11 Absorption Maxima of Difference Spectra (oxidized minus reduced) of Cell-free Extracts from Strain Okib.

	Absorption Maxima (nm)		
	α	β	γ
Okib	554	524	421
Cytochrome c	553	523	423
Cytochrome b	629	556	527

In addition to the three major peaks referred to in table 5.11, a small peak was observed at 630 nm. These results indicate that strain Okib probably possesses both c- and b-type cytochromes.

Growth Response to Sodium Chloride.

Because strain Okib was isolated from an estuarine source and so its dependence on NaCl was tested by inoculating into tubes containing each of the freshwater, estuarine and marine media. Growth curves were obtained for each of the regimes. Results of response to different media are seen in fig 5.5 (over page).

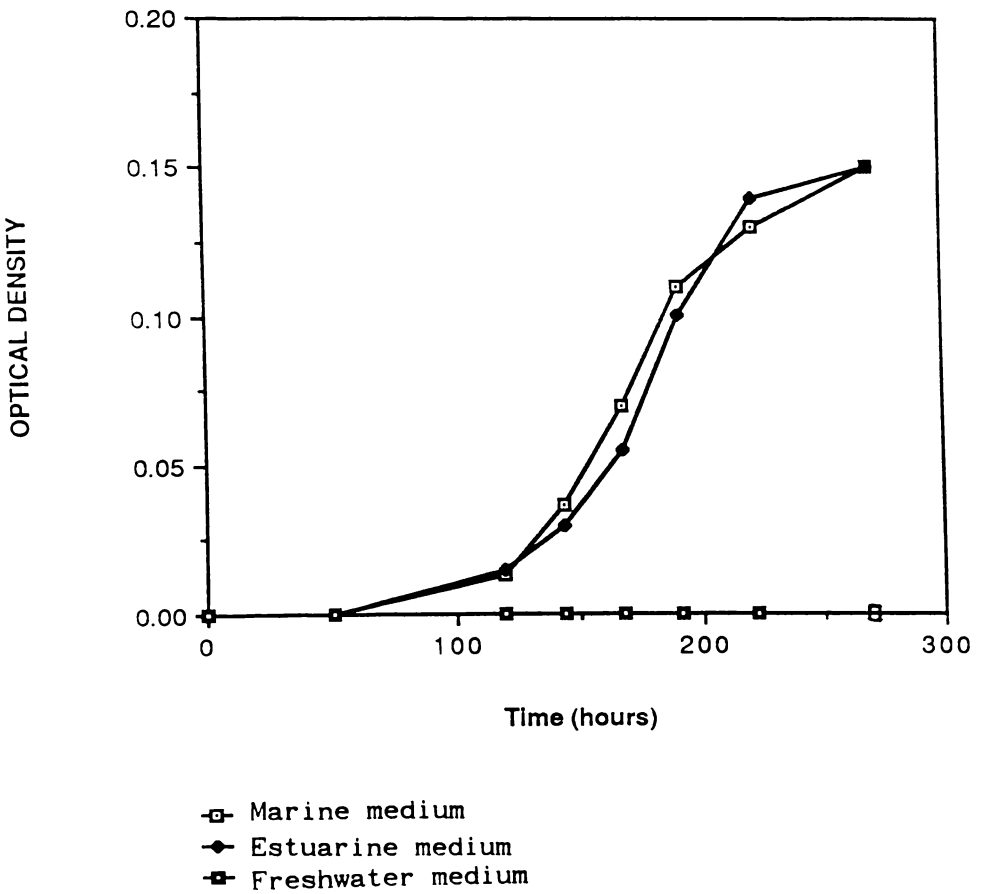
Further Studies on Strain Okib.

Growth on Amino Acids.

Okib was isolated using isobutyrate as the sole carbon and energy source. Having shown that Okib could grow on branched chain fatty acids, it was decided to test Okib for growth on the corresponding branched-chain amino acids. In anaerobic environments, branched-chain fatty acids are usually produced via deamination of the appropriate branched amino acid (i.e. valine yielding isobutyrate, isoleucine yielding 2-methylbutyrate and leucine yielding isovalerate). Having shown that growth occurred on these amino acids, a wider range of amino acids were tested for their ability to support growth. Those tested also included a number of amino substituted fatty acids that do not naturally occur in proteins e.g various amino-substituted valerates. The results are presented in table 5.12. All amino acids were used at 10mM.

The three branched amino acids, (valine, leucine and isoleucine) supported growth. However, there was no obvious pattern to the other amino acids used by Okib either based on their precursors for biosynthesis or on their charge. Of those amino acids used, four (valine, leucine, isoleucine and proline), had non-polar R-groups. It was notable that whereas glutamate was used, aspartate was not.

Figure 5.5 Growth of strain Okib in media with different salinities



This was thought interesting due to similarities in structure and metabolism of these two compounds. Failure to use glutamine was also seen as interesting as glutamine and glutamate are readily interconverted within the cell. Strain Okib presumably lacked the appropriate uptake system for glutamine.

Table 5.12 Amino Compounds Tested For Use As Electron Donors By Strain Okib.

Electron Donor	Growth	Electron Donor	Growth
Glycine	-	Phenylalanine	-
Alanine	+++	Tyrosine	-
Valine	++	Tryptophan	-
Leucine	++	Aspartate	-
Isoleucine	++	Glutamate	+++
Threonine	-	Histidine	-
Serine	-	L-lysine	++
Asparagine	-	Arginine	-
Glutamine	-	Ornithine	++
Cysteine	-	2 amino n-butyrate	-
Methionine	-	2 amino isobutyrate	-
Proline	+++	4 amino butyrate	+++
Hydroxy proline	-	2 amino valerate	-
		5 amino valerate	++

It was thought interesting that Okib should not grow using glycine as it is the simplest amino acid. Glycine is generally metabolized via serine to pyruvate (Bender 1985). Glycine has also been shown to be fermented to acetate by *Peptococcus anaerobius* (Douglas 1951) and was originally thought to be metabolized via pyruvate and serine to acetate. Subsequent studies have led to the suggestion that metabolism of glycine does not proceed via serine and pyruvate but instead via methyltetrahydrofolate intermediates (McInerney 1988).

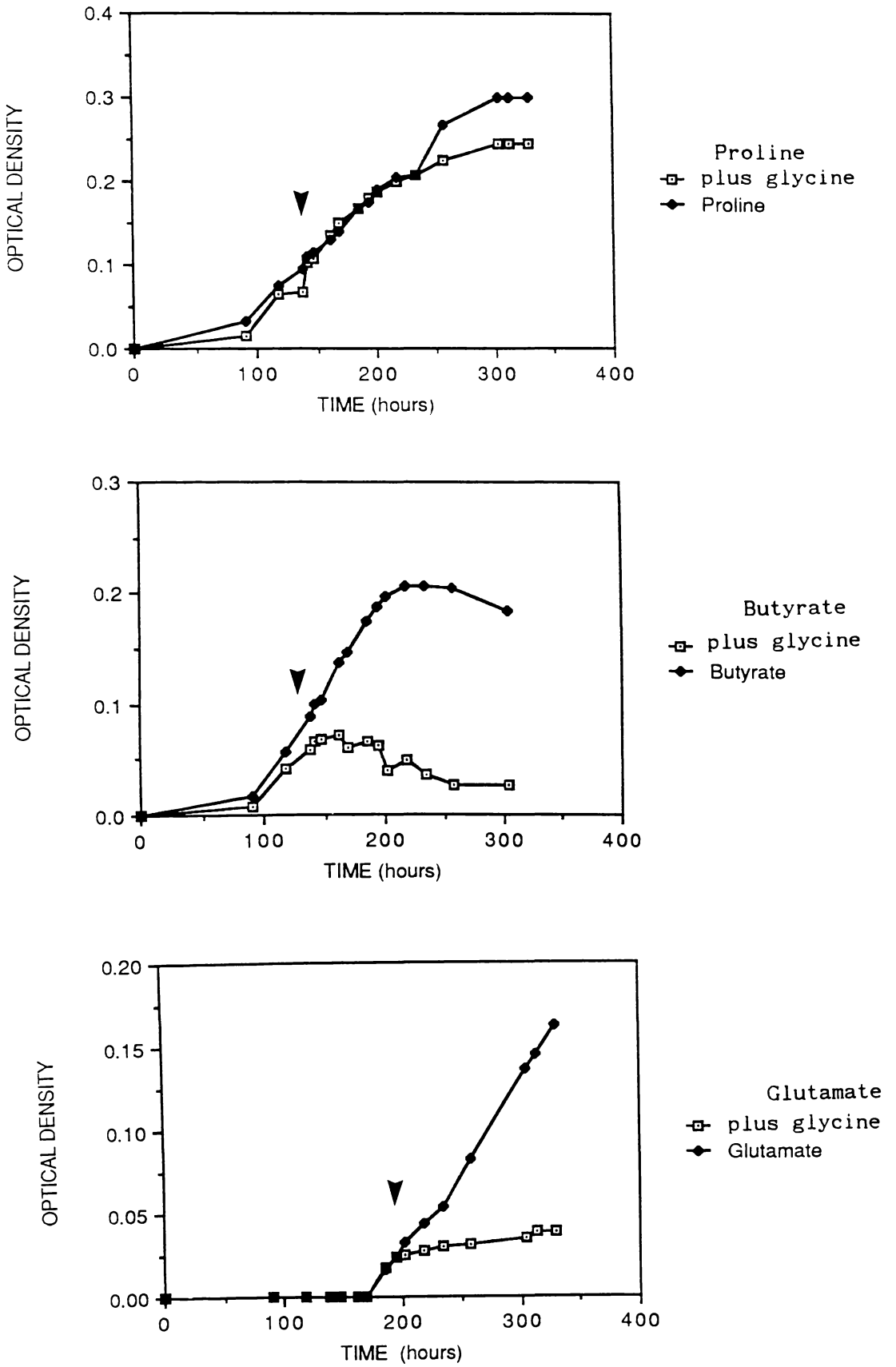
Experiments were carried out to investigate the effect of glycine on growing cells. In the first experiment, media were prepared which

contained glutamate, glycine and glutamate plus glycine. Three tubes of each medium were inoculated, incubated at 28°C and growth rates determined. As expected, growth took place on the medium containing glutamate alone and no growth took place on glycine. That no growth took place on medium containing both glutamate and glycine suggested that glycine had an inhibitory effect on glutamate metabolism, either by glycine competing for absorption sites of glutamate and so blocking glutamate uptake, or the glycine entering the cell and inhibiting glutamate metabolism inside the cell. This phenomenon was investigated further by observing the effects of adding glycine to actively growing cultures. Okib had been shown to grow on glutamate, proline and butyrate and media were therefore prepared with proline (10 mM), butyrate (10 mM) and glutamate (10 mM). Tubes were inoculated and growth rates measured. When cells were in early log phase, glycine was added to give 10 mM final concentration. For each of the compounds, five replicates were used. At the point indicated, glycine was added to one of the series of five tubes containing each of the carbon sources and the growth rates were further monitored. Results are presented in figures 5.6a, b and c.

Addition of glycine to actively-growing cultures produced different results depending on which of the three carbon sources was present. There was no effect on growth when glycine was added to the culture growing on proline. When added to a culture growing on glutamate there was a dramatic reduction in growth rate. The optical density increased very slowly over the remaining experimental time indicating at least some of the cell were still viable. The long lag time with the glutamate culture was possibly due to the inoculum having been grown on proline. Addition of glycine to the tubes containing butyrate resulted in a decline in optical density. This was

Figure 5.6 Effect of glycine added to actively growing cultures of strain Okib

Arrow indicates the time at which glycine was added to one series.



unexpected; indeed, this part of the experiment was originally included as a positive control.

The inhibitory effect that glycine had on cells growing on glutamate or butyrate is difficult to explain. Initially, it was assumed that Okib did not grow on glycine for the simple reason that it lacked an appropriate transport mechanism. Okib had been shown to grow on defined medium containing ammonium ions as the sole source of nitrogen. The isolate therefore possessed the enzymes necessary for the synthesis and turnover of all cellular amino acids, including glycine. It has long been recognized that the metabolism of amino acids is a tightly controlled process and that the metabolism of any one amino acid may be markedly affected by the presence of another amino acid, not necessarily related by either structure or metabolism.

Glutamate metabolism had shown to be inhibited by glycine in *Thermus* sp. (Janssen. personal communication) as was observed with strain Okib. Glutamine synthetase, the enzyme that catalyzes the conversion of glutamate to glutamine, has a number of allosteric inhibitors. Although glycine is not a direct product of the reaction, it is a strong inhibitor of the enzyme. It has been recognized that the mechanism of control of enzymes involved in the metabolism of amino acids, not only depends on the amino acid, but on the individual micro-organism. Biotechnologists have long recognized the complexity and that one cannot extrapolate from one genus to another (Crueger and Crueger 1984).

Fermentation of Amino Acids in the Absence of Sulfate.

Media were inoculated which contained lysine (20mM), proline (10mM) and casamino acids (1g/l) as carbon sources, each in the absence of sulfate. Tubes were left for at least 2-3 weeks at 28°C. The lysine tubes were incubated for 4 weeks as growth on lysine plus sulfate had been shown previously to be slow. No growth was observed with these substrates in the absence of sulfate.

Okib was also tested for its ability to ferment Stickland pairs of amino acids. Sulfate-free medium was prepared to which a variety of potential Stickland pairs of amino acids were added. Numbers in brackets are concentrations in mM. Those tested were alanine(10)/-glycine(20), isoleucine(5)/ proline(10), valine(5)/ proline(10), histidine(10)/ proline(10), cysteine(10)/ serine(10). For the alanine/glycine pair, selenite/tungstate trace element solution (section 3.6) was included as the glycine reductase complex is known to have a selenium requirement (Tanaka and Stadtman 1979). No growth took place on the alanine/glycine, histidine/proline and serine/cysteine pairs. Very faint growth (OD_{660} of about 0.02), took place on the valine/proline pair and also the isoleucine/proline pair. After 3 weeks incubation those tubes showing very faint growth were transferred to fresh medium and incubated for a further 3 weeks. No growth took place in these subcultures. It was concluded that strain Okib could not carry out Stickland fermentation of those amino acid pairs that were tested,

Okib was examined to see if it was capable of fermenting proline in a syntrophic association with *Methanospirillum hungatei* as the hydrogen-utilizing partner. Medium with proline(10mM) and no sulfate

were inoculated and monitored daily for 3 weeks. No growth took place with Okib and proline under syntrophic conditions.

Growth on Proteinaceous Compounds by Okib.

Separate media containing gelatin (5%), soluble casein (0.2%), BBL Gelysate (0.1%), BBL Trypticase (0.1%) and casamino acids (0.1%) were inoculated and incubated at 28°C. Quantitative measurements were made of Okib grown on media containing casamino acids, Trypticase and Gelysate while qualitative measurements were made of growth on the other substrates. The results presented in table 5.13 are averages of five tubes.

Table 5.13 Use of Protein and Protein Lysates by Strain Okib.

Substrate	Growth	OD_{660}
Casein	-	ND
Trypticase	+	0.04
Casamino acids	+	0.14
Gelatin	-	ND
Gelysate	+	0.03

ND = not determined

No growth took place on soluble casein. Trypticase, an enzymatically hydrolyzed casein product supported growth giving an OD_{660} of 0.04. Completely hydrolyzed casein, in this case casamino acids, also supported growth. Approximately 3 times the growth was obtained from the same amount of casamino acids as compared to trypticase. No growth was observed on gelatin. Gelysate, an enzymatically hydrolyzed gelatin product supported growth, giving an OD_{660} of 0.03.

Substrate Oxidation Balances.

Figure 5.7 shows the growth response with respect to time when Okib was grown on 5 mM isobutyrate. Isobutyrate was oxidized with the production of acetate. When all the isobutyrate was used, acetate concentration decreased while optical density and sulfide concentration increased. Quantitative analyses of substrate utilization were carried out with Okib cultures grown on butyrate and isobutyrate which were incubated for six weeks at 28°C. The extended incubation would allow time for complete oxidation of the fatty acid. Results are shown in table 5.14.

Table 5.14 Stoichiometry of Butyrate and Isobutyrate Oxidation By Strain Okib After Six Weeks Incubation.

Substrate	Butyrate	Isobutyrate
Substrate Added	5 mM	5.1 mM
Substrate Remaining	0 mM	0 mM
Substrate Used	5 mM	5.1 mM
Acetate Produced	2.8 mM	0 mM
Sulfide Produced	10.2 mM	10.85 mM

Analysis of oxidation of butyrate showed in the production of acetate while isobutyrate was oxidized with no acetate present after 6 weeks incubation. The product of complete oxidation is CO_2 but was not measured in this experiment.

Oxidation of butyrate by *Desulfobacterium autotrophicum* and acetate oxidation by sulfate-reducing bacteria have been shown to take place according to the following respective formulae 1 and 2 (Schauder *et al.*1986:

1. $1\text{Butyrate} + 1.5SO_4^{2-} \longrightarrow 1\text{Acetate} + 1.5HS^- + 2CO_2 + 2H_2O$

2. Acetate + $SO_4^{2-} \longrightarrow 2HCO_3^- + HS^-$

Okib had been shown to grow slowly on acetate; thus, if it oxidized butyrate according to equation 1 above, it could still grow on the acetate produced when all the butyrate was exhausted. If this were the case, the stoichiometric data above could be linked with the equations 1 and 2 for both butyrate and acetate oxidation to give:

1. $5CH_3(CH_2)_2COOH + 7.5SO_4^{2-} \longrightarrow 5CH_3COOH + 7.5H_2S$

2. $2.2CH_3COOH + 2.2SO_4^{2-} \longrightarrow 4.4HCO_3^- + 2.2H_2S$

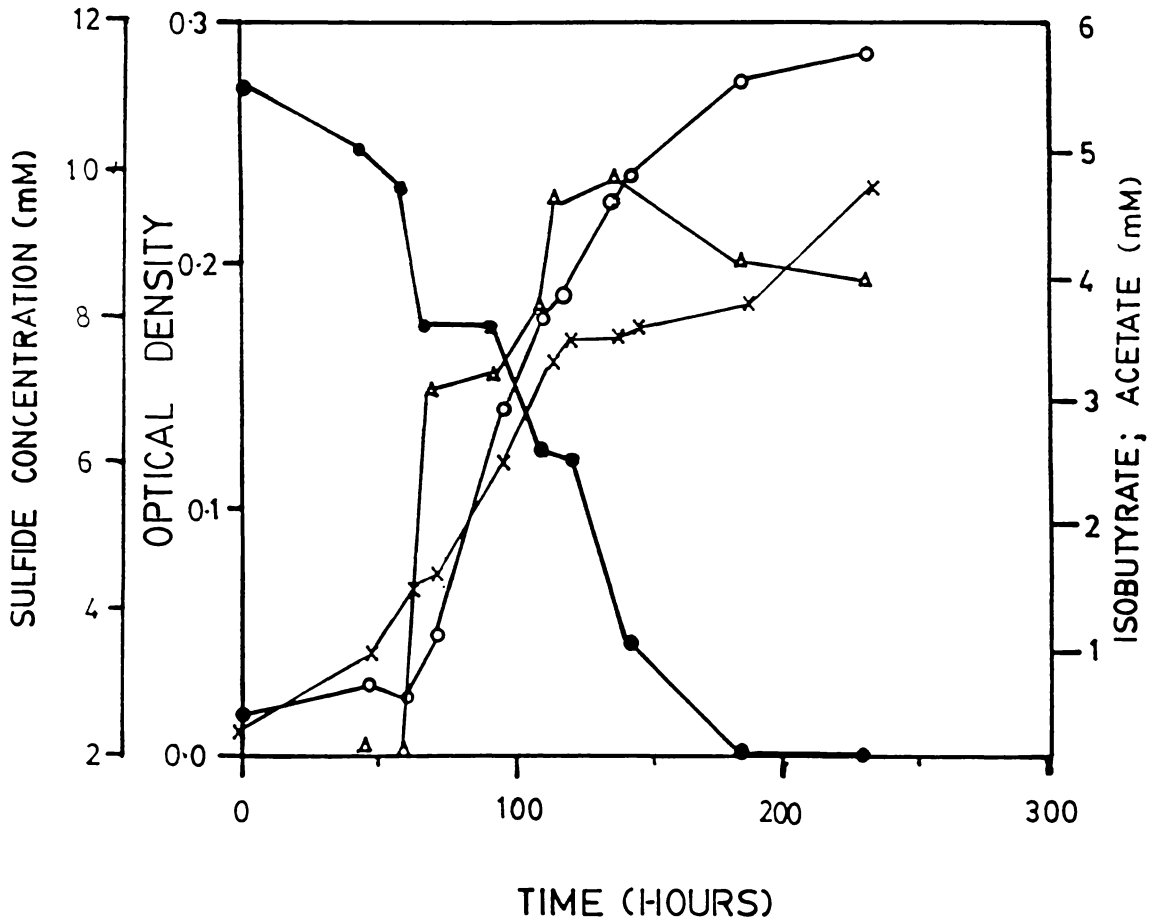
1.+ 2. $5CH_3(CH_2)_2COOH + 9.7SO_4^{2-} \longrightarrow 2.8CH_3COOH + 9.7H_2S$

The measured sulfide produced was 105% that of the theoretical value so the overall equation is in good agreement with the stoichiometric data.

The isobutyrate growth curve (figure 5.7) showed production of acetate which was subsequently used as shown in table 5.14 above. For isobutyrate oxidation to proceed according to the equation of Schauder *et al.* (1986), above, isomerization of isobutyrate to butyrate must first take place. If isobutyrate oxidation proceeded according to the theoretical equations 1 and 2 above, 5.1 mM isobutyrate oxidation should lead to 12.5 mM sulfide; the value obtained in this experiment was only 10.85 mM. The stoichiometric data suggest that oxidation of isobutyrate was in good agreement with the overall equation 3 below as opposed to the combined formulae 1 and 2 above:

3. $(CH_3)_2CHCOO^- + 2SO_4^{2-} \longrightarrow 4CO_2 + 2H_2S + 2H_2O$

Figure 5.7 Growth curve of strain Okib growing on isobutyrate. Isobutyrate (●-●), Acetate (Δ-Δ), Sulfide (O-O), Optical Density (X-X)



This equation does not resemble any other formula described in the literature.

Quantitative measurements were made of proline degradation by Okib. Analysis of the culture supernatant using GC and organic acid HPLC showed no production of organic acids. However, when PITC-derivatized amino acid analysis using HPLC was carried out, in addition to measuring proline disappearance, a metabolic intermediate identifiable as an amino compound was also detected in the culture supernatant at a concentration of 0.2-0.25 mM. Results of stoichiometric analyses are seen in Table 5.15.

Table 5.15 Stoichiometry of Proline Oxidation by Strain Okib.

Proline Added (μmol)	Proline Used (μmol)	Dry Weight (mg)	Sulfide Produced (μmol)	Ammonia Produced (μmol)
881	765	12.4	1400	380

The amount of ammonia produced is less than that expected from the complete oxidation of proline. A metabolic intermediate was detected (20-25 μmol). From the stoichiometric measurements, oxidation of proline appears to proceed according to the formula:

$$C_5H_9NO_2 + 2SO_4^{2-} \longrightarrow 5CO_2 + 2H^+ + 2H_2S + NH_3$$

Identification of Strain Okib.

The general characteristics of strain Okib would indicate it to be a member of the genus *Desulfobacterium*. It is important to compare its characteristics with other species of this genus. Okib was not able

to use phenol, catechol, indole or any of the aromatic compounds used by *D. phenolicum*, *D. catecholicum* or *D. indolicum* (Bak and Pfennig 1986b, Szewzyk and Pfennig 1987, Bak and Pfennig 1986a). Two further species of *Desulfobacterium* have been referred to in the literature, *D. autotrophicum* (Brysch *et al.* 1987) and *D. vacuolatum* (Widdel 1988). The latter is regarded as an unofficial species as no official publication describing its characteristics in a scientific journal has been made. Details of strain Okib, *D. autotrophicum* and *D. vacuolatum* are described in table 5.16. Additional characteristics were determined for these three organisms and are presented in table 5.17.

Table 5.16 Summary Of Characteristics of Okib, *Desulfobacterium autotrophicum* and *Desulfobacterium vacuolatum*.

Characteristic	Okib	*D. vacuolatum**	*D. autotrophicum**
Shape	Oval	Oval/Sphere	Oval
Width (μm)	1.5	1.5-2	.9-1.3
Length (μm)	2.5-3.5	2-2.5	1.5-3
Vacuoles	-	+	-
Motility	+	-	+[1]
G+C Content (mol%)	45	45	48
Desulfoviridin	-	-	-
Complete Oxidation	+	+	+
Enrichment Substrate	ib	ib	H_2/CO_2
Substrates Used			
H_2/CO_2	+	+	+
Formate	-	+	+
Propionate	(+)	+	(+)
C_4-C_{16}	+	+	+
C_{18}	+	-	-
Isobutyrate	+	+	+
2 methylbutyrate	+	(+)	+
Isovalerate	+	(+)	-
Ethanol	+	(+)	+

Symbols: [1], Strain dependant. +, Positive. (+) Poor growth
-, Negative. ib, isobutyrate.
*, From Widdel F (1988)

Table 5.17 Additional Tests Carried Out For Comparison of Okib, *D. vacuolatum* and *D. autotrophicum*. Brackets indicate concentration (mM)

Substrate	Okib	*D. vacuolatum*	*D. autotrophicum*
Substrates Used			
Proline(10)	+++	+++	-
Casamino Acids(0.1%)	+++	+++	-
Glycine (10)	-	-	-
4 amino butyrate(10)	+++	+++	ND
Glutamate(10)	+++	+++	ND
3-Hydroxy benzoate(3)	-	-	ND
α-keto glutarate(10)	+++	+++	ND
5 amino valerate	+++	++	ND
Growth at different salinity*			
Freshwater	-	-	-
Estuarine	+++	-	ND
Marine	++	+++	+++

ND Not determined, * Salinity refers to media in section 3.3

The characteristics indicate Okib is a strain of the species *Desulfobacterium vacuolatum*. The differences between these two organisms are: failure of Okib to produce vacuoles under any of the growth conditions tested, inability of Okib to use formate, presence of motility in Okib and *D. vacuolatum*'s requirement for marine medium whereas Okib can grow equally well in estuarine and marine media. As strain Okib does not produce vacuoles, the specific name for the unofficially described *D. vacuolatum* is undesirable.

Proposed Metabolic Pathways in Strain Okib.

The following pathways for substrate oxidation are based on interpretation of data obtained on Okib as compared to previously published data. Enzymatic analyses and isotope-labelling experiments were not carried out. Determination of end products has been useful in following metabolic pathways in fermentative organisms; however,

this is not so useful with sulfate-reducing bacteria and in particular strain Okib as it oxidizes its substrate completely to CO_2 without the production of easily measurable intermediates.

Oxidation of butyrate.

Butyrate oxidation proceeds via two mechanisms in sulfate-reducing bacteria. Oxidation can proceed by β-oxidation whereby complete oxidation of butyrate occurs according to the formula (Widdel 1980):

$$CH_3(CH_2)_2COO + 3.5SO_4^{2-} \longrightarrow 8HCO_3^- + 3.5HS^- + H^+$$

In *Desulfobacterium autotrophicum*, butyrate oxidation proceeds via a modified pathway (Schauder *et al.* 1986). In *D. autotrophicum*, butyrate is activated via CoA transfer from acetyl-CoA which is formed from butyryl-CoA by β-oxidation. As a consequence, one acetate is formed per butyrate consumed. The oxidation of 1 mol butyrate involves the production of 1.5 mol sulfide.

The stoichiometry of butyrate oxidation in strain Okib (above), suggests that this latter modified β-oxidation pathway is operative as 1 mol acetate and 1.5 mol sulfide were produced per mol butyrate oxidized.

Oxidation of isobutyrate.

Oxidation of isobutyrate has been studied in methanogenic systems where it has been suggested that butyrate is produced as an intermediate in isobutyrate metabolism (Lovley and Klug 1982, Zinder *et al.* 1984). For butyrate to be produced as an intermediate, isomerization of isobutyrate must take place. Tholozan *et al.* (1988) demonstrated a reversible isomerization, which involved the

migration of the carboxyl group. In the methanogenic consortium which they studied, butyrate was detected in the culture supernatant when growing on isobutyrate; they also showed that isobutyrate was produced transiently when the culture was growing on butyrate hence the proposal of a reversible isomerization.

If oxidation of isobutyrate involves its initial isomerization to butyrate, then the end products from oxidation of isobutyrate should be the same those as from butyrate; thus one would expect 1 mol isobutyrate to yield 1 mol acetate plus 1.5 mol sulfide. The growth curve experiment carried out on Okib using isobutyrate showed acetate was produced as an end product. The stoichiomety of isobutyrate oxidation when incubated for six weeks showed this acetate was subsequently oxidized. The amount of sulfide produced from the 510 μmol oxidized isobutyrate was 1085 μmol. The theoretical yield of sulfide production for oxidation if isobutyrate is first isomerized to butyrate is 1250 μmol, the measured amount was some 87% of this theoretical value. It is difficult to draw accurate conclusions as to the probable pathway as the sulfide measured, though inconsistent with the theoretical value, could not be regarded as being too great to discount oxidation via butyrate. The fact that butyrate was not detected in the cultures also should not discount oxidation via butyrate as it may be turned over rapidly with the cell. To determine the exact pathway for isobutyrate oxidation in Okib, a combination of isotope-labelling experiments and enzymatic analyses are required.

Oxidation of amino acids.

Most study of proline catabolism has been carried out under aerobic conditions with study of anaerobic catabolism being restricted to the

study of proline in Stickland pair fermentation. Proline is synthesized from glutamate by oxidation of glutamate to glutamic-γ-semialdehyde which spontaneously cyclizes to Δ'-pyrroline-5-carboxylate. Reduction of this molecule yields proline (Bender 1985). The major pathway for catabolism of proline is oxidation to glutamate. However, the enzymes for proline synthesis do not act in reverse under physiological conditions. Proline is oxidized to Δ'-pyrroline-5-carboxylate by proline oxidase which undergoes dehydrogenation to glutamate. Proline oxidase requires oxygen and cytochrome c and so an alternative pathway must be used by anaerobes. The study of anaerobic metabolism of proline has centered around its role as an electron acceptor in Stickland fermentation where it has been shown to undergo racemization to D-proline followed by reduction to 5-aminovalerate.

Glutamate, glutarate, oxo-glutarate, 5-aminovalerate and proline are all easily interconverted, their oxidation in aerobes proceeding via oxo-glutarate and the TCA cycle. An operative TCA cycle in sulfate-reducing bacteria has been demonstrated only in *Desulfobacter* spp. and so it is likely that oxidation of these compounds proceeds by an alternative pathway. HPLC-amino acid analysis demonstrated the presence of an amino compound in the supernatant of a culture of Okib when grown on proline. As only one amino compound was detected, it was postulated that the proline probably underwent ring cleavage to produce a straight-chain amino compound which was then deaminated. Other related compounds used by Okib include 5-aminovalerate and glutarate whereas 2-aminovalerate was not used. Taken together, these observations suggest that proline oxidation proceeds via an initial reduction to 5-aminovalerate followed by oxidation to

glutarate semialdehyde and then to glutaric acid. The pathway for glutarate oxidation was not studied.

Insufficient data were obtained to speculate as to the possible pathway of the oxidation of branched-chain amino acids. It was shown that each of the corresponding branched fatty acids could support growth and so this is the likely first step in their oxidation. Growth was also shown on tiglic acid, an intermediate in the oxidation of isoleucine. Detailed analyses of enzymes and isotope-labelling experiments would be required to confirm any possible pathways.

CHAPTER SIX

CONCLUSIONS

6.1 THE SULFATE-REDUCING BACTERIA ISOLATED IN THIS STUDY.

During the course of this study, a number of sulfate-reducing bacteria were isolated in pure culture. In the first instance, a number of enrichment techniques were used for the isolation of sulfate-reducing bacteria using palmitate as the carbon-source. The bacteria that were isolated were characterized and identified. The results are summarized in table 6.1. In the second instance, standard enrichments were carried out with branched-chain fatty acids as the carbon-sources. The organisms obtained in pure culture using branched-chain fatty acids are shown in table 6.2

Table 6.1 A Summary of the Sulfate-reducing Bacteria Obtained in Pure Culture Using Palmitate

Enrichment method	Source	Strain designation	Strain assignation
Standard	Am	AmPa2	*Desulfovibrio sapovorans*
	FJ	FJPa1	*Desulfovibrio sapovorans*
	Ok	OkPa1	*Desulfovibrio sapovorans*
Sediment transfer	Am	AmPa1	Insufficient data for identification
	Ho	HoPa1	Insufficient data for identification
Pasteurized sediment	Am	AmPa3	*Desulfotomaculum sapomendens*
13°C	Ok	OkPa1	*Desulfobacterium* sp.
Mud dilution	Ok		*"Desulfovibrio sapovorans"* *"Desulfobacterium* sp."

Table 6.2 A Summary of the Sulfate-reducing Isolated Using Branched-chain Fatty Acids

Substrate	Source	Strain designation	Strain assignation
Isobutyrate	Am	Amib	*Desulfotomaculum acetoxidans*
	Ok	Okib	*Desulfobacterium vacuolatum*
2-methyl butyrate	Am	Am2mb	*Desulfovibrio sapovorans.*
	Ok	Ok2mb	*Desulfosarcina* sp.

6.2 METHODS OF ISOLATION.

The isolation methods used with palmitate showed that it was possible to isolate different organisms using different isolation methods. That palmitate can be used as a compound for selective enrichment of *Desulfovibrio sapovorans* has been well documented, as discussed earlier. This present study has shown that if the enrichment conditions are altered, then sulfate-reducing bacteria other than *Desulfovibrio sapovorans* may be isolated.

Enumeration experiments in this study suggested that *Desulfobacterium*-like bacteria may be numerically dominant. It is also important to consider that the culturing conditions that were used, such as the medium composition etc., may also have some influence as to which organisms might be isolated.

6.3 SUBSTRATES USED IN NATURE.

Desulfovibrio sapovorans is clearly important in the oxidation of palmitate. It is possible however, that in nature, sulfate-reducing

bacteria other than *D. sapovorans* may also be involved in the degradation of palmitate.

Desulfotomaculum acetoxidans was originally isolated using acetate as the carbon source. This study showed that it was also possible to isolate this organism using isobutyrate as the carbon-source. As growth was possible on butyrate, butanol and isobutyrate, *D. acetoxidans* presumably plays a role in the metabolism of C_4-organic compounds

6.4 SUBSTRATES USED BY SULFATE-REDUCING BACTERIA.

This study showed that a more extensive range of amino acids than has hitherto been supposed were used by sulfate-reducing bacteria. These include branched-chain amino acids, proline, amino-substituted fatty acids and keto amino acids. Studies suggested that proline metabolism may proceed via 5-aminovalerate to glutarate.

Sulfate-reducing bacteria were originally thought to grow only on a limited range of compounds such as lactate and pyruvate (Postgate 1984). Widdel (1980) showed that some sulfate-reducing bacteria were able to use fatty acids and some aromatic compounds. Further studies have shown species able to use H_2/CO_2 and acetate which resulted in the suggestion that sulfate-reducing bacteria were important as terminal oxidizers or organic compounds. Results here show that sulfate-reducing bacteria can use an even wider range of compounds, in particular, those normally associated with use by secondary fermenters and acetogenic fermenting bacteria. Sulfate-reducing bacteria can no longer be thought of as simply terminal oxidizers;

they appear also to act in competition for many substrates used by bacteria which would normally ferment such substrates.

6.5 CHARACTERISTICS OF SULFATE-REDUCING BACTERIA ISOLATED.

This study extended the characteristics of the already described 'sapovorans' group. Should strains HoPa1 and AmPa1 be shown to be strains of *Desulfovibrio sapovorans*, then rod-shaped morphologies and differences in metabolism extend the current description of the species. Should these strains be shown to be different from *D. sapovorans*, then a subdivision of the 'sapovorans' group may be required. Any extension to the range of isolates for any of the sulfate-reducing bacteria species is useful; at present we have little idea of the variation of the physical characteristics in many of the "species".

In this study, an organism was isolated which was identified as *Desulfobacterium vacuolatum*. This strain, however, did not form vacuoles and so the species name for this organism would seem inappropriate. Again, with only one isolate of *D. vacuolatum* at present described, we have no idea of the variation of characteristics within the species.

CHAPTER SEVEN

INTRODUCTION TO STUDIES ON THE AGAROLYTIC BACTERIUM

The main aim of this thesis was to investigate aspects of the metabolism of sulfate-reducing bacteria. During the isolation of sulfate-reducing bacteria, an agar shake dilution series showed colonies with a characteristic, spreading, halo-like morphology. The bacteria present were initially assumed to be sulfate-reducing bacteria, but grew more rapidly than did sulfate-reducing bacteria. The organism was rapidly purified and in the course of purification, it became clear that it was not a sulfate-reducing bacterium, but an obligately anaerobic agarolytic bacterium. As there were no reports in the literature concerning such bacteria, it was deemed to be worthy of further study, which was carried out whilst the pure cultures of sulfate-reducing bacteria used for the main part of this study was underway.

The following chapters describe a number of aspects of this study. First is a brief review of the literature concerned with agar; its source, its structure, the uses of agar, the presently known aspects of agar degradation and some further points of interests. Secondly follows an account of the specialist materials and methods that were used or adapted for this study. The experiments and result chapter reports on the characterization of the organism that was obtained in pure culture and the experiments carried out on this organism. Finally, the discussion chapter reports on the identification of the isolated organism and a discussion of the ecology of agar degradation with particular reference to anaerobes.

CHAPTER EIGHT

AGAR AND ITS BACTERIAL DEGRADATION:- A LITERATURE REVIEW

8.1 INTRODUCTION.

The following chapter reports briefly on some of the material that appears in the literature regarding agar. Only information pertaining to this study is reviewed here, such as the sources and use of agar, its degradation by microorganisms and something of the pathway of degradation. A small section on the genetics is also included. It was felt unnecessary to include information on other algal polymers or details of the source seaweeds themselves. A comprehensive review has been published by Chapman and Chapman (1980) on this subject. The structure of agar is complex, and therefore only the basic information is covered here.

8.2 THE SOURCE OF AGAR.

Agar is a polysaccharide obtained from a number of marine red algae. The most important genera of agar-producing algae are species of *Gelidium*, *Gracilaria*, *Pterocladia* spp., *Acanthopeltis japonica* and *Ahnfeltia plicata* (Chapman and Chapman, 1980). The actual sources differ according to the country of origin; for instance Japanese agar is principally obtained from *Gelidium*, New Zealand agar from *Pterocladia* and Russian agar from *Ahnfeltia*.

8.3 AGAR; ITS USE IN MICROBIOLOGY.

Records suggest that Robert Koch was the first micro-biologist to report its use as a gelling agent for microbiological media as opposed to gelatin, which was commonly used at that time. Koch had recognized the need for surface growth of micro-organisms if one was to obtain pure cultures. He realized that the potato surface, the method first used, was very limited in its use and suggested the gelatin plate (Koch 1881). He later mentioned the use of agar-agar (Koch 1882) but only out of interest rather than recognizing its superiority as a gelling agent.

In describing his modification of the plating technique of Koch, Petri presented his dish system (1887). In this paper he especially recommends the dishes for agar-agar plates as the previous system did not use walled dishes and it had been noted that agar-agar stuck poorly to glass. Until this modification then, agar probably was not used to any great extent. From around this time, however, it could be considered that agar has become the most important solidifying agent for microbiological media, though reports of the use of other gelling compounds are occasionally made (Abbott and Chapman 1981) .

The two properties of agar which made it desirable over gelatin were: (i) its stability of gel over a wide temperature range. Gelatin liquefies from 15°-25°C depending on concentration. This is particularly important as bacteria with medical importance are usually incubated at 37°C, (ii) its resistance to bacteriological degradation. Gelatin being proteinaceous suffers degradation and subsequent liquefaction by the action of many proteolytic organisms.

8.4 OTHER USES OF AGAR.

Agar and its components have some very important uses other than microbiological media. Agarose is a purified component of agar (details below) and is used extensively in biochemical and molecular biology studies. The gelling properties of agarose make it an ideal matrix for different electrophoresis procedures such as isoelectric focusing. In another commercial preparation, for example Sepharose, agarose is produced in the form of beads. Such beads are used in molecular exclusion types of column chromatography.

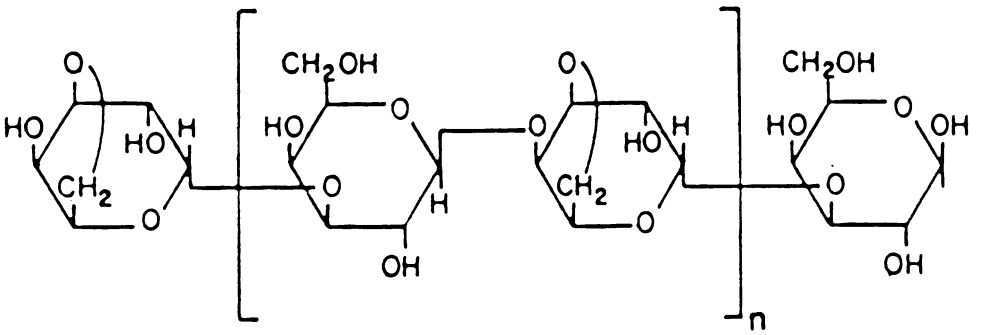
Agar-agar is used directly as a source of food in several Asian countries and as a food additive in some Western countries. In the food industry most use is made of the gel in canned food products, although extensive use is made of agars in baked goods and to some extent in confectionery. There are also other industrial uses of agar. These include sizing of some fabrics, as a lubricant and as a fining agent in brewing. An interesting use of agar is in the manufacture of suppositories.

8.5 STRUCTURE OF AGAR.

The structure of agar has been elucidated to a reasonable extent and has been shown to be a complex mixture of polysaccharide material having the same backbone structure but with different components exhibiting different degrees of side-chain substitution. It is important to realize that as commercial agar is not derived from a single algal species it may be diverse in its characteristics and properties.

The backbone of both agarose and agaropectin consists of alternating units of (1-3)-β-D-galactopyranoside and (1-4)-α-3,6-anhydro-L-galactoside (fig 8.1).

Figure 8.1 General Structure of The Agarose Backbone.



There are three extremes in structure which are non-substituted polymer, pyruvated polymer having little sulfation and a sulfated galactan. As well as pyruvate and sulfate substitutions, the polymer may also have methyl substitutions. The non-substituted neutral polymer is termed agarose while all the substituted chains comprise the component called agaropectin. Both the proportions of agarose to agaropectin and the degree of substitution vary with different agar types. Examples can be seen in table 8.1.

Table 8.1 Percentage Composition of Different Agars

Source	% agarose	6-O-methyl D-galactose
Gelidium amansii	61	1.4
Gelidium subcostatum	89	7.3
Pterocladia tenuis	85	0.8

8.6 BACTERIAL DEGRADATION OF AGAR.

Degradation of agar was first reported by Gran (1902). However, the first systematic survey of agar degradation was not carried out until 1941 when Stanier isolated a number of new species of the genera *Pseudomonas*, *Vibrio* and *Cytophaga* as well as attempting ecological studies relating to these organisms and agar degradation (Stanier 1941). All of Stanier's isolates were obligate aerobes. Since this work appeared in the literature a number of descriptions of agarolytic bacteria representing other bacterial groups have been made including *Bacillus* (Wieringa 1941), *Actinomycetes* (Stanier 1942), as well as unnamed isolates (Hofsten and Malmqvist 1975).

Initial enrichments for agarolytic bacteria were made using saline source materials and even rotting seaweed, giving rise to marine or estuarine strains. Agbo and Moss (1979) isolated and characterized agarolytic bacteria from freshwater samples obtained from the River Wey, England. These isolates degraded a number of polysaccharides of plant origin and it was therefore suggested that the presence of agarolytic bacteria in fresh water was not an indication of the presence of seaweed but more a reflection of the wide number of plant polysaccharides that could support growth.

The agarolytic bacteria that have been described in the literature are for the most part Gram-negative aerobes that are able to degrade a range of carbohydrates. Many are capable of growing on other polymeric carbohydrates such as starch, pullulan, cellulose etc.

Most agarolytic bacteria have been classified in the genera *Pseudomonas*, *Vibrio*, *Alteromonas*, *Streptomyces* *Flavobacterium*,

Cytophaga and allied genera. The true taxonomic status of several of these species is presently in doubt due to the uncertain affiliations these genera have to one another. One such example is *Agarbacterium uliginosum*, described by ZoBell and Upham (1944). Weeks (1974) records this organism as being *Flavobacterium uliginosum* and more recently, this organism is recognized as being a member of the marine agarolytic cytophagas of the *Flavobacterium-Cytophaga* complex (Holmes *et al.* 1984). In general then, agarolytic bacteria have been assigned to the genera *Cytophaga* (Van der Meulen *et al.* 1974), *Pseudomonas* (Yaphe 1957), *Bacillus* (Hunger and Claus, 1978), *Streptomyces* (Bibb *et al.* 1987). *Bacillus* and *Streptomyces* are representatives of agarolytic bacteria that are Gram-positive.

Of the agarolytic bacteria described in the literature *Cytophaga fermentens* var. *agarovorans* and *Cytophaga salminocolor* var. *agarovorans* can grow using agar in both aerobic and anaerobic conditions (Veldkamp 1961). However, best growth occurs in aerobic conditions.

Shieh *et al.* (1988) described several strains of agar degrading bacteria capable of fixing N_2 from seawater and eelgrass-bed sediment (*Zostera marina*). The organisms were Gram-negative, facultatively anaerobic and required NaCl for growth. The characteristics determined placed the organisms in the family Vibrionaceae. *Vibrio* has been something of a "dumping" genus for Gram-negative, facultative vibrios and so is in a similar situation to the *Flavobacterium/ Cytophaga* complex mentioned above. For this reason many of the agarolytic bacteria previously placed in the genus *Vibrio* (Breed *et al.* 1957) are no longer accepted as belonging to the currently recognized family Vibrionaceae (Bauman and Schubert 1984).

The isolates of Shieh *et al.* (1988) are of interest as they make a dual contribution to cycling of organic compounds in nature as i), they show probable involvement in the turnover of polysaccharides such as agar and ii), they contribute to the cycling of nitrogen by way of incorporation of nitrogen into cell material.

Andrykovitch and Marx (1988) isolated a marine bacterium, strain 4-20, that could digest a number of polysaccharides of plant origin. These included laminarin, pullulan and agar. The organism was a Gram-negative, obligately aerobic, pleomorphic rod which possessed a polar flagellum. It sometimes grew as a filamentous helix and produced a melanin-like pigment. Its characteristics conformed to no previously described species but a new taxon was not created to accommodate it.

Irgens (1977) isolated two Gram-negative, gas vacuolated, aerotolerent organisms from an anaerobic digester. One was a rod the other a vibrio. The organisms had a CO_2 requirement and fermented a range of carbohydrates including agar, dextrin, galactose and glucose. The genus *Meniscus* was proposed to accommodate these organisms as the characteristics were such that the organisms could not be assigned to a currently existing genus.

8.7 PATHWAY OF AGAR BREAKDOWN.

The pathway of agar breakdown has been elucidated in a number of agarolytic bacteria. *Cytophaga flevensis* produces an extracellular agarase that cleaves the agarose backbone producing neoagartetraose and neoagarobiose. The organism possesses an enzyme that hydolyses

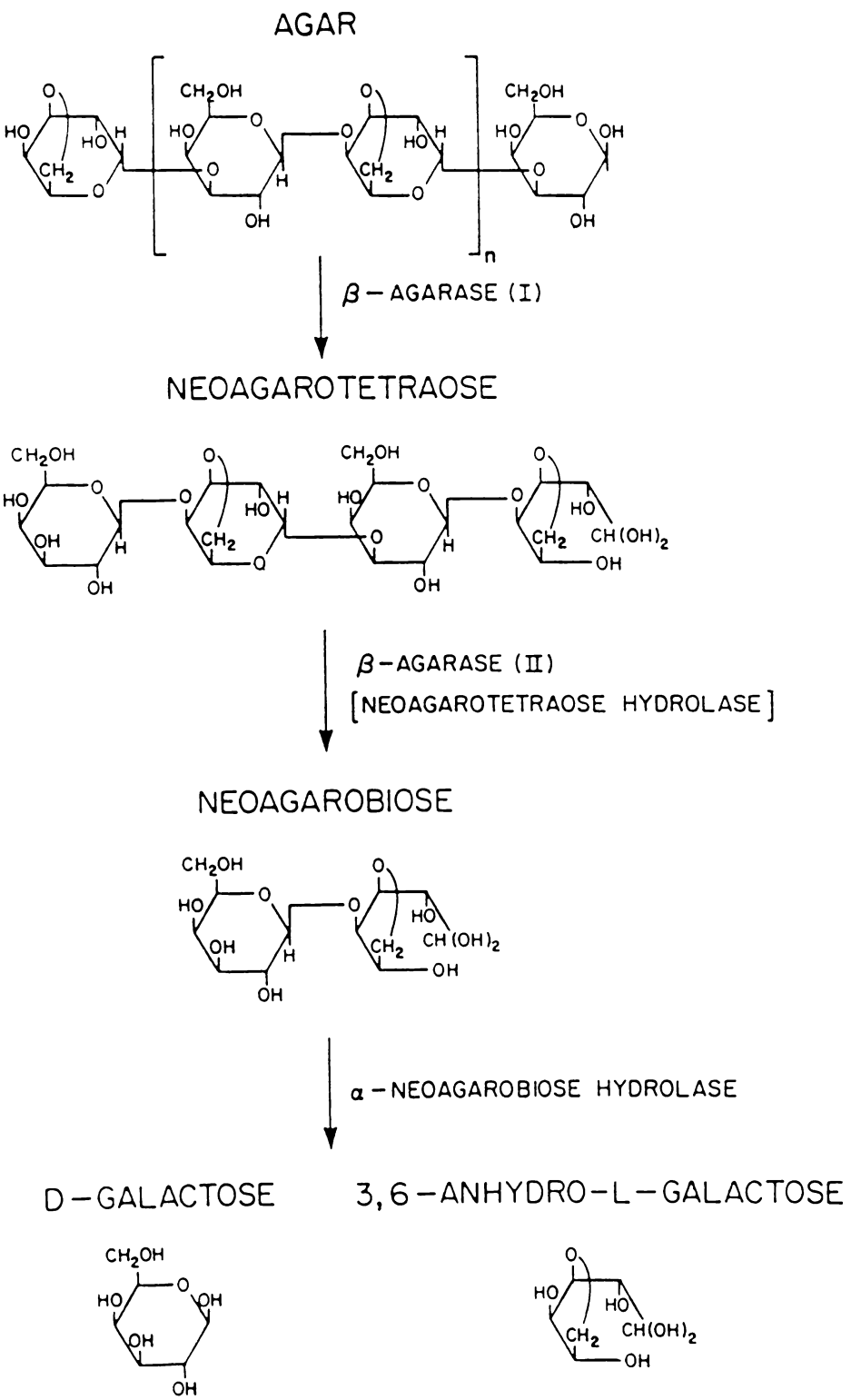
the tetramer by cleavage of its central galactosidic linkage. The products of this reaction are further hydrolyzed enzymatically to D-galactose and 3-6-anhydro-L-galactose. Both enzyme activities of these latter two enzymes are located in the cytoplasm (Van der Meulen and Harder 1975, Van der Meulen and Harder 1976).

The pathway of degradation in *Pseudomonas atlantica* has been well studied. It possesses basically the same enzyme system as described above. The initial cleavage is carried out by an extracellular enzyme called β-agarase, so named as it cleaves the beta linkages. A β-II agarase, cleaving the neoagartetrose, is also extracellular. Disaccharides are transported into the cell where an α-agarase cleaves these units, leaving two monosaccharides as products (Morrice *et al.* 1983). The pathway is summarized in figure 8.2. An *Alteromonas* species (Agbo and Moss 1979) produced an enzyme which produced products with properties of galactose. This work provided little information this pathway.

8.8 GENETIC ASPECTS.

Interest has arisen in the genetic aspects of agarase activity. Study has been based around attempts to clone the responsible genes into other organisms. Bibb *et al.* (1987) successfully cloned the agarase gene (*dag*A) of *Streptomyces coelicolor* in *Streptomyces lividans* 66 on the plasmid vector p1J61. The agarase gene of *S. coelicolor* has been shown to possess interesting regulatory features; consequently the research of Bibb involved a wider study of such phenomena as the mechanisms of catabolite repression. The simple

Figure 8.2 Pathway of agar degradation in *Pseudomonas atlantica*.



purification of agarase to near homogeneity and the fact that the enzyme constitutes over half of the extracellular protein produced by many of the *S. coelicolor* clones makes it potentially useful for the development of expression-secretion vectors for industrial applications of these bacteria.

Belas *et al.* (1988) have cloned the agarase gene (*agr*A) from *Pseudomonas atlantica* into *Escherichia coli.* The aim of their research was to use the agarase gene to probe the genomes of other *P. atlantica* strains. The ability to construct species-specific probes could be a useful tool with which to analyze the distribution of the organism in the marine environment. Finally the cloned gene could be exploited to produce agarase enzyme for scientific research into the chemical structure of polysaccharides such as agar or possibly to produce commercially useful byproducts.

CHAPTER NINE

AGAR DEGRADATION:- MATERIALS AND METHODS

9.1 INTRODUCTION.

While some of the materials and some of the methodology used in the study of agar degrading bacteria were used solely for this part of the thesis, many were also the same as those used in the work on sulfate-reducing bacteria described above. Materials and methods used specifically for the study of agarolytic bacteria are discussed below; all those materials and methods common with the rest of the research program will be cross-referenced, as required, to chapter two.

9.2 MEDIA AND CULTIVATION.

The medium on which 16AV was isolated and cultivated was freshwater medium (section 3.3). Having shown that this isolate was not a sulfate-reducing bacterium, the sulfate was omitted for further experiments. The liquid medium was prepared in the manner described earlier and carbohydrate substrates added to the medium from filter-sterilized stock solutions after it had been autoclaved. When agar or agarose was used as the carbon source the usual amount added was 1-2g/l. At this concentration, it could be cooled to 40°C and dispensed without the medium setting while it was being dispensed.

When plates were used, the medium was prepared in bottles, autoclaved and cooled. It was then reduced with sulfide (final concentration 1-1.5mM), plates poured and inoculated as soon as possible. The

plates were then incubated in an anaerobic jar (GasPak, BBL). Although this was successful, there was a disadvantage in that any volatile hydrogen sulfide derived from the plates reacted irreversibly with the palladium catalyst. The catalyst therefore could not be regenerated and had to be discarded. Thioglycollate (TUSP)medium (Oxoid) could be used by increasing the concentration of agar to 15g/l, with a good degree of success. This medium uses thioglycollate as the reducing agent and although the lag phase was longer than in media reduced with sulfide, the catalyst was not destroyed.

9.3 THE USE OF GELRITE.

A gellan gum produced by a *Pseudomonas* species was marketed in the early 1980's under the trade name GELRITE (Kelco Division of Merck & Co Inc. San Diego, California, USA). GELRITE produces a gel with very good thermal stability and with clarity better than agar when included in media containing divalent cations such as Mg^{2+} or Ca^{2+}. Though not commented on in its description it would clearly be useful in the study of agarolytic bacteria as a gel surface can be readily obtained that is free from possible liquefaction by agarase activity. Most use of this compound has been in the study of thermophilic bacteria, as combinations of GELRITE and $CaCl_2.2H_2O$ in media at concentrations of 1.1%(w/v) and 0.1%(w/v) respectively produced gels that were very stable at 70°C after 48 hours.

GELRITE was used to prepare some plates for taxonomic tests. The medium preparation was carried out in the usual manner with the addition of $MgCl_2.6H_2O$ (0.75g/l) in the medium to act as a source of divalent cations. GELRITE (7g/l) was added and the medium stirred

vigorously. Autoclaving and remaining procedures were standard. The medium with this concentration of GELRITE and magnesium set at approximately 50°C.

9.4 PURITY CHECKS.

Additional tests were devised to test for the presence of contaminant fermentative organisms. Tests were carried out anaerobic conditions in Hungate tubes. The media used were: i), Thioglycollate medium (TUSP) (Oxoid); ii), freshwater salts with yeast extract (1 g/l), pyruvate (10mM) and citrate (5mM); iii), freshwater salts medium plus yeast extract (1 g/l) and glucose (10mM). The usual microscopic examinations were also carried out.

9.5 MOTILITY TESTS.

In addition to using standard phase contrast techniques of wet mounts (section 3.15.3), additional steps were taken to observe for possible gliding motility. Wet mounts were prepared using cultures with partially degraded agar present in order to provide the surface for attachment that is required by gliding organisms. Hanging drop preparations were also made of 16AV cultures using depression slides.

9.6 PIGMENT FORMATION.

In order to induce possible formation of pigments tubes containing agar, as well as those with galactose, were inoculated and incubated as follows. Tubes, each containing agar and galactose, were placed some 30 cm from a 40 watt incandescent light bulb. These tubes were incubated at 28°C. A further series of tubes were incubated on a

window-sill in the laboratory. The temperature of the laboratory was typically 20°C.

9.7 INDUCTION OF SPHERICAL-CELL FORMATION.

An attempt was made to induce the formation of the spherical cells that were typically seen in old cultures of 16AV. This was done by taking a log phase culture, (approximately 72 hours old) grown on galactose, collecting the cells by centrifugation and washing the pellet with sterile salts medium. The cells were recentrifuged and the pellet resuspended in 1 ml of salts medium. This was transferred to 10 ml of reduced medium which contained no carbon source, yeast extract or vitamins. The tubes were checked at regular intervals.

9.8 TAXONOMIC TESTS.

Oxidase: Oxidase was determined using the method of Smibert and Krieg (1981). The pellet from 1ml of centrifuged 16AV culture was applied to reagent test strips containing tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma Chemical Company, St. Louis, MO, USA). A *Bacillus* sp. was used to serve as a positive control.

Phosphatase: The methods of Smibert and Krieg (1981) were used to examine the presence of both acid and alkaline phosphatase.

a) Acid Phosphatase

Reagents: 1) 0.85% NaCl
2) 0.1M citrate buffer pH=4.8 containing 0.01M nitrophenyl-di sodium phosphate
3) 0.04M glycine buffer pH=10.5

Procedure: Spin 10ml culture, resuspend in 0.3ml reagent (1) Add 0.3ml reagent (2) and incubate up to 6 hours. Add 0.3ml reagent (3) and look for colour change. Yellow colour shows cleavage of substrate hence presence of acid phosphatase.

b) Alkaline Phosphatase

Reagents: Reagents (1) above
Reagent (3) above

Procedure: Spin 10ml of culture and resuspend in 0.3 ml saline. Add 0.3ml reagent (3) and incubate. Again, yellow colour indicates free nitrophenol and so presence of enzyme. Medium blanks were also included in these assays.

Gelatin Liquefaction: To test whether gelatin could be liquefied, standard medium was prepared and gelatin added at 5% (w/v). The medium was then dispensed, autoclaved and completed with the usual additions on cooling. After the test incubation period the tubes were placed in a refrigerator at 4°C for several hours after which the presence of a gel could be determined. If the medium gelled on cooling then no gelatin liquefaction took place. No turbidity would also be observed in these tubes.

Urease: Urease production was carried out using Christensen's urea agar with some modifications (Smibert and Krieg 1981). The modified medium had the following composition($g.l^{-1}$); peptone 1.0, galactose 1.0, NaCl 1.0g, KH_2PO_4 2.0g, phenol red 0.012g, yeast extract 1.0g. The pH was adjusted to 6.9, the medium boiled and dispensed under nitrogen into Hungate tubes. Urea was filter-sterilized as a stock solution and added aseptically to give a final concentration of 0.2% (w/v).

Bacteriolytic Test: Two methods were employed to investigate potential bacteriolytic activity. In the first method, GELRITE plates were prepared using standard medium containing yeast extract at 1 g/l. 50 ml of a 24 hour old culture of *E.coli* grown on nutrient broth was centrifuged, washed and resuspended in 100 ml of the medium. Plates were streaked and incubated anaerobically to see whether growth would occur at the expense of the seeded *E.coli* cells. In the second method, liquid media were used. 10 ml of 24 hour old *E.coli* culture grown on nutrient broth and 10 ml of late log phase cells of strain 16AV were centrifuged, washed and resuspended in 5 ml of medium. This medium was dispensed anaerobically into Hungate-tube.

Indole Production: Standard medium was prepared with the inclusion of 2 g/l Casamino acids as a source of tryptophan. Additional tubes were prepared with 5 mM galactose plus Casamino acids. The presence of indole was tested for using the standard Erlich reagent (*p*-dimethylaminobenzaldehyde, 1g; 95% ethanol, 94 ml; concentrated HCl, 20 ml) after Smibert and Krieg (1981).

Lipase: The presence of lipase activity was tested using two different substrates. The test was carried out using GELRITE plates as described: Mineral salts medium was prepared with yeast extract (1g/l), $M_gCl_2.6H_2O$ (0.75g/l) and GELRITE (7g/l) and reduced with thioglycollate (0.5g/l). For tributyrin plates, tributyrin was included at a final concentration of 10ml per litre and the medium was sonicated to effect homogeneity. For Tween 80 Plates the mineral medium was prepared with the further addition of 0.1g/l $CaCl_2.2H_2O$. Tween 80 was prepared as a separate autoclaved solution of 1g/l ml. The medium and Tween 80 solution were cooled to 60°C, mixed and the

plates poured. Control plates were prepared in the form of substrate plus galactose and no substrate plus galactose. Plates were incubated in anaerobic jars and incubated for 2 weeks 28°C.

Hydrogen Sulfide Production: Medium was prepared with the following components ($g.l^{-1}$); trypticase peptone 10, Lab-lemco Peptone 10, ammonium ferric citrate 0.05, $MgCl_2.6H_2O$ 0.15, GELRITE 8.0, pH = 7. Medium was dispensed in the usual manner into Hungate-tubes. Tubes containing medium were autoclaved, cooled to 60°C, inoculated and cooled immediately to 20°C.

9.9 DETERMINATION OF GRAM TYPE.

Two methods were used to determine the Gram-type of cells. Cells were stained using the method already described (section 3.15.1). The second method used was the KOH lysis test of Gregersen (1978). 10 ml of liquid culture was centrifuged to obtain a pellet which was transferred to a glass slide. *E.coli* and *Bacillus circulans* were grown on nutrient agar plates and used as the controls. 10 µl of 3% (w/v) KOH was placed onto each of the samples. Gram-negative cell lyse under these conditions while Gram-positive cells do not. Lysis can be seen by the cell material becoming very viscous due to the release on nucleic acids.

9.10 SODIUM CHLORIDE REQUIREMENT/TOLERANCE.

To test a possible requirement for and tolerance to sodium chloride, 16AV was grown on media containing the following amounts of sodium chloride (% w/v); 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0. No sodium salts were otherwise included in the medium. The buffer was prepared

using $KHCO_3$ as opposed to the sodium salt usually employed though was prepared in the same fashion (section 3.7). Growth was measured directly in the tube using the adapted Spectronic 20 (section 3.13.1).

9.11 β-GLUCOSIDASE.

The assay for β-glucosidase in 16AV was carried out as follows:

Reagents: 1) Na-Phosphate Buffer, 100mM pH = 6.2
2) Assay Buffer = 5mM p-nitrophenol-β-D-glucopyranoside
3) 1M Na_2CO_3

Procedure: 1ml of log phase culture was centrifuged. The supernatant was retained while the pellet was washed in 1ml buffer and recentrifuged. The pellet was resuspended in 1ml of buffer and the cells were lysed by sonication until microscopic examination showed complete lysis. 1ml of supernatant and 1ml of pellet were added to respective 0.5ml amounts of assay buffer and incubated at 30°C for 30 minutes. The reaction was stopped by addition of 1.5ml of carbonate solution 1. Optical density was measured at 400nm.

9.12 AGARASE ASSAY.

The first was a simple qualitative plate assay testing the presence of agarase in the culture supernatant while the second was developed with the aim of using for quantitative assays

9.12.1 PLATE ASSAY.

Plates were prepared using the standard mineral salts base with the pH adjusted to 7 and agarose (Sigma Chemical Co., St Louis, MO., USA)

included at 10 g/l. When used for assays, log phase cultures were centrifuged and 50°l of the cell-free supernatant was placed on the plates. The plates were incubated at 28°C for 48 hours after which the plates were inspected. The presence of agarase was shown by depressions in the agarose at the point at which the sample was placed.

9.12.2 COLOURIMETRIC ASSAY.

This assay involved measuring reducing sugars released for agarose by the action of the enzyme.

Enzyme Assay.

HEPES buffer was prepared at 50 mM and pH 7. Ultra pure agarose was dissolved in the buffer at a concentration of 1 mg/ml providing a substrate for the enzyme. 0.5 ml of substrate plus 0.5 ml of sample and a further 1.0 ml of buffer was added, mixed by vortexing and incubated at 28°C for one hour. The release of reducing sugars was the measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method described below.

p-Hydroxybenzoic Hydrazide Assay For Reducing Sugars.

Reagents were prepared in stock solutions at the following concentrations; Na_2SO_3, 1M; $CaCl_2.2H_2O$, 0.2M; Na_3-citrate.$2H_2O$, 0.5M; NaOH, 5.0M. A working solution was prepared daily by mixing 10 ml of each of the solutions in the following order, citrate, sulfite, calcium and sodium hydroxide. This prevents the formation of a precipitate in the working reagent. 1.52g PAHBAH (Sigma Chemical Co.,

St Louis, MO., USA) was added to the solution (100mM) and the volume made up to 100 ml. To use the working reagent, equal volumes of the working solution and the sample were combined and boiled for 6 minutes. Optical density was measured at 420 nm.

9.13 AGARASE PURIFICATION.

Partial purification of the extracellular agarase was carried out using a culture of 16AV grown on agar. Complete purification of the enzyme was not carried out but those procedures carried out were as follows:

Reagents: Buffer 1 HEPES 50mM pH=7
Buffer 2 HEPES 50mM + 1.5M NaCl, pH=7

Procedure:

Procedures were carried out at 4°C. A 400ml volume of a late log phase culture of 16AV grown on agar was centrifuged at 10000 x *g* for 30 minutes to remove cells and undegraded agar. The supernatant was brought to 75% saturation with ammonium sulfate. The supernatant was stirred gently at 4°C overnight to ensure complete precipitation of the protein. The supernatant was collected by centrifugation at 10000 x *g* for 30 minutes and then dissolved in 10ml Buffer 1.

5g of agarose was soaked in buffer 1 overnight and collected by centrifugation at 1500 x *g* for 5 minutes, washed and recentrifuged once more. The enzyme preparation was mixed with half the collected agarose and stirred gently for 30 minutes. The agarose was collected by centrifugation at 11000 x *g* for 5 minutes, washed once in 10ml buffer 1 and recentrifuged a further 5 minutes at 11000 x *g*. 5ml of buffer 2 was added to the collected agarose and stirred

for 30 minutes. The agarase was spun at 11000 x g for 15 minutes and the supernatant was collected. A further 5ml of buffer2 was added and stirred for 45 minutes to remove all the enzyme from the agarase. A final centrifugation was carried out and the collected supernatants pooled.

This is the stage to which 16AV's enzyme was purified.

9.14 CHEMICAL DETERMINATIONS.

9.14.1 ORGANIC METABOLITES.

Determination of substrates and fermentation products were carried out using HPLC. Running conditions were the same as those described in section 3.18.1.

For determination of metabolites in cultures grown on agar or agarose where the gel structure was still intact, the "freeze and squeeze" technique was used. The appropriate sample (1ml) was frozen at -4°C overnight. The frozen block was wrapped in parafilm (American Can Company, Grenwich, CT, USA) and squeezed between the fingers, causing melting of the block. The liquid collected in a micro centrifuge tube and centrifuged to sediment any agar/agarose particles. The supernatant was removed for HPLC analysis.

9.14.2 DETERMINATION OF GASEOUS PRODUCTS.

Hydrogen gas was determined using a PYE UNICAM PU4500 gas chromatograph fitted with a thermal conductivity detector. The column was stainless steel (2.7m by 3mm outside diameter), packed with Carbosieve B, 120/140 mesh (Supelco Inc., Bellefonte, PA, USA). The

oven temperature was 188°C, detector temperature 76°C and injector temperature was 200°C. The carrier gas was oxygen-free nitrogen and run at a flow rate of 15 ml/min. Carbon dioxide can be measured using the GC as described. However, carbon dioxide was not measured quantitatively due to the presence of the bicarbonate buffer.

9.14.3 PROTEIN DETERMINATION.

Protein was determined using the method of Lowry as modified by Scopes (1982). The reagents were prepared as follows:

Reagent A, 0.5 g $CuSO_4.5H_2O$ + 1.0 g tri-sodium citrate in 100ml H_2O
Reagent B, 20 g Na_2CO_3 + 4 g NaOH in 1000ml water
Reagent C, 50 ml Reagent B + 1 ml Reagent A
Reagent D, Folin-Ciocalteau reagent/Water (50/50 volume)

The assay was carried out as follows: 0.5 ml sample was added to 2.5 ml Reagent C, mixed and left to stand for 10 minutes. 0.25 ml Reagent D was added, mixed well and left to stand for 30 minutes and the optical density measured at 750 nm. Bovine serum albumin was used as the standard.

CHAPTER TEN

ISOLATION AND CHARACTERIZATION OF AN OBLIGATELY ANAEROBIC, AGAROLYTIC BACTERIUM:- RESULTS

10.1 ISOLATION IN PURE CULTURE OF AN AGAROLYTIC BACTERIUM.

Isolation of sulfate-reducing bacteria using shake dilution tubes gave rise to colonies with the apparent ability to cause loss in the gelling properties of the agar. Attention to the presence of bacteria able to carry out degradation of agar under anaerobic conditions was not paid until an isolation experiment showed collapse of the gel structure.

In an agar dilution series containing butyrate as carbon source with sulfate as electron donor, off-white to greyish colonies developed, the typical colour for sulfate-reducing bacteria, but instead of the more usual lenticular morphology close examination showed that they were diffuse. White diffuse colonies were also present. The tubes were broken open and the dark colonies transferred to fresh agar shakes as it was assumed they were sulfate-reducing bacteria. Colonies developed as in the initial shakes i.e. white halo colonies as well as the dark halo colonies. The tubes were left for 3-4 weeks after which examination showed large gas lenses had developed and the colonies had spread. The spreading was associated with liquefaction of the agar. Microscopic examination of the dark colonies showed two bacterial types to be present. The first a large motile vibrio, and the second, a smaller non-motile vibrio. Tubes smelt of sulfide, that is, greater than would be attributed to the sulfide present as the reducing agent. Material from the shake was transferred to fresh

tubes and similar colony types developed. At this stage, all halo colonies showed the two organisms originally observed to occur in the colonies. This led to the suspicion that there was a dependence between one or both of the organisms. The next transfer was made to agar shake tubes that had no added carbon source but sulfate was still included. The only dark colonies to arise were still the halo type originally seen, indicating that the butyrate added to the tubes was not necessary.

Agar shakes were further carried out using lactate as the carbon source with sulfate as electron donor. The colonies that developed were dark lenticular as well as white halo colonies. Both were picked and transferred and gave rise to pure cultures. The culture isolated using lactate was designated 16Avla while the second strain, the agarolytic organism was designated 16AV.

10.2 ENUMERATION OF AGAROLYTIC BACTERIA.

Enumeration of anaerobic agar-fermenting bacteria was carried out using the 5 tube MPN method described in section 3.17. Agar at 2 g/l was added as carbon source. After 6 weeks incubation tubes were checked for complete liquefaction of the agar and for both gaseous and volatile fatty acid products. Results are in Table 10.1

Table 10.1 Most Probable Number Estimates of Agarolytic Bacteria

Site	Number/100ml	Products+
Waste water pond (Am)	23	CH_4, CO_2 No VFA detected
Estuarine sediment (Ok)	920	CH_4, CO_2 No VFA detected

+, VFA = volatile fatty acids

10.3 16Avla AND 16AV DEPENDENCE.

The reason why 16Avla would only grow in mixed culture with 16AV when agar was the only carbon source was studied briefly by looking at the end products of agar fermentation by 16AV and the substrates on which 16Avla was able to grow. The results are shown in table 10.2.

Table 10.2 Products of Agar Fermentation by strain 16AV and Substrates Used by 16Avla

16AV: Products on Agar	16Avla: Substrates used
Acetate	Lactate
Ethanol	Pyruvate
CO_2	Ethanol
H_2	

The likely relationship between 16AV and 16Avla was that 16AV degraded the agar producing ethanol, which was then oxidized by 16Avla using sulfate as the electron acceptor producing sulfide. This resulted in the typically dark colonies. 16Avla was identified as typical *Desulfovibrio* species and was not studied further.

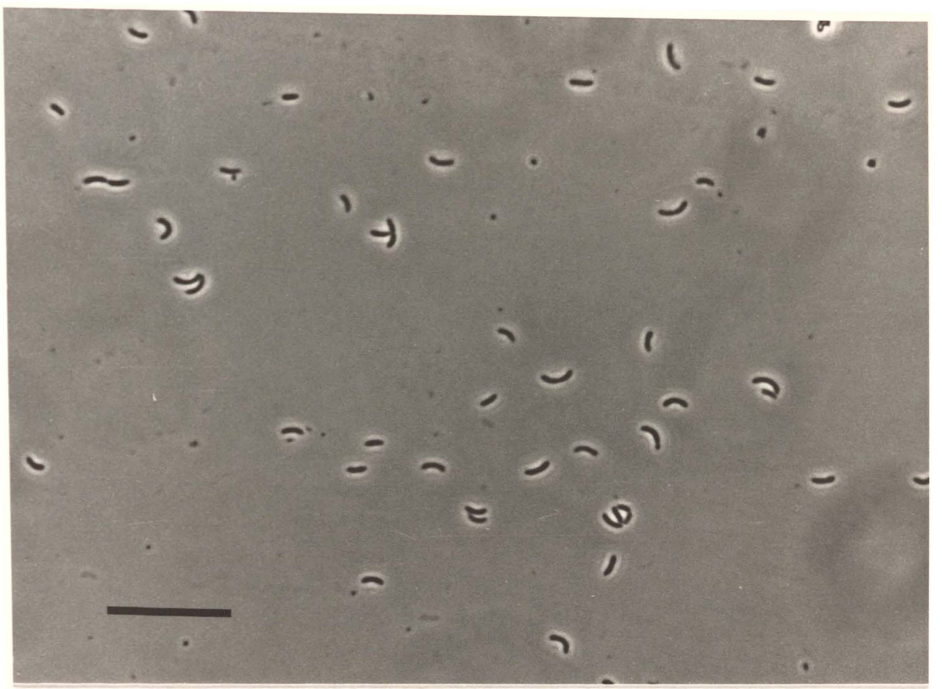
10.4 GENERAL CHARACTERISTICS OF STRAIN 16AV.

16AV was a vibrio measuring 0.25-0.5 μm x 1-2 μm. The morphology of 16AV is seen in plate 6.1 and 6.2. Motility was never observed in cultures examined at a range of ages from very early log phase through to stationary phase. Both typical wet mount preparations and hanging-drop preparations were used. Special attention was paid to

Plate 6.
Photographs of strain 16AV

6.1 Phase contrast micrograph of strain 16AV. (Bar = 10μm)

6.2 Electron micrograph of strain 16AV. Note the absence of flagella. (Bar = 1μm)



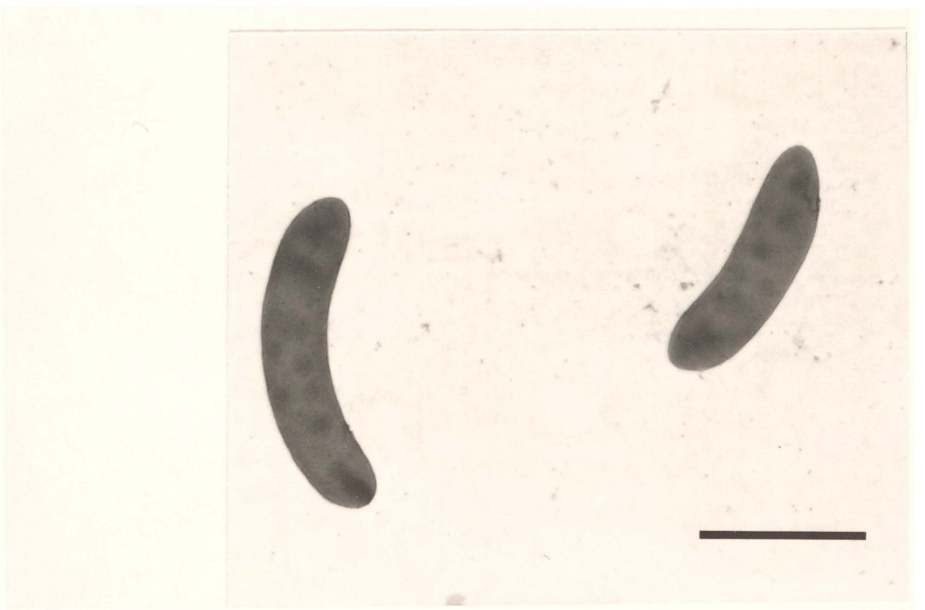
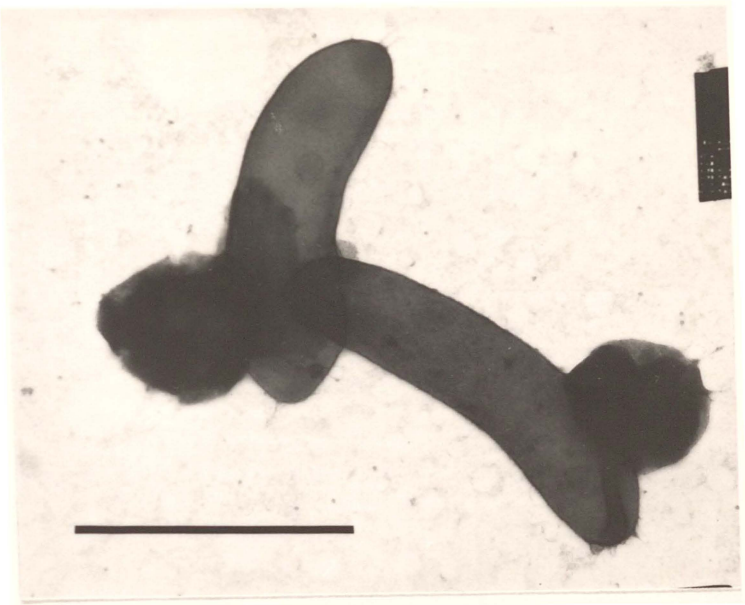
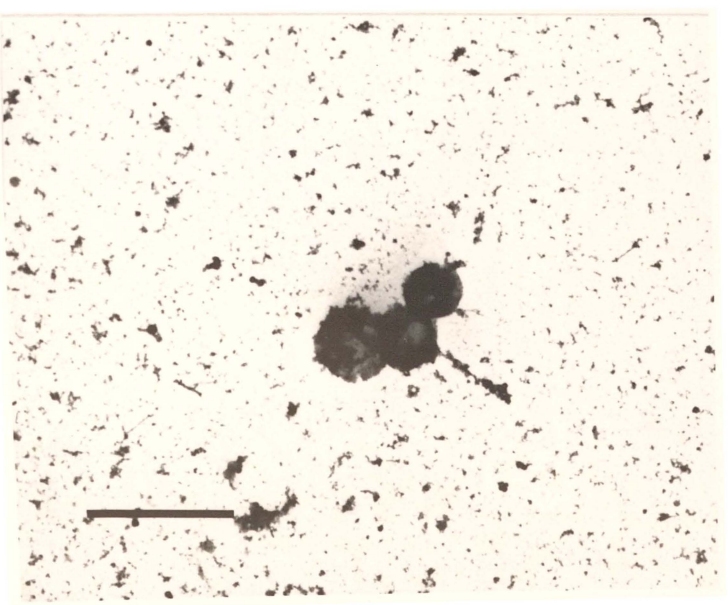


Plate 7.
Resting bodies of strain 16AV.

7.1 Electron micrograph of resting body formation in strain 16AV (Bar = 1μm)

7.2 Electron micrograph of resting bodies. (Bar = 10μm).





any apparent ability to glide as a number of agarolytic bacteria are motile by this means. Gliding was never observed to take place. Examination was further made of a 16AV culture grown on medium with high calcium concentrations as some gliding organisms are known to have a high calcium requirement. Electron micrographs did not reveal the presence of any cells which had flagella nor could any unattached flagella be observed on the grids (plate 6.1).

Refractile bodies resembling spores were never observed. In old cultures however, non-refractile spherical cells were formed. Spherical bodies were apparently formed as "buds" (plate 7.1 and 7.2). These spherical bodies displayed no resistance to heat treatment that is normally associated with spores. These spherical cells did confer a degree of viability on old cultures as tubes with apparently only coccoid cells present that had been stored in the dark at room temperature (typically 20°C) for upwards of 9 to 10 months still retained viability. When such a culture was used as the inoculum a lag period of about two weeks took place before the organism grew. With regard to staining, the spherical cells stained in the usual gram-negative fashion. They did not take up the cyst stain described for *Azotobacter* spp. cysts (Doetsch 1981).

Strain 16AV had a Gram-negative reaction when stained and the KOH lysis test indicated a Gram-negative type of wall. The aminopeptidase reaction was negative, a reaction that one usually associates with a Gram-positive organism.

Experiments were designed to induce pigment production in 16AV (section 9.6). After 8 days both the 16AV cultures failed to produce pigments. Interestingly, the tubes inoculated and incubated on a

window-sill in the laboratory showed no growth even after 4 weeks incubation. The temperature of the laboratory is typically 20°C and so temperature is not the reason for failure to grow as though growth is slower at 20°C the cultures would normally grow in a 4 week incubation period. It would seem that daylight is inhibitory to the growth of 16AV.

10.5 GROWTH CHARACTERISTICS AND NUTRITION.

Strain 16AV required the presence of 1g/l yeast extract for growth. At this concentration no growth occurred on the yeast extract alone. The yeast extract could not be substituted with the vitamin mix including riboflavin and vitamin B_{12} (section 3.5). Strain 16AV was routinely grown on the bicarbonate-buffed sulfide-reduced mineral salts medium with the 1 g/l yeast extract as the organic nutrient supplement and the fermentable carbon source supplied in the form of 2 g/l agar. The cultures were maintained in sealed Hungate-tubes. 16AV was subjected to a wide range of growth media and differing degrees of anaerobiosis. When the inoculum of 16AV was pregrown on agar the immediate point of contact the loop made on the plate being inoculated often showed small depressions after a 24 hour incubation indicating agar lysis. The depression was never observed over the whole streaked pattern, only the first few millimeters. The area of depression was used as an inoculum for subculturing on a further plate of the same medium. No growth or degradation took place. Microscopic examination of these depressions showed no bacteria present. The depressions were thus attributed to carry over of the agarolytic enzyme.

Growth did not take place on Veldkamp's agarolytic *Cyptophaga* medium (Veldkamp 1961), in either of the plate or stab tubes. Slight growth took place on the TUSP medium. This medium has thioglycollate as a reducing agent and also contains 0.5 g/l agar to aid in maintaining anaerobic conditions so the slight growth is most likely attributable to the degradation of the small amount of agar present.

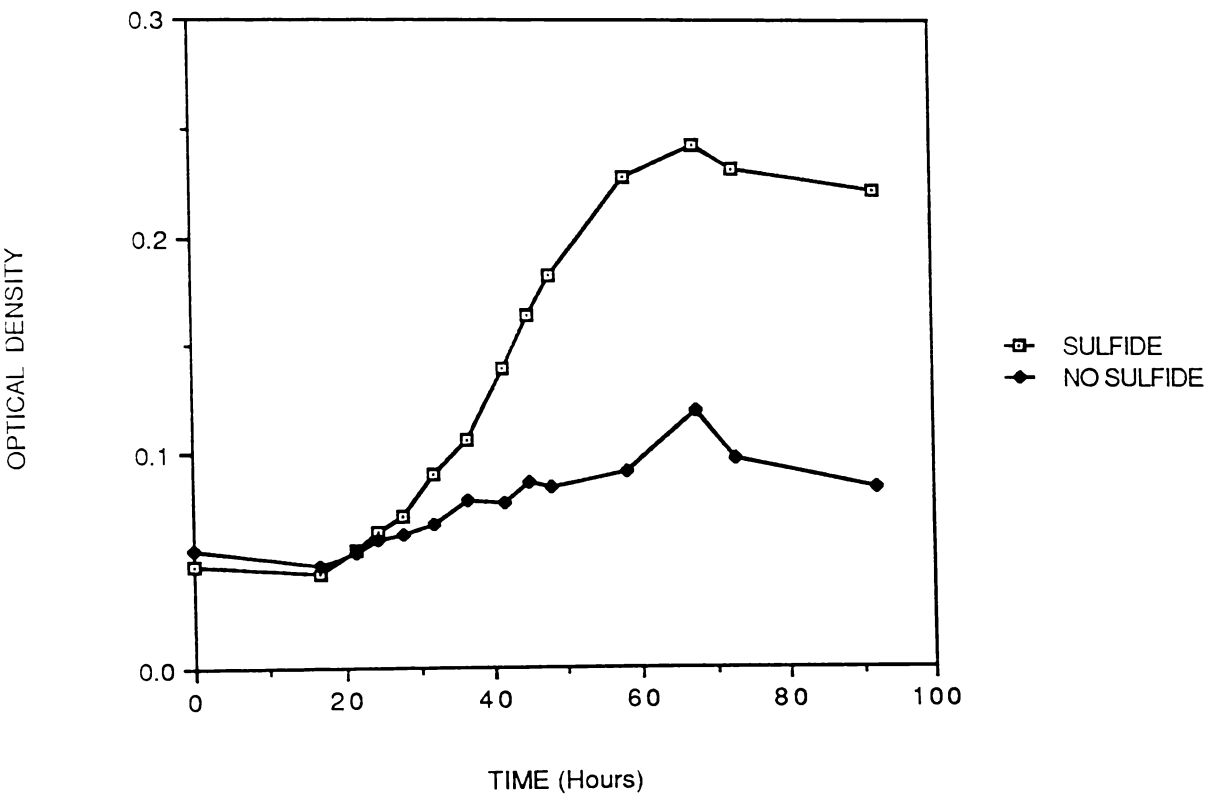
Growth was obtained on plates when the mineral medium was prepared and reduced on cooling, the plates poured, inoculated then incubated immediately in an anaerobic jar. When plates were used in the fashion, extensive agarolytic activity could be seen with ease.

When freshwater medium containing yeast extract and galactose was added to McCartney bottles in 10 or 20 ml volumes (total volume 25ml), inoculated and sealed tight no growth took place. Further to this, if the same medium was not boiled prior to dispensing into Hungate-tubes left with an air headspace, bicarbonate added but no sulfide included after autoclaving, no growth took place. Best growth occurred on the bicarbonate-buffered sulfide-reduced mineral medium. To determine the importance of sulfide, growth curves were obtained with 16AV growing on galactose in the presence or absence of sulfide. (When used sulfide was at 1.5 mM final concentration). Results are seen in figure 10.1.

10.6 TEMPERATURE OPTIMUM AND RANGE.

16AV grew fastest at 28°C reaching stationary phase after 72 hours incubation. The same rate of growth was obtained at 20°C, 25°C and 37°C, all slightly less than at 28°C. No growth took place after 1 week incubation at 10°C and 42°C.

Figure 10.1 Growth of 16AV in the presence and absence of sulfide.



10.7 SUBSTRATE TESTS

16AV was subjected to a vast range of compounds to determine those that could act as substrates for growth when added as single carbon sources. The range included monosaccharides, disaccharides, trisaccharides and polysaccharides. Pentose sugars were also tested. In some cases, both the D and L isomers were used. As well as sugars, a number of sugar alcohols, organic and amino acids were also included. Some polymeric compounds were included such as pectin and gum xanthan, carrageenan etc.. The details of compounds tested and their use appears in table 10.3.

Of all the compounds tested for ability to support growth only agar, agarose, D-galactose and cellobiose were positive. No other carbohydrate, organic acid or alcohol could support growth. A number of types of agar were used as substrate tests, all allowing the same good growth. Those tested were Oxoid No 3 and Oxoid Purified Agar (Oxoid, Basingstoke, England), Merck Purified Agar (E. Merck, Darmstadt, FRG) and Coast Biologicals Agar (Coast Biologicals, Opotiki, New Zealand). Ultra pure agarose (electrophoresis grade, BRL Laboratories, Bethesda, MA, USA) and Sigma Type 1 agarose (Sigma Chemical Co., St. Louis, MO, USA) were both used by 16AV.

A number of peptones and extracts were used for substrate tests. These were tested at 1% (w/v) final concentration. Those used were; Bacto peptone, Malt extract (Difco, Detroit, USA) ;Phytone, Trypticase, Gelysate, Polypeptone (BBL, Cockeysville, USA); Proteosepeptone, Lab-lemco peptone (Oxoid, Basingstoke, England). All peptones failed to support growth.

Table 10.3 List of Substrates Tested as Carbon and Energy Source by Strain 16AV

Compound	Growth	Compound	Growth
D-Glucose	-	Starch	-
D-Galactose	+++	Inulin	-
L-Galactose	-	Cellulose	-
		Carboxy Methyl	
L-Fucose	-	Cellulose	-
Rhamnose	-	Glucuronic Acid	-
L-Arabinose	-	Galacturonic Acid	-
D-Arabinose	-	Mannose	-
Pectin	-	Polygalacturonic	
Lactose	-	Acid	-
Maltose	-	Gum Xanthan	-
Cellobiose	+++	Gum Locust Bean	-
Melibiose	-	Gum Karaya	-
Trehalose	-	Carrageenan	-
Raffinose	-	Agar	+++
Melizitose	-	Agarose	+++
D-Xylose	-	Inositol	-
L-Xylose	-	Mannitol	-
Sorbitol	-	Citrate	-
Glycerol	-	Fumarate	-
Galactitol	-	Malate	-
Mucic Acid	-	Succinate	-
Salicin	-	Lactate	-
Gluconate	-	Pyruvate	-
Glycollate	-	Malonate	-
Methyl Galacto-			
Pyranoside	-	L-Proline	-
2-3,Butanediol	-	Glutamate	-
Ethanediol	-	L-Lysine	-
1-3,Propanediol	-	L-Glycine	-

10.8 GROWTH OF 16AV UNDER DIFFERENT CONDITIONS.

Because 16AV was maintained on agar or galactose, all inocula came from cultures grown on these substrates. A range of substrates was studied to see whether they would support growth of 16AV following its culture on cellobiose. Results are presented in table 10.4.

Table 10.4 Substrates Tested as Carbon and Energy Source With a Cellobiose-grown Inoculum

Carbon Source	Growth
Cellulose	-
Carboxy methyl-Cellulose	-
Starch	-
Sucrose	-
D-Glucose	-
D-Galactose	+++
Cellobiose	+++
Fructose	-
Lactose	-

Pregrowing the inoculum on cellobiose had no effect on the range of carbon sources used. It should be noted the range tested was by no means extensive and only those substrates considered likely to be used were chosen. Attempts were made to see whether some substrates could be used if a small amount of galactose was present. Results are presented in table 10.5. Optical density values are averages obtained with 3 tubes while numbers in brackets represent the concentration used (mM).

When galactose was added with the other carbohydrates, the only growth which took place was that which could be attributed to the presence of the galactose. No growth took place when galactose was not added.

Table 10.5 Effect of Addition of Trace Galactose To Growth Tubes

Substrates	Optical Density	Ratio OD/[gal]
Galactose (0.5)	0.05	0.10
Glucose (8.3)+ Galactose(2.7)	0.35	0.13
Glucose(10)	0.00	NA
Glucose(10)+ Galactose(0.5)	0.025	0.05
Lactose(5)+ Galactose (0.5)	0.05	0.10
Lactose(5)	0.00	NA

An experiment was carried out to investigate the possibility that 16AV might use further carbohydrates or organic acids when grown in the presence of a H_2-utilizing sulfate-reducing bacterium. The medium contained sulfate and was inoculated with *Desulfovibrio* sp. strain HL21 (5% inoculum) and 16AV (1% inoculum). Results are presented in table 10.6.

Table 10.6. Growth of 16AV in Co-culture with *Desulfovibrio* Species.

Substrate	Growth of Culture
Melibiose	-
Lactose	-
Fructose	-
Glucose	-
Galactose	+
Maltose	-
Malate	-
Lactate	+
Citrate	-
Galacturonic Acid	-
Fumarate	-

Microscopic examination of the lactate-grown culture showed only the *Desulfovibrio* to be present, apart from the very few 16AV cells attributable to the inoculum. Both organisms were present in the galactose culture. This qualitative test showed that co-cultures of 16AV and a hydrogen-utilizing species could not grow on other substrates.

10.9 MISCELLANEOUS TESTS.

Phosphatase: This was carried out as described in section 9.8. No yellow colour developed when looking for acid phosphatase indicating 16AV does not possess acid phosphatase. In the alkaline phosphatase experiment, slight yellow colour was observed but not greater than occurred in the reagent blank indicating it does not have an alkaline phosphatase.

Catalase: The standard hydrogen peroxide test for catalase was applied to plate cultures of 16AV (Smibert and Krieg 1981). No oxygen evolution took place showing the absence of catalase. *Bacillus* sp. acted as a positive.

Oxidase: The standard test was used on 16AV (section 9.8). The purple colour associated with oxidase positive organisms developed in the control but not in the negative control or 16AV. 16AV was therefore found to be oxidase negative.

Indole Production: The standard test was applied to 16AV. No growth took place on the Casamino acids so indole could not be produced.

Urease: The method described in section 9.8 was used to test for the production of urease. There was no colour change indicating no breakdown of urea to ammonia was taking place. 16AV was therefore found to be negative.

Hydrogen Sulfide Production: No dark colonies developed in the medium described in section 9.8 and therefore 16AV was found to be negative.

Gelatin Liquefaction: The test for gelatin liquefaction was carried out under a number of conditions. The media compositions and atmospheres are described in Table 10.7 along with the results of those growth conditions.

Table 10.7. Test For Gelatin Liquefaction in Strain 16AV

Medium*	Atmosphere/ Conditions	Growth	Liquefaction
BHI + Gelatin	O_2	-	-
BHI + Gelatin	Anaerobic jar	-	-
FM + Gelatin	N_2 in Hungate Tubes	-	-
FM + Gelatin + Galactose	N_2 in Hungate Tubes	+	-

* BHI, Brain heart infusion; FM, fresh water salts (standard medium above).

16AV was unable to liquefy gelatin under aerobic or anaerobic conditions. The growth that occurred in the final test above can be attributed to growth on the galactose as 16AV failed to grow under the same conditions with the exclusion of galactose.

10.10 BACTERIOLYTIC ACTIVITY.

Electron micrographs of a stationary phase culture of 16AV that was forming spherical bodies occasionally gave the appearance the cells were undergoing lysis and so it was decided to investigate the possibility that 16AV possessed bacteriolytic activity. The methods used for this test are described in section 9.8. 16AV did not show bacteriolytic activity under either of the experimental conditions.

10.11 ESCULIN HYDROLYSIS.

Esculin, (6,7-dihydroxycoumarin 6-glucoside), was included in Hungate-tubes with 1g/l yeast extract and 0.5 % ferric citrate. Esculin hydrolysis is typically used as a simple taxonomic test for classification of bacteria, but was used here to test 16AV for the ability to hydrolyse a glycoside-substituted compound of plant origin. Tubes inoculated with 16AV along with tubes or plates inoculated with appropriate controls were incubated and regularly checked for the presence of darkening, the signs for hydrolysis of esculin. No darkening was observed in the culture tube while the positive control, a *Bacillus* sp., showed darkening. 16AV could not be shown to hydrolyse esculin.

10.12 LIPASE ACTIVITY.

The plates prepared as described were examined after a 2 week incubation period. No growth was observed on either of the tributyrin or Tween 80 plates with only lipid present. 16AV grew on the plates with lipid plus galactose and no lipid plus galactose. Under these conditions 16AV shows no lipase activity.

10.13 *Campylobacter* TEST.

16AV's morphology resembles that of *Campylobacter*, spp. particularly those classed in the group which are able to reduce elemental sulfur. 16AV was therefore tested for its ability to reduce sulfur using formate/acetate, malate or pyruvate as electron donor/carbon source. When using formate as an electron donor acetate was included as a source of carbon. Electron donors were added at 10 mM final concentration. Nitrate (10 mM) was also included as a test terminal electron acceptor from a sterile stock solution, while sulfur was added to the Hungate-tubes with a small spatula prior to dispensing and autoclaving the medium. 16AV did not grow in any of the test media.

10.14 *Syntrophococcus* TEST.

Syntrophococcus sucromutans oxidizes carbohydrates using formate as the terminal electron acceptor, acetate being the product (Krumholz and Bryant 1986). 16AV was tested for this type of metabolism as it was shown to have a carbohydrate-based metabolism. Glucose, lactose, pyruvate, lactate and citrate were added as electron donors at 10 mM final concentration and formate was included in the medium at 2mM. 16AV was unable to oxidize those carbohydrates tested with formate as the electron acceptor.

10.15 FERMENTATION STOICHIOMETRY.

When grown on galactose, agar, agarose or cellobiose, 16AV produced acetate, ethanol CO_2 and H_2. The growth yields and stoichiometric balances for growth on galactose and cellobiose are seen in table

Table 10.8a. Fermentation Balances of 16AV Grown on Galactose.

Substrate	Amount of substrate degraded (μmol)	Cell dry weight formed (mg)	Substrate* assimilated (μmol)	Products				Growth Yield (mg/mmol)	Carbon Recovery (%)
				Acetate (μmol)	Ethanol (μmol)	CO_2	H_2		
Galactose	1001	13.46	277	726	728	+	+	13.4	93
Galactose plus H_2	1520	20.00	412	1060	1230	+	ND	13.1	92

Table 10.8b. Fermentation Balances of 16AV Grown on Cellobiose.

Substrate	Amount of substrate degraded (μmol)	Cell dry weight formed (mg)	Substrate* assimilated (μmol)	Products					Growth Yield (mg/mol)	Carbon Recovery %
				Acetate (μmol)	Ethanol (μmol)	Glucose (μmol)	H_2	CO_2		
Cellobiose	645	12.8	206	695	771	133	+	+	22	108

*. See text for details on the method used to calculate the amount of substrate assimilated.

10.8a and b. CO_2 and H_2 were determined only qualitatively as CO_2 was involved in the buffering of the medium and meant that it would be difficult to determine CO_2 present as buffer compared with CO_2 produced by the organism. When calculating balances for galactose and cellobiose the particular substrate, acetate and ethanol were determined accurately using HPLC. Elution traces showed no other organic acids or alcohols to be produced as the Bio-Rad HPX.87H column and detector system that was used can detect most compounds usually found as end products of fermentation. A theoretical maximum of 2 moles of H_2 and CO_2 can be obtained from fermentation of a monosaccharide and if this value was used in the balance a 93 % carbon recovery was obtained on the galactose balance and 108% carbon recovery on the cellobiose balance.

Substrate assimilated was calculated using the emperical formula for a cell, $<C_4H_7O_3>$. (Widdel 1980). Using this formula, the following equation can be derived:

$$17CH_3COOH \qquad 8<C_4H_7O_3> + 2CO_2 + 6H_2O$$

It follows then that 0.0206 mmol acetate is required for 1 mg cell dry weight. This can be taken a step further as 1 mol monosaccharide is fermented to 3 mol acetate.

Table 10.8a shows the fermentation balance of 16AV grown on galactose under nitrogen and hydrogen atmospheres. The ratio of products formed did not change significantly when grown under either of these conditions (table 10.9). The ratio of ethanol to acetate produced when 16AV was grown under nitrogen or hydrogen were 1.0 and 1.2 respectively.

Table 10.9 Ratio of Product Formation When Grown Under Nitrogen or Atmospheres

Atmosphere	Substrate Used / Acetate Produced	Substrate Used / Ethanol Produced	Ethanol / Acetate
Nitrogen	1.4	1.4	1.0
Hydrogen	1.4	1.2	1.2

The fermentation balance when grown on cellobiose shows presence of a monosaccharide, probably glucose. This must have leaked from the cells as it has been shown 16AV has an intracellular β-glucosidase (below). The amount of glucose that leaked from the cells had to be taken into consideration when calculating the carbon recovery as it would be observed as substrate disappearance but have no contribution to formation of endproducts of fermentation.

The results of growth on different amounts of agar and agarose are seen in table 10.10. The table shows some interesting observations. 16AV probably does not degrade all the components of agar. When equal amounts of agar and agarose were added, some 2-3 times the amount of products were formed on agarose as compared to growth on agar. Optical density could not be determined due to the background that is attributable to agar and agarose as opposed to cells.

The amounts of ethanol and acetate produced increased with agar concentration to a point where 30g/l resulted in only marginally more than at 10g/l. At 10g/l and 30g/l two further products were detected. A peak was observed on the HPLC trace that eluted at the same time as galactose. Another peak eluted at a slightly faster time, that associated with disaccharides. It is important to realize

that identification of these peaks is only supposition and no attempt was made to identify them using such techniques as mass spectrometry.

Table 10.10 Total Products of 16AV on Different Agars/Agarose

Substrate (g/l)*	Products (mM) Acetate	Ethanol	"Mono-saccharide"	"Disacc-haride"
Σ Agarose (1)	3.2	3.1	0.0	0.0
UP Agarose (1)	3.1	3.0	0.0	0.0
Agar (1)	0.74	0.88	0.0	0.0
Agar (2)	5.0	6.9	0.0	0.0
Σ Agarose (2)	14.1	23.2	0.0	0.0
Agar (5)	13.9	23.0	0.0	0.0
Agar (10)	19.2	29.0	'3.5'	'1.0'
Agar (30)	18.7	34.2	'3.4'	'1.0'

Abbreviations used:
Σ = Sigma Chemical Co, St. Louis, MO, USA; UP = Ultra pure. BRL Laboratories, Bethesda, USA; Agar = Coast Biologicals, Opotiki, New Zealand.

10.16 DNA COMPOSITION.

The mole% G+C was determined on DNA extracted from 16AV using thermal denaturation according to the method in section 3.22. Determinations were carried out using DNA from 2 separate purification procedures. The values obtained were 39 mol%G+C and 40 mol%G+C.

10.17 ENZYMATIC STUDIES.

Aspects of enzymology were investigated to varying degrees with 16AV.

10.17.1 β-GLUCOSIDASE.

β-glucosidase is the enzyme that cleaves the β,(1→4) linkages of the cellobiose molecule leaving two free glucose molecules per cellobiose molecule. 16AV could grow on cellobiose but not glucose and so 16AV was assayed for the presence and location of this enzyme. The method described in section 9.11 was used for the assay and gave the results as follow in table 10.11.

Table 10.11. β-glucosidase Activity in 16AV

Sample	OD_{400}
Control	0.07
Culture Supernatant	0.06
Culture Pellet	0.18

Though only a qualitative experiment, in that activity is not expressed in terms of amount of protein, there is clearly β-glucosidase activity associated with the cells which does not exist in the supernatant whose optical density was the same as the control.

10.17.2 AGARASE ACTIVITY.

It was found that when using the PAHBAH assay as described, that high grade agarose was necessary if one was to obtain a relatively low background. For example, when Oxoid Purified agar or Merck Purified agar were prepared at 1 mg/ml and used as 1 ml samples with PAHBAH reagent, the optical density after boiling 6 minutes was greater than 2.0, making assays with the addition of an enzyme impossible. When ultrapure agarose was used at the same concentration, the optical

density after treatment was 0.3, still quite high but at a manageable level as compared to optical density after action of the enzyme.

The activity of the partially purified enzyme was carried out as described in section 9.13 and shown to be 0.55 mg gal/mg protein/hour. Activity is expressed as galactose equivalents (reducing sugar) released per per mg of protein.

10.17.3 IS AGARASE CONSTITUTIVE OR INDUCED?

To determine whether the enzyme was induced by the presence of agar/agarose 16AV was taken through several passages on different substrates. Each transfer was a 1% inoculum. The substrates used were agar, galactose and cellobiose. After 6 transfers into fresh medium with agar and galactose as substrate and 3 transfers with cellobiose, the presence of agarase activity was determined using the plate assay. Liquefaction of the agar plate surface only occurred using supernatant from cultures grown on agar indicating absence of agarase in cultures growing on cellobiose and galactose; agarase in not constitutive.

CHAPTER ELEVEN

ISOLATION AND CHARACTERIZATION OF AN OBLIGATELY ANAEROBIC, AGAROLYTIC BACTERIUM:- DISCUSSION AND CONCLUSIONS

11.1 GENERAL CHARACTERISTICS OF 16AV.

11.1.1 GRAM TYPE.

16AV had a negative Gram-stain reaction and showed a Gram-negative reaction with 2.0 M KOH. As further supportive evidence, 16AV was observed to lyse very easily with SDS when extracting DNA, a phenomenon associated with Gram-negative cells. The aminopeptidase test showed no reaction with the test reagent, a result that one associates with Gram-positive cells. This contradiction of results has been observed in *Sporomusa* where the staining procedure and KOH test showed the cells to be Gram-negative but the aminopeptidase reaction indicated a Gram-positive wall (Moller *et al.* 1984). Wall analyses of *Sporomusa* demonstrated a Gram-negative type of wall.

11.1.2 MOTILITY.

Despite all attempts, motility via flagella was never shown in 16AV. Examination of cells using electron microscopy demonstrated the absence of flagella. The possibility remained that 16AV was a *Cytophaga*-like organism. *Cytophaga* spp. are all flexible slender rods. Gliding motility is a characteristic of some members of this genus, several members of which degrade agar. As gliding motility could not be demonstrated, it is unlikely that 16AV belongs to this

group. Additional characteristics also exclude 16AV from this genus (discussed in detail later).

11.1.3 SPHERICAL BODIES.

In the discussion which follows (and elsewhere in the thesis) the term "resting stages" is used to describe the spherical bodies seen in old cultures. This avoids the use of specific terms such as 'spores', 'microcysts' etc. It is not known whether these bodies are truly dormant or merely starved cells.

as

he

og

phase culture did not increase the rate at which they were formed. The exact nature of the spherules was not determined though it was shown they were not refractile and conferred no heat resistance on a culture. Clearly though, they did promote viability of cultures as a culture left in the dark at room temperature for eight to ten months was still viable. As a consequence, further reference to these spherical bodies will be made as resting cells. The relationships of these spherical bodies to other spore-like and resistant bodies is discussed below.

Endospores.

Bacterial endospores are formed entirely within the parent cell. Their exact form varies with different genera, but all have basically the same composition and characteristics of resistance to heat, desiccation, a degree of UV radiation and certain chemicals. Endospores are produced by members of the genera *Clostridium*, *Bacillus*, *Desulfotomaculum* and *Sporosarcina* and appear as highly refractile cellular inclusions under phase contrast microscopy. Endospore morphology differs in different species, for example, in *Clostridium botulinum*, endospores are oval-shaped, formed centrally and cause no swelling of the cell, whereas in *Clostridium*

thermocellum, the endospores are spherical, situated terminally and are 4-5 times the width of vegetative cells (Gottschalk *et al.* 1981).

The resting bodies of 16AV are clearly not endospores as they were not formed as intracellular bodies, nor were they refractile.

Exospores.

Another organism known to form a type of spore is *Methylosinus trichosporium*. This organism sporulates by budding off an exospore which is similar to typical endospores in that they demonstrate resistance to desiccation and heat (Wittenbury *et al.* 1970). The exospores had a thick electron dense wall but they did not have a well-defined cortex. The resting bodies of 16AV are seemingly formed in a similar manner to *M. trichosporium* exospores but do not have the same complex structure. They also do not appear refractile and so are not exospores of the *M.trichosporium* type.

Myxospores.

A group of bacteria forming resting bodies are the order Myxobacterales, many members of which form structures termed myxospores. Migrating vegetative cells aggregate, at which point, the cells differentiate to form fruiting bodies. Each fruiting body is made up of slime and cells. Within these fruiting bodies, vegetative cells are converted to resting cells; the myxospores. This contrasts with endospores which are formed inside cells (Stanier *et al.* 1977). Different genera produce a range of structures which are responsible for the production of myxospores and the myxospores themselves vary in structure with different genera. However, they

are all generally conspicuous by appearing refractile when viewed with phase contrast microscopy (Reichenbach and Dworkin 1981). Two exceptions exist, namely *Polyangium* and *Chondromyces* which both form non-refractile myxospores. These organisms still produce specialist fruiting bodies for the production of myxospores. The spores of *Myxococcus* have been studied in some detail and show more heat resistance than vegetative cells, though not to the extent of endospores. Myxospores are considerably more resistant to desiccation than are vegetative cells

16AV does not produce its resting body in a complex fruiting structure, nor do they appear refractile; they are thus unlikely to be myxospores.

Microcysts.

Sporocytophaga is the only genus in the *Cytophaga* group that forms resting bodies. *Sporocytophaga myxococcoides* produces spherical refractile cysts by vegetative cells undergoing transformations, similar to those occurring in the myxobacteria, although fruiting bodies are not formed (Stanier 1940).

16AV produces its resting bodies in a similar manner to *Sporocytophaga* but they lack refractility. The resting cells of 16AV also seem not to have the thick wall associated with microcysts

Other Spherical Bodies.

Spherical bodies are produced by other bacteria. One such example is *Arthrobacter crystallopoietes*. When typical rods and spherules of

A.crystallopoietes were starved by shaking at 30°C in phosphate buffer for 30 days both maintained 100% viability. After this period the rods had degraded 40% of their carbohydrate while only 20% of that in the spheres was degraded. The RNA contents dropped by 85% and 32% respectively. The DNA content decreased in both spheres and rods immediately on starving but then remained almost the constant in both (Boylen and Ensign 1970b). Despite these relative decreases both rods and spheres retained viability after 70 days (Boylen and Ensign, 1970a). It is possible that spheres might confer even longer viability on a culture, say of up to several months compared with the 70 days tested.

The cellular organization of the resting bodies in strain 16AV was not studied and so detailed comparisons with the resting bodies of *A. crystallopoietes* cannot be made.

The formation of spherical bodies has been observed in a number of aged bacterial cultures. Two such examples are *Cytophaga* species (Stanier 1942, Bachman 1955, Veldkamp 1961) and *Treponema zuelzerae* (Veldkamp 1960). In all these organisms, sphere formation seemed to occur when cultures were about to lose viability making it unlikely that they were cysts of any type.

In summary, the resting bodies of 16AV are unlike all those described above. Detailed electron microscopy, including thin section analysis, along with analysis of the walls of the resting bodies is required to determine the true nature of the resting bodies.

11.1.4 SUBSTRATE METABOLISM PATTERNS.

The most notable feature of 16AV's substrate metabolism is the very restricted range of compounds on which it is able to grow. Only agar, agarose, galactose and cellobiose were used as substrates. Considering that its metabolism was that of carbohydrate fermentation, it was thought unusual that compounds like glucose, fructose and sucrose were not used. It was shown that 16AV possesses an intracellular β-glucosidase; this being the case, presumably cellobiose is taken into the cell and then cleaved into the two glucose molecules which are then utilized. The carbon balance studies of cultures grown on cellobiose showed traces of glucose in the supernatant, presumably having 'leaked' from the cells. The reason why glucose and other monosaccharides are not used is presumably due to lack of a the appropriate transport systems. For lactose, 16AV may not only lack the transport system but also lack a β-galactosidase. That no other polymers were used shows inability to produce depolymerases such as amylase or cellulases.

No organic acids were used by 16AV. Organic acids such as pyruvate and citrate are fermented by some *Clostridium* species and by genera like *Ilyobacter* (Stieb and Schink 1984) and *Eubacterium* (Moore and Holdeman 1986). Such compounds are not always used by some organisms which have otherwise extensive carbohydrate metabolism.

On the four substrates used by 16AV, the products of metabolism were ethanol, acetate, H_2 and CO_2. The yield of cell dry matter on galactose to substrate fermented was 13 g/mol. Stouthamer (1979) calculated a theoretical mean cell yield of 10 g cell dry matter per mol ATP when bacteria were growing on defined mineral medium. During

the conventional glycolytic fermentation of carbohydrates, monomers are degraded to pyruvate which maybe split to acetyl-CoA plus formate. Formate can be split by hydrogen formate lyase to H_2 and CO_2 and the acetyl-CoA metabolized to acetate and ethanol. The net energy conservation of glycolytic fermentation is 3 ATP/mol; thus, using the Stouthamer (1979) value above, the theoretical cell yield would be 30g/mol substrate oxidized. The yield of 13 g/mol obtained with 16AV is only one third to a half of that which one would expect.

The fermentation balances on galactose did not changed when grown under a positive pressure of H_2. The latter has been shown to alter the ratio of acetate and ethanol production in some micro-organisms by "pushing" the metabolic pathway from acetyl-CoA to acetyl-phosphate instead of acetaldehyde. A cell therefore generates more ATP per substrate oxidized and produces less ethanol.

When growing on agarose and agar at concentrations greater than 5g/l additional compounds were formed as products of the fermentation. These were tentatively identified as being a disaccharide and a monosaccharide. This is perhaps not surprising, as the agarase enzyme was shown to have activity for a period of time outside the cell, as seen when carrying out the plate assays. If growth of 16AV was inhibited by product formation, then agarase activity in the culture would result in release of carbohydrates. When 16AV was grown on cellobiose small amounts of glucose were found in the culture supernatant indicating leakage from the cells. Given that leakage of monomers from cells can take place, the extracellular enzyme may still be a typical β-agarase. The β-agarase cleaves the β,(1→4) linkages of the agarose backbone producing neoagarobiose and

neoagarotetraose. When the dimer or tetramer is taken into the cell, monomers, the product of their cleavage, could leak from the cells.

On one occasion only, a growth experiment on cellobiose showed the production of small amounts of lactic acid (approximately 1mM from 10mM cellobiose). This production of lactate by 16AV was not seen on any of the several other occasions on which growth on cellobiose was studied. Janssen (1989) showed that *Rumminococcus pasteurii* produced increased lactate upon lowering pH. In the description of this organism Schink (1984) did not report production of any lactate. It is thought unlikely that in 16AV the production of lactate was due to low pH as the pH of the medium at the time of stationary phase of 16AV was typically 6.2-6.5.

11.1.5 AGARASE ASSAYS AND MEASURED AGAROLYTIC ACTIVITY.

The plate assay for enzyme activity showed that agarase was only produced when 16AV was grown on agar. The culture supernatants of cellobiose- and galactose-grown cultures showed no activity so presumably the enzyme is not constitutive.

Assay for agarase activity was carried out using the PAHBAH method for determination of reducing sugars (Lever 1973). The usual procedure used to measure agarase activity involves incubating the enzyme preparation with an agarose solution and measuring the reducing sugars produced as galactose equivalents. Most publications in the literature involving agarase activity use the method of Dygert (1965) in which reducing sugars are estimated by chelation of oxidized copper ions, from reaction of the reducing sugars, with neocuproine, to form a coloured complex. The Lever method involves

reaction of *p*-hydroxybenzoic acid hydrazide with the reducing sugar. The exact mechanism of the reaction is not fully understood but is thought to involve the production of a hydrazone. This method was preferred as it is quick, sensitive and considerable use of this assay procedure had been made by colleagues.

The extracellular enzyme was partially purified during this study. The method used for purification was that of Bibb *et al.* (1987). Activity was retained during the steps taken to purify the agarase though no attempt was made to monitor purification steps.

11.2 IDENTIFICATION OF 16AV.

Ability to grow on agar as a sole carbon source has never been observed in an obligate anaerobe. However, a single characteristic such as this should not exclude the isolate from being recognized as a strain of an existing species.

16AV was observed to be a Gram-negative obligate anaerobe fermenting agar agarose, galactose and cellobiose to acetate, ethanol, carbon dioxide and hydrogen. No other substrates supported growth.

Obligately anaerobic, non-sporeforming, Gram-negative straight, curved or helical rods are assigned to the family Bacteroidaceae (Holdeman *et al.* 1984). A number of genera have been included in this family and their relationships to 16AV are as follows.

The metabolic patterns of 16AV mean it cannot be assigned to the genera *Bacteroides*, *Fusobacterium* or *Leptotrichia* which ferment a range of carbohydrates and produce a complex mixture of butyric, lactic, succinic, acetic and formic acids, as well as producing other

organic acids. For similar reasons, 16AV cannot be assigned to the genera *Butyrivibrio*, *Succinomonas*, *Succinovibrio*, *Pectinatus*, *Anaerobiospirillum*, *Wolinella* and *Selenomonas*. Members of these genera also produce a range of fatty acids as fermentation products from carbohydrates and organic acids; the major acid produced depending on the particular genus, for example *Succinomonas* spp. produce predominantly succinate. The substrate utilization patterns and fermentation products of 16AV exclude it from being assigned to either of the genera *Ilyobacter* or *Pelobacter*. Members of these two genera ferment a range of organic acids and often have diverse metabolism. The DNA base composition of *Pelobater* spp. are typically 50 % G+C whereas the G+C content in 16AV is 39 mol% (Schink 1982).

Aerobic, non-sporeforming, Gram-negative vibrios with DNA composition of approximately 40 % molG+C could possibly be assigned to the *Cytophaga/Flavobacterium* group. Members of the *Cytophaga/-Flavobacterium* group are also known to have species which degrade agar. Almost all species are obligately aerobic and all motile by way of gliding. Veldkamp (1960) described anaerobic growth of two *Cytophaga* species, however, these isolates were facultative, with best growth occurring in aerobically grown cultures. Leadbetter *et al.* (1979) isolated Gram-negative anaerobic rods from periodontic lesions that were saccharolytic, required CO_2 for growth and produced acetate as the major product of fermentation. These organisms were assigned to the genus *Capnocytophaga*. Important features of the genus *Cytophaga* are its ability to glide, its flexibility and aerobic nature, although facultative species are known and related species such as *Capnocytophaga* can also grow anaerobically. 16AV did not glide and never showed any growth under aerobic conditions.

Gram-negative bacteria able to degrade agar include *Meniscus glaucopsis* (Irgens 1977). It carries out fermentative metabolism on all the substrates that it uses, producing acetate, propionate, succinate but no gas as endproducts of fermentation. It is however an aerotolerant anaerobe, but growth only takes place if CO_2 is present. An additional characteristic of *M. glaucopsis* is the ability to produce gas vacuoles. 16AV should not be assigned to the genus *Meniscus* as it is an obligate anaerobe and ferments a very limited range of substrates producing acetate, ethanol, H_2 and CO_2. Succinate and propionate are not formed.

The general description of the genus *Acetivibrio* is that of a Gram-negative obligately anaerobic vibrio producing acetate as a major end product of fermentation. Two species have been described in the literature. The first, *Acetivibrio cellulolyticus* ferments only cellulose, cellobiose and salicin to acetate, hydrogen and carbon dioxide with traces of ethanol formed (Patel *et al.* 1980). The second species, *Acetivibrio ethanolgignens*, as the name indicates, produces greater amounts of ethanol as well as producing acetate, hydrogen and carbon dioxide. This species is capable of growth on a larger number of carbohydrates (Robinson and Ritchie 1981). The DNA composition of the two species are 38 and 40 %mol G+C respectively. Both the species are motile, but having different flagella arrangements. *A. cellulolyticus* possesses a single subterminal flagellum while *A. ethanolgignens* possesses several flagella, attached laterally in the concave sides of the cells.

Considering all the characteristics determined, 16AV most closely resembles members of the genus *Acetivibrio*. A summary of comparative characteristics is seen in table 11.1.

Table 11.1 Comparison of Characteristics of *Acetivibrio cellulolyticus*, *Acetivibrio ethanolgignens* and Strain 16AV

Characteristic	*A. ethanolgignens**	*A. cellulolyticus**	*16AV*
Flagella	Fascile	Subterminal	None
H_2S Production	+	-	-
Urease	-	-	-
Gelatin	+	-	-
Indole	-	-	-
Substrates Used			
Agar	-	-	+
Arabinose	-	-	-
Cellobiose	-	+	+
Cellulose	-	+	-
Esculin Hydrolysis	-	-	-
Fructose	+	-	-
Galactose	+	-	+
Glucose	+	-	-
Glycerol	-	-	-
Lactate	-	-	-
Lactose	+	-	-
Maltose	+	-	-
Mannitol	+	-	-
Pyruvate	+	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	-	-	-
Salicin	+	+	-
Starch	(+)	-	-
Sucrose	-	-	-
Xylose	-	-	-
Heat Resistant 80°C for 10 min	-	NR	-
Fermentation Products			
Formate	(x)	-	-
Acetate	+	+	+
Propionate	-	-	-
Lactate	-	-	-
Succinate	-	-	-
Ethanol	+	(x)	+
H_2	+	+	+
CO_2	±	+	+
DNA Base Composition mol% G+C	40	38	39

Symbols: +, growth; (+), weak growth; ± some strains positive; NR, not reported; (x), trace production
*, data from Patel *et al.* (1980), Robinson and Ritchie (1981).

16AV differs from the general description of the genus in that it is non-motile and from the two species in the range of compounds that can support growth. After considering the differences between the two *Acetivibrio* species and 16AV, they would not seem enough to exclude 16AV from inclusion in this genus. 16AV is similar to *A. cellulolyticus* in that it is very specialized with respect to the substrates that it can use, principally a polysaccharide and its monomeric units. 16AV then is probably best regarded as a new species of the genus *Acetivibrio*.

11.3 ECOLOGICAL ASPECTS OF AGAR DEGRADATION BY 16AV.

16AV was isolated from an anaerobic waste water pond with salinity corresponding to that of a freshwater source and with a carbon input, (the main polymers being cellulose and protein), more associated with terrestrial, as opposed to marine environments. That agarolytic bacteria occur in freshwater environments has been observed by Agbo and Moss (1979), isolating *Cytophaga saccharophilia* from a river, Van der Meulen *et al.* (1974), isolating *Cytophaga flevensis* from the IJsselmeer, a large freshwater lake in The Netherlands and Hunger and Claus (1978), isolating *Bacillus agar-exedens* from soil. The presence of agarolytic bacteria in freshwater environments has been difficult to explain. Agbo and Moss reasoned that their isolates probably were not from some contamination of the fresh water source with marine material, but the ability to degrade agar by their isolates was perhaps simply a reflection of the organisms' ability to degrade a great range of plant polysaccharides. This conclusion was supported by the fact the agarase they purified had activity to varying degrees on other polygalactans. This supportive evidence does not hold for *C.flevensis* as its agarase was

specific in its substrate requirements. These authors considered that the agarase activity of this organism was a reflection of a past association of the IJsselmeer with the North Sea and adaptation of *C. flevensis* to freshwater environments. They also observed that there appeared to be an increased tolerance of a red alga to freshwater conditions by living near a stream connecting the IJsselmeer with the sea.

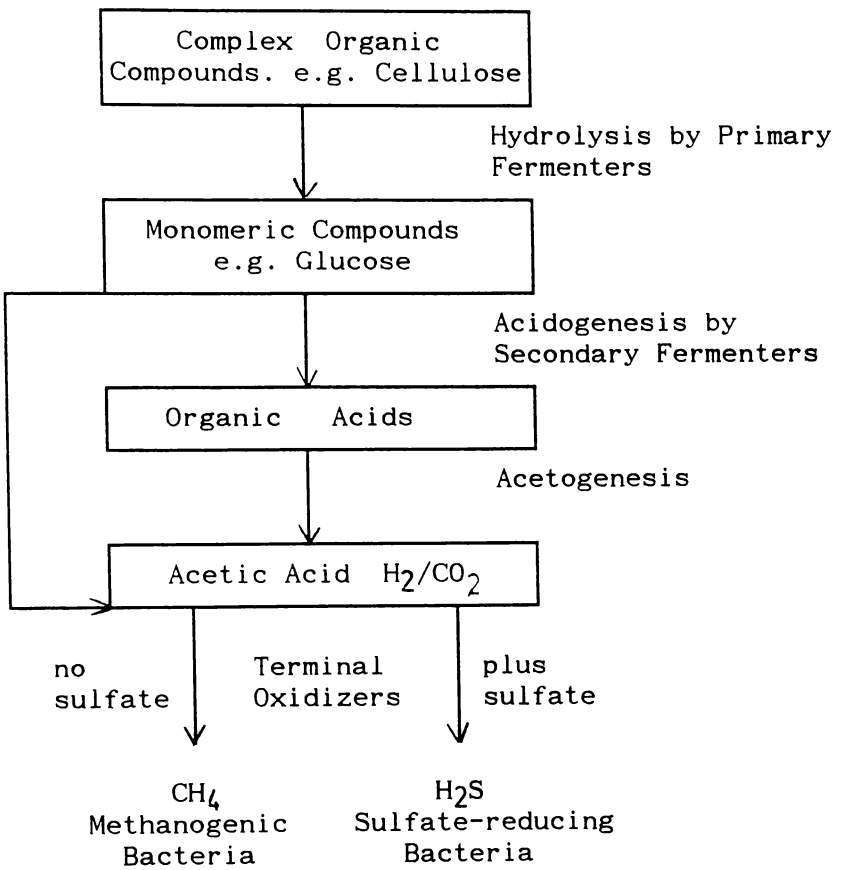
Similar problems arose when trying to to rationalize the isolation of 16AV for a freshwater source. While the reasons for the presence of agarolytic bacteria in the IJsselmeer appear valid, the arguments do not apply to the isolation of 16AV from a abattoir waste water pond. 16AV may utilize compounds other than those tested in this study or the agarase may be non-specific and attack other polysaccharides of plant origin, such as gum arabic.

11.4 ECOLOGY OF AGAR DEGRADATION.

Most probable enumeration of anaerobic agar degrading bacteria showed there were 23 cells per 100 ml of treatment pond from where 16AV was obtained and 920 per 100 ml in the estuarine sample. Enumeration was carried out simply using the mud collected from the sites; no attempt was made to include decaying seaweed in the estuarine sample.

Interesting questions arise from the isolation of an anaerobe able to ferment an algal polymer. Anaerobic systems have been studied in great detail with the generalized representation of anaerobic mineralization of polymers being derived as described in detail earlier see figure 11.1. Detailed information is available on the

Figure 11.1 Generalized Scheme For Anaerobic Mineralization of Carbon Compounds in Nature



ecology of anaerobic bacterial interactions in freshwater sediment, anaerobic digesters and the rumen. In figure 11.1, polymers entering the scheme are normally considered to be cellulose and hemicelluloses. For the freshwater environments, this is clearly the case and ecological studies of such environments have led to the isolation of a wide range of cellulolytic anaerobes. Culture collections offer species of the genera *Clostridium*, *Bacteroides* and *Butyrivibrio*, among others, all able to degrade cellulose. The degradation pathways of cellulose compounds have been elucidated, as well as determination of the products of fermentation. With the knowledge that volatile fatty acids, acetate, H_2 and CO_2 are produced, overall microbial metabolic strategies like that shown in figure 11.1 have been shown to exist.

However, estuarine and marine environments may receive very different polymer inputs. Although mangrove swamps receive cellulose from leaf fall, shorelines and inshore marine waters presumably receive large inputs of other polymers of algal origin.

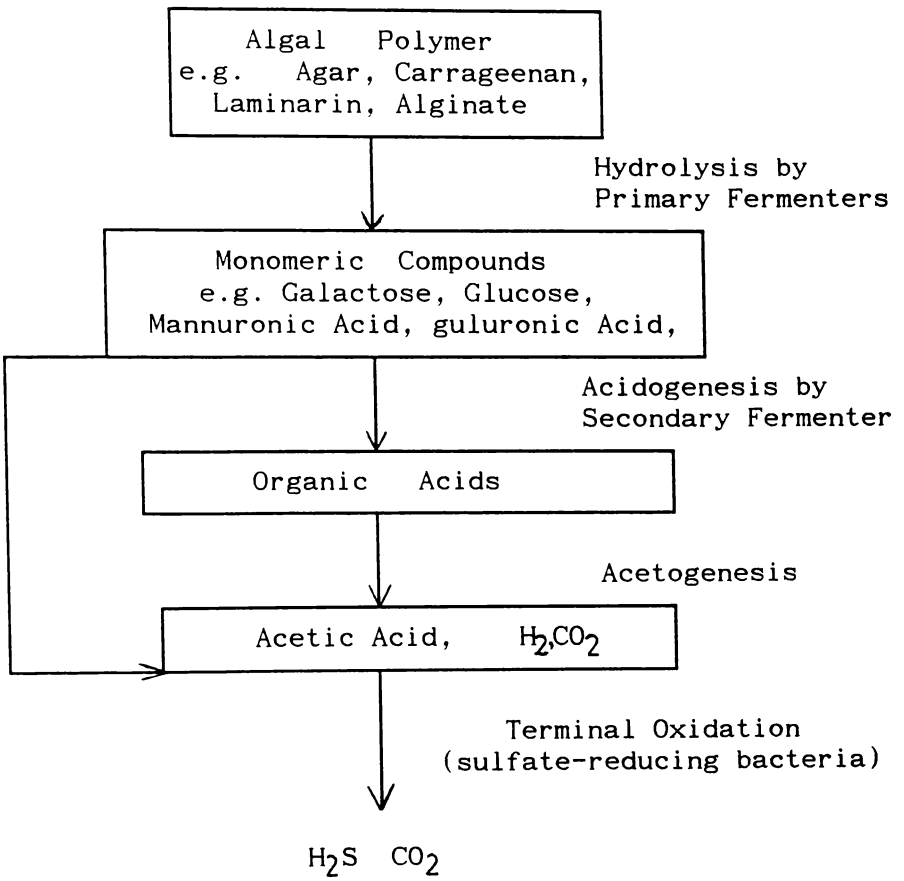
Studies of total seaweed production have been inconsistent in both their approach and the site studied and so detailed information on input are not available for many coastal regions. Furthermore, the type of shoreline dictates the type and amount of seaweed that can develop. As a result, figures on the amounts of seaweed production in nature show considerable variability.

On the northeast coast of Canada, estimates of seaweed in the littoral zone are 1-2 $kg.m^{-2}$ and up to 4-7 $kg.m^{-2}$ in the sub-littoral (Chapman and Chapman 1980). In the Arctic sea around the Murman coast the amount of seaweed is high with up to 28 $kg.m^{-2}$. In New

Zealand, *Microcystis* beds in Cook Strait are thought to yield up to 8500 tonnes wet weight per annum (Chapman and Chapman 1980). Another interesting situation occurs with the annual seaweeds. Wallentius (1984) studied standing crops of some annual seaweeds in the Baltic. It was estimated that in June there were some 1100 tonnes dry weight in the 160 km^2 study area; this weight fell to 100 tonnes in December. From these data, it is clear a large input of algal polymers must take place by a process analogous to seasonal leaf-fall in terrestrial ecosystems. The actual amounts of algal polymer released into nature are difficult to determine as different polymers occur in different proportions in different species. In addition to this, there are also seasonal fluctuations in composition. Examples of compositions have been determined for *Chondrus*, which is typically about 80 % weight water. Of the 20% dry weight, 50-60% are polygalactans. Yields of commercial preparations of agar from *Gracilaria* are typically 20-30% of the dry weight though it is thought the true amount may be up to 60% of the dry weight. Laminarin and fucoidan account for some 10-30% of the dry weight of *Laminaria* species (Chapman and Chapman 1980).

This study further demonstrated the potential for anaerobic degradation. Agar was fermented to smaller organic compounds (acetate, ethanol) plus H_2 and CO_2. During isolation procedures, the ethanol produced from the agar fermentation was used by a sulfate-reducing bacterium, while in nature the acetate, along with the H_2 could also be used by sulfate-reducing bacteria. A diagram for metabolism of organic material under anaerobic conditions in estuarine or marine environments where cellulose is not the major polysaccharide, but instead agar could be proposed (figure 11.2). In this situation, agar is degraded, leading to production of anhydro-L-

Figure 11.2 Proposed Scheme For Anaerobic Mineralization of Algal Polymers



galactose and galactose which are fermented to produce acetate plus other oxidized products; terminal oxidation is carried out by sulfate-reducing bacteria. This leads to further speculation; could other algal polymers such as carrageenan, algin and laminarin also have a place as the primary polymers for anaerobic degradation in estuarine or coastal marine environments in a manner like that shown in figure 11.2? There have been very few reports of anaerobes able to degrade algal polymers. Salyers *et al.* (1977) examined a number of *Bacteroides* strains found in the colon for their ability to ferment a range of plant polysaccharides including laminarin, alginate and fucoidin. Laminarin was fermented by a very small number of species, alginate was fermented by *B. ovatus* and fucoidin was not fermented by any of the test strains. Studies on methane generation using *Laminaria*-fed digesters have taken place but these investigations have been from a biotechnological viewpoint and the microorganisms involved have not been characterized (Flowers and Bird 1984, Schram and Lehnberg 1984).

CHAPTER TWELVE

SUGGESTIONS FOR FURTHER WORK

Having completed the research of this project, a number of avenues for further work have become apparent. Suggestions for further work follow two direction; a) the further study of the bacteria that were isolated in this study, b) the study of wider ecological aspects relating to processes in the sediment.

12.1 WORK RELATING TO SULFATE-REDUCING BACTERIA.

The first priority is to carry out a number of experiments on the sulfate-reducing bacteria that were isolated in this study. Many species descriptions of sulfate-reducing bacteria are based on characteristics that have been determined on a few strains or more often, a single strain. Characterization of these isolates will improve, to some degree, the knowledge of strain variability. This is important for strains AmPa1 and HoPa1 as they show several differences from *Desulfovibrio sapovorans*, but insufficient data were obtained on these strains to justify the creation of a new species. The current description of the genus *Desulfosarcina* was made following the determination of the characteristics of one strain. Strain Ok2mb was identified as strain of the genus *Desulfosarcina* and so warrants complete characterization. Further experiments on the bacteria isolated in this study should include:

- Attempts to determine the DNA composition of strains HoPa1 and AmPa1 in order to give an accurate identification.
- Completion of the characterization of strain Ok2mb, a *Desulfosarcina* species.

- Completion of the characterization of those strains isolated using the diluted mud transfer in order to give more details as to the different organisms that might be isolated using enrichments with mud from a dilution series of mud.

Secondly, further experiments resulting from this study include the study of sulfate-reducing bacteria and the compounds which they can oxidize. Work relating to this should include:

- The screening of sulfate-reducing bacteria held in culture collections for the ability to use amino acids.
- Studies on the physiological aspects of amino acid metabolism in sulfate-reducing bacteria. The degradation of amino acids is still a poorly-understood phenomenon and it would be of interest to investigate further the pathways of amino acid oxidation in sulfate-reducing bacteria.

A number of longer-term projects could be carried out which result from observations made in this thesis. These concern the wider ecological aspects of sulfate-reducing bacteria and their roles in sediments. The sediment dilution method for isolating sulfate-reducing bacteria indicated that it might be useful for the isolation of novel bacteria. Suggested work might include:

- Carrying out the isolation of sulfate-reducing bacteria with electron donors such as H_2, acetate and aromatic compounds using the mud dilution method to see if this technique was useful for the isolation of novel bacteria.
- A comparison of the growth rates of *Desulfovibrio sapovorans* and *Desulfobacterium* sp. growing on palmitate. These were the two different bacteria isolated from the different dilutions of mud. Such studies might be useful in explaining the reason for the

isolation of such bacteria and may perhaps aid in assigning more precise ecological roles to the two different populations.

Further long-term studies might also include *in situ* studies of carbon metabolism. One approach would be to carry out colonization experiments using an insoluble substrate, such as palmitate. This could be in the form of continuous enrichment or by the addition to sediment of microscope slides which have been coated with palmitate. This would give more data on the bacteria that are responsible for palmitate degradation in sediments.

12.2 WORK RELATING TO SYNTROPHIC CULTURES.

Defined syntrophic cultures degrading fatty acids were not obtained in this study; even so, further work would be profitable with these cultures. Suggested work should include:

- Complete characterization of the syntrophic culture isolated from the marine source should be carried out in order to compare the bacteria responsible for the degradation of palmitate with those responsible for palmitate degradation in freshwater systems.

It would also be of interest to determine what are the advantages of sulfate-reducing bacteria over syntrophic bacteria with regard to the degradation of fatty acids. This would involve measuring and comparing K_s values for substrates such as palmitate.

12.3 WORK RELATING TO AGAR DEGRADATION.

A number of studies lead on from the study that was carried out on agar degradation. As with the sulfate-reducing bacteria, one priority is to continue work on the obligately anaerobic, agarolytic

bacterium that was isolated in this study. A second priority involves those wider aspects of research that arose from this study. Suggestions for further work on strain 16AV could include ecological, physiological and biochemical studies:

-Isolation of further strains of 16AV in order to determine the range of physiologies present.

- DNA homology studies on 16AV and related genera in order to determine its relationship with described related genera.

- Investigation of the nature of the resting bodies formed by 16AV.

- Characterization of the agarase enzyme system of 16AV in order to allow comparison with the agarase of aerobic bacteria.

Longer-term studies should be carried out on agarolytic bacteria; it would be interesting, for instance, to study the distribution of anaerobic agarolytic organisms, in different environments, particularly estuarine and coastal marine sediments.

As an additional longer-term study, it would be of interest to study the degradation of other structural polymers of algal origin, such as carrageenan, alginic acid and laminarin. As compounds like these represent the primary input into coastal and estuarine environments, and since their degradation is the first step in carbon degradation in such environments, (see figure 11.2), it is of interest to determine the extent of anaerobic degradation of such compounds.

REFERENCES

Abbott IA, Chapman FA (1981) Evaluation of kappa carrageenan as a substitute for agar in microbiological media. Archives of Microbiology 128:355-359

Agbo JAC, Moss MO (1979) The isolation and characterization of agarolytic bacteria from a lowland river. Journal of General Microbiology 115:355-368

Allison MJ (1969) Biosynthesis of amino acids by ruminal microorganisms. Journal of Animal Sciences 29:797-807

Allison MJ (1978) Production of branched-chain volatile fatty acids by certain anaerobic bacteria. Applied and Environmental Microbiology 35:872-877

American Public Health Association (1971) Standard Methods for the examination of water and waste water, 13th edition.

Andrykovitch G, Marx I (1988) Isolation of a new polysaccharide-digesting bacterium from a salt marsh. Applied and Environmental Microbiology 54:1061-1062

Bachman BJ (1955) Studies on *Cytophaga fermentens* n. sp., a facultatively anaerobic lower myxobacterium. Journal of General Microbiology 13:541-551

Bak F, Cypionka H (1987) A novel type of energy metabolism involving fermentation of inorganic sulphur compounds. Nature 326:891-892

Bak F, Pfennig N (1987) Chemolithotrophic growth of *Desulfovibrio sulfodismutans* sp. nov. by disproportionation of inorganic sulfur compounds. Archives of Microbiology 147:184-189

Bak F, Widdel F (1986a) Anaerobic degradation of indolic compounds by sulfate-reducing enrichment cultures, and description of *Desulfobacterium indolicum*. Archives of Microbiology 146:170-176

Bak F, Widdel F (1986b) Anaerobic degradation of phenol and phenol derivatives by *Desulfobacterium phenolicum* sp. nov.. Archives of Microbiology 146:177-180

Balas R, Bartlett D, Silverman M (1988) Cloning and gene replacement mutagenesis of a *Pseudomonas atlantica* agarase gene. Applied and Environmental Microbiology 54:30-37

Barker HA (1940) Studies upon the methane fermentation IV. The isolation and culture of *Methanobacterium omelianskii*. Antonie van Leeuwenhoek Journal of Microbiology and Serology 6:201-220

Barker HA (1981) Amino acid degradation by anaerobic bacteria. Annual Reviews of Biochemistry 50:23-40

Bauman P, Schubert RHW (1984) Family II. Vibrionaceae. In: Krieg NE (ed) Bergey's manual of systematic bacteriology, volume 1. Williams and Wilkins:Baltimore. pp 516-538

Blackburn TH, Hobson PN (1962) Further studies on the isolation of proteolytic bacteria from the sheep rumen. Journal of General Microbiology 29:69-81

Beaty PS, McInerney MJ (1987) Growth of *Syntrophomonas wolfei* in pure culture on crotonate. Archives of Microbiology 147:389-393

Beijerink MW (1895) Uber *Spirillum desulfuricans* als ursache von sulfatreduktion. Zentralblatt fur Bakteriologie und Parasitenkunde 2, Abt 1: 1-9, 49-50, 104-114. Cited in Postgate JR (1984) The sulfate-reducing bacteria, 2nd edition. Cambridge University Press:Cambridge

Beerens H, Romond H (1977) Sulfate-reducing bacteria in human feces. American Journal of Clinical Nutrition 30:1770-1776

Bender DA (1985) Amino acid metabolism, 2nd edition. Wiley and Sons:London, New York, Sydney, Toronto

Bibb MJ, Jones GH, Joseph R, Buttner MJ, Ward JM (1987) The agarase gene (*dag* A) of *Streptomyces coelicolor* A3(2): Affinity purification and characterization of the cloned gene product. Journal of General Microbiology 133:2089-2096

Blackburn TH, Hobson PN (1962) Further studies on the isolation of proteolytic bacteria from sheep rumen. Journal of General Microbiology 29:69-81

Boone DR, Bryant MP (1980) Propionate-degrading bacterium, *Syntrophobacter wolinii* sp.nov. gen.nov., from methanogenic ecosystems. Applied and Environmental Microbiology 40:626-632

Boone DR, Whitman WB (1988) Proposal of minimum standards for describing new taxa of methanogenic bacteria. International Journal of Systematic Bacteriology 38:212-219

Boylen CW, Ensign JC (1970a) Long term starvation survival of rods and spherical cells of *Arthrobacter crystallopoietes*. Journal of Bacteriology 103:569-577

Boylen CW, Ensign JC (1970b) Intracellular substrates for endogenous metabolism during long-term starvation of rod and spherical cells of *Arthrobacter crystallopoietes* Journal of Bacteriology 103:578-587

Breed RS, Murray EGD, Smith NR (1975) Bergey's manual of determinative bacteriology, 7th edition. Williams and Wilkins:Baltimore

Bryant MP, Boone DR (1987) Emended description of strain MS^T (DSM 800^T), the type strain of *Methanosarcina barkeri*. International Journal of Systematic Bacteriology 37:169-170

Brysch K, Schneider C, Fuchs G, Widdel F (1987) Lithotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum*. Archives of Microbiology 148:264-274

Campbell LL, Jr., Frank HA, Hall ER (1957) Studies on the sulfate-reducing bacteria. I. Identification of *Sporovibrio desulfuricans* as *Clostridium nigrificans*. Journal of Bacteriology 73:516-521

Campbell LL, Kasprzycki MA, Postgate JR (1966) *Desulfovibrio africanus* sp. nov., a new dissimilatory sulfate-reducing bacterium. Journal of Bacteriology 92:1122-1127

Campbell LL, Postgate JR (1965) Classification of the spore-forming sulfate-reducing bacteria. Bacteriological Reviews 29:359-363

Campbell LL, Singleton Jr R (1986) Genus IV. *Desulfotomaculum*. In: Sneath PHA, Mair NS, Sharpe ME (eds) Bergey's manual of systematic bacteriology, volume 2. Williams and Wilkins: Baltimore. pp 1200-1202.

Cappenberg TE (1974) Interactions between sulfate-reducing and methane producing bacteria in bottom deposits of a fresh water lake 1. Field observations. Antonie van Leeuwenhoek 40:285-295

Cardon BP, Barker HA (1947) Amino acid fermentation by *Clostridium propionicum* and *Diplococcus glycinophilus*. Archives of Biochemistry 12:165-180

Chapman VJ, Chapman DJ (1980) Seaweeds and their uses, 3rd edition. Chapman and Hall: London, New York

Chartrain M, Zeikus JG (1986) Microbial ecophysiology of whey biomethanation: characterization of trophic populations and prevalent species composition in continuous culture. Applied and Environmental Microbiology 51:1147-1156

Coleman GS (1960) A sulfate-reducing bacterium from the sheep rumen. Journal of General Microbiology 22:423-436

Cord-Ruwisch R, Garcia JL (1985) Isolation and characterization of an anaerobic benzoate-degrading spore-forming sulfate-reducing bacterium, *Desulfotomaculum sapomendens*. FEMS Microbiology Letters 29:325-330

Cord-Ruwisch R (1984) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. Journal of Microbiological Methods 4:33-36

Crueger W, Crueger A (1984) Biotechnology, a textbook of industrial microbiology. Science Tech:Madison

Daumas S, Cord-Ruwisch R, Garcia JL (1988) *Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H_2 from geothermal ground water. Antonie van Leeuwenhoek 54:165-178

DeLey J (1970) Re-examination of the association between melting point, buoyant density and chemical base composition of deoxyribonucleic acid. Journal of Bacteriology 101:738-754

Doetsch RN (1981) Determinative methods in light microscopy. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of methods for general bacteriology. American Society of Microbiology:Washington D.C. pp 21-33

Douglas HC (1951) Glycine fermentation by nongas-forming anaerobic micrococci. Journal of Bacteriology 62:517-518

Dygert S, Li LH, Florida D, Thoma JA (1965) Determination of reducing sugars with improved precision. Analytical Biochemistry 13:367-374

Eichler B, Schink B (1985) Fermentation of primary alcohols and diols and pure culture of syntrophically alcohol-oxidizing anaerobes. Archives of Microbiology 143:60-66

Esnault G, Caumette P, Garcia JL (1988) Characterization of *Desulfovibrio giganteus* sp. nov., a sulfate-reducing bacterium isolated from a brackish coastal lagoon. Systematic and Applied Microbiology 10:147-151

Ferry JG, Smith PH, Wolfe RS (1977) *Methanospirillum*, a new genus of methanogenic bacteria and characterization of *Methanospirillum hungatii* sp. nov.. International Journal of Systematic Bacteriology 24:465-469

Flowers A, Bird K (1984) Marine biomass: A long-term methane supply option. Hydrobiologia 116/117:272-275

Fowler VJ, Widdel F, Pfennig N, Woese CR (1986) Phylogenetic relationships of sulfate-and sulfur-reducing eubacteria. Systematic and Applied Microbiology 8:32-41

Gottschalk G, Andreesen JR, Hippe H (1981) The genus *Clostridium* (nonmedical aspects). In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG (eds) The prokaryotes. A hand book on habitats, isolation and identification of bacteria. Springer-Verlag:Berlin, Heidelberg, New York. pp 1767-1803

Gogotova GI, Vainshtein MB (1983) Spore-forming, sulfate-reducing bacterium *Desulfotomaculum guttoideum* sp.nov.. Microbiology 52:789-793. English translation of Mikrobiologiya

Gran HH (1902) Studien uber Meeresbakterien II. Ueber die Hydrolyse des Agar-agar durch ein neues Enzym, die Gelase. Bergens Museums Aarbog no 2, 1-16. In: Stanier RY (1941) Studies on marine agar-digesting bacteria. Journal of Bacteriology 115:355-368

Hanaki K, Matsuo T, Nagase M (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnology and Bioengineering 23:1591-1610

Handley DA, Olsen RR (1979) Butvar B-98 as a thin support film. Ultramicroscopy 4:479-480

Harfoot CG (1981) Lipid metabolism in the rumen. In: Christie WW (ed) Lipid metabolism in ruminant animals. Pergamon Press: Oxford. pp 21-55

Harwood CS, Canale-Parola E (1983) *Spirochaeta isovalerica* sp. nov., a marine anaerobe that forms branched-chain fatty acids as fermentation products. International Journal of Systematic Bacteriology 33:573-579

Hazelwood GP, Edwards R (1981) Proteolytic activities of a rumen bacterium, *Bacteroides ruminicola* R8/4. Journal of General Microbiology 125:11-15

Henderson C (1971) A study of the lipase produced by *Anaerovibrio lipolyticus*, a rumen bacterium. Journal of General Microbiology 65:81-89

Hofsten Bv, Malmqvist M (1975) Degradation of agar by a Gram-negative bacterium. Journal of General Microbiology 87:150-158

Holdeman LV, Moore WEC (1974) Genus I *Bacteroides*. In: Buchanan RE Gibbons NE (eds) Bergey's manual of determinative bacteriology, eigth edition. Williams and Wilkins:Baltimore pp 385-404

Holdeman LV, Kelley RW, Moore WEC (1984) Family I. Bacteroidaceae. In: Krieg NE (ed) Bergey's manual of systematic bacteriology, volume 1. Williams and Wilkins:Baltimore. pp 602-603

Holmes B, Owen RJ, M^{c}Meekin TA (1984) Genus *Flavobacterium*. In: Kreig NE (ed) Bergey's manual of systematic bacteriology, volume 1. Williams and Wilkins:Baltimore. pp 353-360

Hobson PN, Mann SO (1961) The isolation of glycerol fermenting and lipolytic bacteria from the rumen of sheep. Journal of General Microbiology 25:227-240

Huising J, McNeill JJ, Matrone G (1974) Sulfate reduction by *Desulfovibrio* species isolated from sheep rumen. Applied Microbiology 28:481-497

Hunger W, Claus D (1978) Reisolation and growth conditions of *Bacillus agar-exedens*. Antonie van Leeuwenhoek 44:105-113

Iizuka H, Okazaki H, Seto N (1969) A new sulfate-reducing bacterium isolated from Antarctica. Journal of General and Applied Microbiology 15:11-18

Imhoff-Stuckle D, Pfennig N (1983) Isolation and characterization of a nicotinic acid-degrading sulfate-reducing bacterium. Archives of Microbiology 136:194-198

Ingvorsen K, Brock TD (1982) Electron flow via sulfate reduction and methanogenesis in the anaerobic hypolimnion of Lake Mendota. Limnology and Oceanography 27:559-564

Ingvorsen K, Jorgensen BB (1984) Kinetics of sulfate uptake by freshwater and marine species of *Desulfovibrio*. Archives of Microbiology 139:61-66

Ingvorsen K, Zehnder AJB, Jorgensen BB (1984) Kinetics of sulfate and acetate uptake by *Desulfobacter postgatei*. Applied and Environmental Microbiology 47:403-408

Ingvorsen K, Zeikus JG, Brock TD (1981) Dynamics of bacterial sulfate reduction in a eutrpohic lake. Applied and Environmental Microbiology 42:1029-1036

Irgens RL (1977) *Meniscus*, a new genus of aerotolerant, gas-vacuolated bacteria. International Journal of Systematic Bacteriology 27:38-43

Janssen PH (1989) Isolation and characterization of organic acid-fermenting bacteria from anaerobic sediments. D.Phil Thesis. University of Waikato. In preparation

Jones HE (1972) Cytochromes and other pigments of dissimilatory sulfate-reducing bacteria. Archiv fur Mikrobiologie 84:207-224

Keith SM, Herbert RA (1983) Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*. FEMS Microbiology Letters 18:55-59

Keith SM, Herbert RA, Harfoot CG (1982) Isolation of new types of sulfate-reducing bacteria from estuarine and marine sediments. Journal of Applied Bacteriology 53:29-33

Klemps R, Cypionka H, Widdel F, Pfennig N (1985) Growth with hydrogen and further nutritional characteristics of *Desulfotomaculum* species. Archives of Microbiology 143:203-208

Koch R (1881) Zur Untersuchung von pathogenen Organismen. Mittheilungen aus dem Kaiserlichen Gesundheitsamte 1:1-48. In: Brock TD (1961) Milestones in Microbiology. Prentice-Hall:London. pp 101-108

Koch R (1882) Die Atiologie der Tuberkulose. Berliner Klinischen Wochenschrift No.5:221-230. In: Brock TD (1961) Milestones in Microbiology. Prentice-Hall:London. pp 109-115

Kristjansson JK, Schönheit P, Thauer RK (1982) Different k_s values for hydrogen of methanogenic bacteria and sulfate-reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Archives of Microbiology 131:278-282

Krumholz LR, Bryant MP (1986) *Syntrophococcus sucromutans* sp. nov. gen. nov. uses carbohydrates as electron donors and formate, methoxymonobenzoids or *Methanobrevibacter* as electron acceptor systems. Archives of Microbiology 143:313-318

Laanbroek HJ, Abee T, Voogd IL (1982) Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. Archives of Microbiology 133:178-184

Leadbetter ER, Holt SC, Socransky SS (1979) *Capnocytophaga*: New genus of gram-negative gliding bacteria. I. General

characteistics, taxonomic considerations and significance. Archives of Microbiology 122:9-16

Lee MJ, Zinder SH (1988) Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H_2-CO_2. Applied and Environmental Microbiology 54:124-129

LeGall J (1963) A new species of *Desulfovibrio*. Journal of Bacteriology 86:1120

Lever M (1973) Colormetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. Biochemical Medicine 7:274-281

Lovley DR, Dwyer DF, Klug MJ (1982) Kinetic mechanisms for the ability of sulfate reducers to outcompete methanogens for hydrogen in freshwater and marine sediments. Abstracts for the Annual meeting of the ASM, Abstract 194

Lovely DR, Klug WJ (1982) Intermediary metabolism of organic matter in the sediments of a eutrophic lake. Applied and Environmental Microbiology 43:552-560

Mah RA, Kuhn (1984) Transfer of the type species of the genus *Methanocccus* to the genus *Methanosarcina* naming it *Methanosarcina mazei* (Barker 1941) comb. nov. et emend. and conservation of the genus *Methanococcus* (Approved Lists 1980) with *Methanococcus vannielii* (Approved Lists 1980) as the type species. International Journal of Systematic Bacteriology 34:263-265

Massey LK, Sokatch JR, Conrad RS (1976) Branched-chain amino acid catabolism in bacteria. Bacteriological Reviews 40:42-54

McInerney MJ (1988) Anaerobic hydrolysis and fermentation of fats and proteins. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. John Wiley and Sons:New York. pp 373-415.

McInerney MJ, Bryant MP, Pfennig N (1979) Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Archives of Microbiology 122:129-135

McInerney MJ, Bryant MP, Hespell RB, Costerton JW (1981) *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Applied and Environmental Microbiology 41:1029-1039

Miller JDA, Wakerley DS (1966) Growth of sulphate-reducing bacteria by fumarate dismutation. Journal of General Microbiology 43:101-107

Moller B, Oßmer R, Howard BH, Gottschalk G, Hippe H (1984) *Sporomusa*, a new genus of gram-negative anaerobic bacteria including *Sporomusa sphaeroides* spec. nov. and *Sporomusa ovata* spec. nov. Archives of Microbiology 139:388-396

Moore WEC, Johnson JL, Holdeman LV (1976) Emendation of *Bacteroidaceae* and *Butyrivibrio* and descriptions of

Desulfomonas gen. nov. and ten new species in the genera *Desulfomonas*, *Butyrivibrio*, *Eubacterium*, *Clostridium* and *Ruminococcus*. International Journal of Systematic Bacteriology 26:238-252

Moore WEC, Holdeman LV (1986) *Eubacterium*. In: Sneath PHA Mair NS, Sharpe ME (eds) Bergey's manual of systematic bacteriology, volume 2. Williams and Wilkins:Baltimore. pp 1353-1373.

Morrice LM, McLean MW, Long WF, Williamson FB (1983) b-agarase I and II from *Pseudomonas atlantica*. Substrate specifities. European Journal of Biochemistry 137:149-154

Mountfort DO, Asher RA (1981) Role of sulfate reduction versus methanogenesis in terminal electron flow in polluted intertidal sediment of Waimea inlet, Nelson, New Zealand. Applied and Environmental Microbiology 42:252-258

Mountfort DO, Asher RA (1986) Isolation from a methanogenic ferulate degrading consortium of an anaerobe that converts methoxy groups of aromatic acids to volatile fatty acids. Archives of Microbiology 144:55-61

Mountfort DO Brulla WJ, Krumholtz LR, Bryant MP (1984) *Syntrophus buswellii* gen. nov. sp. nov., a benzoate catabolizer from methanogenic ecosystems. International Journal of Systematic Bacteriology. 34:216-217

Mountfort DO, Bryant MP (1982) Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Archives of Microbiology 113:249-256

Nanninga HJ, Gottschal (1987) Properties of *Desulfovibrio carbinolicus* sp. nov. and other sulfate-reducing bacteria isolated from an anaerobic-purification plant. Applied and Environmental Microbiology 53:802-809

Ollivier B, Cord-Ruwisch R, Hatchikian EC, Garcia JL (1988) Characterization of *Desulfovibrio fructosovorans*. Archives of Microbiology 149: 447-450

Opperman RA, Nelson WO, Brown RE (1961) *In vitro* studies of methanogenesis in the bovine rumen: dissimilation of acetate. Journal of General Microbiology 25:103-111

Owen RJ, Hill LR, Lapage SP (1969) Determination of DNA base composition from melting profiles in dilute buffers. Boipolymers 7:503-506

Owen RJ, Lapage SP (1976) The thermal denaturation of partially purified bacterial deoxyribonucleic acid and its taxonomic applications. Journal of Bacteriology 41:335-340

Patel GB, Kahn AW, Agnew BJ, Colvin JR (1980) Isolation and characterization of an anaerobic, cellulolytic microorganism, *Acetivibrio cellulolyticus* gen. nov., sp. nov.. International Journal of Systematic Bacteriology 30:179-185

Patel BKC (1984) Extremely thermophilic bacteria in New Zealand hot pools. D.Phil. Thesis. University of Waikato, Hamilton.

Patel BKC, Morgan HW, Daniel RM (1985) A simple and efficient method for preparing and dispensing anaerobic media. Biotechnology Letters 7:277-278

Petri RJ (1887) Eine kleine Modifikation des Koch'schen Plattenverfahrens. Zentralblatt fur Bacteriologie und Parasitenkunde. In: Brock TD (1961) Milestone in Microbiology. Prentice-Hall:London. pp 218-219.

Pfennig N, Trüper HG (1981) Isolation of members of the families Chromatiaceae and Chlorobiaceae. In: Starr M, Stolp H, Truper HG, Balows A, Schlegel HG (eds) The prokaryotes, a hand book on habitats, isolation and identification of bacteria, volume 1. Springer-Verlag:Berlin, Heidelberg, New York. pp 279-289.

Pfennig N, Wagener S (1986) An improved method of preparing wet mounts for photomicrographs of microorganisms. Journal of Microbiological Methods 4:303-306

Pfennig N, Widdel F, Trüper HG (1981) The dissimilatory sulfate-reducing bacteria. In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG (eds) The prokaryotes, a handbook on habitats, isolation and identification of bacteria, volume 1. Springer-Verlag:Berlin, Heidelberg New York. 926-940

Porschen RK, Chan P (1977) Anaerobic vibrio-like organisms cultured from blood: *Desulfovibrio desulfuricans* and *Succinivibrio* species. Journal of Clinical Microbiology 5:441-447

Postgate JR (1956) Cytochrome c_3 and desulphoridin; pigments of the anaerobe *Desulphovibrio desulphuricans*. Journal of General Microbiology 14:545-572

Postgate JR (1959) A diagnostic reaction of *Desulphovibrio desulphuricans*. Nature 183:481-482

Postgate JR (1984) The sulphate-reducing bacteria, 2nd edition. Cambridge University Press:Cambridge

Postgate JR, Campbell LL (1966) Classification of *Desulfovibrio* species, the nonsporulating sulfate-reducing bacteria. Bacteriological Reviews 29:359-363

Postgate JR, Kent HM (1985) Diazotrophy within *Desulfovibrio*. Journal of General Microbology 131:2119-2122

Rees GN (1986) Isolation and characterization of sulfate-reducing bacteria. M.Sc. Thesis. University of Waikato

Rees GN, Janssen PH, Harfoot CG (1986) An unusual strain of *Desulfovibrio* sp. from an Antarctic lake. FEMS Microbiology Letters 37:363-366

Reichenbach H, Dworkin M (1981) The order myxobacterales. In: Starr MP, Stolp H, Truper HG, BAlows A, Schlegel HG (eds) The prokaryotes, A handbook on habitats, isolation and

identification of bacteria Volume 1. Springer-Verlag:Berlin, Heidelberg, New York

Robinson IM, Ritchie AE (1981) Emendation of *Acetivibrio* and description of *Acetivibrio ethanolgignens*, a new species from the colon of pigs with dysentery. International Journal of Systematic Bacteriology 31:333-338

Romesser JA, Wolfe RS, Mayer F, Spiess E, Walther-Mauraschat A (1979) Methanogenium, a new genus of marine methanogenic bacteria, and characterization of *Methanogenium cariaci* sp. nov., and *Methanogenium marisnigri* sp. nov.. Archives of Microbiology 121:147-153

Roy F, Albagnac G, Samain E (1985) Influence of calcium on growth of highly purified syntrophic cultures degrading fatty acids. Applied and Environmental Microbiology 49:702-705

Roy F, Samain E, Dubourguier HC, Albagnac G (1986) *Synthrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated fatty acids. Archives of Microbiology 145:142-147

Rozanova EP, Khudyakova AI (1973) A new nonsporeforming thermophilic sulfate-reducing organism, *Desulfovibrio thermophilus*. Microbiology 43:1069-1075. English translation of Mikrobiologiya.

Rozanova EP, Nazina TN (1976) A mesophilic, sulfate-reducing, rod-shaped, nonsporeforming bacterium. Microbiology 45:825-830. English translation of Mikrobiologiya.

Salyers AA, Vercellotti JR, West SEH, Wilkins TD (1977) Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. Applied and Environmental Microbiology 33:319-322

Samain E, Dubourguier HC, Albagnac G (1984) Isolation and characterization of *Desulfobulbus elongatus* sp. nov. from a mesophilic industrial digester. Systematic and Applied Microbiology 5:391-401

Schink B, Pfennig N (1982) Fermentation of trihydroxybenzenes by *Pelobacter acidigallici* gen. nov. sp. nov., a new strictly anaerobic, non-sporeforming bacterium. Archives of Microbiology 133:195-201

Schink B (1984) Fermentation of tartrate enantiomers by anaerobic bacteria and description of two new species of strict anaerobes, *Ruminococcus pasteurii* and *Ilyobacter tartaricus*. Archives of Microbiology 139:409-414

Schönheit P, Kristjansson JK, Thauer RK (1982) Kinetic mechanism for the ability of sulfate-reducers to out-compete methanogens for acetate. Archives of Microbiology 132:285-288

Schramm W, Lehnberg W (1984) Mass culture of brackish-water-adapted seaweeds in sewage-enriched sea water. II Fermentation for biogas production. Hydrobiologia 116/117:282-287

Seitz H-J, Cypionka H (1986) Chemolithotrophic growth of *Desulfovibrio desulfuricans* with hydrogen coupled to ammonification of nitrate or nitrite. Archives of Microbiology 146:63-67

Shelton DR, Tiedge JM (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Applied and Environmental Microbiology 48:840-848

Sheih WY, Simudu U, Maruyama Y (1988) Nitrogen fixation by marine agar-degrading bacteria. Journal of General Microbiology 134:1821-1825

Sleytr U, Adam H, Klaushofer H (1969) Die Feinstruktur der Zellwand und Cytoplasmamembran von *Clostridium nigrificans*, dargestellt mit Hilfe der Gefrieratz und Ultradunnschnittechnik. Archives of Microbiology 66:40-58

Smibert RM, Krieg NR (1981) General characterization. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of determinative bacteriology. American Society of Microbiology: Washington. pp 409-443

Smith LDS, Hobbs G (1974) Genus III *Clostridium*. In: Buchanan RE, Gibbons NE (eds) Bergey's manual of determinative bacteriology, eigth edition. Williams and Wilkins:Baltimore. pp. 551-572

Smith RL, Klug MJ (1981) Electron donors utilized by sulfate-reducing bacteria in eutrophic lake sediments. Applied and Environmental Microbiology 42:116-121

Stams AJM, Hansen TA (1984) Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen.nov. sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing bacteria. Archives of Microbiology 137:329-337

Stams AJM, Hansen TA, Skyring GW (1985) Utilization of amino acids as energy substrates by two marine *Desulfovibrio* strains. FEMS Microbiology Ecology 31:11-15

Stanier RY (1940) Studies on the Cytophagas. Journal of Bacteriology 40:619-636

Stanier RY (1941) Studies on marine agar-digesting bacteria. Journal of Bacteriology 42:527-558

Stanier RY (1942) Agar decomposing strains of the *Actinomyces coelicolor* species of the group. Journal of Bacteriology 44:555-570

Stanier RY, Adelberg EA, Ingraham JL (1977) General Microbiology, 4th edition. Macmillan Press:London

Stetter KO, Lauerer G, Thomm M, Neuner (1987) Isolation of extremely thermophilic sulfate-reducers:evidence for a novel branch of archaebacteria. Science 236:822-824

Stetter KO (1988) *Archaeglobus fulgidis* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. Systematic and Applied Microbiology 10:172-173

Stickland (1935a) Studies in the metabolism of the strict anaerobes (genus *Clostridium*). II. The reduction of proline by *Clostridium sporogenes*. Biochemical Journal 29:288-290

Stickland (1935b) Studies in the metabolism of strict anaerobes (genus *Clostridium*). III. The oxidation of alanine by *Clostridium sporogenes*.IV The reduction of glycine by *Clostridium*. Biochemical Journal 29:889-898

Stieb M, Schink B (1984) A new 3-hydroxybutyrate fermenting anaerobe, *Ilyobacter polytropus*, gen. nov., sp. nov., possessing various fermentation pathways. Archives of Microbiology 140:139-146

Stieb M, Schink B (1985) Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Archives of Microbiology 140:387-390

Stieb M, Schink B (1986) Anaerobic degradation of isovalerate by a defined methanogenic coculture. Archives of Microbiology 144:291-295

Stouthhamer AH (1979) The search for correlation between theoretical and experimental growth yields. In: Quayle JR (ed) International review of biochemistry, volume 21, Microbial biochemistry. University Park Press:Baltimore

Szewzyk R, Pfennig N (1987) Complete oxidation of catechol by strictly anaerobic sulfate-reducing *Desulfobacterium catecholicum* sp. nov.. Archives of Microbiology 147:163-168

Tanaka H, Stadtman TC (1979) Selenium-dependent clostridial glycine reductase. Purification and characterization of the two membrane-associated protein components. Journal of Biological Chemistry 254:447-452

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriological Reviews 41:100-180

Thiele JH, Chartrain M, Zeikus JG (1988) Control of interspecies electron flow during anaerobic digestion: Role of floc formation in syntrophic methanogenesis. Applied and Environmental Microbiology 54:10-19

Thiele JH, Zeikus JG (1988) Control of interspecies electron flow during anaerobic digestion: Significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Applied and Environmental Microbiology 54:20-29

Tholozan JL, Samain E, Grivet JP (1988) Isomerization between n-butyrate and isobutyrate in enrichment cultures. FEMS Microbiology Ecology 53:187-191

Trüper HG. Kelleher JJ, Jannasch HW (1969) Isolation and characterization of sulfate-reducing bacteria from various marine environments. Archiv fur Mikrobiologie 65:208-217

Trüper HG, Schlegel HG (1964) Sulphur metabolism in Thiorhodaceae. I. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek Journal of Microbiology and Serology. 30:225-238

Van der Meulen HJ, Harder W, Veldkamp H (1974) Isolation and characterization of *Cytophaga flevensis* sp. nov., a new agarolytic bacterium. Antonie van Leeuwenhoek 40:329-346

Van der Meulen HJ, Harder W (1975) Production and characterization of the agarase of *Cytophaga flevensis*. Antonie van Leeuwenhoek 41:431-447

Van der Meulen HJ, Harder W (1976) Characterization of the neoagarotetra-ose and neoagarobiose of *Cytophaga flevensis*. Antonie van Leeuwenhoek 42:81-94

Veldkamp H (1960) Isolation and characteristics of *Treponema zeulzerae* nov. spec., an anaerobic, free-living spirochete. Antonie van Leeuwenhoek Journal of Microbiology and Serology 26:103-125

Veldkamp H (1961) A study of two marine agar-decomposing, facultatively anaerobic myxobacteria. Journal of General Microbiology 26:331-342

Wallentius I (1984) Partitioning of nutrient uptake between annual and perennial seaweeds in a Baltic archipelago area. Hydrobiologia 116/117:363-370

Weeks OB (1974) Genus *Flavobacterium*. In: Buchanan RE, Gibbons NE (eds) Bergey's manual of determinative bacteriology, eighth edition. Williams and Wilkins:Baltimore. pp 357-363

Werkman CH, Weaver HJ (1927) Studies in the bacteriology of sulphur stinker spoilage of canned sweet corn. Iowa State College Journal of Science 2:57-69

Widdel F (1980) Anaerober Abbau von Fettsauren und Benzosaure durch neu Isolierte Arten Sulfat-reduzierender Bakterien. Dissertation. Georg-August Universitat zu Gottingen. Lindhorst/Schaumberg:Gottingen

Widdel F (1987) New types of acetate-oxidizing, sulfate-reducing *Desulfobacter* species, *D. hydrogenophilus* sp. nov, *D. latus* sp. nov., and *D. curvatus* sp. nov.. Archives of Microbiology 148:286-291

Widdel F (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. John Wiley & Sons:New York. pp 469-585.

Widdel F, Kohring GW, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids III. Characterization of the filamentous gliding *Desulfonema*

limicola gen. nov. sp. nov., and *Desulfonema magnum* sp. nov.. Archives of Microbiology 134:286-294

Widdel F, Pfennig N (1977) A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum* (emend) *acetoxidans*. Archives of Microbiology 112:119-122

Widdel F, Pfennig N, (1981a) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids I. Isolation of a new sulfate-reducing bacterium enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov.. Archives of Microbiology 129:395-400

Widdel F, Pfennig N (1981b) Sporulation and further nutritional characteristics of *Desulfotomaculum acetoxidans*. Archives of Microbiology 129:401-402

Widdel F, Pfennig N (1982) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. II Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov. sp. nov.. Archives of Microbiology 131:360-365

Widdel F, Pfennig N (1984) Dissimilatory sulfate- or sulfur-reducing bacteria. In Krieg NE (ed) Bergey's manual of systematic bacteriology, volume 1. Williams and Wilkins:Baltimore. pp 663-679.

Wieringa KT (1941) *Bacillus agar-exedens*, a new species, decomposing agar. Antonie van Leeuwenhoek 7:121-127

Wildengruber G, Thomm M, Konig H, Ober K, Ricchiuto T, Stetter KO (1982) *Methanoplanus limicola*, a plate-shaped methanogen representing a novel family, the *Methanoplanaceae*. Archives of Microbiology 132:31-36

Winfrey MR, Ward DM (1983) Substrates for sulfate reduction and methane production in intertidal sediments. Applied and Environmental Microbiology 83:193-199

Winfrey MR, Zeikus JG (1977) Effect of sulfate and carbon and electron flow during microbial methanogenesis in fresh water sediments. Applied and Environmental Microbiology 33:275-281

Wittenbury RS, Davies SL, Davey JF (1970) Exospores and cysts formed by methane-utilizing bacteria. Journal of General Microbiology 61:219-226

Yaphe W (1957) The use of agarase from *Pseudomonas atlantica* in the identification of marine algae (Rhodophyceae). Canadian Journal of Microbiology 3:987-993

Zeikus JG, Dawson MA, Thompson TE, Ingvorsen K, Hatchikian EC (1983) Microbial ecology of volcanic sulfidogenesis: Isolation and characterization of *Thermodesulfobacterium commune* gen. nov. and sp. nov.. Journal of General Microbiology 129:1159-1169

Zhilina TN (1983) New obligate halophilic methane producing bacterium. Microbiology 52:290-297. English translation of Mikrobiologiya

Zindel U, Freudenberg W, Rieth M, Andreesen JR, Schell J, Widdel F (1988) *Eubacterium acidaminophilum* sp. nov., a versatile amino acid-degrading anaerobe producing or utilizing H_2 or formate. Archives of Microbiology 150:254-266

Zinder SH, Cardwell SC, Anguish T, Lee M, Koch M (1984) Methanogenesis in a thermophilic digester. *Methanothrix* sp. as an important acetoclastic methanogen. Applied and Environmental Microbiology 47:796-807

ZoBell CE, Upham HC (1944) A list of marine bacteria including descriptions of sixty new species. Bulletin of the Scripps Institute of Oceanography 5:239-251