The physiological characterisation of a novel *Thermomicrobia* strain WKT50.2, and a review of the characteristics of the class *Thermomicrobia*

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Abstract

Thermomicrobia is a class of heterotrophic, obligate aerobes within the phylum *Chloroflexi.* At the time of writing, it contained four distinct species, *Thermomicrobium roseum*, *Sphaerobacter thermophilus*, *Nitrolancetus hollandicus* and WKT50.2, the novel strain described in this study. The physiology and ecology of this strain, and the description of the class *Thermomicrobia* was investigated as part of this study.

Strain WKT50.2 was isolated from the hot springs at Waikite, New Zealand. We investigated temperature and pH range and optima, analysed PLFA, and determined preferred carbon and energy sources, N-fixation, modes of growth and antibiotic sensitivity. As a general phenotype, WKT50.2 is a heterotrophic and thermophilic obligate aerobe with a preference for moderately alkaliphilic pH conditions. The possible ecology of WKT50.2 was discussed.

WKT50.2 was able to oxidise methane to gain energy. This was an unexpected result as methanotrophy is a highly specialised phenotype restricted to the phyla *Proteobacteria* and *Verrucomicrobia.* Methanotrophs also tend to be metabolic specialists with no capability to oxidise substrates other than C1 compounds. In contrast, WKT50.2 was also able to oxidise ammonia and nitrite, along with a range of saccharides and protein rich substrates. WKT50.2 was sensitive to most of the antibiotics tested in this study, but metronidazole and trimethoprim both stimulated growth in a dosage-dependent manner. WKT50.2 is clearly a distinct genus from *T. roseum* and *S. thermophilus*, based on 16S rRNA gene sequence similarities (91 % and 88 % respectively), G+C content and metabolic capabilities.

T. roseum and *S. thermophilus* were confirmed as heterotrophic, thermophilic, moderately alkaliphilic, obligate aerobes. The temperature and pH optima and ranges for *T. roseum* and *S. thermophilus* were amended, along with expanding the known metabolic capabilities of both species. Of particular note was the discovery that both *T. roseum* and *S. thermophilus* were also able to oxidise both methane and ammonia for energy, and that both species possessed cellulolytic activity.

In summary, the class *Thermomicrobia* can be redefined as comprising obligately aerobic, facultative heterotrophs. Cells are non-motile, non-spore forming rods. This study has shown that the cells have proteinaceous cell walls containing ornithine, and no lipopolysaccharide. The major fatty acid is 12-Me-18:0, and lipids include ester-linked diols. The major isoprenoid quinone is MK-8.

Several of the observations in this study merit further research. The discovery of methanotrophy in this class was unexpected and significant, and future work should include the identification of the enzymes involved in methanotrophy, and those involved in the oxidation of ammonia for energy. The mechanism for growth stimulation of WKT50.2 and *T. roseum* by antibiotics was also an interesting observation and warrants further analysis to determine the mode of action.

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1 Literature Review

1.1 Introduction

It is now generally accepted that there is a vast "uncultured microbial majority" of both Bacteria and Archaea that are abundant in the environment and may play significant roles in biogeochemical cycles [1, 2]. It is estimated that there are around 50 phylum-level divisions in the domain *Bacteria* [3] yet only 29 have so far been formally recognised (LPSN [http://www.bacterio.cict.fr/classifphyla.html] retrieved October 2012) with one or more cultivated and characterised strains. Of those 29 phyla, over 95 % of characterised species belong to only four phyla, *Bacteriodetes*, *Firmicutes*, *Proteobacteria* and *Cyanobacteria*. Over half of the recognised phyla have less than 10 species, including the ubiquitous, but difficult to cultivate, *Armatimonadetes* and *Acidobacteria phyla*.

Extremophiles

Extremophiles are organisms that grow in what is considered non-anthropogenic conditions, usually of one or more environmental extremes, such as temperature, pH or salinity. Thermophiles have an optimum temperature for growth above about 45 ^{o}C [4], hyperthermophiles an optimum above 80 ^{o}C, while psychrophiles have optima below 20 ^{o}C. Other extremophiles include those resistant to, or requiring, extremely high or low pH, high sodium chloride concentrations (halophiles), and extremes of pressure in deep-sea vents (barophiles). There are also microorganisms which are resistant to radiation and high heavy metal concentrations such as mercury, arsenic or cadmium.

The phylum *Chloroflexi* is a widely distributed phylum, with few cultivated species. It currently contains 125 phylotypes [5] and six recognised classes, but only 22 cultivated and characterised strains. *Chloroflexi* is thought to be one of the earliest branching lineages within Bacteria [6, 7]. The isolated species cover a wide range of morphologies and phenotypes, and are found in a multitude of environments. However, most of the uncultivated strains fall within several subdivisions with no cultivated members. Considering their apparent wide distribution, it is probable that these bacterial strains have a significant role in microbial ecosystems, although very little is known about them. This literature review will focus on the phylum *Chloroflexi*, in particular the class *Thermomicrobia*. This MPhil research centres around the description of a novel extremophile, strain WKT50.2, from the class *Thermomicrobia*.

1.2 The *Chloroflexi* phylum

1.2.1 History

The phylum *Chloroflexi* has undergone a number of changes since its original description by Woese in 1985 [8]. At the time, Woese defined *Chloroflexi* as the Green Non-Sulfur (GNS) phylum, and included four isolates; *Chloroflexus aurantiacus*, *Thermomicrobium roseum*, and two *Herpetosiphon* species. It was noted that the 16S rRNA gene sequence of *T. roseum* had only a 77 % homology to the other three isolates [9], and that it may represent a separate division. *Thermomicrobia* was described as a separate phylum in 2001 [10], before being reclassified as *Chloroflexi* again in 2004, when 16S rRNA gene homology suggested that *S. thermophilus* grouped within *Thermomicrobia* [11]. At this point three additional classes had been proposed; *candidatus* "Chloroflexi", Anaerolineae and *candidatus* "Dehalococcoidetes" [11].

The class *Anaerolineae* was split in 2006, following the isolation of three more novel species by Yamada *et al*. [12]. *Caldilinea aerophila* is a single species representing the class *Caldilineae* of thermophilic facultative aerobes, while the six species within *Anaerolineae* are obligate anaerobes. Another class, *Ktedobacter* (now *Ktedonobacteria*) was also proposed in 2006, after the discovery of several strains of spore-forming filamentous bacteria from soil that were most closely related to other members of the *Chloroflexi* [13] (Figure 1.1).

With the advent of DNA sequencing techniques a number of other bacteria have been reclassified to *Chloroflexi*. These include *Herpetosiphon geysericola* which was first described in 1936 and was originally thought to be a thermophilic blue-green alga [14]. It was not reclassified as *Chloroflexi* until 1970 [14]. *Sphaerobacter thermophilus* was originally placed in the *Actinobacteria* division [15]. Conversely, *Thermoleophilum minutum* was originally named *Thermomicrobium fosterii* [16] and placed in the phylum *Chloroflexi*, but was later renamed and moved to the *Actinobacteria* [17].

Figure 1.1 - Timeline of events in the *Chloroflexi* phylum

		Reference
1936	*Herpetosiphon geysericola* identified	[18]
1968	*Herpetosiphon aurantiacus* isolated	[19]
1971	*Chloroflexus aurantiacus* described	[20, 21]
1973	*Thermomicrobium roseum* isolated	[22]
1976	New family name *Chloroflexaceae* within *Chlorobiineae* for gliding, filamentous phototrophs	[23]
1985	Green Non-Sulfur bacterial phyla defined	[7, 8]
1997	"*Dehalococcoides ethenogenes*" isolated	[24]
2001	Phylum *Thermomicrobia* described	[25]
2003	C*aldilinea aerophila* and *Anaerolinea thermophila* isolated	[26]
2004	Phylum *Chloroflexi* split into four classes: *Anaerolineae, "Chloroflexi", "Dehalococcoidetes" and Thermomicrobium*, plus an unnamed clade	[11]
2006	Class *Ktedobacteria* (*Ktedonobacteria*) proposed *Anaerolineae* class split into *Anaerolineae* and *Caldilineae*	[13] [12]
2011	WKT50.2 isolated	

1.2.2 Diversity

The phylum *Chloroflexi* is incredibly diverse, with species exhibiting a wide range of phenotypes and sequence diversity. Members may be mesophilic or thermophilic; display phototrophic or chemoheterotrophic growth; be obligate aerobes or anaerobes; are detected in marine or freshwater environments; and may be gliding filaments or non-motile rods and cocci [10, 27].

The phylum was first described in 1985, as the Green Non-Sulfur group, and from the first phylogenetic analyses it was apparent that this was an ancient, deep branching line of descent (Figure 1.2).

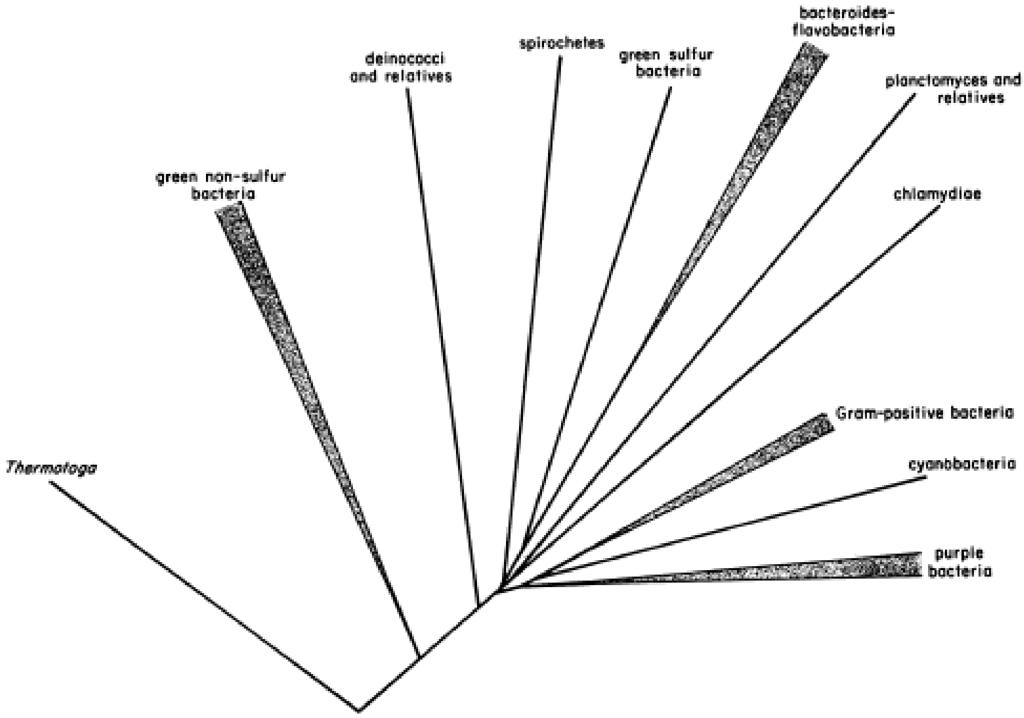

Figure 1.2. An early view of the phylogenetic position of the phylum *Chloroflexi* (Green Non-Sulfur).

(taken from Woese [9])

There are currently six recognised classes within the phylum *Chloroflexi*: *Anaerolineae* [11], *Caldilineae* [12], *candidatus* “Chloroflexi” [10], *candidatus* “Dehalococcoidetes” [11], *Ktedonobacteria* [13] and *Thermomicrobia* [11], and at least one more class-level division with no representative strains [5] (Table 1.1 and Figure 1.3).

Table 1.1- Comparison of selected growth characteristics of the six classes within the phylum *Chloroflexi*

	Anaerolineae	***Caldilineae***	**“Chloroflexi”**	**“Dehalococcoidetes”**	***Ktedonobacteria***	***Thermomicrobia***
Validly named species	6	1	8 *	1	4	2
Morphology	Multicellular filaments	Multicellular filaments	Multicellular motile filaments	Cocci	Sporulating filaments	Rods
Isolation site	Anaerobic sludge; rice paddy soil; artificial wastewater	Hot spring	Hot springs; freshwater lake	Anaerobic sludge; river sediment; groundwater	Soil; compost; geothermal soil	Hot spring; sewage sludge
Metabolism	Chemo-organotrophy	Chemo-organotrophy	Photo or chemohetero-trophy	Chemolitho-heterotrophy	Heterotrophy	Heterotrophy
Temperature range for growth (^{o}C):	25 - 65	37 – 65	20 - 60	20 - 34	17 - 70	n.d. – 85$^{\#}$
pH range for growth:	7.0 – 9.0	7.0 - 9.0	6.0 – 9.0	6.0 – 8.0	3.1 – 8.7	6.0 – 9.4$^{\#}$
Respiration Aerobic	-	+	+	-	+	+
Anaerobic	+	-	+	+	-	-
Facultative	-	-	+	-	-	-
Major fatty acids	varied	18:0, 16:0, 17:0, *i*17:0	16:0, plus various others	18:1ω9c, 16:1ω9c, 16:0	*i*17:0, plus various others	12-Me-18:0, 18:0 $^{\#}$
Major quinone	None	MK-10	MK-10 (some MK-4)	n.d.	MK-9 (H_2)	MK-8, MK-10 $^{\$}$
G+C mol%: Range	48.2 – 59.5	n.d.	48.1 – 62.0	n.d.	53.9 – 60.8	64.0 – 68.0

n.d.: not determined, *: only five of the eight species in “Chloroflexi” have been used in this table. The remaining strains *Heliothrix oregonensis*, *Chloronema giganteum* and *Chloronema spiroideum* have not been isolated in pure culture. # determined for *T. roseum* only. $ determined for *S. thermophilus* only.

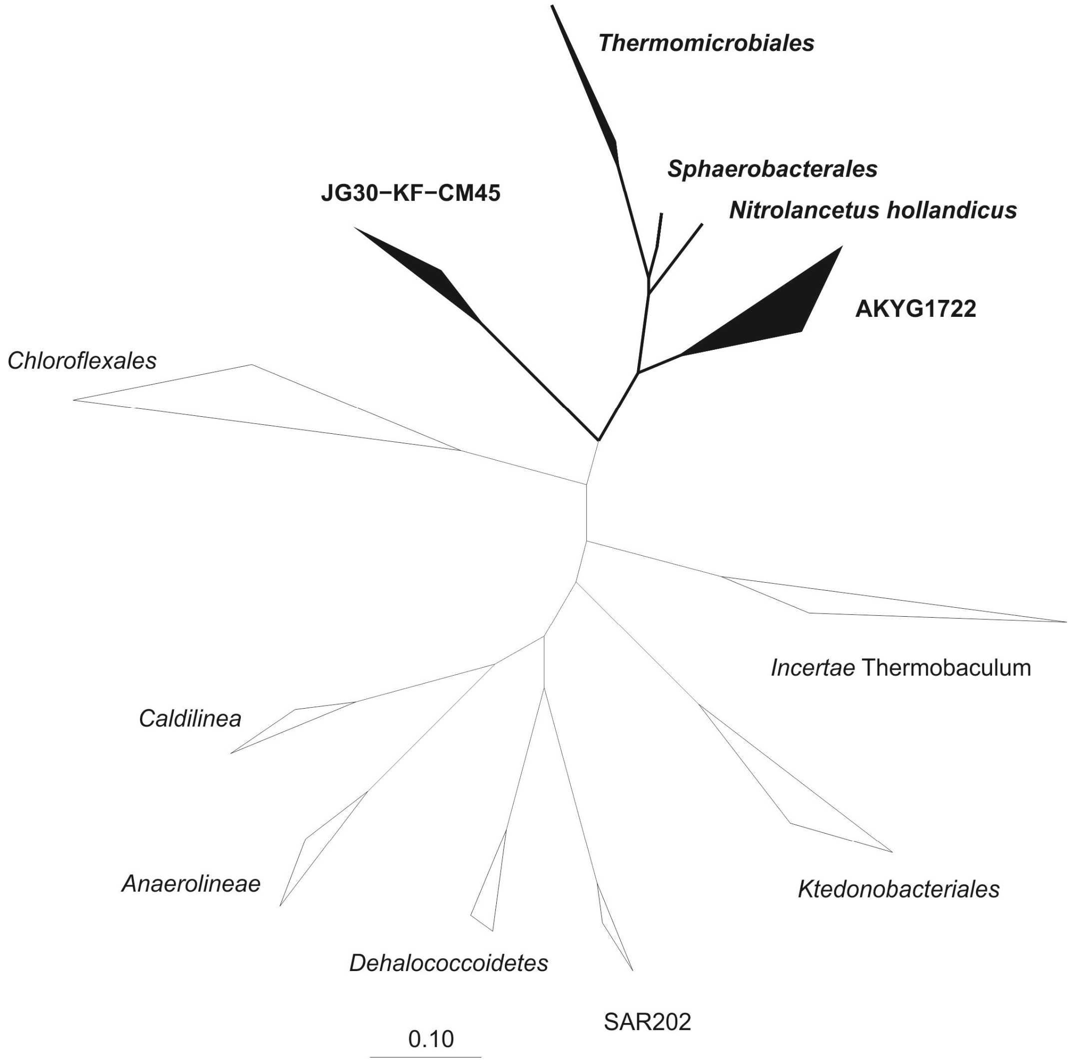

Figure 1.3 - Phylogenetic tree of the *Chloroflexi* classes

Unrooted maximum-likelihood tree showing class groupings within the phylum *Chloroflexi (*n=80). Possible order level groupings within the class *Thermomicrobia* have been highlighted. The tree was constructed using 16S rRNA gene sequences from only cultivated strains with the exception of *Thermomicrobia*, *Caldilinea* and SAR202, where a combination of cultivated and/or environmental clones were used. The scale bar represents 0.10 substitutions per nucleotide site.

In addition to displaying major differences in phenotype across and even within the classes, there is a significant level of sequence diversity in the 16S rRNA genes with over 26 % sequence dissimilarity across the phylum (Table 1.2). A full pairwise comparison of all *Chloroflexi* isolates is in Appendix A.

Table 1.2 - Pairwise percent 16S rRNA gene sequence dissimilarity table of *Chloroflexi* classes

	Caldilineae	*Anaerolineae*	“Dehalococcoidetes”	*Ktedonobacteria*	*Thermomicrobia*	“Chloroflexi”
Caldilineae	0					
Anaerolineae	17	0				
“Dehalococcoidetes”	19	21	0			
Ktedonobacteria	20	22	22	0		
Thermomicrobia	20	24	23	21	0	
“Chloroflexi”	24	24	24	23	21	0

The average percent dissimilarity across all isolated species in each class is shown.

Other clades and species (outside the recognised classes)

There are several clusters of environmental 16S rRNA gene sequences within the *Chloroflexi*, such as the OPB and OPT sequences found in Octopus Spring, Yellowstone National Park [28], or the SAR202 and SAR307 groups found 250m deep in the Sargasso Sea [29], which form separate clades. Ward *et al.* [30] have also found related rRNA gene sequences in a hot spring microbial mat dominated by cyanobacterial species.

Thermobaculum terrenum is a member of the *Chloroflexi* phylum isolated from Yellowstone National Park in 2004 [31]. It is not officially recognised as validly named, as it has only been deposited in culture collections within the USA. *T. terrenum* is a thermophilic obligate aerobe, produces non-spore-forming rods and grows heterotrophically. Following publication of the full genome in 2010 [32], Kunisawa compared gene order from the genomes of 11 species within the phylum *Chloroflexi* [33]. It was found that *T. terrenum* was most closely related to *Sphaerobacter thermophilus*, putatively placing it in the class *Thermomicrobia*.

Nitrolancetus hollandicus [34] was partially described in 2012. It is a chemolithoautotrophic nitrite oxidizer, isolated from a bioreactor. Comparison of 16S rRNA gene sequences show it is also most closely related to *S. thermophilus*.

Difficulty in isolating species

It is apparent that *Chloroflexi* species appear to be relatively difficult to isolate and characterise. Considering the number of phylotypes associated with *Chloroflexi*, the number of cultivated strains is relatively low (Table 1.2). *Chloroflexus aurantiacus* was first described in 1971 [20], but it was not until 1974 that it was isolated and fully characterised [21]. *Heliothrix oregonensis* was described at the same time, but was never able to be isolated, and was only deposited as a co-culture with *Isocystis pallida* in 1985 [35]. Similarly, *Chloronema giganteum* was observed in a Russian/Finnish lake in 1975, but has also never been obtained in pure culture [36]. The reason for this paucity in cultivated *Chloroflexi* strains is unknown, but highlights the potential for novel species discovery and isolation.

Difficulty in resolving species

The candidate class "Dehalococcoidetes" currently contains only one validly named isolate, *Dehalogenimonas lykanthroporepellens* [37], although several other strains have been described [24] [38] [39]. Related 16S rRNA gene sequences have been detected in anaerobic digesters [40], contaminated groundwater [41], river sediments [42], and landfill sites [43]. This group of obligate anaerobes are able to dechlorinate a variety of halogenated compounds, using H_2 as an electron donor.

In 2002, Hendrickson *et al.* [43] analysed the 16S rRNA gene sequences of environmental samples taken from a wide range of chloroethene-contaminated sites. They proposed splitting the "Dehalococcoidetes" into three sub-classes, Cornell, Pinellas and Victoria, based on differences in variable regions II and VI.

He *et al.* [38] found that species with very similar 16S rRNA gene sequences had different metabolic capabilities for electron acceptors; the sequence obtained from their enrichment which used chloroethenes had only one nucleotide difference from that of strain CBDB1, a member of the Pinellas clade, which could only respire chlorobenzenes.

This problem was also demonstrated by Sung *et al.* [39], when the microscopic and molecular (16S rRNA gene) analysis of an enrichment suggested a pure culture, but a qPCR analysis suggested a mixed culture of different *Dehalococcoides* populations. These different strains were resolved only by the use of reductive-dehalogenase (RDase) functional gene qPCR.

The same group also determined that the sum of the known RDase genes in an enrichment of *Dehalococcoides* cells was between 10 to 30 % of cell numbers predicted by 16S rRNA gene qPCR [44], suggesting that other *Dehalococcoides* cells contained RDase genes not detected by the specific PCR primers used in this study. In addition, Holscher *et al.* [45] found that each strain tested contained at least 14 different RDase genes. It has also been shown that multiple RDase genes are induced by a single substrate and transcribed at the same time in a strain [46]. Cells of the same species may also contain different cohorts of a range of RDase genes.

Given the range of environments that *Dehalococcoides* strains have been detected in, and the difficulty in assigning species through the amplification of 16S rRNA or RDase genes, it is likely that there are many more as-yet unidentified species in this class waiting to be discovered.

1.2.3 Ecology

The members of the *Chloroflexi* phylum do not appear to have a specific ecological niche. The isolated species alone have been found in a wide variety of environments, including: mesophilic and geothermal soils [13, 47]; compost [48]; anaerobic sludge [26]; hot springs [21, 22, 26]; freshwater lakes [36]; artificial wastewater [12] and contaminated groundwater [37]. As there are so few isolated species within each class, it is difficult to determine if there are any common niches. *Chloroflexi* 16S rRNA gene sequences also appear frequently in clone libraries constructed from marine water column bacterioplankton samples [29] and hydrothermal sediments [49].

1.2.4 Common features of *Chloroflexi*

There are few features that are common to all members of *Chloroflexi*. These include characteristic cell wall structures, signature nucleotides within the 16S rRNA gene, and specific secondary structures within the 16S rRNA. This section will cover common characteristics of the phylum *Chloroflexi*, with more detail on the *Thermomicrobia* in Section 1.3.

Cell Walls

A rapid and widely accepted means to characterise microbial species is the use of Gram staining, developed by Christian Gram in 1884. It can be used to characterise microorganisms via their cell wall structure. Bacteria can be broadly defined in two groups based on cell wall structure and accordingly, the Gram stain reaction; Gram-positive, which appear purple following staining, and Gram-negative, which stain pink. As a general observation, the generic cell wall structure is conserved across an individual phylum, and thus the Gram-staining reaction is also expected to be similarly conserved. Interestingly however, members of the *Chloroflexi* stain both Gram-positive and -negative.

Classical Gram-positive bacteria, such as *Staphylococcus* species, generally contain a thick (20-80nm) peptidoglycan layer outside the plasma membrane of the cell [50]. Gram-negative bacteria, such as *E. coli*, have a much thinner (1-3nm) peptidoglycan layer, and an outer membrane composed of lipopolysaccharide (LPS).

The Gram-staining procedure consists of an initial stain with crystal violet, which dissociates to form ions that are able to penetrate the cell wall and cell membrane. Iodine is then added as a mordant, reacting with the crystal violet ions to form large purple complexes which precipitate from solution. Alcohol is used to decolourise the cells; in Gram-negative cells, the outer layer of LPS is lost, and the crystal violet complexes are washed from the cell. However, in Gram-positive cells, the thick outer layer of peptidoglycan becomes dehydrated, forcing the pores to close. This traps the crystal violet within the cell and negates the proceeding secondary counter stain. The secondary stain, such as safranin, stains any Gram-negative cells on the slide pink and allows them to be seen easily under a microscope [4].

There are several groups of bacteria that do not fit neatly into these two groups. Some may appear Gram-variable, whilst other bacteria do not retain either stain well. Some species have a double envelope architecture but lack LPS; *Sphingomonas* species, within the *Proteobacteria* which are generally considered to be Gram-negative, have an outer membrane consisting of glycosphingolipids (GSL) rather than LPS [51]. Many members of the *Chloroflexi* phylum stain Gram-negative but appear to have cell walls more similar to those of Gram-positive cells; they lack LPS [52], and electron micrographs generally do not show a well-defined outer membrane [21, 53, 54].

It is more useful to consider the architecture of the cell envelope specific to particular species rather than staining properties. Sutcliffe [52] has recently suggested that bacteria should be divided into monoderm and diderm groups, referring to the actual cell architecture rather than to how cells are stained. The classical Gram-negative species where the outer leaflet of the outer membrane is composed mainly of the lipid A anchor of LPS would then be referred to as diderm-LPS. Under this classification system, the *Chloroflexi* species would be considered monoderm, although they may have complex layered structures deriving from polysaccharides or proteins bound to the peptidoglycan.

Comparative genomics has also given insight into the cell wall architecture of the *Chloroflexi*. Sutcliffe [52] compared the fully sequenced genomes of 11 members and searched for genes involved in key steps of LPS biosynthesis. Across all 11 genomes, there were no orthologues of the genes for proteins involved in the biosynthesis of the lipid A anchor (*E.coli* LpxB, LpxC or KdsA). There were also no gene homologues of LptA or LptD family proteins, which are responsible for the transport of LPS across the periplasm. However, some phyla such as *Chlamydiae* are known to produce LPS but lack the Lpt pathway. The *Chloroflexi* genomes examined did have homologues of genes for proteins involved in lipoprotein biogenesis, such as Lgt and Lsp, as well as those for the *Sec* and *Tat* translocation channels. No homologues of outer membrane assembly proteins (e.g. the BamA protein) were found, or specific components for any of the eight known secretory systems associated with protein translocation across the outer membrane [55].

This suggests that proteins are secreted using only the *Sec or Tat* translocation channels, which translocate proteins across lipid bilayers but not through LPS.

The peptidoglycan in bacterial cell walls is composed of short peptides cross-linked to glycan strands. The glycan strands consist of alternating amine sugar residues, β,-1,4-linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with few variations across species. The peptide subunit is made of alternating L- and D- amino acids, usually L-alanine linked to muramic acid, then D-alanine, then links to a L-diamino acid such as meso-diaminopimelic acid (DAP or Dpm), lysine or ornithine [56]. There is some variation in the specific amino acids across microbial species. In those *Chloroflexi* species where the cell wall has been investigated, the DAP is replaced with L-ornithine [13, 15, 53, 57].

The amino group from the diamino acid forms a peptide linkage to the carboxyl group of the C-terminal D-alanine or muramic acid of an adjacent peptide subunit [58]. There is considerable variation within linkages, and peptidoglycan can be classified into groups depending on the cross-linking of the peptide subunits, types and biosynthesis of interpeptide bridges, and the amino acid sequence [56]. The peptidoglycan of some members of the *Chloroflexi* phylum has been described as type A3β [15, 53] (Figure 1.4).

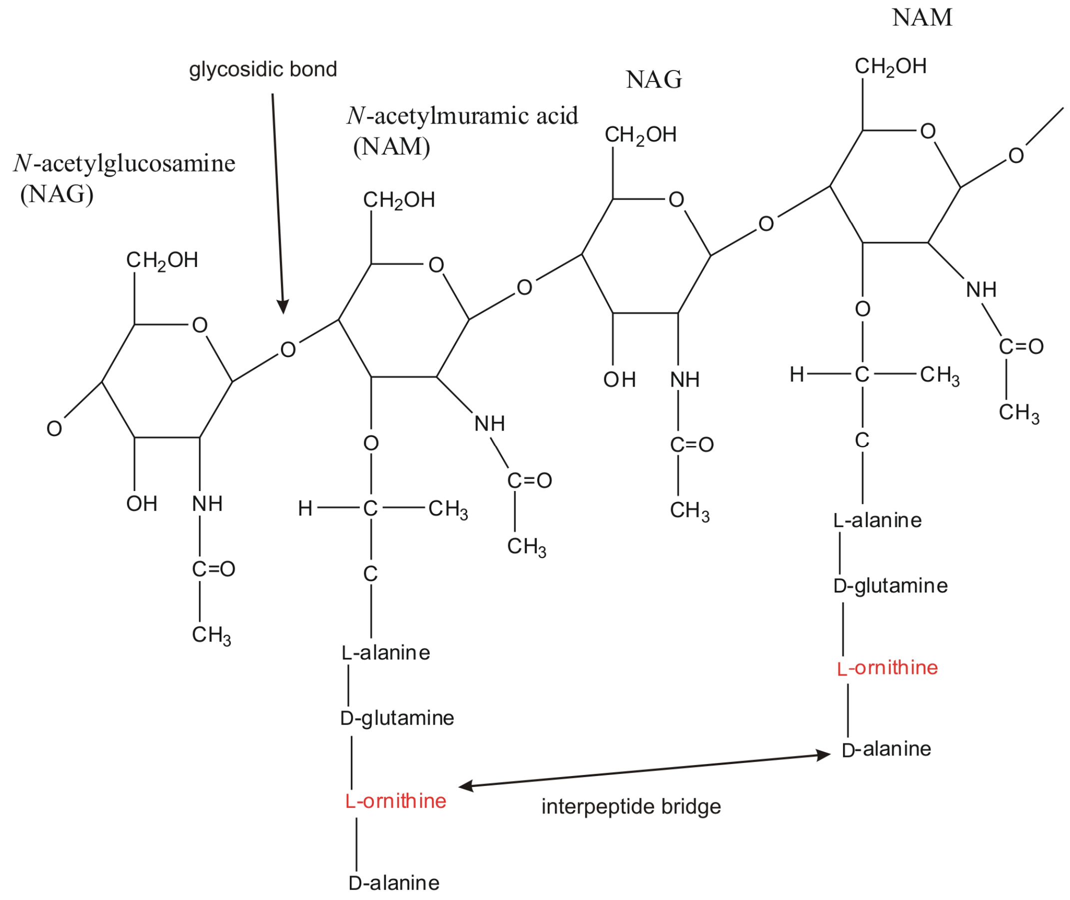

Figure 1.4 – Structure of the A3β form of peptidoglycan.

In this form, DAP has been replaced with ornithine. The stability of the molecules is enhanced by interpeptide bridges connecting the L-ornithine in position 3 of one strand of N-acetylmuramic acid with the D-alanine in position 4 of the second. Adapted from [58].

Merkel [57] found that the cell wall of *Thermomicrobium roseum* did not contain glucosamine. Instead, muramic acid and galactosamine were present in an equimolar ratio, and it was suggested that galactosamine may be substituted for glucosamine in the glycan strands in this species.

Extracellular polysaccharides (EPS), sheaths or S-layers

The synthesis (or lack of) of EPS, cell sheaths and/or S-layers are characteristics which can be used to describe *Chloroflexi*. EPS usually consists of a heteropolysaccharide, with a variety of sugars as well as uronic acids, although some bacteria may produce EPS that is predominately protein. EPS helps surface adhesion and biofilm production, and protects cells from extremes of pH or temperature. EPS can also act as carbon and energy reserves, bind nutrients and metal ions, and serve as a buffer between the cell and anti-microbial agents or an accumulation of toxic compounds [59, 60]. The production of EPS has not been described in *Chloroflexi* species.

Filamentous bacteria are known to produce sheaths, an additional permeability layer made of either polysaccharides or proteins, covalently linked to the peptidoglycan [61]. Sheaths may allow groups of cells to break off to form new colonies when resources become limited. *Herpetosiphon aurantiacus* and *H. geyersicola* were originally described as possessing sheaths [19], but as electron microscopy provided greater resolution, the genus description was later amended [62]. The other filamentous species within the phyla were described as either having a thin sheath, or none [21, 26, 35, 63]. It is unlikely that these are classical sheaths such as those found in cyanobacteria, which surround all cells, are visible using light microscopy, and have a fine structure of fibres running parallel to the cell surface [61].

S-layers are regularly structured, proteinaceous layers, sometimes with minor carbohydrate constituents, which provide protection against parasites including *Bdellovibrio bacteriovorus,* and enzymes such as lysozyme [64]. They are found in almost all bacterial and archaeal phyla, and may be arranged to form narrow channels, which allow the flow of nutrients and by-products into and out of the cell [64]. Some S-layers are also capable of binding metal cations which may be useful for stockpiling, and minerals such as gypsum which may provide a protective coat for the bacteria. S-layers can then be shed when the load of minerals becomes excessive [65].

Within the *Chloroflexi*, "Dehalococcoides ethenogenes" was described as having a cell envelope that resembled the S-layer protein subunit type of cell wall [24], while *Thermomicrobium roseum* has an outer layer consisting of a regular repeating unit arrayed over the cell surface, with an amino acid composition similar to that of subunit cell wall polymers [22], suggesting it is also an S-layer.

Signature nucleotides

Woese *et al.* [9] analysed signature nucleotides for each proposed phylum, compared to an overall consensus sequence of the 16S rRNA gene. The Green Non-Sulfur group (*Chloroflexi*) had five signature nucleotides that differed from the consensus:

Position (*E. coli* 16S rRNA gene numbering)	Consensus nucleotide	*Chloroflexi* / Green Non-Sulfur
53	A	G
906	G	A
1202	U	G
1224	U	G
1410	A	G

An exception is the *Chloroflexi* SAR202 sub-divisional grouping from the Sargasso Sea, which does not have a G at position 1202 or 1224 – instead a uracil residue replaces the guanidine as the consensus nucleotide [29]. A survey of freshwater lakes in the North American Great Lakes region found over half of the *Chloroflexi* 16S rRNA gene sequences retrieved also contained a U at position 1224 [66]. Finally, there are also regions within the 16S rRNA gene sequence which show a high degree of similarity across the phylum *Chloroflexi* [66]. These regions (538-555; 941-957; 948-964; 1328-1344 and 1340-1365 – *E. coli* numbering) have been used to design phylum-specific primer pairs [66].

Secondary structure

Oyaizu *et al.* [6] analysed the secondary structure of the 16S rRNA and found several specific characteristics of the *Chloroflexi* group. For example, a helical element between 1126 and 1146 in hypervariable region V7, was absent in the *Chloroflexi* group but found in all other bacterial groups. Another helix between position 455 and 477 was also missing, but this is also absent in some other bacterial groups.

H. aurantiacus was also shown to have an unusual loop structure in the V4 region (607 – 630) [6], which was also seen in the SAR202 clone cluster [29], but not in most other *Chloroflexi* species (Figure 1.5).

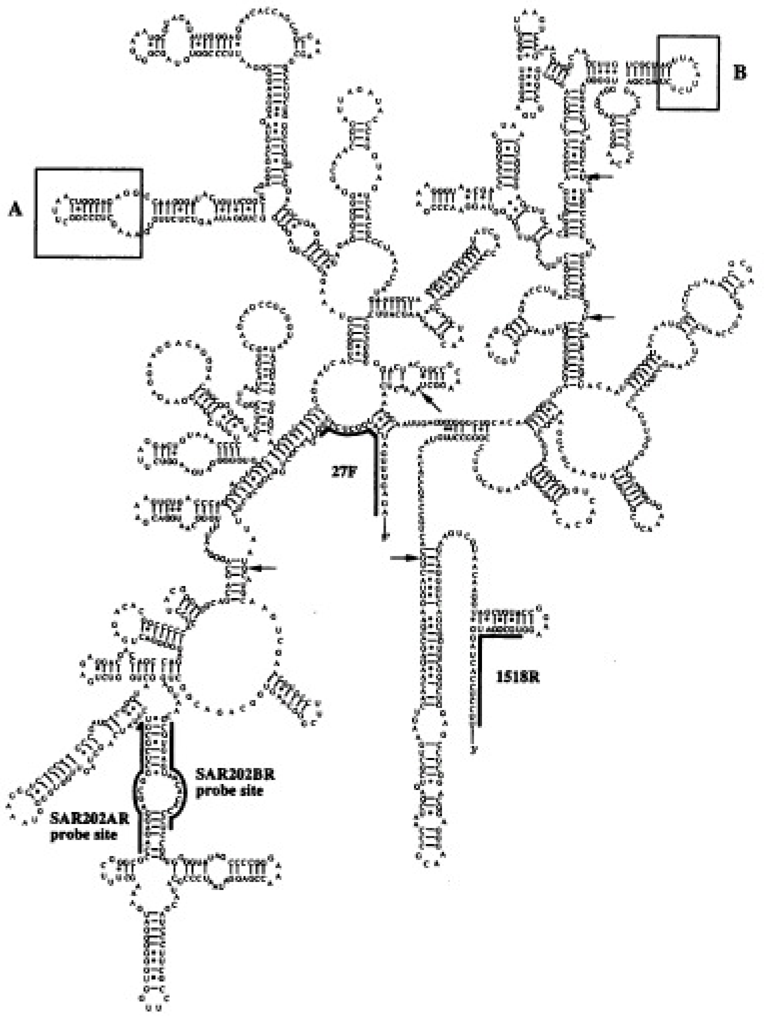

Figure 1.5 – 16S rRNA secondary structure in *Chloroflexi*

The boxed areas indicate features unique to the phylum *Chloroflexi*. A: Loop structure at position 607-630. B: Deletion of helix at 1126. Taken from Giovannoni *et. al.* [29]

1.3 *Thermomicrobia*

The class *Thermomicrobia* contains two isolated and characterised members, *Thermomicrobium roseum* [22], and *Sphaerobacter thermophilus* [15]. Based on the described characteristics of these two isolates, the group appears to have a thermophilic, chemoheterotrophic and obligately aerobic phenotype (Table 1.3). Recently a manuscript describing the ecology of a third *Thermomicrobia* strain has been published as "*Nitrolancetus hollandicus*" [34]. However, at this time it has not been formally characterised or officially accepted.

Table 1.3 - Comparison of selected growth characteristics of *Thermomicrobia*

Differences in phenotype (where known) are noted in bold.

	T. roseum	***S. thermophilus***	***"N. hollandicus"***
Isolation site	Yellowstone National Park, USA	Thermophilic sewage sludge, Germany	Bioreactor, The Netherlands
Morphology	Dumbbell or irregular shaped rods; singles or in pairs	Irregular rods or dumbbells	Lancet shaped cells
Motility	Not observed	Not observed	Not observed
Colonies	**Pink,** entire	**Opaque,** entire	n.d.
Gram stain	**-ve**	**+ve**	+ve
Temperature range for growth (^{o}C) Optimum	n.d. – 85 **70 - 75**	n.d. **55**	20 – 46 40
pH range for growth Optimum	6.0 – 9.4 8.2 – 8.5	n.d. 8.5	6.2 – 8.3 6.8 – 7.5
Atmosphere	Aerobic	Aerobic	Aerobic
Carbon monoxide oxidation	Yes	Yes	No
Major fatty acids	12-Me-18:0 (68 %) 18:0 (16 %)	n.d.	12-Me-18:0
Fatty acids ester-linked to diols	Yes	n.d.	Yes
Major quinone	n.d.	MK-8 (75 %), MK-10 (12 %), MK-7 (8 %), MK-6 (5 %)	MK-8
DNA	2 chromosomes: 2 Mbp and 1Mbp	2 chromosomes: 2.7 Mbp and 1.2 Mbp	n.d.
G+C content	63.6 mol% and 65.7 mol%	68.1 mol%	62.6 mol%
Cell wall structure	75,000 kDA protein Small amount of peptidoglycan DAP replaced by ornithine	High in protein Small amount of peptidoglycan DAP replaced by ornithine	Small amount of peptidoglycan DAP replaced by ornithine

n.d. not determined

Ecology

T. roseum was isolated from a bacterial mat in Toadstool Spring, Yellowstone National Park, USA [22], while *S. thermophilus* was isolated from high temperature sewage sludge treatment facility in Germany [15]. The 16S rRNA gene sequences of *Thermomicrobia* species have also been detected in a wide variety of environments including soils, compost and skin. A number of phylotypes have also been detected in environments as diverse as freshwater lakes, oil seeps, bioreactors, and invertebrate intestines (summarised in Figure 1.6).

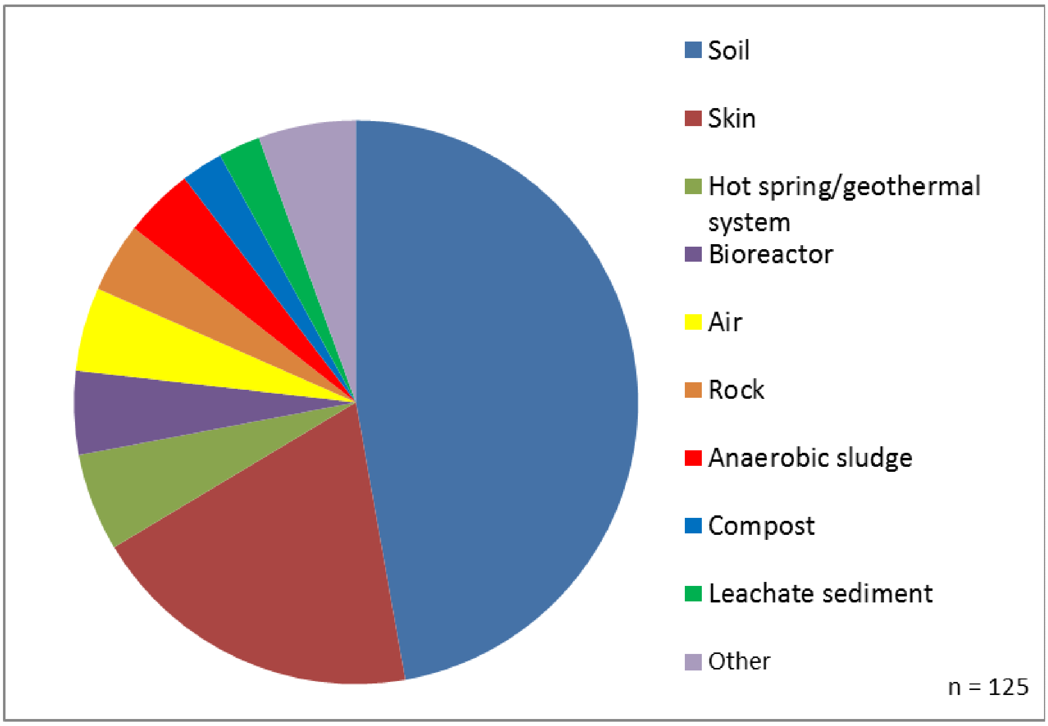

Figure 1.6 - A summary of *Thermomicrobia* habitats

Habitats where *Thermomicrobia* sequences have been detected. The ecological niche classifications were determined using the 16S rRNA gene sequences descriptions (NCBI Nucleotide database [http://www.ncbi.nlm.nih.gov/nuccore]), retrieved October, 2012.

Recently, a fourth strain (WKT50.2) of *Thermomicrobia* was isolated from steam affected soil above a hot spring at the Waikite Geothermal Valley, New Zealand (Figure 1.7). The Waikite hot springs produce water above 90 ^{o}C, with a slightly alkaline pH (7.2 – 8.9) [Appendix F] . The water is high in silica, but low in both calcium and magnesium [67].

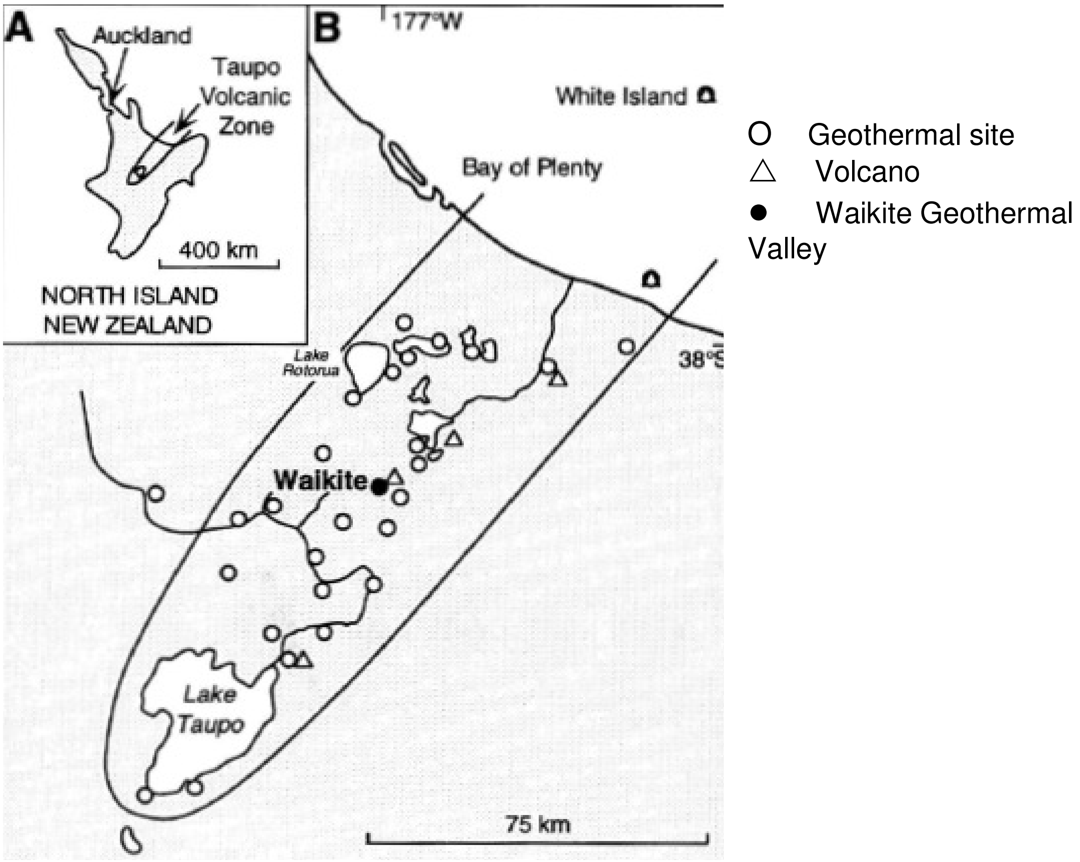

Figure 1.7 - Location of the sampling site

(a) Map of the North Island, New Zealand showing the location of the Taupo Volcanic Zone. **(b)** Location of the Waikite hot springs in the Taupo Volcanic Zone. Taken from Jones *et al.* [67]

Like the previously described strains of *Thermomicrobia,* WKT50.2 appears to conserve the thermophilic, chemoheterotrophic and obligate aerobic characteristics of the class.

1.4 Aims and Objectives

An initial characterisation of strain WKT50.2 indicates that it is thermophilic, an obligate aerobe, and has a pale pink pigmentation (similar to that of *T. roseum*). Pairwise analysis of the 16S rRNA gene sequences of these strains indicates that WKT50.2 shares 91 % and 88 % sequence similarity with *T. roseum* and *S. thermophilus* respectively.

The primary aim of this study was to describe the physiological characterisation of strain WKT50.2. This included determining temperature and pH optima, preferred carbon and energy sources, N-fixation, modes of growth, and phospholipid fatty acid analysis. In particular, utilisation of carbon monoxide as an energy source by WKT50.2 was investigated, as the other members of the class have this capability. Direct observation of the pure colonies and microscopic analyses of the bacterium under phase contrast and transmission electron microscopy (TEM) was used to define morphological characteristics. Phenotypic characterisation was used to make initial inferences of the ecology of this strain.

The other members of the *Thermomicrobia* class, *T. roseum* and *S. thermophilus* have been only partially characterised, although the genomes of both are available. The secondary aim of this study is to complete the characterisation of *T. roseum* and *S. thermophilus*, using strains from the DSMZ culture collection. This will increase our understanding of the ecology and metabolic capabilities of the class *Thermomicrobia.*

2 Materials and methods

All methods in this section were applied in the analysis of WKT50.2, *Thermomicrobium roseum* and *Sphaerobacter thermophilus*, unless otherwise stated.

Enrichment and isolation background

As part of a general culturing programme to isolate novel extremophiles from geothermal areas, environmental samples from soil at Waikite (Figure 2.1) were aseptically collected with a sterile spatula and transferred to sterile 50 mL Falcon™ tubes. Temperatures of the sample sites were measured on-site with a Fluke 50S thermocouple, and sample pH was measured in the laboratory at 25 ℃ by averaging the pH of 1 g of soil suspended in DI water.

Figure 2.1 - The isolation site of WKT50.2

Steam affected soil above the hot spring. At the point of sampling, the reddish-pink soil was 64.8 ^{o}C and pH 4.5.

Soil crumbs were spread on AOM1 plates [68], a weakly acidic, minimal media with gellan as a solidifying agent. These were incubated at 60 °C in an aerobic atmosphere, in Oxoid anaerobic jars to prevent desiccation. Plates were viewed after 3–4 days, and then at 1 week intervals for at least 4 weeks. Colonies that formed around soil crumbs within 4 weeks were re-streaked onto fresh medium. Individual colonies growing from these secondary streaks were selected with the aid of a stereo microscope and serially streaked onto new plates until a pure culture, named WKT50.2, was obtained. Enrichment and isolation was conducted by Dr. Xochitl Morgan.

WKT50.2 gDNA was extracted from a single colony from solid AOM1 medium using the Nucleospin Tissue kit (Macherey-Nagel GmbH and Co., Düren, Germany) and the 16S rRNA gene was amplified using PCR with universal bacterial primers 9f (originally named fD1) and 1492r (rP1) [69].

2.1 Medium preparation

All media were prepared as described in sections 2.1.1 to 2.1.3, and pH adjusted prior to sterilisation. pH was measured at room temperature using a PHM250 Ion Analyser pH probe (Radiometer Analytical SAS, Lyon, France) and adjusted, unless otherwise stated, using 1 M NaOH. All media was sterilised at 121 ^{o}C at 15 lb/in^2 for 20 minutes in a P52014 Pressure Vessel (Mercer Stainless Limited, Henderson, New Zealand). In some cases, where components of the medium were unable to be sterilised as above, they were instead aseptically passaged into the medium after cooling, using a 0.22 µm syringe filter.

2.1.1 AOM1 media

WKT50.2 was originally isolated on AOM1 media [68], although this was no longer used after initial cultivation tests showed much better growth on CPS media.

$(NH_4)_2SO_4$	4.000 g	
$MgSO4.7H_20$	0.600 g	
K_2HPO_4	0.450 g	
$CaCl_2.2H_2O$	0.005 g	
FeEDTA solution	3 mL	See Appendix B
Methanogens trace metal solution	3 mL	See Appendix B
Methanotrophs trace metal solution	1 mL	See Appendix B

All components were dissolved in approximately 950 mL ddH2O. The pH was adjusted to 6.5, before the volume was brought up to 1 L with ddH_2O. The pH dropped to around 6.0 following autoclaving.

2.1.2 Solid modified Castenholz medium with peptone and sucrose (CPS)

Thermomicrobium roseum was originally cultured in Castenholz medium D [22], which consisted of Castenholz Salts Solution with 0.1 % (w/v) yeast extract and tryptone (see Appendix B). The recipe was modified for optimal growth of WKT50.2.

Castenholz Salts Solution	see Appendix B	[70]
Peptone	0.50 g	
Sucrose	0.25 g	
$MgCl_2.6H_2O$	1.00 g	
Phytagel™	15.00 g	

All components, except Phytagel™, were dissolved in approximately 950 mL ddH_2O. The pH was adjusted to 8.2, before the volume was brought up to 985 mL with ddH_2O. The media was stirred rapidly before addition of Phytagel™ and autoclaved. The pH dropped to around 6.7 following autoclaving.

Media was aseptically transferred into sterile petri dishes, and allowed to cool for several hours to reduce condensation, before storage at 4 °C.

2.1.3 Liquid 10xCPS media

Liquid 10xCPS (Castenholz media as above with ten times the amount of peptone and sucrose) was used for some growth experiments, and for growing large amounts of biomass.

Castenholz Salts Solution	see Appendix B
Peptone	5.0 g
Sucrose	2.5 g

All components were dissolved in approximately 950 mL ddH_2O. The pH was adjusted to 8.2, before the volume was brought up to 1 L with ddH_2O, and autoclaved. The pH dropped to around 7.2 following autoclaving.

Liquid media was kept at room temperature in serum bottles or Schott™ bottles sealed with butyl rubber septa.

2.2 Initial cultivation and storage

Frozen stocks of WKT50.2 were revived via aseptic transfer of stock culture stored at -80 °C. Approximately 0.2 mL of defrosted stock were added to liquid 10xCPS medium and cultivated aerobically at 68 °C in a shaking incubator at 125 rpm. Unless otherwise stated, WKT50.2 was maintained via subculture every 7-10 days on solid CPS media, cultivated in Oxoid Anaerobic chambers at 60 °C and an aerobic atmosphere.

2.3 Measurement of growth

During characterisation experiments, unless otherwise specified, cells were grown in liquid 10xCPS, with an aerobic atmosphere, in an orbital incubator (Ratek Instruments Pty. Ltd., Boronia, Australia) set to 125 rpm and 65 ^{o}C. Growth was determined by measurement of optical density (O.D.) at 600 nm on a Lambda 35 UV/Vis Spectrometer (Perkin Elmer, Waltham, M.A., USA). O.D. was measured using 2 mL polystyrene cuvettes with a pathlength of 1 cm (Thermo Fisher Scientific Inc., Waltham, M.A., USA) or a 17 mm test tube holder (Perkin Elmer).

2.4 Microscopy

Phase contrast Microscopy

Cells were grown using both solid and liquid CPS media and observed using a 1000x Eclipse Ni-U phase-contrast microscope (Nikon, Tokyo, Japan).

Transmission Electron Microscopy

Cells were grown in liquid 10xCPS media and analysed externally by staff at the Advanced Microscopy Facility, University of Melbourne, Australia. Two different methods were used to fix and visualise cells.

Manual Cryo Plunging

A portion of the cells were manually snap-frozen in liquid N_2 and viewed using a cryo-preservation stage and low electron doses.

Negative staining

Cells were placed on a Formvar-Carbon coated copper 400-mesh grid and fixed with 2 % (w/v) uranyl acetate stain before visualisation on the TEM.

2.5 Temperature range and optimum

Cells were grown in 10xCPS media in 18 mm anaerobic tubes sealed with grey butyl stoppers, which allowed the passage of oxygen into the tubes. The tubes were incubated in duplicate in a Temperature Gradient Incubator (Terratec Corporation, Hobart, Australia) set from 40 to 90 ^{o}C, and agitated at 30 oscillations per minute. The optimum temperature for growth was determined based both on the fastest growth rate and the highest O.D. obtained.

2.6 pH range and optimum

Cells were grown in 10xCPS with the addition of either 30 mM sodium acetate/acetic acid buffer, 60 mM Tris(hydroxymethyl)aminomethane (Tris) buffer or with no buffer as a control. pH measurements were calibrated and measured at room temperature (20 ^{o}C). pH was measured before and after autoclaving, and after the growth experiment was terminated. pH determination experiments were conducted in triplicate using 20 mL serum vials sealed with grey rubber septa. An additional non-inoculated control was included to allow for abiotic pH shifts during incubation.

1M Tris Buffer

Tris(hydroxymethyl)aminomethane	12.11 g
ddH$_2$O	to 100 mL

Tris was dissolved in approximately 90 mL of ddH$_2$O. The pH was adjusted as needed with 1M HCL, before the volume was brought up to 100 mL with ddH$_2$O.

Table 2.1 - 1M Sodium acetate/acetic acid buffer recipe

Desired pH	Sodium acetate	Glacial acetic acid
4.5	2.95 g	3.84 g
5.0	5.25 g	2.16 g
5.5	6.96 g	0.91 g
6.0	7.77 g	0.32 g
6.5	8.06 g	0.10 g

Glacial acetic acid was dissolved in approximately 80 mL of ddH_2O. The sodium acetate was then dissolved and the volume brought up to 100 mL with ddH_2O.

Table 2.2 - pH values tested

30 mM sodium acetate / acetic acid buffer	4.5	5.0	5.5	6.0	6.5	
60 mM Tris buffer	6.9	7.5	7.9	8.2	8.9	9.2

2.7 Phylogenetic tree construction

16S rRNA genes belonging to *Thermomicrobia* and *Chloroflexi* were selected from the SILVA SSU NR (non-redundant) Release 111 database [5] for initial sequence alignment. Phylogenetic trees were constructed using either FastDNAml maximum likelihood algorithm or TREEPUZZLE, a quartet-puzzling maximum-likelihood algorithm, in Figures 1.3 and 3.7 respectively. Trees were constructed using ARB. 16S rRNA gene sequence similarity between *Thermomicrobia* isolates was determined with near-full length sequences in the ARB environment using the Neighbour-Joining distance matrix function.

2.8 Peptidoglycan

Cells were grown in 10xCPS media and harvested by centrifugation. The cell pellet was analysed externally by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Briefly, cells were disrupted in a Vibrogen cell mill before digestion with 0.5 mg/mL trypsin and a 2 % (v/v) solution of sodium dodecylsulfate (SDS) [71]. The extracted peptidoglycan was then hydrolysed by either 4N HCl at 100 ^{o}C for 16 hours; 6N HCl at 120 ^{o}C for 16 hours; or 4N HCl at 100 ^{o}C for 45 minutes. The hydrolysates were then analysed by TLC, using a methanol:pyridine: formic acid: water (80:10: 1: 19 v/v/v/v) solvent system [72]; and 2D-TLC, using isopropanol and α-picoline as solvents [56].

2.9 Fatty Acid Methyl Ester (FAME) analysis

Fatty acid analysis was performed under supervision by K. Lagutin at Callaghan Innovation, Lower Hutt.

Whole cell extract

Total fatty acids were extracted using a modified two step method [73]. Around 2 mg of biomass, from cells grown in liquid 10xCPS media, was placed in a 2 mL scinter vial with a teflon screw-cap. 300 µL of base reagent (1 % (w/v) NaOH in methanol) was added, and the mixture was incubated at 60 °C for 30 minutes. A small amount of toluene was added and the mixture was incubated for a further 10 minutes at 60 °C. 600 µL of acidic reagent (5 % (w/v) HCl in methanol) was then added and the mixture incubated at 60 °C for 30 minutes. Sodium methoxide in the solutions acted as a catalyst for the methylation of fatty acids (free or bound) to form fatty acid methyl esters (FAMEs).

200 µL ddH_2O and 500 µL hexane were added and mixed well before incubation at room temperature for 30 minutes. The top hexane layer was removed to a new vial, and the liquid evaporated under a stream of argon. The residue was redissolved in 50 µL hexane and transferred to a GC insert in a new vial.

TMS derivitisation

50 µL from the methyl ester vial was taken and evaporated under a stream of argon to around 5 µL. 100 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and mixed. This contains trimethylchlorosilane (TMCS), which replaces free hydroxyl groups, to enable better resolution on the GC. The mixture was incubated at 60 °C for 10 minutes, before the liquid was evaporated again under a stream of argon. The residue was redissolved in 50 µL hexane before being transferred to a GC insert in a fresh vial.

GC analysis of FAME was performed on a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and ZB-5MS (30m x 0.25mm i.d., 0.25µm) capillary column (Phenomenex, Torrance, CA, USA). Helium was used as a carrier gas, split 1:50. Injector and detector temperatures were both 280°C, and the oven temperature was held at 195°C for 60 min. Individual peaks of FAME were identified by comparison with standards of FAME and by equivalent chain length (ECL) values [74].

2.10 Identification of polar lipids

Some polar lipid analysis was carried out under supervision by K. Lagutin at Callaghan Innovation, Lower Hutt. Further analysis was conducted by Dr. Andrew Mackenzie (Callaghan Innovation).

Approximately 5 g (wet weight) of biomass from cells grown in liquid 10xCPS media was added to a conical flask. 150 mL of methanol was added and mixed, before being placed in a sonicator at ~40 °C for 30 minutes to lyse the cells. 80 mL of chloroform was added, and incubated in the warm sonicator for another 30 minutes. The mixture was vacuum filtered through Whatman™ 589[1] ashless filter paper, and the filtrate poured into a separating funnel. 130 mL of water was added to make a 1:1:0.9 (v/v) methanol:chloroform:water solution, mixed well, and left in the cold room overnight.

The clear fluid from the bottom phase, a 86:14:1 (v/v) chloroform:methanol:water solution containing most of the lipids, was removed to a conical flask, and evaporated using a rotary evaporator. 50 mL of the same bottom phase solution and 4 mL brine was added to the separating funnel. This was mixed well, to allow removal of more polar lipids such as phosphatidylserine which may be partly carried into the upper 3:48:47 (v/v) chloroform:methanol:water phase. Following settling and separation, the bottom phase was removed again, added to the conical flask and evaporated again. The flask was finally vacuumed in a desiccator to ensure removal of all solvents.

Polar lipids were separated by two dimensional thin layer chromatography (silica gel, Macherey- Nagel Art. No. 818 033, cut to 10 x 10 cm) using the solvent systems chloroform:methanol:hexane:25 % ammonium hydroxide solution (65:30:10:6, v/v) in one direction, followed by chloroform:methanol:acetic acid:water:benzene (70:30:5:4:1:10, v/v) for the second dimension. After thorough drying, plates were sprayed with either molybdate solution for detection of phospholipids, ninhydrin for detection of free amino groups, or diphenylamine or α-napthol-sulfuric acid for the detection of glycolipids. Another duplicate plate was charred by spraying with 10% v/v sulfuric acid in methanol and incubating at 120 ^{o}C for 30 minutes, in order to show total lipids.

2.10 Quinones

Cells were grown in 10xCPS media and harvested by centrifugation. The cell pellet was lyophilised and analysed externally by DSMZ.

Respiratory quinones were extracted using the two step method described by Tindall [75]. Briefly, lipoquinones were extracted from the lyophilised pellet by addition of methanol:hexane (2:1 v/v), stirring for 30 minutes, followed by incubation on ice for ten minutes. Ice-cold hexane was added to give a final ratio of methanol:hexane (1:1, v/v). The hexane phase was then taken off and reserved. More hexane and 0.3 % (w/v) NaCl were added to the methanolic phase to produce a methanol:hexane: 0.3 % NaCl (1:1:1) biphasic mixture. After centrifugation, the hexane phase was removed and combined with the hexane phase removed earlier, and used for lipoquinone analysis.

Respiratory quinones were separated by thin layer chromatography on a silica gel (Macherey-Nagel Art. No. 805 023) using hexane:*tert*-butylmethylether (9:1 v/v) as solvent. Quinones were removed from the plate and analysed using HPLC, on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18) using methanol:heptane 9:1 (v/v) as the eluant. Quinones were detected at 269 nm.

2.11 Substrate utilisation

Substrate utilisation tests were performed using Castenholz Salts Solution (Appendix B) in 20 mL serum vials sealed with grey rubber septa. Substrates tested were added at a concentration of 0.2 % (w/v), either before autoclaving or filtered using a sterile 0.22 µm syringe filter. Each substrate was tested in triplicate, with an additional non-inoculated control. A negative control with no carbon source was inoculated with the same volume of WKT50.2, to determine if there was any growth from carry-over.

2.12 HPLC analysis

Following initial substrate tests, six polysaccharide substrates were selected for further characterisation. Cells were grown in Castenholz Salts Solution with either Avicel™, CMC, raffinose, starch, trehalose or xylan at 0.2 g/L. Samples were taken for analysis at days 0, 3, 5, 6, 7 and 9.

HPLC analysis was performed using an UltiMate 3000 (Dionex, Sunnyvale, CA, USA), equipped with a RI-101 Refractive Index Detector (Shodex, New York, USA). A reversed phase Aminex HPX-87H Ion Exclusion Column (300 mm x 7.8 mm) and Micro-Guard Cation H guard column (30 mm x 4.6 mm) (Bio-Rad, Hercules, CA, USA) were used. The column was maintained at 60 ^{o}C, and the RI detector was set to 50 ^{o}C. 50 µl of the sample was injected, and eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Integration was performed using Chromeleon Chromatography Data System V6.80. Individual peaks were compared to peaks from stock standard solutions prepared at 5 g/L in 5 mM H_2SO_4 and stored at 4 ^{o}C in sealed serum bottles.

2.13 Carbon monoxide utilisation

For testing induced oxidation of carbon monoxide, cells were grown in Castenholz Salts Solution (Appendix A) with 0.05 % (w/v) yeast extract. The headspace contained 100 mg/L of CO in air. For testing constitutive oxidation, cells were grown in 10xCPS media (section 2.1.3) initially with an aerobic headspace, then 100 mg/L of CO was added once cells had grown to form visible biomass.

For CO analysis, gas samples were removed from vials and analysed immediately using a Peak Performer 1 Gas Analyser (Peak Laboratories, Mountain View, C.A., USA) equipped with Unibeads 1S and Molecular Sieve 13X columns, and a Reducing Compound Photometer. The temperature was set at 105 ^{o}C, and compressed air was used as a carrier. Detection limits were about 2 mg/L. The instrument was standardized using a certified CO standard (1044 ppm, Airgas Gulf States, Theodore, USA), and laboratory dilutions of this standard.

PCR was performed using primers specific to the large subunit of carbon monoxide dehydrogenase genes [76] to amplify putative *coxL* fragments (see Appendix E). Further primer pairs were designed on the basis of conserved motifs, including the active site, using the genomes of *T. roseum* (http://www.ncbi.nlm.nih.gov/nuccore/NC_011959.1) and *S. thermophilus* (http://www.ncbi.nlm.nih.gov/nuccore/NC_013524.1), using Geneious version 6.0.5 (Biomatters, http://www.geneious.com/).

2.14 Methane, nitrite and ammonia utilisation

For methane tests, cells were grown in a Castenholz Salts Solution (Appendix A), without energy sources, and $MgSO_4.7H_2O$ replaced with 0.08 g/L $MgCl_2.6H_2O$. The aerobic headspace of the vials then had CH_4 added at final ratios of either 5, 10 or 20 % (v/v) aseptically via a syringe. One set of vials with 5 % CH_4 also had 1 % CO_2 (v/v) added to the headspace. Controls included a vial with a nitrogen headspace and 10 % CH_4 or 10 % CO_2, and a vial with sucrose added as carbon source.

PCR was carried out using the primers m661 [77] and A189f [78] for amplification of the pmoA gene from pMMO (see Appendix F).

For nitrite oxidation tests, cells were grown in a Castenholz Salts Solution with $MgSO_4.7H_2O$ replaced with $MgCl_2.6H_2O$ as above, with 10 mM KNO_2 added through a sterile filter following autoclaving. 10 % CO_2 (v/v) was added to the aerobic headspace as a carbon source.

For ammonia oxidation tests, cells were grown in a Castenholz Salts Solution with $NaNO_3$ and KNO_3 replaced with 0.58 g/L NH_4Cl, and $MgSO_4.7H_2O$ replaced with 0.08 g/L $MgCl_2.6H_2O$. CO_2 was added to the aerobic headspace at final ratios of either 5, 10 or 20 % (v/v).

2.15 Anaerobic growth

Cells were grown in a modified Castenholz Salts Solution with nitrate replaced by 0.58 g/L NH_4Cl, and $MgSO_4.7H_2O$ replaced with 0.08 g/L $MgCl_2.6H_2O$. Sucrose (2.5 g/L) was used as a carbon source. Anaerobic growth was tested using either elemental S^0 (6.5 g/L), Na_2SO_4 (0.5 g/L) or $NaNO_3$ (0.5 g/L). Resazurin (1 mg/L) and $Na_2S.9H_2O$ (1 mM) were added to the medium before autoclaving. The headspace comprised (v/v): 85 % N_2, 10 % H_2 and 5 % CO_2. Vials were incubated at 65 ^{o}C.

2.16 Nitrogen sources

Nitrogen source tests were performed in 20 mL serum vials using a modified version of Castenholz Salts Solution without $NaNO_3$ or KNO_3, and sucrose (0.5 g/L) as carbon source. Initially, dinitrogen gas as an N-source was tested by incubating strains in Castenholz Salts medium minus KNO_3. As no N_2-fixation was observed for any of the strains (Section 3.4), no manipulation of the headspace was required to test alternate N-sources. Nitrogen sources tested were either KNO_3 (0.689 g/L), $(NH_4)_2SO_4$ [1.48 g/L], KNO_2 [1.7g/L], Urea [0.23 g/L], alanine [0.70 g/L], serine [0.80 g/L], trimethoprim [30 µg/mL], or metronidazole [30 µg/mL].

2.17 Photosynthesis test

Cells were grown in Castenholz Salt Solution with no carbon source other than carbon dioxide from the air. The cells were inoculated in six 300 mL conical flasks, sealed with foam bungs and foil caps and incubated in the light. Three flasks were wrapped completely in foil as negative controls.

2.18 Enzymatic tests

Catalase

Cells from a seven day culture on CPS solid media were collected using a sterile inoculating loop and placed on a microscope slide. A drop of 3 % (v/v) H_2O_2 solution was added to the cells, and the cells were observed for signs of bubble formation for three minutes.

Oxidase

Oxidase activity was tested using Microbact™ Oxidase Detection Strips (Oxoid, Basingstoke, UK) following the manufacturer's protocol.

Other enzymatic activities

A selection of enzymatic activities was tested using the api®ZYM 25-200 strips (bioMérieux, Marcy l'Etoile, France), following the manufacturer's protocol.

2.19 Salinity tolerance

Cells were grown in 10xCPS, with the addition of either 0.5, 1, 2, 3, 4 or 5 % (w/v) NaCl. Growth was determined by the visible formation of biomass and measurement of O.D. at 600 nm.

2.20 Antibiotic resistance and sensitivity

T. roseum had previously been found to be resistant to chloramphenicol and streptomycin, and sensitive to kanamycin, neomycin and penicillin [22]. WKT50.2 was also tested on these antibiotics, with the exception of penicillin being replaced with ampicillin. In addition, WKT50.2 was tested on metronidazole, polymyxin B, trimethoprim and vancomycin, shown to be relatively heat stable at 72 ^{o}C for at least three days [79]. Antibiotic resistance tests were performed using 10xCPS in 20 mL serum vials sealed with grey rubber septa. Antibiotics were added after autoclaving through a sterile 0.22 µm syringe filter, at concentrations of 3 µg/mL and 30 µg/mL. Positive controls with no addition of antibiotics were inoculated simultaneously and all tests were performed in triplicate. Cultures were checked visually every day, and the OD was measured on days three and seven. The mean OD of the test cultures was compared to the mean of the controls, and the result was expressed as a percentage of growth compared to the control.

Following the initial resistance tests, two antibiotics were tested further using solid media. CPS media (Section 2.1.2) was made following the standard recipe, autoclaved and allowed to cool to around 60 °C. Trimethoprim or metronidazole were then added to the media at concentrations of either 30, 50, 100 or 150 µg/mL, before the media was aseptically poured into sterile petri dishes and allowed to cool. WKT50.2 was streaked onto five plates of each concentration, for each antibiotic and incubated at 60 °C for seven days, being visually checked for growth every day.

The efficacy of trimethoprim was confirmed by using *Micrococcus luteus* as an indicator organism, grown on Todd Hewitt Agar plates either without antibiotic or with 3 or 30 µg/mL trimethoprim, at 32 °C overnight. The efficacy of metronidazole was tested by using a *Bacteroidetes* strain from our culture collection, P294, grown on R2A plates either without antibiotic or with 3 or 30 µg/mL metronidazole, at 60 °C in a nitrogen-only atmosphere for 3 days.

2.21 G+C content

Cells were grown in 10xCPS media at 65 °C and harvested by centrifugation. The cell pellet was analysed externally for GC content using HPLC by DSMZ. Cells were lysed using lysozyme, and the DNA extracted using phenol:chloroform with ethanol precipitation, before RNAse T1 treatment. The DNA was hydrolysed with P1 nuclease and dephosphorylised with bovine alkaline phosphatase [80]. The deoxyribonucleosides were then analysed by HPLC [81]. G+C values were calculated from the ratio of deoxyguanosine and deoxythymidine [80]. Non-methylated Lambda-DNA and three DNAs with published genome sequences (*Bacillus subtilis*, 43.52 mol% G+C; *Xanthomonas campestris*, 65.07 mol%; and *Streptomyces violaceoruber*, 72.12 mol%) were used as standards.

2.22 Further characterisation of *Thermomicrobium roseum*

Thermomicrobium roseum DSM 5159 was purchased from DSMZ, and initially grown on DSM 592 media (Appendix A) at 70 ^{o}C. Following initial cultivation, cells were transferred to CPS media. Unless otherwise stated, *T. roseum* was maintained via subculture every 7-10 days on solid CPS media, cultivated in Oxoid Anaerobic chambers at 70 ^{o}C and an aerobic atmosphere.

Characterisation experiments were carried out in 10xCPS media, adjusted to pH 8.2. For substrate utilisation tests, a Castenholz Salts Solution with the addition of 0.1 g/L glutamic acid was used, as the published data [22] and initial tests indicated an absolute requirement for glutamate. For nitrogen source tests, *T. roseum* was tested for assimilation of nitrogen in the form of nitrate, nitrite, ammonia, yeast extract or N_2 gas. For antibiotic sensitivity tests, *T. roseum* was tested for growth in 10XCPS media containing either metronidazole or trimethoprim.

2.23 Further characterisation of *Sphaerobacter thermophilus*

S. thermophilus DSM 20745 was purchased from DSMZ, and initially grown on DSM 467 media (Appendix A) at 55 ^{o}C. Following initial cultivation, cells were transferred to CPS media. Unless otherwise stated, *S. thermophilus* was maintained via subculture every 7-10 days on solid CPS media, cultivated in Oxoid Anaerobic chambers at 55 ^{o}C and an aerobic atmosphere.

Characterisation experiments were carried out in 10xCPS media, adjusted to pH 8.2, in an orbital incubator set to 125 rpm and 60 ^{o}C. For nitrogen source tests, *S. thermophilus* was tested for assimilation of nitrogen in the form of nitrate, nitrite, ammonia, yeast extract or N_2 gas. For antibiotic sensitivity tests, *S. thermophilus* was tested for growth in 10XCPS media containing either chloramphenicol, kanamycin, metronidazole, neomycin or trimethoprim.

3 Results – WKT50.2

3.1 General physiology

WKT50.2 colonies appeared two days after inoculation onto CPS plates. The colonies were small (2 - 3 mm), convex, pale pink in colour and with an entire margin (Figure 3.1). The colour deepened slightly as the colonies aged. WKT50.2 formed a white turbid solution when grown in a shaken liquid culture.

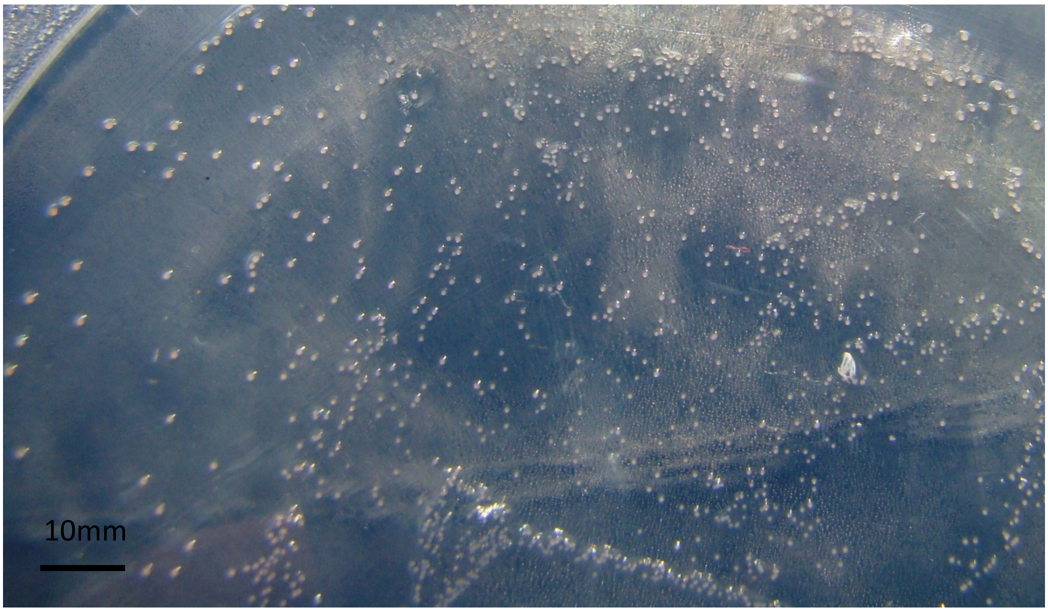

Figure 3.1 - Colony formation of WKT50.2 on CPS media

WKT50.2 cells have a characteristic dumb-bell, or very short rod morphology (Figure 3.2 and 3.3). As the cultures aged, pairs or chains of cells were frequently observed. The cells were not motile under any growth conditions. The cells appear to divide by binary fission (Figure 3.3).

Figure 3.2 - Phase-contrast micrograph of WKT50.2 cells

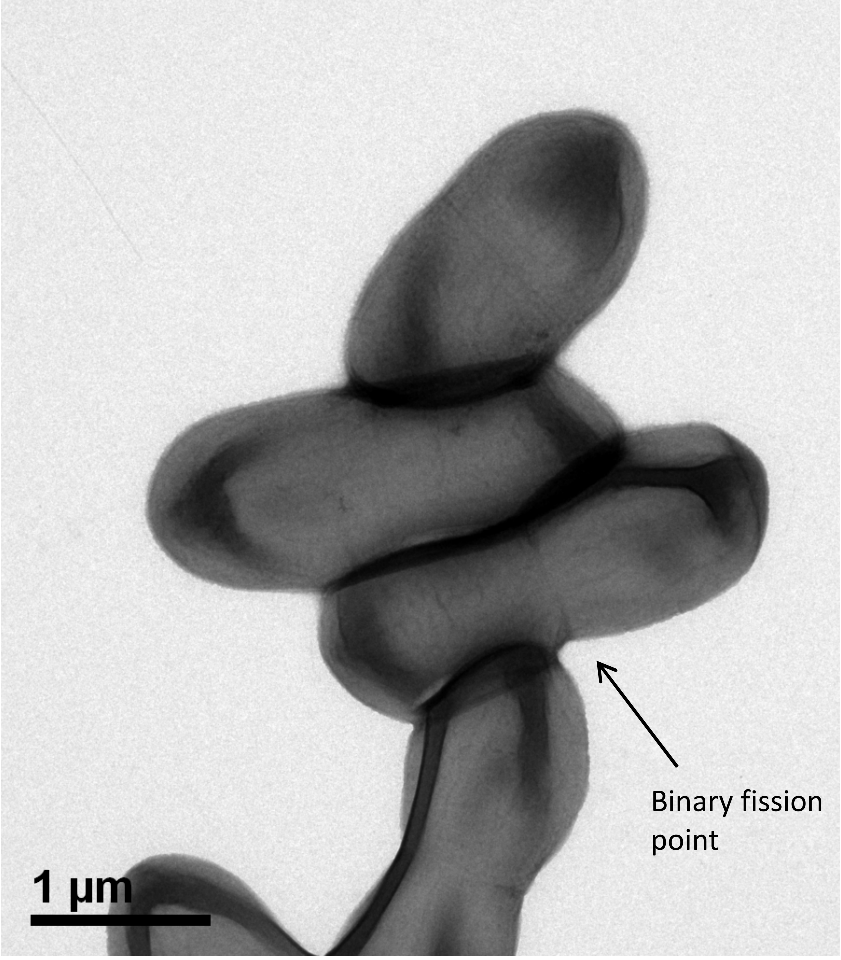

Figure 3.3 - Negative stain TEM image showing cell morphology

WKT50.2 cells were between 2.1 and 2.8 µm long, and 0.9 to 1.1 µm wide, and stained Gram-negative. TEM images suggested the existence of two lipid bilayers separated by a thin layer of peptidoglycan (Figure 3.4).

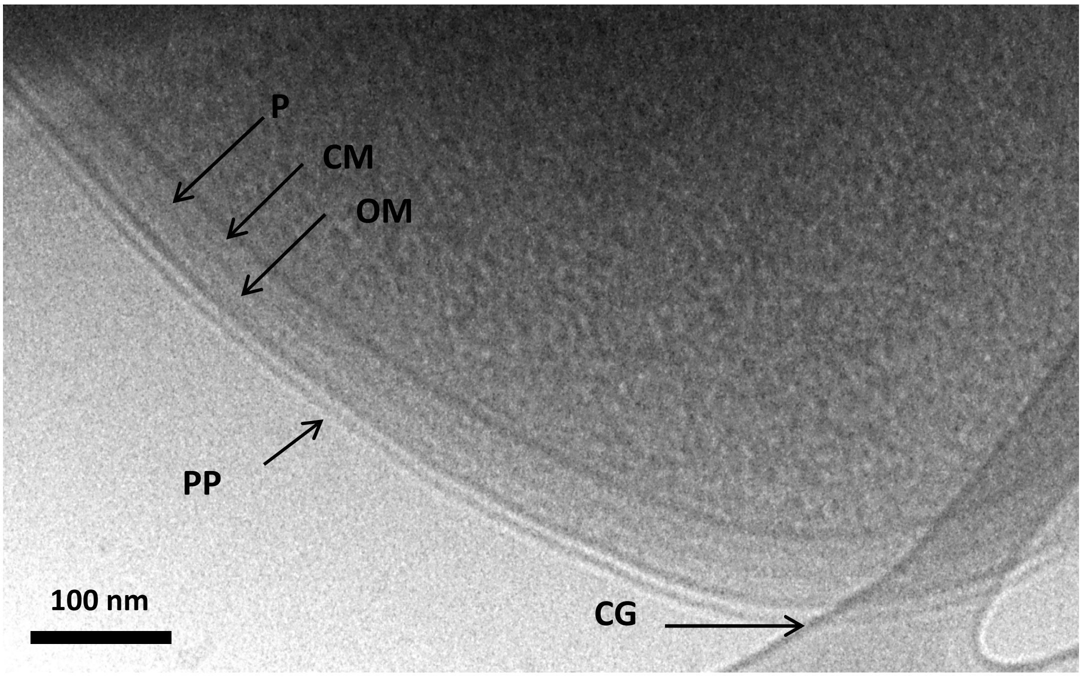

Figure 3.4 - Cryo TEM image showing membrane structure

Only part of the cell is shown. CM: cytoplasmic membrane; OM: outer membrane; P: peptidoglycan; PP: possible proteinaceous and/or polysaccharide outer layer; CG: copper grid

TEM imaging using negative staining showed that a small amount of extracellular polymeric substances (EPS) was sometimes associated with the cell (Figure 3.5). Although WKT50.2 does not form a visible biofilm in liquid culture, EPS may be used to connect bacteria together or adhere to a substratum. No staining of the EPS was attempted.

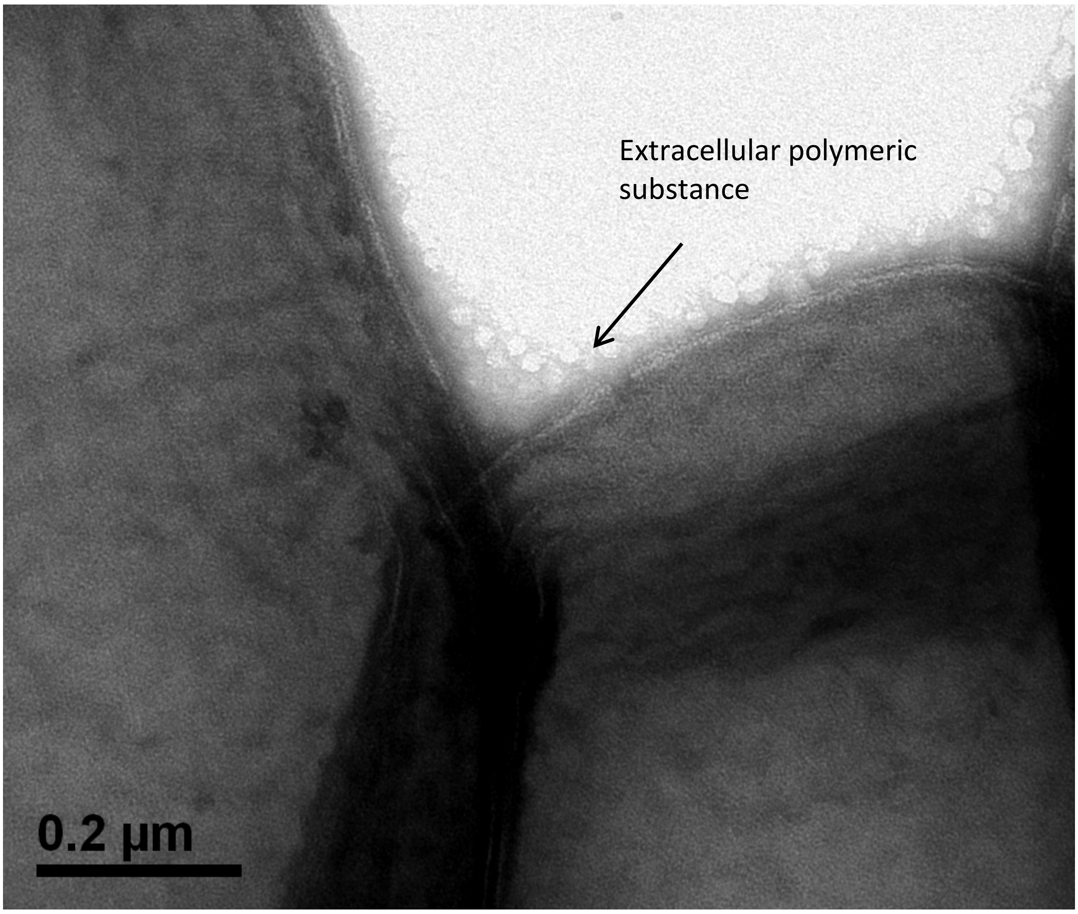

Figure 3.5 - Negative stain TEM image showing EPS

Pili were occasionally observed during TEM imaging of cells (Figure 3.6). Some bacterial species produce thousands of pili (also called fimbriae) and may use them for attachment to solid surfaces [82]. TEM images only showed single pili on WKT50.2 cells.

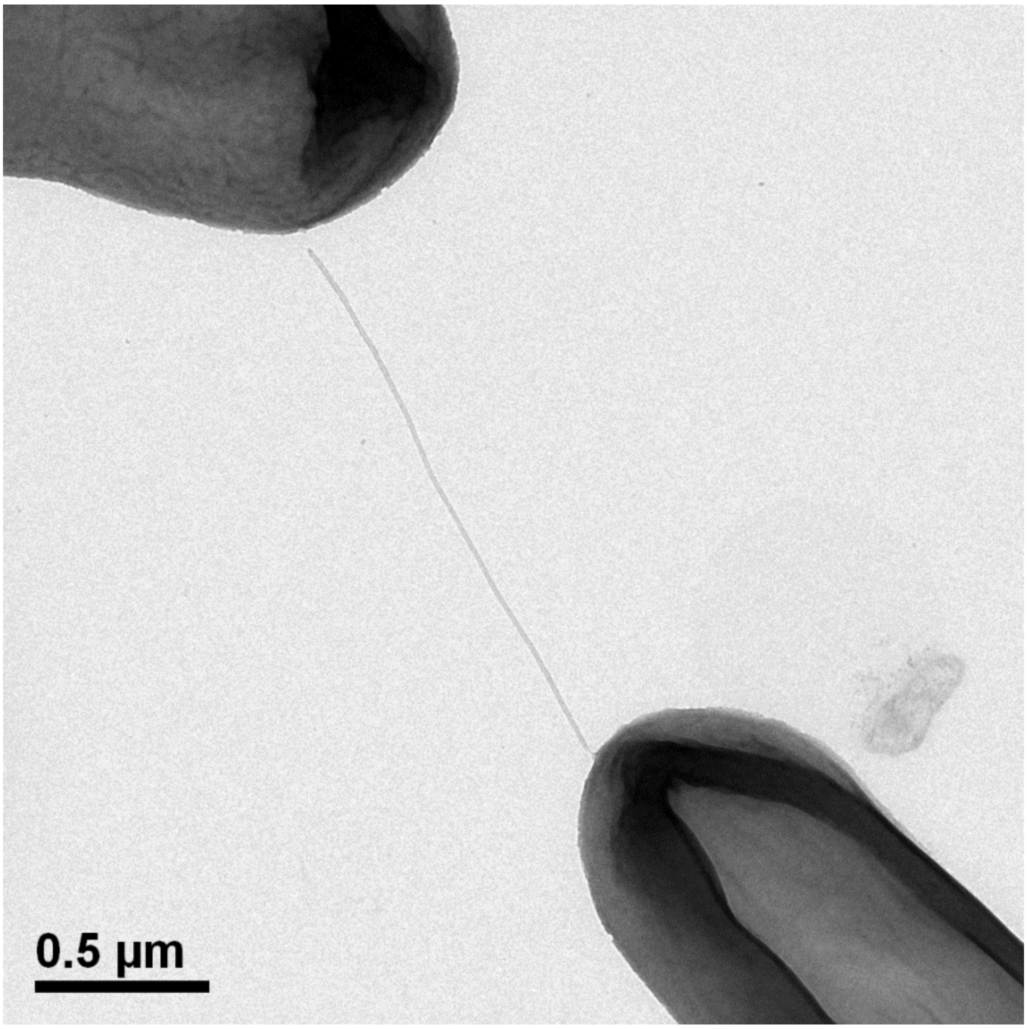

Figure 3.6 – Negative stain TEM image showing a pilus

On standard 10xCPS medium, WKT50.2 grew between 53 °C and 76 °C, but not at 51.9 °C and 77.1 °C. Maximum growth was observed between 68 °C and 73 °C. The optimum growth pH was 6.8 – 7.0, with a range of pH 5.9 to 8.2. No growth was observed at or below pH 5.5, or at or above pH 8.9. The pH was measured at room temperature before inoculation and after seven days growth; on average the pH of inoculated bottles dropped by 0.2.

3.2 Phylogeny

Phylogenetic analysis of the 16S rRNA gene sequence shows that WKT50.2 groups within the class *Thermomicrobia* in the phylum *Chloroflexi* (Figure 3.7). WKT50.2 was most closely related to *T. roseum* sharing a 91.4 % sequence similarity over a near full length 16S rRNA gene sequence (Table 3.1). WKT50.2 was more distantly related to the other *Thermomicrobia* isolates, *S. thermophilus (*88.2 % sequence similarity*)* and *N. hollandicus* (86.0 % similarity). High support values for the branching nodes confirms the placement of WKT50.2 within the deeper branches of the order *Thermomicrobiales*. The most closely related environmental phylotype sequences FN545867 and GU437584 (both 98 % sequence similarity) were detected from geothermal sediments. Interestingly, all *Thermomicrobiales* phylotypes appear to have a geothermal and/or a thermophilic phenotype, which may turn out to be a defining characteristic of the order.

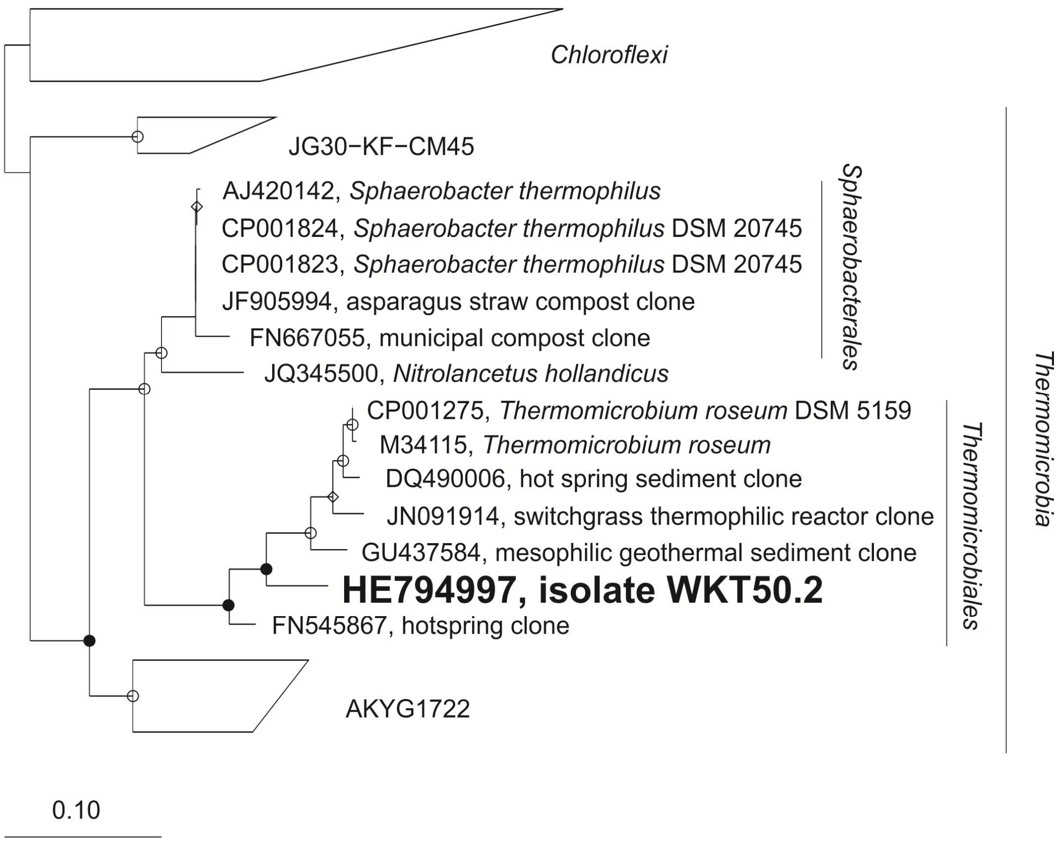

Figure 3.7 - Phylogenetic tree of *Thermomicrobi*a

Maximum-likelihood quartet-puzzling phylogenetic tree showing the position of cultivated isolates and environmental 16S rRNA gene sequences from isolates and clones belonging to the class *Thermomicrobia*. The tree was rooted against 23 16S rRNA gene sequences from other cultivated strains from *Chloroflexi*. Quartet-puzzling support values (10,000 resamples) are represented by the following symbols: ○ 90%,● 80%, ◊ 70% at each internal branch. The scale bar represents 0.1 change per nucleotide position.

Table 3.1 - Pairwise 16S rRNA gene sequence similarity in *Thermomicrobia* strains

Species	**WKT50.2**	***T. roseum***	***S. thermophilus***
WKT50.2	-		
T. roseum	91.4 %	-	
S. thermophilus	88.2 %	89 %	-
N. hollandicus	86.0 %	87 %	98 %

16S rRNA gene sequence similarities

The 16S rRNA gene sequence of WKT50.2 was analysed using the ARB environment [5] for the presence of signature nucleotides as described in Section 1.2.4 and [9]. The nucleotide in each position checked was identical to that of the *Chloroflexi* group, rather than the consensus nucleotide for the *Bacteria* domain (Table 3.2).

Table 3.2 - Signature nucleotides within the 16S rRNA gene sequences of *Chloroflexi* and WKT50.2

Position (*E. coli* 16S rRNA gene numbering)	**Consensus nucleotide**	***Chloroflexi***	**WKT50.2**
53	A	G	G
906	G	A	A
1202	U	G	G
1224	U	G	G
1410	A	G	G

The 16S rRNA gene sequence of WKT50.2 also showed a high level of similarity with all other *Chloroflexi* isolates in several regions (see Section 1.2.4 and [66]).

3.3 Cell Walls

Peptidoglycan

Analysis of the WKT50.2 peptidoglycan layer showed a substantial amount of protein was present in the cell membrane, which was unable to be removed from the cell hydrolysate and prevented full analysis of the peptidoglycan. However, the results indicate that cells of WKT50.2 contained only a small amount of peptidoglycan. No diaminopimelic acid (DAP) was detected, but ornithine was identified. Previous reports have indicated that the cell envelopes of *T. roseum* [57] and *S. thermophilus* [15] are composed largely of protein with only small amounts of peptidoglycan, and is consistent with the WKT50.2 observations. The peptidoglycan identified in WKT50.2 was of the A3β type with DAP replacing the diamino acid ornithine. The partial hydrolysate of WKT50.2 contained the peptide L-Ala-D-Glu which indicated that it was type A peptidoglycan.

The cell membrane of *C. aurantiacus* consists of a large polysaccharide complex bound to the peptidoglycan [53, 83]. The cell wall sugars of WKT50.2 were investigated to see if evidence of a polysaccharide complex could also be found in this species; sugars included xylose, mannose, glucose, galactose and rhamnose which are not normally found in cell walls but were identical to those found in the *C. aurantiacus* polysaccharide complex. The aldopentose ribose was also identified which was not found in the *C. aurantiacus* complex, although the complex did contain the isomer arabinose.

Fatty Acids

The fatty acid methyl ester profile of WKT50.2 (Table 3.3) was dominated by saturated long chain fatty acids (FA) of chain length >15 carbons with 12-Me-18:0, as a major component of the cell membrane. 12-Me-18:0 was also the predominant fatty acid in *T. roseum,* and in the newly isolated *N. hollandicus*, suggesting that the presence of this lipid could be a marker for the *Thermomicrobia* class. An interesting characteristic of the WKT50.2 FA profile was the presence of methyl groups in the iso- or anteiso- format of the long chain fatty acids. Without exception, all C15 and greater FA had at least one methylated form. Long chain fatty acids, particularly with 15 to 18 carbons, and with a single methyl group, are common in thermophiles [84], although the *iso*- (penultimate carbon) and *anteiso*- (3 carbons from the end) forms are more common than mid-chain branching [85], such as the 12-Me-18 and 10-methyl-16 forms seen in WKT50.2.

Table 3.3 - Fatty Acid Methyl Ester (FAME) profile of WKT50.2

Fatty acids	% of total fatty acids
12-Me-18	22.6
18:0	16.4
*i*17:0	14.2
*i*15:0	14.0
*i*16:0	9.4
16:0	7.8
*ai*17:0	5.4
20:0	3.4
*ai*15:0	2.0
14:0	1.0
15:0	0.8
*i*18:0	0.7
17:0	0.5
10-Me-16:0	0.4

Lipids

Thin layer chromatography of the lipids from WKT50.2 indicated five glycerolipids and six phospholipids, as well as two lipids that contained both glycerol and phosphate groups (Figure 3.8).

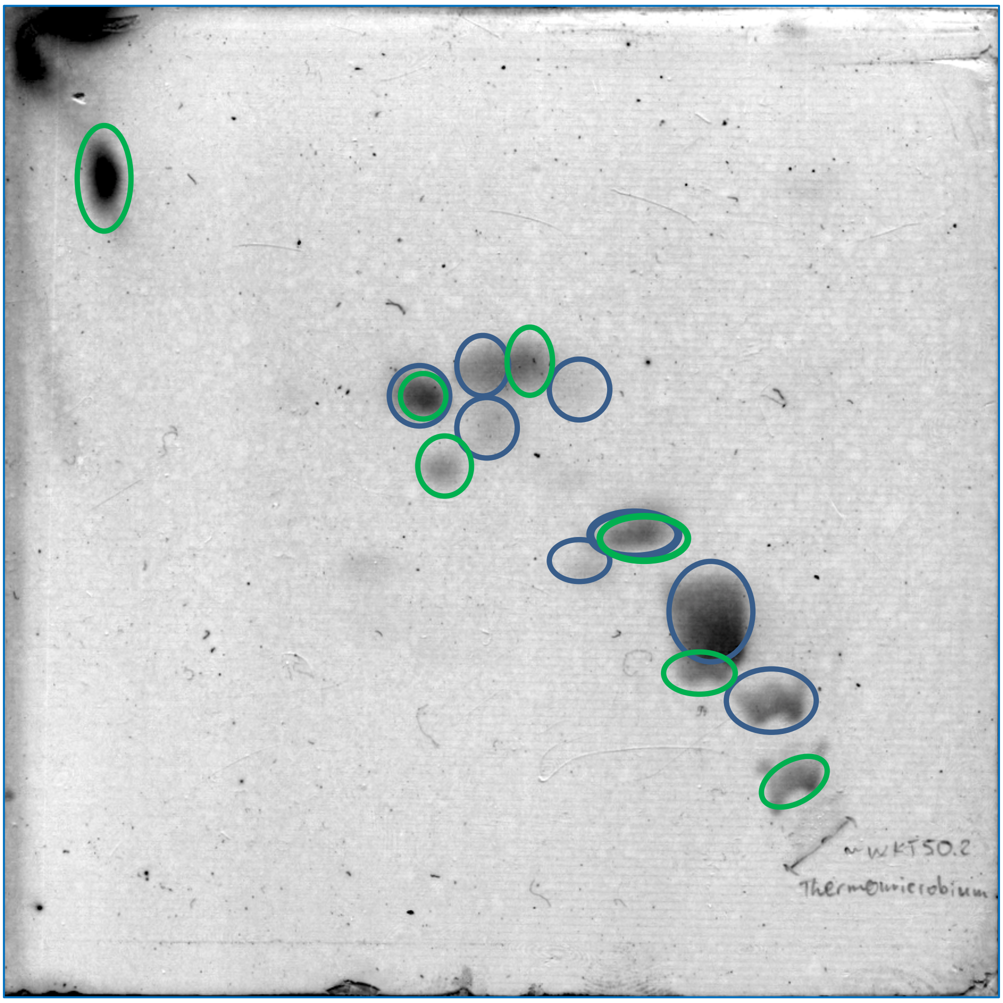

Figure 3.8 – TLC analysis of lipids from WKT50.2

Blue circles: phospholipids (molybdate stain); green circles: glycerolipids (diphenylamine stain).

^{31}P-NMR analysis lipids from WKT50.2 showed the presence of four phospholipids, including at least two diol phospholipids with inositol and/or sugar headgroups (Figure 3.9). ESI-MS and MSMS analysis of deacylated WKT50.2 lipids confirmed the presence of diol phospholipids in the extract (Appendix D). No further analysis of the lipid and/or diols were completed at the time of writing.

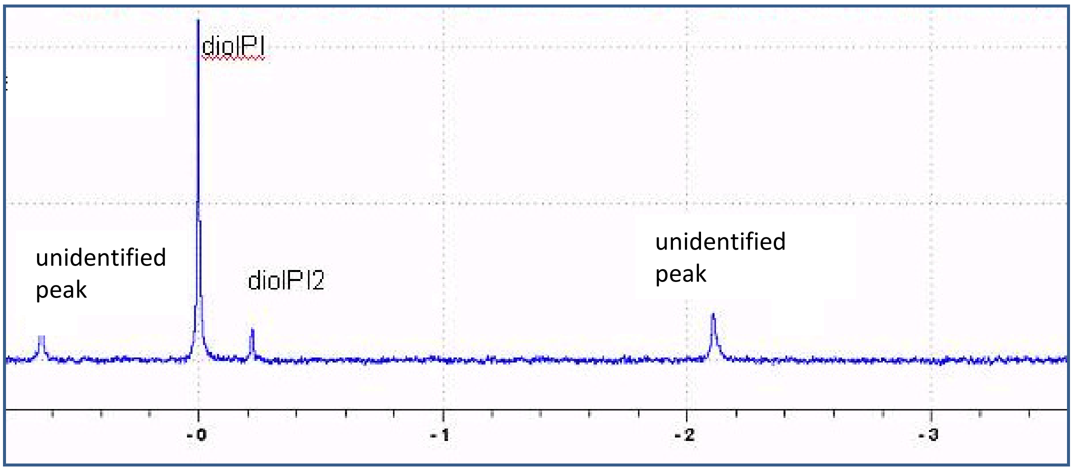

Figure 3.9 - NMR analysis of lipids from WKT50.2

Two partially characterised diol peaks are labelled at -0 and 0.2 ppm. Peaks at +0.6 and -2.1 ppm are unknown phospholipids. diolPI: diol based phosphotidylinositol; diolPI2: diol based hexosyl-phosphotidylinositol. Analysis and image courtesy of Andrew MacKenzie, Callaghan Innovation.

Quinones

The only quinone found in WKT50.2 following growth on 10x CPS media was MK-8, a menaquinone which contains a side chain with eight fully unsaturated isoprenoid units. This was also the major quinone found in *S. thermophilus* [15] and *N. hollandicus* [34]. *E. coli* is capable of synthesising both ubiquinone-8 (Q-8) and MK-8. The bacterium predominantly synthesises Q-8, but increases the synthesis of MK-8 following growth on non-fermentable carbon sources such as succinate, or under anaerobic conditions [86, 87]. Quinones were not investigated in WKT50.2 following growth on other carbon sources.

Quinones act as pools for protons delivered by various enzymes during metabolic processes. For example, succinic dehydrogenase transfers protons to quinones during the breakdown of acetate in the tricarboxylic acid cycle to form CO_2. The quinones transfer protons to cytochromes within the respiratory chain to generate ATP [88].

3.4 Metabolism

WKT50.2 was tested for growth on various substances including sugars, organic acids, cellulosic substrates, alcohols and protein derivatives (listed in Table 3.4). As a general observation, WKT50.2 is an aerobic heterotroph capable of growing on a limited range of both carbohydrates and protein containing compounds as energy source. Unusually, WKT50.2 appeared to be capable of using oligosaccharides but not monosaccharides, and in addition, the only disaccharides for which positive growth was only observed were sucrose and trehalose. WKT50.2 exhibited good growth on the polysaccharides crystalline cellulose (Avicel™), CMC, starch and xylan, but was unable to use chitin, dextrin, pectin, Phytagel™ or xanthan. HPLC analysis of growth of WKT50.2 on the polysaccharides Avicel™, CMC, raffinose, starch, trehalose or xylan did not detect the production of monosaccharides in solution.

Alcohols and organic acids are a common product of carbohydrate fermentation, and as such may be present in the Waikite environment. WKT50.2 was able to grow on methanol and ethanol but not 1- or 2-propanol, and grew well on all organic acids tested except citrate (Table 3.4). WKT50.2 was also able to utilise a range of complex protein derivatives, growing well on tryptone, casamino acids and yeast extract, and particularly well on peptone, but none of the single amino acids tested (Table 3.4).

Table 3.4 - Substrate utilisation of WKT50.2

Group	Substrate	Result
Aldopentoses	DL-Arabinose	-
	D-Ribose	-
	D-Xylose	-
Methylpentose	Rhamnose	-
Aldohexoses	D-Galactose	-
	D-Glucose	-
	D-Mannose	-
Deoxyhexose	L-Fucose	-
Ketohexose	D-Fructose	-
Disaccharides	D-Cellobiose	-
	Lactose	-
	D-Maltose	-
	Sucrose	+
	D-Trehalose	+
Trisaccharide	D-Raffinose	+
Sugar derivatives	D-Galacturonic acid	-
	D-Glucuronic acid	-
	D-N-acetylglucosamine (NAG)	-
Glucose-based polymers	Avicel™	+
	Carboxymethyl cellulose (CMC)	+
	Dextrin	-
	Starch	+
	Whatman™ filter paper	±
Algal and plant-derived polymers	Agarose	-
	Sodium alginate	+
	Galactomannan (Locust bean gum)	-
	Glucomannan	-
	Lignin	-
	Pectin	-
	Xylan	+
Bacterial exopolysaccharides	Phytagel™	-
	Xanthan	-

++ very good growth; + growth; ± poor growth; - no growth after at least seven days incubation

Group	Substrate	Result
Animal-derived polymers	Chitin	-
Organic salts	Sodium acetate	+
	Trisodium citrate	-
	Sodium formate	+
	Sodium fumarate	+
	Sodium lactate	+
	Sodium pyruvate	+
	Sodium succinate	+
Alcohols (0.05%)	Methanol	+
	Ethanol	+
	1-propanol	-
	2-propanol	-
Sugar alcohols	Sorbitol	+
	Mannitol	-
Protein derivatives / amino acids	Yeast extract	+
	Peptone	++
	Tryptone	+
	Casamino acids	+
	Alanine	-
	Cysteine	-
	Glutamic acid	-
	Glycine	-
	Leucine	-
	Lysine	-
	Methionine	-
	Serine	-
	Tryptophan	-
	Valine	-
Others	B-vitamins	-
	Ascorbic acid	-
	Sodium benzoate	-
	Glycerol	++
	Methylamine (0.05%)	-
	Trimethylamine (0.05%)	-
	Trimethoprim (30 µg/mL)	-
	Metronidazole (30 µg/mL)	-

++ very good growth; + growth; ± poor growth; - no growth after at least seven days incubation

Carbon Monoxide Oxidation

Carbon monoxide oxidation has been observed in both *T. roseum* [89] and *S. thermophilus* [personal communication, G. King]. However, no measurable CO uptake by WKT50.2 was observed in either of the two methods tested.

PCR was performed using primers specific for the CoxL carbon monoxide monooxygenase large subunit gene, based on the sequences from the published genomes of *T. roseum* and *S. thermophilus*. No product was amplified from WKT50.2, although CoxL genes were amplified successfully from both *S. thermophilus* and *T. roseum*. Combined, these data suggest that WKT50.2 does not have the capability for growth on carbon monoxide.

Methane, nitrite and ammonia oxidation

Following the observation that WKT50.2 grew well on methanol, a C1 carbon source, it was decided to test growth on methane. WKT50.2 was able to utilise 5, 10 or 20 % (v/v) methane, and demonstrated increased growth when 1 % CO_2 was added to the headspace (data not shown).

PCR was performed using primers specific for the pmoA gene of MMO. Primers m661[77] and A189f [78] amplified a band of approximately 470bp using DNA extracted from *Methylobacter, Methylocystis* and *Methylococcus* species from our culture collection, but there was no amplification with DNA from WKT50.2, *S. thermophilus* or *T. roseum*. This suggests that if a particulate MMO is present in *Thermomicrobia species,* it has a low sequence homology to the MMO of known methanotrophs.

N. hollandicus has been shown to oxidise nitrite to nitrate, using CO_2 as a sole carbon source, although it is unable to assimilate nitrogen from nitrite [34]. WKT50.2 was tested for growth on both nitrite and ammonia. WKT50.2 was able to oxidise nitrite, using CO_2 as the carbon source, and nitrate as the source for nitrogen. WKT50.2 was also able to oxidise ammonia, using it both as an energy and nitrogen source (Table 3.5).

Nitrogen sources

There are various ways for bacteria to import the nitrogen needed to survive. WKT50.2 was able to utilise nitrate, ammonia or alanine as sole sources of nitrogen, but not nitrite, nitrogen gas, yeast extract, urea, serine, trimethoprim or metronidazole.

Table 3.5 - Nitrogen, carbon and energy sources of WKT50.2

	WKT50.2
Nitrogen sources	Nitrate, ammonia, alanine
Carbon sources	Methane, carbon dioxide, polysaccharides, protein derivatives, alcohols, organic acids
Energy sources	Methane, ammonia, nitrite, polysaccharides, protein derivatives, alcohols, organic acids

Anaerobic growth

WKT50.2 was tested for the ability to grow anaerobically using nitrate, sulfur or sulfate as electron acceptors, using sucrose as an energy and carbon source. WKT50.2 did not exhibit anaerobic growth using any of these acceptors.

Other features

Enzymatic tests

WKT50.2 did not show catalase or oxidase activity. WKT50.2 showed positive results for the following enzymatic activities: esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, and trypsin (api®ZYM, bioMérieux).

Salinity tolerance

WKT50.2 does not require NaCl for growth, but tolerates up to 1 % (w/v) NaCl. No growth was observed at or above 2 % (w/v) NaCl.

Antibiotic resistance and sensitivity

T. roseum had previously been found to be resistant to chloramphenicol and streptomycin, and sensitive to kanamycin, neomycin and penicillin [22]. WKT50.2 was also tested on these antibiotics, with the exception of penicillin being replaced with ampicillin. WKT50.2 was completely inhibited by ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin and vancomycin (3 and 30 µg/mL). Metronidazole and trimethoprim both improved the growth of WKT50.2 (Table 3.6) when added to CPS media, but were unable to act as either energy or nitrogen sources in Castenholz Salt Solution.

Table 3.6 - Growth response of WKT50.2 to metronidazole and trimethoprim

Antibiotic	**% growth compared to positive control***
Metronidazole (3 µg/mL)	113
Metronidazole (30 µg/mL)	343
Trimethoprim (3 µg/mL)	119
Trimethoprim (30 µg/mL)	135

* The mean O.D. of the test cultures was compared to the mean of the controls, and the result is expressed as a percentage of growth compared to the control.

G+C content

The G+C content of WKT50.2 was determined by HPLC to be 58.4 mol%. *T. roseum* has a G+C content of 64 mol% [89] while *S. thermophilus* is between 66 and 68 mol% [15, 90]. High G+C contents often correlate with high temperature optima in bacteria [91] although there are many other factors which may affect G+C content, such as nitrogen utilisation and availability of nucleotides [92].

4 Results – *Thermomicrobium roseum*

4.1 General physiology

T. roseum grew between 52.3 °C and 77.2 °C. Maximum growth was observed between 65.0 and 69.6 °C. No growth was observed at 50.6 °C and 79.2 °C. The optimum growth pH was 8.0 to 8.4, with a range of pH 5.7 to 9. No growth was observed at or below pH 5.5 or at or above 9.2. pH was measured at room temperature.

4.2 Metabolism

T. roseum was tested for growth on a series of sugars, organic acids, cellulosic substrates, alcohols and protein derivatives, particularly those which WKT50.2 was shown to utilise. Glutamate was shown to be necessary for growth (as previously reported [22]), but *T. roseum* was unable to use this as a sole carbon and energy source. *T. roseum* was able to use all monosaccharides and many polysaccharides tested but was unable to hydrolyse Avicel™ or CMC (Table 4.1).

Table 4.1 - Substrate utilisation of *Thermomicrobium roseum*

Group	Substrate	Result
Aldopentoses	D-Xylose	+
Aldohexoses	D-Galactose	+
	D-Glucose	+
	D-Mannose	+
Ketohexose	D-Fructose	+
Disaccharides	D-Cellobiose	+
	D-Maltose	+
	Sucrose	+
	D-Trehalose	+
Trisaccharide	D-Raffinose	+
Glucose-based polymers	Avicel™	-
	Carboxymethyl cellulose (CMC)	-
	Dextrin	+
	Starch	+
Plant-derived polymer	Xylan	+
Bacterial exopolysaccharides	Phytagel™	+
	Xanthan	+
Organic salts	Sodium acetate	+
	Trisodium citrate	-
	Sodium formate	+
	Sodium fumarate	+
	Sodium lactate	+
	Sodium pyruvate	+
	Sodium succinate	+
	Glutamic acid	-
Sugar alcohols	Sorbitol	-
	Mannitol	+
Alcohols (0.05%)	Methanol	+
	Ethanol	+
	1-propanol	+
	2-propanol	+
Other	Glycerol	+
Protein derivatives	Yeast extract	+
	Peptone	+
	Tryptone	+
	Casamino acids	+

++ very good growth; + growth; ± poor growth; - no growth after at least seven days incubation

Methane, nitrite and ammonia oxidation

T. roseum was able to utilise methane or ammonia aerobically as sole energy sources, but did not exhibit growth using nitrite.

Nitrogen sources

T. roseum was able to utilise nitrate, ammonia or yeast extract as sole sources of nitrogen. It was unable to utilise nitrite or nitrogen gas as an N-source.

4.3 Other features

Salinity tolerance

T. roseum did not require NaCl for growth, but tolerated up to 1.5 % (w/v) NaCl. No growth was observed at or above 2% (w/v) NaCl.

Antibiotic resistance and sensitivity

The growth of *T. roseum* was stimulated by 3 or 30 µg/mL of metronidazole or trimethoprim, with higher dosages having a slightly more stimulatory effect (Table 4.2).

Table 4.2 - Growth response of *T. roseum* to metronidazole and trimethoprim

Antibiotic	Concentration (µg/mL)	Growth compared to the positive control (%) *
Metronidazole	3	196
	30	202
Trimethoprim	3	121
	30	134

* The mean O.D. of the test cultures was compared to the mean of the controls, and the result is expressed as a percentage of growth compared to the control.

5 Results – *Sphaerobacter thermophilus*

5.1 General physiology

S. thermophilus grew between 49.3 °C and 66.9 °C, but not at 47.6 °C or 69.3 °C. Maximum growth was observed between 55 °C and 60 °C. The optimum growth pH was 8.0 to 8.4, with a range of pH 6.1 to 9.0. No growth was observed at or below pH 5.7 or at or above 9.2.

5.2 Metabolism

S. thermophilus was tested for growth on the same series of substrates as *T. roseum* (Table 5.1). *S. thermophilus* was able to use only fructose and cellobiose of the oligosaccharides tested, but was able to utilise the polysaccharides Avicel™, CMC, Phytagel™, starch and xylan. *S. thermophilus* was unable to use dextrin or xanthan. In addition, *S. thermophilus* could use peptone, tryptone and casamino acids but not yeast extract as energy sources. All organic salts tested were utilised, and several alcohols.

Table 5.1 - Substrate utilisation of *Sphaerobacter thermophilus*

Group	Substrate	Result
Aldopentoses	D-Xylose	-
Aldohexoses	D-Galactose	-
	D-Glucose	-
	D-Mannose	-
Ketohexose	D-Fructose	+
Disaccharides	D-Cellobiose	+
	D-Maltose	-
	Sucrose	-
	D-Trehalose	-
Trisaccharide	D-Raffinose	-
Glucose-based polymers	Avicel™	+
	Carboxymethyl cellulose (CMC)	+
	Dextrin	-
	Starch	+
Plant-derived polymer	Xylan	+
Bacterial exopolysaccharides	Phytagel™	+
	Xanthan	-
Organic salts	Sodium acetate	+
	Trisodium citrate	+
	Sodium formate	+
	Sodium fumarate	+
	Sodium lactate	+
	Sodium pyruvate	+
	Sodium succinate	+
Sugar alcohols	Sorbitol	-
	Mannitol	+
Alcohols (0.05%)	Methanol	-
	Ethanol	+
	1-propanol	+
	2-propanol	+
Others	Glycerol	+
Protein derivatives	Yeast extract	-
	Peptone	+
	Tryptone	+
	Casamino acids	+
Broths	Nutrient broth	+

++ very good growth; + growth; ± poor growth; - no growth after at least seven days incubation

Methane, nitrite and ammonia oxidation

S. thermophilus was able to oxidise methane and ammonia aerobically, but it was unable to use nitrite as an energy source.

Nitrogen sources

S. thermophilus utilised nitrate, ammonia or yeast extract as sole sources of nitrogen. It was unable to utilise nitrite or nitrogen gas from the air.

5.3 Other features

Salinity tolerance

S. thermophilus did not require NaCl for growth, but tolerated up to 1 % (w/v) NaCl. No growth was observed at or above 2 % (w/v) NaCl.

Antibiotic resistance and sensitivity

S. thermophilus was completely inhibited by chloramphenicol and neomycin, and partially inhibited by trimethoprim and kanamycin (Table 5.2). The growth of S*. thermophilus* was inhibited by 3 µg/mL metronidazole, but was not inhibited by 30 µg/mL of the same antibiotic.

Table 5.2 Growth response of *S. thermophilus* to antibiotics

Antibiotic	Concentration (µg/mL)	Growth compared to the positive control (%)*
Chloramphenicol	3	0
	30	0
Kanamycin	3	68
	30	1
Metronidazole	3	39
	30	105
Neomycin	3	0
	30	0
Trimethoprim	3	13
	30	2

* The mean O.D. of the test cultures was compared to the mean of the controls, and the result is expressed as a percentage of growth compared to the control.

6 Discussion

WKT50.2 is a novel strain from the *Thermomicrobia* class within the phylum *Chloroflexi*, isolated from steam affected soil at Waikite, New Zealand. The primary aim of this study was to describe the physiological characterisation of strain WKT50.2, and make initial inferences about its ecology. The second aim was to complete the characterisation of the two species most closely related to WKT50.2, *T. roseum* and *S. thermophilus*. The results from WKT50.2 will be discussed in Section 6.1. An overall discussion of the common features and differentiating characteristics between the three species will form Section 6.2.

6.1 WKT50.2

The optimum growth temperature for WKT50.2 was 68 - 73 ^{o}C, with a range of 53 to 76 ^{o}C, and was consistent with the soil temperature at the Waikite sample site at the time of sampling which was 64.8 ^{o}C (Section 2). The optimum growth pH was 6.8 - 7.0, with a range of 5.9 to 8.2. At the time of sampling, the soil pH was measured at 4.5, although in pure culture WKT50.2 failed to grow at pH 5.5 or below. However, soil pH analysis is conducted by averaging the pH of 1 g of suspended soil in DI water and, as such, the measured sample pH may not be representative of WKT50.2 environmental niche. Previous analysis of the bacterial diversity at the Waikite site [68] via a 16S rRNA gene clone library (n~100) constructed using DNA extracts from the Waikite sample site detected a *Chloroflexi* OTU making up ~31 % of the clones, but this phylotype sequence was only 81 % similar to that of *Ktedonobacter racemifer*, and no *Thermomicrobia* clones were found. WKT50.2 did not appear to produce spores which may aid survival in these conditions. These observations suggest that WKT50.2 is in low abundance at this sampling site and possibly resides in small micro-environments within the Waikite sample site soil-matrix.

Ecology and phylogeny

Thermomicrobia strains have been detected in a wide variety of environments. Approximately 47 % of *Thermomicrobia* 16S rRNA gene sequences in the NCBI database were found in soil environments, and 19 % from skin samples (Figure 1.7). WKT50.2 was isolated from a geothermal soil environment, and the other phylotypes found in soil samples generally cluster in two clades, indicating that this may be a common habitat for *Thermomicrobia.* However, the relatively high proportions of *Thermomicrobia* phylotypes found on skin may be an artefact of the sequences deposited in the NCBI database as part of the human skin and mouse wound microbiome projects; at the time of writing, there were 431,432 nucleotide sequences deposited with the keywords “bacteria” and “skin”.

The *Thermomicrobia* 16S rRNA gene sequences recovered from skin samples almost all cluster within one clade, the JG30-KF-CM45 group. This may indicate that there is a clade of mesophilic *Thermomicrobia* that are able to colonise human skin, or simply that these bacteria are components of dust and are transferred onto human skin only briefly, and the large number of skin samples in the database has over-represented this class.

The phylogenetic placement of WKT50.2 within *Thermomicrobia* (Figure 3.7), and the 16S rRNA gene sequence similarity of WKT50.2 to *T. roseum* (91 %), *S. thermophiles* (88 %), and *N. hollandicus* (86 %) (Table 3.1), indicates that WKT50.2 can be considered to represent a new genus within the class *Thermomicrobia*. Results of phenotypic analysis also confirmed the differentiation of WKT50.2 from previously isolated species. Further comparison of the phylogenetic and physiological similarities of the three described *Thermomicrobia* strains are shown in Section 6.2.

Cell walls

WKT50.2 stained Gram-negative, indicating the presence of layered cell envelopes, which could be seen on the TEM images (Figure 3.4). Gram-negative bacteria usually have cell walls consisting of a plasma membrane enclosing the cytoplasm, surrounded by a thin layer of peptidoglycan, and then an outer membrane with phospholipids on the inner face and lipopolysaccharide (LPS) on the outer surface. LPS has not been found in *Chloroflexus aurantiacus* [83] or in any of the other described species within the phylum *Chloroflexi* [52]. Homologues of LPS biosynthesis genes, or of genes involved in transport across LPS, were not found in any of the 11 published *Chloroflexi* genomes [55]. Instead, evidence of a complex polysaccharide bound to the peptidoglycan has been described in *C. aurantiacus* [53, 83], indicated by the presence of the neutral sugars rhamnose, mannose, glucose, galactose, xylose and arabinose in the rigid layer fraction of the cell membrane.

The cell wall sugars of WKT50.2 were investigated to determine if the cell membrane also contained a polysaccharide complex. The results indicated that the sugars rhamnose, mannose, glucose, galactose, xylose and ribose all formed part of the cell wall, suggesting a rigid layer very similar to that of *C. aurantiacus*.

The peptidoglycan in bacterial cell walls is composed of short peptides cross-linked to alternating strands of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (Figure 1.5). In members of the phylum *Chloroflexi*, the peptidoglycan is usually of type A3, with ornithine as the diamino acid instead of the more common meso-diaminopimelic acid (DAP) [13, 15, 53, 57]. The cell wall of the most closely related species to WKT50.2, *T. roseum,* which also stained Gram-negative, showed several layers, with the outermost layer consisting of a regular repeating unit arranged in an array over the surface [22]. This layered cell envelope is composed primarily of protein, with only 8 % carbohydrate and 9 % lipid [57]. The cell wall of WKT50.2 was also found to contain a large amount of protein. This may indicate that the layered cell envelope seen in WKT50.2 may be comprised of polysaccharide and/or protein layers, but is unlikely to be LPS as found in the classical Gram-negative bacteria such as *E. coli*.

The analysis of the cell wall in WKT50.2 indicates that there was only a small amount of peptidoglycan, consistent with a Gram-negative bacterium. The large amount of protein hampered full analysis, but the presence of ornithine and a L-Ala-D-Glu peptide indicated that the peptidoglycan is also of type A3.

Lipids

The cell membrane of WKT50.2 contained primarily long chain, branched fatty acids, ester-linked to diols rather than to glycerol. The major fatty acid found was 12-Me-18:0, an unusual lipid which made up 23 % of the total fatty acids. This was also the major fatty acid found in *T. roseum*, where it comprises up to 68 % of the total fatty acids [93, 94]. The predominant membrane fatty acid in the newly isolated *N. hollandicus* was also reported to be 12-Me-18:0 [34]. Although the fatty acids of *S. thermophilus* have not yet been analysed, the consistency with which 12-Me-18:0 was observed in the other three strains suggests that it could be used as a biomarker for the class *Thermomicrobia*.

In other phyla, 12-Me-18:0 has only been found in the bacterial species *Rubrobacter xylanophilus* [95], and two strains of *Rubrobacter taiwanensis* [96], but in both these species the 12-Me-18:0 was less than 5% of the total fatty acids. This lipid was not found in the closely related species *Rubrobacter radiotolerans* [97]. 12-Me-18:0 has also been reported in small amounts in marine sponges from the Great Barrier Reef [98] and the Mediterranean Sea [85]. Sponges can host significant numbers of prokaryotes within the intracellular matrix, and bacteria may be up to 60 % of the total biomass [85]. Mid-chain branched fatty acids are thought to originate from heterotrophic bacteria within the sponge rather than from the cell membranes of the sponge itself [99]. This may indicate the presence of *Thermomicrobia* or related bacteria in these marine sponges.

Other major fatty acids found in WKT50.2 included 18:0 (16%), *i*17:0 (14%), *i*15:0 (14%) and i16:0 (9%), which are ubiquitous across many phyla. The elevated proportion of fatty acids with chains > C16 may be in response to its thermophilic growth range. Several authors have shown that many species of bacteria synthesise longer chain fatty acids (> C16) as the growth temperature increases [93, 100, 101], as longer length chains have higher melting points [93]. The permeability of the membrane is therefore temperature dependent, and bacteria need to regulate their fatty acid composition as growth temperatures alter [102].

Interestingly, WKT50.2 contained a significant proportion of iso-containing fatty acids. Branched fatty acid chains have lower melting points than their straight chain equivalents [93], particularly ante-iso and mid-chain branched fatty acids, yet many thermophiles contain high percentages of branched fatty acids [84, 102]. The proportions of iso-containing and straight chain fatty acids may be actively regulated by WKT50.2 in response to temperature fluctuations [93, 100, 101].

Fatty acids are normally found ester-linked to glycerol in bacterial lipids, as in Figure 6.1A. The lipid complement of WKT50.2 was not fully analysed at the time of writing. However, initial analysis showed a lipid profile containing five glycerolipids and, in addition, two diol-type lipids linked to inositol and/or sugar groups. Diols contain two hydroxyl groups, and *iso*-branched, long chain 1,2 diols were also identified in lipid analysis of *T. roseum* [94], with the two –OH groups on the first and second carbons. In this configuration, a diol could replace glycerol as the backbone of a lipid, acting as a structural homolog (Figure 6.1B).

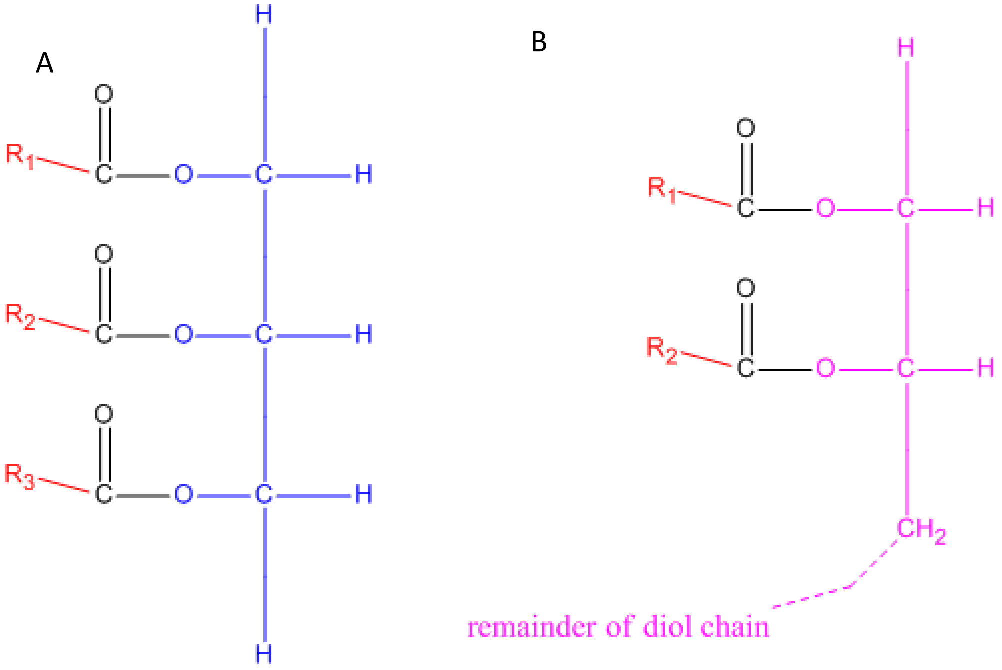

Figure 6.1 – A) Partial lipid structure B) Partial 1,2 diol structure

Blue: glycerol; Black: ester linkage; Red: fatty acids; Pink: Diol
R_1 = Polar head group, such as phosphate, sugars or inositol; R_2 and R_3 = Fatty acid chains

The principal diols found in *T. roseum* were each three carbons longer than the major fatty acids, suggesting that the diol is bent at the third carbon position to allow acylation with the fatty acid connected to the second carbon position, producing a membrane bilayer. Figure 6.2 shows a 1,2-diol based phospholipid found in WKT50.2. The structure of both identified diols indicates a C21 diol attached to a hexose group and/or an inositol group, consistent with being three carbons longer than the major fatty acids, 12-Me-18:0 and 18:0.

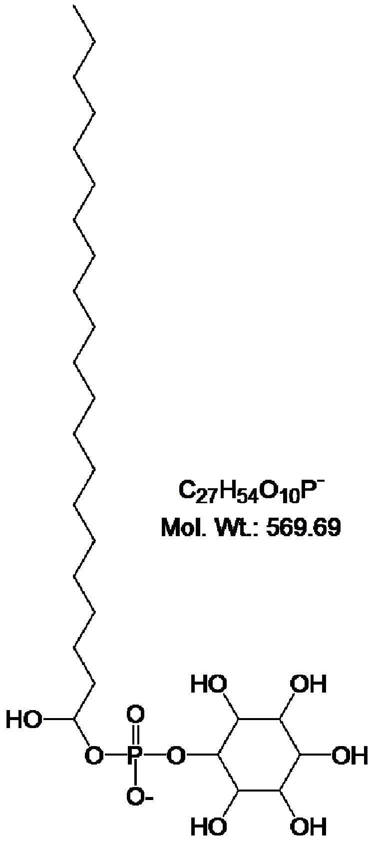

Figure 6.2 - Structure of 1,2-diol based phosphoinositol in WKT50.2

Other bacterial species such as *Roseiflexus castenholzii* [103] and some *Thermus* [104] and *Meiothermus* [105] species also have mixed glycerol and diol backbone structures in their lipids. Diols have so far only been found in thermophilic organisms, which suggests that they may play a role in adaptations to high temperature.

Initial analysis of the WKT50.2 diols suggest that they were linked to inositol and/or sugar groups (Figure 6.2 and Appendix D). In many bacterial cells phosphatidylinositol (PI) is found in cell membranes. This glycerophospholipid has a glycerol backbone, an inositol polar head group and two fatty acid tails, and has an important role in binding and regulating proteins in the membrane [106].

Metabolism

WKT50.2 had a primarily proteolytic metabolism being able to use substrates such as peptone, tryptone, casamino acids and yeast extract as sole energy sources. However, it was also able to use various cellulosic substrates such as carboxymethyl cellulose (CMC), starch, Whatman™ filter paper, xylan, and crystalline cellulose (Avicel™).

The ability to grow on cellulosic substrates as observed by WKT50.2 has not previously been reported for *Thermomicrobia* (see Section 6.2 for further discussion). However, cellulolytic growth is not an unusual observation amongst characterised species in the phylum *Chloroflexi.* Examples of cellulolytic strains include *Anaerolinea thermophila* which hydrolysed starch and xylan [26], and *Thermogemmatispora onikobensis* and *T. foliorum* which were both able to grow on Avicel™, CMC and xylan but not starch [47]. In addition, *Thermomicrobia* phylotypes (Figure 3.7) closely related to WKT50.2 were recovered from habitats rich in cellulose; two clones in the *Sphaerobacterales* clade were detected in compost (FN667055 and JF905994), and a clone from a switchgrass reactor used for biofuels production (JN091914) clustered with the *Thermomicrobiales*.

Avicel™, CMC and filter paper all have a linear structure composed of glucose monomers linked with β(1→4) bonds and supported the growth of WKT50.2, yet WKT50.2 was unable to utilise the monosaccharide D-glucose for growth. In the same manner, WKT50.2 was able to utilise the polysaccharide xylan (the xylan structure includes xylose subunits also linked by β(1→4) bonds) but not the xylose monomers as an energy source. WKT50.2 was also able to use sucrose, which contains α(1→2) bonds, but neither of the monomers glucose or fructose. HPLC analysis of growth of WKT50.2 on Avicel™, CMC, raffinose, starch, trehalose or xylan did not detect the production of monosaccharides in solution, as would be expected if the bacterium was releasing extracellular glycosyl hydrolases to break down the polysaccharides before transport into the cell.

WKT50.2 was able to use starch as an energy source, a semi-crystalline cellulose which contains α(1→4) and α(1→6) bonds between glucose monomers, but was unable to utilise dextrin which contains the same type of bonds and also contains glucose sub-units. This is an unusual observation considering that one would expect that in order for WKT50.2 to utilise these cellulosic substances, they would need to be hydrolysed for the sugars to be imported across the cell wall. A number of polysaccharides were unable to be metabolised, such as chitin, which contains N-acetylglucosamine residues, or xanthan, which contains glucose, glucuronic acid and mannose monomers.

The inability to utilise the monomer form of sugars by WKT50.2, but demonstration of positive growth on a number of polysaccharides including crystalline cellulose is an unusual observation. One possible explanation for these observations is that WKT50.2 lacks, or does not express, exo-acting glycosyl hydrolases, and instead hydrolyses cellulosic polymers into short-chain oligosaccharides using endo-acting glycosyl hydrolases. This hypothesis is supported by the lack of monosaccharides detected in HPLC analysis of the leachate from cellulose hydrolysis. However, this explanation would require WKT50.2 to possess an array of oligosaccharide transporters. The most closely related species, *T. roseum*, has a large number of transporters; around 10% of the genome codes for membrane transport functions [89], and a large number of these are ABC superfamily transporters, which use the hydrolysis of ATP to drive transport. *T. roseum* also has 76 genes for secondary-type transporters, which use gradients such as proton motive force to drive transport, and may be associated with sugar importation. A review of the *T. roseum* genome for sugar transporters revealed a number of C5 sugar transport systems, but few comprehensive transporters targeting C6 oligo- or monomeric transport systems.

Alternatively, WKT50.2 may be able to transport monosaccharides only after a long lag time, meaning that the uptake may not be observed in the described experiments. In a similar manner, *Clostridium thermocellum* has been shown to use an ATP-dependent transport system for the uptake of D-glucose [107], but a phosphoenolpyruvate-protein (PEP)-phosphotransferase transport system for mannitol and D-fructose [108]. This means that the uptake of D-glucose is energetically unfavourable over the uptake of fructose. The ATP-dependent transport system was only expressed in cells grown on D-glucose, and needed an extended lag time to be expressed.

Methane and ammonia oxidation

One of the most surprising observations was that WKT50.2 was able to grow aerobically with methane as a sole energy and carbon source. In addition, it was also able to utilise ammonia as an energy source and use CO_2 as the sole carbon source. The discovery of methanotrophy by WKT50.2 is unusual for the following reasons.

Methanotrophy to date has been only observed in phyla *Proteobacteria* or *Verrucomicrobia* [109]. There are currently 14 described genera of aerobic methanotrophs [110], the majority of which are in either the α- or γ-classes of *Proteobacteria*. Methanotrophy is not a characterised trait of either of the two most closely related bacteria to WKT50.2, *T. roseum* or *S. thermophilus*. Methanotrophs are generally, but not exclusively mesophilic [110]. Most species of methanotrophs which have been isolated are mesophilic, although there are some exceptions, such as *Methylacidiphilum infernorum* V4 [111], *Methylocaldum szegediense* [112], and some *Methylothermus* species which can grow at up to 72 ^{o}C [113]. Methanotrophic bacteria have also been detected in hot springs in Kamchatka, Russia [114].

Methanotrophs are predominately C1 specialists being only able to utilise compounds containing one carbon [115]. WKT50.2 was able to grow on methane and methanol but also a wide variety of sugars, organic acids, and cellulosic and proteinaceous substrates. Some methanotrophs have been shown to oxidise CO [116] or ethanol and organic salts [115], but are unable to obtain energy from the process. However, a few facultative methanotrophic species have been shown to utilise other carbon sources as well; some *Methylomonas* species can use succinate as an energy source, and *Methylobacterium organophilum* utilised a range of sugars, organic acids and even rich media such as nutrient agar for growth [117]. *M. organophilum* could use methane and glucose simultaneously, but growth was more rapid on succinate or glucose than on methane [118]. However, after repeated culture on glucose alone, the organism lost the ability to oxidise methane [109].

It would be very unusual for a methanotroph to also have the ability to gain energy from ammonia oxidation. The key enzyme involved in methanotrophy, methane monooxygenase (MMO), facilitates the oxidation of the C-H bond in methane to form methanol. MMO can also oxidise ammonia [119] and a range of other substrates including alkanes, alkenes and aromatics [110], but does so via co-metabolism, with the resulting products not able to be used as energy sources. Currently, there are no known methanotrophs able to grow via the dissimilatory oxidation of ammonia.

Future work includes identification of the enzymes involved in methanotrophy. Specific PCR primers have been designed for the detection of both the *pmoA* gene of the membrane-bound particulate form (pMMO) [77] and the *mmoX* gene of the cytoplasmic soluble form sMMO [120]. However, there is a level of variability within MMO genes [120], and if the MMO genes are not well conserved in WKT50.2, they may be difficult to detect with previously designed primers.

In addition, Southern blot hybridisation with probes that are heterologous for the products of pMMO and sMMO would allow identification of enzymes that have little sequence homology but still possess the same activity.

Sequencing the genome of WKT50.2 would enable the discovery of specific genes involved in the methane oxidation pathway, including MMO but also genes such as those encoding methanol dehydrogenase, which catalyses the reaction of methanol, a key intermediate of methane oxidation, to formaldehyde.

It would also be worthwhile to investigate the presence of ammonia monooxygenase (AMO) in WKT50.2. AMO is used to gain energy through the oxidation of ammonia, and is similar in structure and sequence identity to the particulate form of MMO (pMMO) [121]. PCR primers have previously been designed which detect both the *pmoA* gene of pMMO and the *amoA* gene of AMO [78].

In addition, the ability to fix CO_2 and use it as a sole carbon source suggests the ability for autotrophic growth. There are several different mechanisms WKT50.2 could be using to fix CO_2. First, the Calvin-Benson-Bassham (CBB) cycle, which is the most commonly found pathway in plants but is also found in algae and bacteria, including the *Chloroflexi* species *Oscillochloris trichoides*. *N. hollandicus* also appears to have a complete CBB pathway [34], and *T. roseum* has genes encoding possible homologs of many of the enzymes involved [89]. However, this pathway is not usually found in thermophiles, as its upper temperature limit is around 70 ^{o}C [122].

Another pathway for the fixation of CO_2 is the Wood-Ljungdahl, or reductive acetyl CoA pathway, which is often found in methanogens and acetogens [122]. This pathway fixes two molecules of CO_2 to form acetyl CoA. *T. roseum* has genes for part of this pathway, which would enable the assimilation of CO_2 via methionine rather than acetyl CoA [89].

The final possibility is the 3-hydroxypropionate cycle, first discovered in *C. aurantiacus*. The energy costs for this cycle are high, but the key enzyme involved uses bicarbonate as an inactive inorganic carbon species, which would be beneficial to an organism living in alkali or neutral conditions, as WKT50.2 does. In addition, the pathway can be used to assimilate a wide variety of compounds such as acetate and succinate, and would be useful for a mixotroph like WKT50.2.

Other features

Antibiotic resistance

WKT50.2 was completely inhibited by 3 µg/mL ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin and vancomycin, but metronidazole and trimethoprim both improved growth (Section 3.4). An increase in the amount of these antibiotics added led to an increase in cell biomass, and this effect was dosage dependent up to the 150 µg/mL tested. WKT50.2 was unable to use either trimethoprim or metronidazole as sole carbon or nitrogen source. Resistance to antibiotics may confer a selective advantage to WKT50.2 if the antibiotics or their analogues are found naturally in the soil environment. A mechanism to improve growth in the presence of antibiotics would increase the competitiveness of WKT50.2.

Trimethoprim Resistance

Trimethoprim is a dihydrofolate reductase inhibitor, which blocks the synthesis of deoxythymidine monophosphate (dTMP), an essential DNA precursor, in susceptible organisms. Classical thymidylate synthases use N^5N^{10} methylene tetrahydrofolate and deoxyuridine monophosphate (dUMP) to produce dihydrofolate and dTMP. The dihydrofolate is then recycled, by being reduced back to tetrahydrofolate by the dihydrofolate reductase enzyme [123]. These thymidylate synthases are encoded by the ThyA gene.

In 2002 another form of flavin-dependent thymidylate synthase (FDTS) was discovered, encoded by a ThyX gene [124]. These synthases share no sequence or structural homology with classical synthases. FDTSs transform dUMP, N^5N^{10} methylene tetrahydrofolate and NADPH to dTMP, tetrahydrofolate and NADP+. Organisms with a ThyX pathway do not require a tetrahydrofolate reductase in order to product thymidylate, and so are resistant to the action of trimethoprim.

The ThyX gene is found in 30 % of all microorganisms, and in ~70 % of organisms with a temperature optimum above 60 °C [125]. The ThyX gene has been found in the genome of *S. thermophilus*, although neither ThyA nor ThyX has been identified in the *T. roseum* genome, and it seems likely that the resistance of WKT50.2 to trimethoprim has been conferred by the presence of a ThyX pathway.

Growth stimulation

Two isoforms of serine transhydroxymethylase, an enzyme required for the assimilation of C1 units, have been purified from the facultative methanotroph *M. organophilum*. One form is upregulated when the cells are grown on methane or methanol, and the other isoenzyme is produced when cells are grown on succinate or glucose [126]. Trimethoprim in the growth medium was shown to cause an increased level of serine transhydroxymethylase in both methanol- and succinate-grown cells by stimulating the synthesis of the first (methanol/ methane-activated) enzyme. This could lead to the increased uptake of carbon and hence an increase in biomass for WKT50.2, if it also produces these enzymes.

Metronidazole

Metronidazole is a 5-nitroimidazole compound, which is added to media in an inactive form and becomes activated within anaerobic cells. The nitro group is reduced by metabolic pathways of low redox potential; in aerobic cells there are no electron transport proteins with sufficiently negative potential to activate the antibiotic [127].

There have been at least two reports of the stimulation of growth of *Lactobacillus* strains using certain concentrations of metronidazole. Eight strains of *Lactobacillus* from healthy vaginal microbiota showed that growth was stimulated by metronidazole concentrations of between 128 and 256 µg/mL, but growth was partially inhibited by concentrations of between 1000 and 4000 µg/mL. Concentrations of less than 128 µg/mL, or between 256 and 1000 µg/mL, had no effect [128].

Another research group showed that the growth of *L. plantarum* KCA, a strain from healthy vaginal microbiota, was stimulated by 256 µg/mL of metronidazole and partially inhibited by 1000 µg/mL. In contrast, *L. rhamnosus* GR-1, a probiotic strain used to treat vaginal infection, was stimulated by 64 and 256 but not 128 µg/mL of metronidazole [129]. It was suggested that that the lactobacilli could be using the antibiotic as a carbon source, but this possibility was ruled out with WKT50.2. The mechanism for growth stimulation was not determined. Testing the supernatant from WKT50.2 cells grown in the presence of metronidazole or trimethoprim, on bacterial strains sensitive to either antibiotic, would help to determine if the antibtiocs have been degraded by WKT50.2.

Future work to elucidate the mechanism for growth stimulation could include denaturing the antibiotics by heating to 95 ^{o}C before adding them to the culture media and determining if this still had a stimulatory effect.

It would be interesting to establish if using both antibiotics together created an additive effect, and also if using an antibiotic such as vancomycin, which WKT50.2 is very sensitive to, would cancel out the effect of the growth stimulation on cells. Higher doses of the antibiotic should also be investigated, as the work done on *Lactobacillus* strains [128, 129] used up to 4000 µg/mL, and showed that the stimulation of growth was not a simple dose-response. Sequencing the genome of WKT50.2 could also be useful to elucidate possible resistance mechanisms and/or genes involved in pathways that facilitate growth in response to the use of this antibiotic. It may also be of interest to grow WKT50.2 cells on methane with trimethoprim in the culture medium, to determine if there is an additive effect on growth. Serine transhydroxymethylase is upregulated in *M.organophilum* cells grown on methane, and trimethoprim upregulated the production of this enzyme in both methane- and succinate-grown cells [126]. This may lead to an increased carbon uptake and increased growth in cells.

6.2 Commonalities and differences

The other members of the *Thermomicrobia* class, *T. roseum* and *S. thermophilus* had been only partially characterised at the time of writing. This study completed the characterisation of these species in order to increase our understanding of the ecology and metabolic capabilities of the class, and to determine if there are common features which define the class (Table 6.1).

Table 6.1 - Comparison of characteristics in the class *Thermomicrobia.*

Features common to all four species are highlighted in bold.

	WKT50.2	***T. roseum***	***S. thermophilus***	***N. hollandicus***
Isolation site	Soil at Waikite Springs, New Zealand	Hot spring, Yellowstone National Park, USA	Thermophilic sewage sludge, Germany	Mesophilic bioreactor, The Netherlands
Morphology	Dumbbells; singles or in chains	Dumbbell or irregular shaped rods; singles or in pairs	Irregular rods or dumbbells	Lancet shaped cells
Motility	**Not observed**	**Not observed**	**Not observed**	**Not observed**
Colonies	Pink, entire	Pink, entire	Opaque, entire	n.d.
Gram stain	-ve	-ve	+ve	+ve
Temperature range for growth (Optimum growth temp.)	53 – 76 °C (68 - 73 °C)	52 - 77 °C (65 - 70 °C)	49 - 67 °C (55 - 60 °C)	20 – 46 °C * (40 °C *)
pH range for growth (Optimum growth pH)	**5.9 – 8.2** (6.8 – 7.0)	**5.7 – 9.0** (8.0 – 8.4)	**6.1 – 9.0** (8.0 - 8.4)	**6.2 – 8.3** (6.8 – 7.5)
Atmosphere	**Aerobic**	**Aerobic**	**Aerobic**	**Aerobic**
CO oxidation	No	Yes	Yes	No
CH_4 oxidation	**Yes**	**Yes**	**Yes**	n.d.
NH_3 oxidation	**Yes**	**Yes**	**Yes**	n.d.
NO_2^- oxidation	Yes	No	No	Yes
Growth on trimethoprim & metronidazole	Yes	Yes	No	n.d.
Major fatty acids (proportion)	**12-Me-18:0** (23 %), 18:0 (16 %), i17:0, i15:0	**12-Me-18:0** (68 %) 18:0 (16 %)	n.d.	**12-Me-18:0**
Fatty acids ester- linked to diols	**Yes**	**Yes**	n.d.	**Yes**
Major quinone	**MK-8**	n.d.	**MK-8** (75 %), MK-10 (12 %), MK-7 (8 %), MK-6 (5 %)	**MK-8**
G+C content (mol%)	58.4	64.0	68.1	62.6
Cell wall structure	**High in protein** **Small amount of peptidoglycan** **DAP replaced by ornithine**	**High in protein** **Small amount of peptidoglycan** **DAP replaced by ornithine**	**High in protein** **Small amount of peptidoglycan** **DAP replaced by ornithine**	**Small amount of peptidoglycan** **DAP replaced by ornithine**

* previously published data.

Physiology

WKT50.2, *T. roseum* and *S. thermophilus* share many common physiological features, such as small rod-shaped morphology, and similar temperature and pH ranges for growth. The T_{max} of *T. roseum* was not the same as the published data; although originally the maximum temperature for growth was described as 85 °C, in this study growth was only observed up to 78 °C under the conditions used.

All three species form small entire colonies on solid media, although *S. thermophilus* formed white colonies while those of WKT50.2 and *T. roseum* were pink pigmented. None of the species have been observed to produce spores or motile cells.

Cell walls

The cell envelopes of all three species show a multi-layered membrane , typical of Gram-negative bacteria, although *S. thermophilus* actually stains Gram-positive, and LPS is missing from at least *T. roseum* and *S. thermophilus* [52, 55]. The outer envelope layer is likely to be either a proteinaceous [15, 57] and/or polysaccharide [53] complex. The thin peptidoglycan layer of the three species is of the A3β form, and has DAP replaced with ornithine. Studies of the fatty acids of *S. thermophilus* have yet to be investigated, but WKT50.2 and *T. roseum* both contain 12-Me-18:0 as their major fatty acid, linked to long chain diols instead of glycerol [93, 94]. The major quinone found in both WKT50.2 and *S. thermophilus* is MK-8. Quinones have not yet been analysed in *T. roseum*.

Metabolism

S. thermophilus was only able to utilise cellobiose and fructose of the saccharides tested, but it could hydrolyse crystalline cellulose, CMC, starch and xylan, as well as Phytagel™ which WKT50.2 could not (Table 6.2). On the other hand, *T. roseum* could use all mono-, di- and trisaccharides tested. *T. roseum* could use Phytagel™, starch and xylan as well, but was unable to grow on Avicel™ or CMC. Glycosyl hydrolases from several families have been found in the genomes of both of these species (Appendix G).

Table 6.2 - Differences in substrate utilisation by WKT50.2, *Thermomicrobium roseum* and *Sphaerobacter thermophilus*

	WKT50.2	***T. roseum***	***S. thermophilus***
Monosaccharides	-	+	±
Avicel	+	-	+
CMC	+	-	+
Dextrin	-	+	-
Phytagel	-	+	+
Starch	+	+	+
Xanthan	-	+	-
Xylan	+	+	+
Methanol	+	+	-
Ethanol	+	+	+
1-propanol	-	+	+
2-propanol	-	+	+
Sodium citrate	-	-	+
Casamino acids	+	+	+
Peptone	+	+	+
Tryptone	+	+	+
Yeast extract	+	+	-

T. roseum [89] and *S. thermophilus* (Gary King, personal communication) have both been shown to oxidise carbon monoxide, but this capability was not demonstrated in WKT50.2 or *N. hollandicus* [34], suggesting that this unusual property cannot be used as a marker for the class *Thermomicrobia* as originally thought (Section 1.4).

All three species could oxidise methane aerobically in a minimal media, and growth of WKT50.2 was improved with the addition of 1 % (v/v) CO_2 to the headspace. This unusual observation (discussed in Section 6.1), suggests that *Thermomicrobia* species may be mixotrophic, and that although capable of oxidising methane, they were using alternate energy sources at the low-methane sites where each was isolated.

Future experiments include determining the rate of methane oxidation using a gas chromatograph to analyse levels of methane in the headspace of vials. Initial work indicates that WKT50.2 grows well on methane, but there is a longer lag time than with growth on proteinaceous substrates (data not shown). The molecular basis for methane oxidation could be investigated using trace metal availability, HPLC analysis of supernatants following growth, and transcriptomic experiments.

Future work could also focus on the detection of other novel *Thermomicrobia* strains from environments rich in methane, using PCR primers or probes designed using conserved sections of the 16S rRNA gene sequences and/or any MMO analogs discovered in any investigation of the methane oxidising enzymes of the known isolates.

A 16S rRNA gene clone library (n~100), constructed using DNA extracts from the Hell’s Gate area, Tikitere, New Zealand [68], detected two OTUs that were 81 - 82 % similar to *S. thermophilus*, making up 9 % of the total clones. In the layer of soil from where the DNA was extracted the methane concentration was measured at less than the detection limit of 100 ppm methane in the atmosphere [101]. However, in the layer just below, there was a large amount of methane being consumed by novel acidophilic and thermophilic verrucomicrobial methanotrophs in a subsurface sink. Other areas of Tikitere also showed high levels of methane (1.9 – 3.8 % total gas) (unpublished data from samples analysed at the New Zealand Water and Gas Laboratory). These OTU’s may represent a novel *Thermomicrobia* strain, possibly using the high concentrations of methane at this site as an energy source.

WKT50.2, *T. roseum* and *S. thermophilus* could all oxidise ammonia for growth. Methane monooxygenase is known to oxidise ammonia as well as methane, but no known methanotrophs are able to use both ammonia and methane for energy. MMO and AMO (ammonia monooxygenase) genes were not found in the genomes of either *T. roseum* or *S. thermophilus*. Ammonia concentrations were very low at Waikite, but were not measured at the isolation sites of *T. roseum* or *S. thermophilus*. Tikitere sample analysis also showed high concentrations of ammonia in various areas, ranging from 86 µg/mL at Mini Cooking Pot to 764 µg/mL at Inferno Crater. WKT50.2, *T. roseum* and *S. thermophilus* were all able to gain energy from oxidising ammonia, using CO_2 as the sole carbon source. No known methanotrophs can oxidise both methane and ammonia and gain energy for growth from both substrates [110, 119].

These data may suggest that the *Thermomicrobia* strains are also facultative ammonia oxidisers. As discussed in Section 6.1, future work should focus on identifying the genes involved in both methane and ammonia oxidation.

WKT50.2 was also able to use nitrite at 10 mM as an energy source, with CO_2 as the carbon source. Neither *T. roseum* nor *S. thermophilus* were able to gain energy in this manner, but *N. hollandicus* has been shown to tolerate and oxidise up to 75 mM nitrite [34].

WKT50.2 was able to utilise nitrate, ammonia and alanine as nitrogen sources, but not yeast extract. *T. roseum* and *S. thermophilus* were both able to use ammonia and nitrate, but they could also use yeast extract as a nitrogen source. None of the three species could assimilate nitrogen from nitrite or nitrogen gas.

WKT50.2 was strictly aerobic, unable to use nitrate, sulfate or sulfur as electron acceptors in an anaerobic atmosphere with sucrose as a carbon and energy source. *T. roseum* has previously been classified as an obligate aerobe [25], and *N. hollandicus* was unable to grow under the anaerobic conditions tested [34]. *S. thermophilus* has yet to be tested for anaerobic growth.

Other features

WKT50.2, *T. roseum* and *S. thermophilus* all showed similar levels of salinity tolerance; WKT50.2 and *S. thermophilus* could both only grow in media with less than 1.5 % NaCl, while *T. roseum* could grow in media with 1.5 %, but not 2 %, NaCl. This is consistent with the low salinity levels associated with the environments in which each species was isolated.

WKT50.2 and *T. roseum* both showed the same interesting reaction to antibiotics; both grew noticeably better when given either 3 or 30 µg/mL metronidazole or trimethoprim (Tables 3.6 and 4.2). *S. thermophilus* was inhibited by trimethoprim and 3 µg/mL metronidazole, but also showed a non-significant stimulation of growth (105 % of the growth of the positive control) when 30 µg/mL metronidazole was added (Table 5.2).

The mechanism of growth stimulation is currently unknown, but may be related to the upregulation of serine transhydroxymethylase in cells. For further discussion, see Section 6.1.

Species and class descriptions

WKT50.2 is clearly a distinct species from *T. roseum* and *S. thermophilus*. The 16S rRNA gene sequence of WKT50.2 is 91 % similar to that of *T. roseum* and only 88 % similar to that of *S. thermophilus* (Table 3.1). In addition, there are several other differences between the three species (Table 6.1), such as metabolic capabilities, G+C content, and Gram stain reaction.

WKT50.2 is unable to oxidise CO, although both *T. roseum* and *S. thermophilus* have this capability, and is also unable to hydrolyse Phytagel™, which has been observed with the other two species. However, WKT50.2 was able to utilise Avicel™ and CMC as energy sources, while *T. roseum* could not.

The G+C content of WKT50.2 is 58.4 mol%, compared with 64.0 mol% in *T. roseum* and 68.1 mol% in *S. thermophilus.* This correlates with high temperature bacteria often, although not always, having high G+C contents [91]. Finally, WKT50.2 stains Gram-negative, as does *T. roseum*, but *S. thermophilus* stains Gram-positive.

However, there are also several characteristics that are common to WKT50.2, *T. roseum*, *S. thermophilus* and the partially characterised *N. hollandicus* (Table 6.1). Commonalities include an apparent alkaliphilic phenotype (pH ranges 5.7 to 9.0), an ability to utilise ammonia and methane as energy sources, as well as cellulolytic and proteolytic metabolisms. These common features can be used to amend the class description for *Thermomicrobia*, which currently only states that strains are "Pleomorphic, non-motile, non-spore forming rods", with "atypical proteinaceous cell walls" [11, 25].

Possible IJSEM Class Descriptor

Cells are pleomorphic, non-motile, non-spore forming rods. Cells grow under strictly aerobic conditions. May be mesophilic or thermophilic. MK-8 is the predominant isoprenoid quinone. The major fatty acid is 12-Me-18:0, and lipids include ester-linked diols. Cells possess atypical proteinaceous cell walls containing ornithine. Lipopolysaccharide-containing outer membrane not present in studied representatives. Cells are facultative heterotrophs displaying the ability to utilise carbohydrates and protein-rich substrates.

Possible Genus Descriptor

Gram-negative, catalase negative, oxidase negative. Cells are non-motile, rod-shaped. Spores are not formed. Cells grow under aerobic conditions. Thermophilic, moderately alkaliphilic. Cells divide by binary fission. G+C content of the type species is 58.4 mol %. The major respiratory quinone is MK-8. The main fatty acids are 12-Me-18:0, 18:0, *i*17:0 and *i*15:0.

Possible Species Descriptor

Cells are non-motile, non-spore-forming, rods (approx. 0.9 - 1.1 µm in diameter and 2.1 - 2.8 µm in length). Gram-negative, oxidase and catalase negative, exhibits an oxidative, heterotrophic metabolism. Cell division by binary fission. No growth observed on monosaccharides. Sucrose, trehalose and raffinose support growth; lactose and cellobiose do not. Avicel™, carboxymethylcellulose, starch, and xylan are hydrolysed, but chitin, dextrin, gellan gum, galactomannan, glucomannan, pectin and xanthan are not. Cells also grow on Whatman™ filter paper, glycerol, sorbitol and 0.05 % (v/v) methanol or ethanol. Sodium acetate, formate, fumarate, lactate, pyruvate and succinate support growth; sodium citrate, mannitol, 1-propanol, 2-propanol, and galacturonic acid do not. Cells grow readily on yeast extract, peptone, tryptone and casamino acids. Cells can oxidise methane, ammonia and nitrite for growth.

Growth inhibited by ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin and vancomycin; metronidazole and trimethoprim stimulate growth. Ammonium ion, nitrate ion or alanine can act as sole nitrogen sources. Optimal growth at 68 - 73 °C (range: 53 - 76 °C) and pH 6.8 – 7.0 (range: 5.9 – 8.2). NaCl tolerance up to 1 % (w/v). Growth on solid gellan-based medium produces pink opaque colonies with circular and entire edges. G+C content of strain WKT50.2^T is 58.4 mol% and the quinone system consists of MK-8 (100 %). The major fatty acids are 12-Me-18:0 (22.6 %), 18:0 (16.4 %), *i*17:0 (14.2 %) and *i*15:0 (14:0 %) with minor amounts of *i*16:0 (9.4 %), 16:0 (7.8 %) and *ai*17:0 (5.4 %).

The type strain, WKT50.2^T was isolated from geothermally heated soils at Waikite Springs, New Zealand.

7 Summary

The primary aim of this study was to conduct a physiological characterisation of novel *Thermomicrobia* strain WKT50.2, and make initial inferences about its ecology. The two most closely related species to WKT50.2, *Thermomicrobium roseum* and *Sphaerobacter thermophilus*, had not been fully characterised at the time of writing. The secondary aim of this study was therefore to complete the characterisation of the *T. roseum* and *S. thermophilus* species, to increase our understanding of the class *Thermomicrobia* and to amend the class description.

Strain WKT50.2 is an obligate aerobic heterotroph from the class *Thermomicrobia* in the phylum *Chloroflexi*. It was isolated from the hot springs at Waikite, New Zealand. This study showed that WKT50.2 had a dumbbell or small rod morphology, with cells between 2.1 to 2.8 µm long, and 0.9 to 1.1 µm wide. The G+C content was 58.4 mol%. WKT50.2 grew at temperatures between 53 °C and 76 °C and maximum growth was observed between 68 °C and 73 °C. The optimum growth pH was 6.8 – 7.0 with a range of pH 5.9 to 8.2. The fatty acid profile was comprised primarily of branched long chain fatty acids, with the principal fatty acids being 12-Me-18:0 (22.6%), 18:0 (16.4%), i17:0 (14.2%), i15:0 (14.0%) and i16:0 (9.4%). The initial lipid analysis showed the presence of two diol phospholipids with inositol and/or sugar groups attached. The only quinone found was MK-8.

WKT50.2 was able to oxidise methane, ammonia and nitrite to gain energy. This was an unexpected result as methanotrophy is a highly specialised phenotype restricted to the phyla *Proteobacteria* and *Verrucomicrobia.* Methanotrophs also tend to be metabolic specialists with no capability to oxidise substrates other than C1 compounds. In contrast, WKT50.2 was able to oxidise ammonia and nitrite, along with a range of saccharides and protein rich substrates. Specifically, substrate utilisation tests showed WKT50.2 was incapable of using monosaccharides, but good growth was exhibited on polysaccharides, including crystalline cellulose (Avicel™), carboxymethyl cellulose, starch and xylan. Cellulolytic activity had not previously been described for this class. In addition, WKT50.2 was able to use a range of complex protein derivatives, organic acids and alcohols as energy sources. WKT50.2 was sensitive to most of the antibiotics tested in this study, but metronidazole and trimethoprim both stimulated growth in a dosage-dependent manner. WKT50.2 is clearly a distinct genus from *T. roseum* and *S. thermophilus*, based on 16S rRNA gene sequence similarities (91 % and 88 % respectively), G+C content and metabolic capabilities.

This study gives some indications to the possible ecology of WKT50.2. It is a thermophilic, obligate aerobe, which survives in neutral to moderately alkaline environments. All closely related 16S rRNA gene sequences from clone libraries have been detected in hot springs and/or a thermophilic bioreactor environments suggesting that the thermophilic trait is conserved in closely related microorganisms. Importantly, WKT50.2 displays a mixotrophic phenotype with the capability of utilising a wide variety of substrates, including sugars, protein as well as methane, nitrite and ammonia. This broad metabolic capability may confer WKT50.2 the ability to survive in low numbers under non-ideal environments. At Waikite, for example, which is low in both methane and ammonia, WKT50.2 may be using cellulosic substrates as energy sources, or protein derivatives as these are likely to be present in the soil as breakdown products from plants and other microorganisms.

T. roseum and *S. thermophilus* are both heterotrophic, moderately alkaliphilic, obligate aerobes. The temperature and pH optima and ranges for *T. roseum* and *S. thermophilus* were amended, along with expanding the known metabolic capabilities of both species. Of particular note was the discovery that both *T. roseum* and *S. thermophilus* were also able to oxidise both methane and ammonia for energy, and that both species possessed cellulolytic activity. All three species could grow on a range of polysaccharides and complex protein derivatives, although there were slight differences in substrate utilisation. For example, *T. roseum* grew on all monosaccharides tested, but *S. thermophilus* could only utilise two monosaccharides, fructose and cellobiose. In addition, *T. roseum* was able to use dextrin but not crystalline cellulose (Avicel™) or carboxymethyl cellulose, which was the opposite to what was observed for both WKT50.2 and *S. thermophilus*. The growth of *T. roseum* was stimulated by 3 or 30 µg/mL of metronidazole or trimethoprim, but *S. thermophilus* was inhibited by trimethoprim, and by 3 but not 30 µg/ml of metronidazole.

In summary, the class *Thermomicrobia* can be redefined as comprising obligately aerobic, facultative heterotrophs. Cells are non-motile, non-spore forming rods. This study has shown that the cells have proteinaceous cell walls containing ornithine, and no lipopolysaccharide. The major fatty acid is 12-Me-18:0, and lipids include ester-linked diols. The major isoprenoid quinone is MK-8. The ability to oxidise methane, ammonia and nitrite, along with cellulosic and protein-rich substrates was a common trait in all three species studied. However, more strains need to be isolated and characterised before these metabolic capabilities can be classed as a conserved feature across *Thermomicrobia*.

Several of the observations in this study merit further research. The discovery of methanotrophy in this class was unexpected and significant, and future work should include the identification of the enzymes involved in methanotrophy, and those involved in the oxidation of ammonia for energy. The reaction kinetics of methane and ammonia oxidation should be analysed to determine if this is consistent with other known methanotrophs. The molecular basis for methane oxidation could be investigated using trace metal availability, HPLC analysis of supernatants following growth, and transcriptomic experiments.

The mechanism for growth stimulation of WKT50.2 and *T. roseum* by antibiotics was also an interesting observation and warrants further analysis to determine the mode of action.

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9 Appendix A – *Chloroflexi* sequence similarity

Table 9.1 - Pairwise 16S rRNA gene sequence dissimilarity table of *Chloroflexi* isolated species

	Ca	At	Atl	Bc	La	Lt	Ls	Dl	To	Tf	Kr	Th	St	Tr	Hg	Ha	Ot	Cag	Cau
Ca	0																		
At	14	0																	
Atl	18	7	0																
Bc	18	11	11	0															
La	19	12	11	7	0														
Lt	19	12	12	8	8	0													
Ls	19	12	11	6	8	8	0												
Dl	19	21	21	20	21	22	21	0											
To	22	22	22	22	23	24	24	22	0										
Tf	21	22	22	22	23	24	24	22	4	0									
Kr	21	23	22	21	22	23	22	22	17	17	0								
Th	21	22	21	22	23	23	22	23	14	15	12	0							
St	20	22	22	22	23	23	22	23	20	20	20	20	0						
Tr	22	22	23	23	25	25	24	23	22	21	22	22	11	0					
Hg	24	25	25	24	25	26	24	24	24	25	23	23	20	23	0				
Ha	23	23	24	23	24	25	23	23	23	23	22	22	19	22	2	0			
Ot	23	24	24	23	24	24	24	24	23	23	23	22	20	21	20	18	0		
Cag	25	24	25	24	25	25	25	26	23	23	24	22	19	22	21	19	12	0	
Cau	24	24	25	25	25	25	25	26	24	24	25	22	20	23	21	20	12	6	0
Rc	23	24	25	24	25	25	25	24	21	22	23	22	20	22	20	19	15	16	17

16S rRNA gene sequences were compared using the ARB environment [5].

Abbreviations:

Ca: Caldilinea aerophila; At: Anaerolinea thermophila; Atl: Anaerolinea thermolimosa; Bc: Bellilinea caldifistulae; La: Longilinea arvoryzae; Lt: Leptolinea tardivitalis; Ls: Levilinea saccharolytica; Dl: Dehalogenimonas lykanthroporepellens; To: Thermogemmatispora onikobensis; Tf: Thermogemmatispora foliorum; Kr: Ktedonobacter racemifer; Th: Thermosporothrix hazakensis; St: Sphaerobacter thermophilus; Tr: Thermomicrobium roseum; Hg: Herpetosiphon geysericola; Ha: Herpetisphon aurantiacus; Ot: Oscillochloris trichoides; Cag: Chloroflexus aggregans; Cau: Chloroflexus aurantiacus; Rc: Roseiflexus castenholzii

10 Appendix B - Media components

Methanogens Trace Metal Solution

Modified from Wolin et. al. [130]

Nitrilotriacetic acid	1.500 g
$Na_2SeO_4.10H_2O$	0.440 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.200 g
$CoCl_2 \cdot 6H_2O$	0.100 g
$MnSO_4 \cdot 2H_2O$	0.100 g
$Na_2MoO_4 \cdot 2H_2O$	0.100 g
$Na_2WO_4 \cdot 2H_2O$	0.100 g
$ZnSO_4 \cdot 7H_2O$	0.100 g
$Al_2(SO_4)_3 \cdot 18H_2O$	0.055 g
$NiCl_2 \cdot 6H_2O$	0.025 g
H_3BO_3	0.010 g
$CuSO_4 \cdot 5H_2O$	0.010 g

To prepare the trace metal solution, dissolve the nitrilotriacetic acid in 800 ml of water and adjust the pH to 6.5 with KOH. Then dissolve the minerals in order, adjust the pH to 7.0, and bring the volume to 1 litre.

Methanotrophs Trace Metal Solution

$ZnSO_4 \cdot 7H_2O$	0.44 g
$CuSO_4 \cdot 5H_2O$	0.20 g
$MnCl.4H_2O$	0.19 g
$Na_2MoO_4 \cdot 2H_2O$	0.06 g
H_3BO_3	0.10 g
$CoCl_2 \cdot 6H_2O$	0.08 g

ddH_2O to 1 L

FeEDTA solution

$FeSO_4 \cdot 7H_2O$	1.54 g
Na_2EDTA	2.06 g

ddH_2O to 1 L

Castenholz Salts Solution [70]

$NaNO_3$	0.689 g
$Na_2HPO_4.12H_2O$	0.111 g
KNO_3	0.103 g
Nitrilotriacetic acid	0.100 g
$MgSO_4.7H_2O$	0.100 g
$CaCl_2.2H_2O$	0.060 g
NaCl	0.008 g
$FeCl_2$ solution	1 mL
Nitsch Element Solution	1 mL

Nitsch Element Solution

98 % H_2SO_4	50 µL
$MnSO_4$	220.0 mg
$ZnSo_4.7H_2O$	50.0 mg
H_3BO_3	50.0 mg
$CuSO_4.5H_2O$	16.0 mg
$CoCl_2.6H_2O$	4.6 mg
$Na_2MoO_4.2H_2O$	3.0 mg
ddH_2O to 100 mL	

$FeCl_2$ solution

$FeCl_2.4H_2O$	0.44 g
ddH_2O to 1 L	

DSM592 media for *Thermomicrobium roseum*

Yeast extract	1.000 g
Tryptone	1.000 g
$(NH_4)_2SO_4$	1.300 g
$MgSO_4.7H_2O$	0.247 g
KH_2PO_4	0.280 g
$CaCl_2.2H_2O$	0.074 g
FeCl2.4H2O	0.020 g
Salt solution	1.000 ml
ddH2O to 1L	
Adjust pH to 8.5	

Salt solution for DSM 592

MnCl2.4H2O	1.80 g
$Na_2B_4O_7.10H_2O$	4.40 g
$ZnSO_4.7H_2O$	0.22 g
$CuSO_4.5H_2O$	0.08 g
Na_2MoO_4.2H2O	0.03 g
$VoCl_2.2H_2O$	0.04 g
ddH_2O to 100 mL	

Adjust pH to 2.0 with H_2SO_4

DSM 467 for *Sphaerobacter thermophilus*

Nutrient broth	12.0 g
Yeast extract	2.5 g
Casamino acids	2.5 g
Glucose	1.0 g
NaCl	5.0 g
Tap water to 1 L	

Adjust pH to 8.5.

11 Appendix C - Chemical suppliers

All chemicals used were of Analar or Technical grade, unless otherwise stated.

Table 11.1 - List of chemicals used

Chemical	**Manufacturer**	**Product Code**
Acetic acid	BDH Chemicals	10001
Agar, Bacto™	BD Chemicals	214010
Agarose, SeaKem LE	Lonza	50004
L-Alanine	BDH Chemicals	370065B
Aluminium sulfate, $Al_2(SO_4)_3.18H_2O$	Ajax Fine Chemicals	24
Ammonium chloride, NH_4Cl	Ajax Fine Chemicals	32
Ammonium sulfate, $(NH_4)_2SO_4$	APS Chemicals	56
Ampicillin	Sigma-Aldrich	A9518
DL-Arabinose	Sigma-Aldrich	A9524
Ascorbic acid	Ajax Fine Chemicals	A79
Avicel® PH-101, Ph Eur grade	Fluka	11365
Boric acid, H_3BO_3	Ajax Fine Chemicals	A101
B-vitamins, B Complex Forte	Clinicians	N/A
Calcium chloride, $CaCl_2.2H_2O$	APS Chemicals	A127
Casamino acids, Bacto™	BD Chemicals	223120
D-Cellobiose	Sigma-Aldrich	C7252
Chitin, from crab shells	Sigma-Aldrich	C7170
Chloramphenicol	Sigma-Aldrich	C0378
Cobalt (II) chloride, $CoCl_2.6H_2O$	APS Chemicals	A986
Copper (II) sulfate, $CuSO_4.5H_2O$	APS Chemicals	171
L-Cysteine	Sigma-Aldrich	W326305
Dextrin, from corn, type I	Sigma-Aldrich	D2006
Ethanol, 200 proof, molecular biology grade	Sigma-Aldrich	E7023
Ferrous ammonium sulfate, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	BDH Chemicals	100223T
Filter paper, Whatman™ No. 1	Whatman	1001
D-Fructose	Sigma-Aldrich	F0127
L-Fucose	Sigma-Aldrich	F2252
Galactomannan (Locust bean gum), from *Ceratonia siliqua* seeds	Sigma-Aldrich	G0753
D-Galactose	Sigma-Aldrich	G0750
D-Galacturonic acid	Fluka	48280
Glucomannan	Vita Cost	NSI 3001903
D-Glucose	Ajax Fine Chemicals	A783
D-Glucuronic acid	Sigma-Aldrich	G5269
L-Glutamic acid	Sigma-Aldrich	G1251
Glycerol	Ajax Fine Chemicals	1003203
Glycine	Fisher	G/0800/60
Hydrogen peroxide, H_2O_2	Ajax Fine Chemicals	260
Iron (II) chloride, $FeCl_2.4H_2O$	Merck	1.03861
Iron (II) sulfate, $FeSO_4.7H_2O$	Ajax Fine Chemicals	226
Kanamycin	Sigma-Aldrich	K1377

Lactose	Difco	215620
L-Leucine	BDH Chemicals	371213W
Lignin	Pure Power Technologies	N/A
L-Lysine	Fluka	62930
Magnesium chloride, $MgCl_2.6H_2O$	BioLab	BSPML950
Magnesium sulfate, MgSO4.7H20	BDH Chemicals	10151
Manganese chloride, $MnCl_2.4H_2O$	Ajax Fine Chemicals	A307
Manganous sulfate, $MnSO_4.4H_2O$	BDH Chemicals	1990690
D-Maltose	Sigma-Aldrich	M5895
Mannitol	BDH Chemicals	10330
D-Mannose	Fluka	63582
Methanol, HPLC grade	Fisher	A452-4
L-Methionine	BDH Chemicals	371315E
Methylamine	Fluka	65580
Metronidazole	Sigma-Aldrich	M1547
D-*N*-acetylglucosamine (NAG)	Sigma-Aldrich	A1628-9
Neomycin	Sigma-Aldrich	N6386
Nickel chloride, $NiCl_2{\cdot}6H_2O$	Ajax Fine Chemicals	A829
Nitrilotriacetic acid	Sigma-Aldrich	N9877
Nutrient Broth	Sigma-Aldrich	N7519
Pectin, type 115	CP Kelco	N/A
Peptone, Bacto™	BD Chemicals	211677
Phytagel™	Sigma-Aldrich	P8169
Polymyxin B	Sigma-Aldrich	P0972
Potassium nitrate, KNO_3	Ajax Fine Chemicals	A412
Potassium nitrite, KNO_2	Ajax Fine Chemicals	1073
di-Potassium hydrogen orthophosphate, K_2HPO_4	Ajax Fine Chemicals	A2221
1-propanol, HPLC grade	Merck	1-01024
2-propanol, molecular biology grade	Sigma-Aldrich	I9516
R2A agar, Difco™	BD Chemicals	218263
D-Raffinose	Fluka	83400
Resazurin	Sigma-Aldrich	R7017
L-Rhamnose	Sigma-Aldrich	R3875
D-Ribose	Sigma-Aldrich	R7500
DL-Serine	Sigma-Aldrich	S5386
Sodium acetate	Sigma-Aldrich	S52889
Sodium alginate	Sigma-Aldrich	180947
Sodium benzoate	Ajax Fine Chemicals	A459
Sodium carboxymethyl cellulose (CMC)	Hercules	N/A
Sodium chloride, NaCl	Ajax Fine Chemicals	465
Sodium EDTA, Na_2EDTA	Sigma-Aldrich	E5134
Sodium formate	Merck	1.06443
Sodium fumerate	Sigma-Aldrich	F1506
Sodium hydroxide, NaOH	Sigma-Aldrich	S8045
Sodium lactate	Ajax Fine Chemicals	A738
Sodium molybdate, $Na_2MoO_{4}.2H_2O$	BDH	1.06537
Sodium nitrate, $NaNO_3$	Merck	A397737
Sodium phosphate dibasic, $Na_2HPO_4.12H_2O$	Fluka	71663
Sodium pyruvate	Sigma-Aldrich	P2256

Sodium selenate, $Na_2SeO_4.10H_2O$	BDH Chemicals	10262
Sodium succinate	Fluka	14160
Sodium sulfate, Na_2SO_4	BDH	10264
Sodium sulfide, $Na_2S.9H_2O$	Ajax Fine Chemicals	A508
Sodium tungstate, $Na_2WO_4.2H_2O$	BDH Chemicals	1830580
D-Sorbitol	Sigma-Aldrich	S1876
Starch, soluble	Sigma-Aldrich	S9765
Streptomycin	Sigma-Aldrich	S6501
Sucrose	Fluka	84097
Sulfur	APS Chemicals	A1686
Sulfuric acid, H_2SO_4	Merck	1.00731
Todd Hewitt Broth, Difco™	BD Chemicals	249240
D-Trehalose	Sigma-Aldrich	T0167
Trimethoprim	Sigma-Aldrich	T7883
Trimethylamine	Fluka	92262
Tris(hydroxymethyl)aminomethane [Trizma™]	Sigma-Aldrich	T1503
Trisodium citrate	Ajax Fine Chemicals	A467
Tryptone, Bacto™	BD Chemicals	211705
L-Tryptophan	Sigma-Aldrich	T0254
Urea, molecular biology grade	BDH Chemicals	443876Y
L-Valine	BDH Chemicals	371605L
Vanadyl chloride, $VoCl_2.2H_2O$	BDH Chemicals	561030
Vancomycin	Sigma-Aldrich	V2002
Whatman		
Xanthan®CC	CP Kelco	N/A
Xylan, from beechwood, >90 % xylan residue	Sigma-Aldrich	X4252
D-Xylose	Sigma-Aldrich	X1500
Yeast extract, BBL™	BD Chemicals	211929
Zinc sulfate, $ZnSo_4.7H_2O$	APS Chemicals	583

12 Appendix D – ESI-MS and MSMS analysis of deacylated WKT50.2 lipids

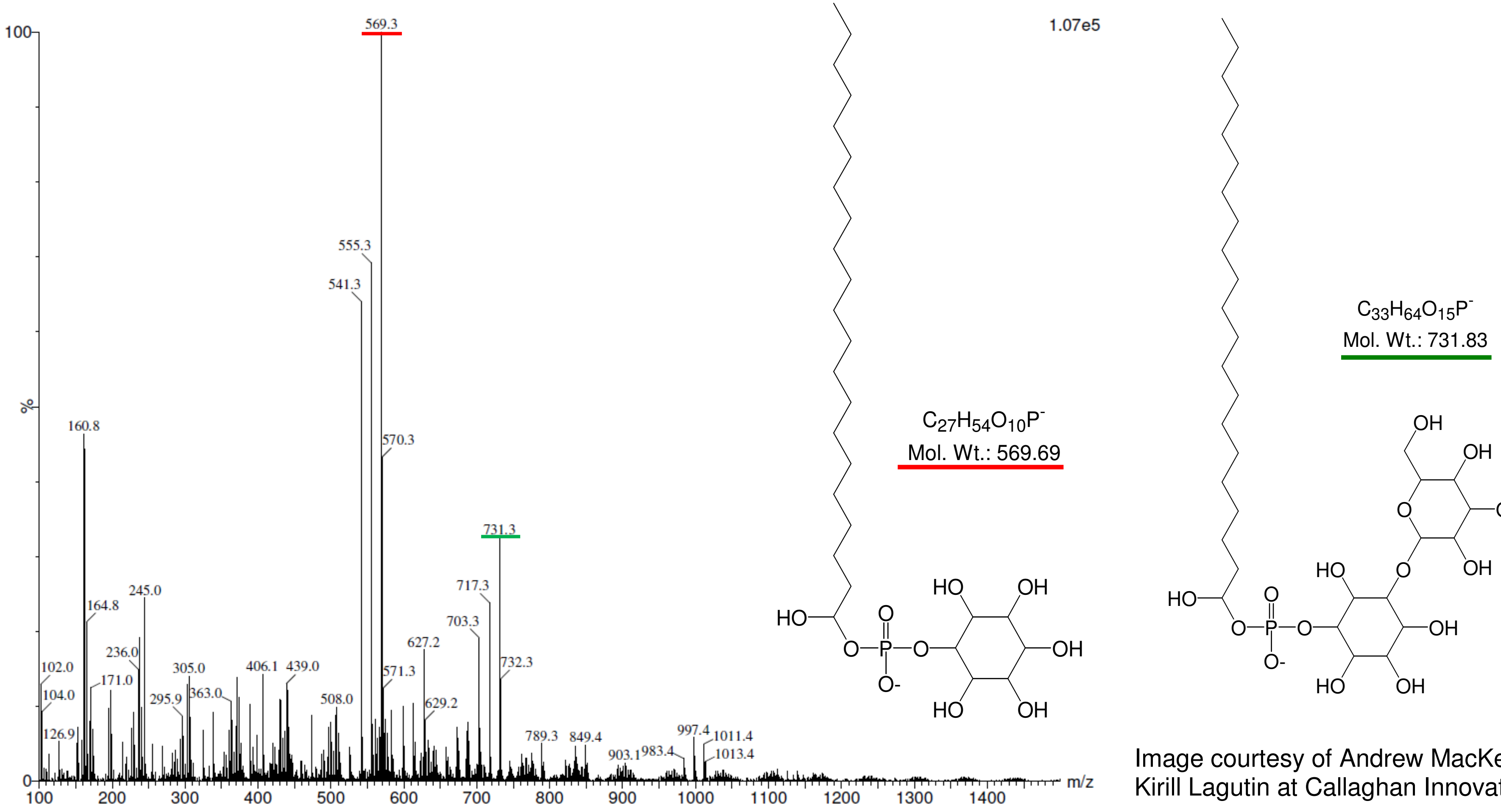

Image courtesy of Andrew MacKenzie and Kirill Lagutin at Callaghan Innovation.

13 Appendix E – PCR amplification of *coxL* fragments

PCR was carried out in 50 µL reaction volumes in 200 µL tubes containing 100 µM dNTPs, 0.5 µM primers and 1U Intron i-Taq™. The final concentration of $MgCl_2$ was 1.5 mM. DNA extracts from cultures were used at final concentrations of 10 – 50 ng.

PCR amplifications were performed using a CGI-96 thermal cycler (Corbett Research, Sydney, Australia) with PalmCycler™ software, v2.1.10.

Table 13.1 - Amplification conditions for *coxL* PCR

°C	Conditions	Time	Number of cycles
94	Initial denaturation	3 minutes	1
94 **62↓** 72	1 °C decrease in annealing temperature every 2 cycles	45 seconds 60 seconds 90 seconds	10
94 58 72	Annealing temperature remains constant	45 seconds 60 seconds 90 seconds	30
72	Final elongation	20 minutes	1

Table 13.2 - Primer sequences for *coxL* PCR

Name	Sequence 5' – 3'
OMPf	GGC GGC TTY GGS AAS AAG GT
BMSf	GGC GGC TTY GGS TCS AAG AT
O/Br	YTC GAY GAT CAT CGG RTT GA
cox F1	TAC GTC GAC GAC GTC AAA CT
cox F2	GTC GAG TAC GAG CCG CTG
cox R1	CAR GGG CAG GGA CAC GAG AC
cox R2	TTY GCS GCC TAC ACG AAT TTC CCG C
Thermo cox F	TGC CGG TCT ACC CCG GCT AC
Thermo cox R	CTG TCG TAC TCC CAG CCG GT

OMPf, BMSf and O/Br were previously published [76]; other primers were designed as described in Section 2.15.

14 Appendix F - PCR amplification of *pmoA* fragments

PCR was carried out in 50 µL reaction volumes in 200 µL tubes containing 100 µM dNTPs, 0.5 µM primers and 1U Intron i-Taq™. The final concentration of $MgCl_2$ was 1.5 mM. DNA extracts from cultures were used at final concentrations of 10 – 50 ng.

PCR amplifications were performed using a CGI-96 thermal cycler (Corbett Research, Sydney, Australia) with PalmCycler™ software, v2.1.10.

Table 14.1 - Amplification conditions for *pmoA* PCR

°C	Conditions	Time	Number of cycles
94	Initial denaturation	5 minutes	1
94 55 72		1 minute 1 minute 1 minute	30
72	Final elongation	5 minutes	1

Table 14.2 - Primer sequences for *pmoA* PCR

Name	Sequence 5' – 3'
mb661	CCG GMG CAA CGT CYT TAC C
A189f	GGN GAC TGG GAC TTC TGG

15 Appendix G – Chemical analysis of Waikite Springs

Samples of water from the stream immediately below the soil sample site were taken in November 2009, and analysed at the New Zealand Water and Gas Laboratory (GNS Science, Wairakei).

Table 15.1 - Chemical analysis of water at Waikite Springs

	Average of duplicate analyses	**Units of measurement**
pH	8.18	
Conductivity	1010	µS/cm
Bicarbonate (total)	314.74	mg/L
Ammonia (total as NH_3)	< 0.01	mg/L
Arsenic (As)	0.34	mg/L
Boron (B)	1.38	mg/L
Calcium (Ca)	6.83	mg/L
Chloride (Cl)	142.11	mg/L
Magnesium (Mg)	0.17	mg/L
Phosphate (PO_4)	< 0.05	mg/L
Potassium (K)	8.36	mg/L
Sodium (Na)	212.90	mg/L
Sulfate (S)	33.83	mg/L
Sulfide (total as H_2S)	< 0.01	mg/L
Carbon dioxide	84.9	% of total gas
Hydrogen sulfide	1.6	% of total gas
Hydrogen	0.4	% of total gas
Methane	0.1	% of total gas
Nitrogen	10.0	% of total gas
Oxygen	2.4	% of total gas

16 Appendix H – Glycosyl hydrolases from *Thermomicrobia*

Glycosyl hydrolases were identified from the genomes of *T. roseum* (http://www.ncbi.nlm.nih.gov/nuccore/NC_011959.1) and *S. themophilus* (http://www.ncbi.nlm.nih.gov/nuccore/NC_013523.1 and NC_013524.1, accessed using JGI IMG/ER [131].

Known activities for enzymes within these families were identified using the Carbohydrate-Active EnZymes database (CAZy, [132]). Selected activities are shown in Tables E.1 and E.2.

Table 16.1 - *T. roseum* glycosyl hydrolases

Family	**Selected known activities from CAZy**
15	glucoamylase; glucodextranase; α, α-trehalase
20 *	β-hexosaminidase; β-1,6-N-acetylglucosaminidase;
57 *	a-amylase; 4-α-glucanotransferase; a-galactosidase; amylopullulanase

Table 16.2 - *S. thermophilus* glycosyl hydrolases

Family	**Selected known activities from CAZy**
13 *	α-amylase; pullulanase; trehalose-6-phosphate hydrolase; α-glucosidase; 4-α-glucanotransferase; sucrose phosphorylase; maltotetraose-forming α-amylase; oligo-α-glucosidase; glucodextranase
15	glucoamylase; glucodextranase; α, α-trehalase
18 *	chitinase; endo-β-N-acetylglucosaminidase
20	β-hexosaminidase; β-1,6-N-acetylglucosaminidase;
57 *	a-amylase; 4-α-glucanotransferase; a-galactosidase; amylopullulanase
76	α-1,6-mannanase

* not a complete list