

2017

Taxonomy, physiology and biochemistry of the sulfur Bacteria

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<http://hdl.handle.net/10026.1/8612>

<http://dx.doi.org/10.24382/619>

University of Plymouth

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Taxonomy, physiology and biochemistry of the sulfur *Bacteria*

By

Lee Philip Hutt

A thesis submitted to Plymouth University in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences, Faculty of Science and Technology

July 2016

Thesis Abstract

Inorganic sulfur-oxidising *Bacteria* are present throughout the *Proteobacteria* and inhabit all environments of Earth. Despite these facts they are still poorly understood in terms of taxonomy, physiology, biochemistry and genetics. Using phylogenetic and chemotaxonomic analysis two species that were erroneously classified as *Thiobacillus trautweinii* spp. in 1921 and 1934 are in fact novel chemolithoheterotrophic species for which the names *Pseudomonas trautweiniana* sp. nov. and *Achromobacter starkeyanus* sp. nov. are proposed, respectively. These species were found to oxidise thiosulfate in a “fortuitous” manor when grown in continuous culture and increases in maximum theoretical growth yield (Y_{MAX}) and maximum specific growth rate (μ_{MAX}) were observed. Cytochrome *c* linked thiosulfate-dependent ATP production was confirmed in both species, confirming “true” chemolithoheterotrophy. Evidence is presented that the ATP concentration governs the benefits of chemolithoheterotrophy. There were significant changes in enzyme activities, including enzymes of the TCA cycle that might be affecting amino acid synthesis. This is strong evidence that chemolithoheterotrophy gives a strong physiological boost and evolutionary advantage over strictly heterotrophic species. An autotrophic species that was historically placed in *Thiobacillus* was also shown to be a novel species for which the name *Thermithiobacillus parkerianus* sp. nov. is proposed. The enzyme profiles of *Thermithiobacillus parkerianus* differed significantly between different inorganic sulfur growth substrates and was the first time all TCA cycle enzymes were assayed in a member of the *Acidithiobacillia*. The properties of thiosulfate dehydrogenase varied significantly between *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM both in terms of optimal parameters and the effect of inhibitors. This evidence adds to the increasing body of work indicating there to be at least two thiosulfate dehydrogenases present in the *Bacteria*.

List of abbreviations

| | |
|--------------------|--|
| α | Bunsen coefficient |
| ϵ | Molar extinction coefficient |
| λ | Wavelength |
| μ | Specific growth rate |
| μ_{MAX} | Maximum specific growth rate |
| A_x | Absorbance at x nm |
| ADP | Adenosine 5'-Diphosphate |
| AMP | Adenosine 5'-Monophosphate |
| ATP | Adenosine 5'-Triphosphate |
| BLAST | Basic local alignment search tool |
| BSA | Bovine serum albumin |
| CFE | Cell-free Extract |
| Cyt | Cytochrome |
| D | Dilution rate |
| DCPIP | 2,6-Dichlorophenolindophenol |
| DNA | Deoxyribonucleic acid |
| e^- | Electron |
| EC number | Enzyme Commission number |
| EBS | E-Basal Salts |
| EDTA | Ethylenediamine tetraacetic acid |
| ETC | Electron transport chain |
| g | Earth's gravitational acceleration at sea level |
| h | hours |
| K | Kelvin |
| m | Maintenance energy coefficient |
| m_s | Maintenance energy coefficient with respect to substrate |

| | |
|-----------------------|---|
| M | Molar |
| Mol | Mole |
| Mol% | Molar percentage |
| NAD ⁺ | Nicotinamide adenine dinucleotide (oxidised form) |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADP ⁺ | Nicotinamide adenine dinucleotide phosphate (oxidised form) |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| <i>N.D.</i> | Not detected |
| NEM | <i>N</i> -ethylmaleimide |
| <i>N.G.</i> | No growth |
| <i>OD_x</i> | Optical density at <i>x</i> nm |
| PBS | Phosphate Buffered Saline |
| PCA | Perchloric acid |
| pCMB | <i>p</i> -chloromercuribenzoic acid |
| PCR | Polymerase chain reaction |
| PGA | 3-phosphoglycerate |
| PSO | <i>Paracoccus</i> sulfur oxidation |
| SEM | Standard Error of the Mean |
| <i>q</i> | Specific rate of substrate uptake |
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| S ₄ I | Tetrasulfur intermediate |
| T | Type strain |
| TCA | Tricarboxylic acid |
| TEM | Transmission electron microscopy |
| TOMES | Thiosulfate-oxidising multi-enzyme system |
| UV | Ultraviolet |

| | |
|------------------|--------------------------|
| V | Rate of reaction |
| V_{MAX} | Maximum rate of reaction |
| v/v | Volume to volume |
| w/v | Weight to volume |
| x | Dry weight of biomass |
| Y | Yield |
| Y_{MAX} | Maximum yield |

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Acknowledgements

This research was funded by a Ph.D. studentship from the School of Biological Sciences, Plymouth University. Further support was provided by Royal Society Research Grant RG120444 awarded to Rich Boden.

I would like to thank Matt Emery who provided advice, support and technical guidance throughout the Ph.D.. Thanks also go to Andy Atfield, Nick Crocker, Will Vevers and Sarah Jamieson for there constant support. Thanks go to Dr. Carly Benefer for help with producing this Thesis. I would like to thank Dr. John Moody for many years of direction and motivation. In particular I would like to thank Dr. Rich Boden for his outstanding supervision, patience and inspiration.

Special thanks go to my family, my dad for being the best dad anyone could wish for, my beautiful children for bring me so much happiness and especially to the love of life; my Hayley.

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

This study was financed with the aid of a Ph.D. studentship from the School of Biological Sciences.

Relevant scientific seminars and conferences were regularly attended at which work was presented

Presentations and conferences attended

Poster presentation: EMBO Workshop: Microbial sulfur metabolism, Helsingør, Denmark, April 2015 “Physiological and biochemical examination of the moderately thermophilic chemolithoautotroph *Thermithiobacillus* sp. ParkerM”.

FEMS 6th Congress of European Microbiologists, Maastricht, The Netherlands, June 2015.

Platform presentation: “New insights into physiology and biochemistry of inorganic sulfur-oxidising *Bacteria*” Ecology, behaviour and Evolution (EBE) and Environment, Food and Biotechnology (ENFOB) launch workshop.

Word count of thesis

50,489

Signed

Date

04/07/2016

Chapter 1

Introduction

1.1 The biogeochemical sulfur cycle

Many elements essential to life undergo a global cycling process involving both biotic and abiotic processes. The sulfur cycle is an important example as sulfur itself is an essential component of proteins (*via* cysteine and methionine), co-factors and vitamins (Roy & Trudinger, 1970), making sulfur vital to all life on Earth. Sulfur is also an important component in compounds used for energy yielding metabolism in many different species of microorganisms. Many organisms rely on such sulfur compounds, organic and inorganic, for survival and growth and many other organisms metabolise such compounds in addition to other organic substrates. Microbial activity (both anabolic and catabolic) is a major driving force in the sulfur cycle but precisely to what extent remains largely a mystery.

The oxidation of reduced inorganic sulfur compounds is a key step in the sulfur cycle. To date, two major pathways have been identified in the *Bacteria*, the Kelly-Friedrich (Sox) pathway and the Kelly-Trudinger (S₄I) pathway. The biochemistry of the latter pathway is poorly understood as is the taxonomy of organisms that utilise it. Many species of sulfur oxidising *Bacteria* have been reassigned in recent years based on their 16S rRNA gene sequence highlighting the diversity of organisms that use these pathways; however, many species of Kelly-Trudinger *Bacteria* have yet to be tested for their correct taxonomic positions. The level of activity that Kelly-Trudinger organisms have in the environment is also a major question yet to be addressed as is the level of contribution they have on the biogeochemical sulfur cycle.

The sulfur cycle is driven by redox reactions of sulfur between its extensive range of oxidation states, -2 at its most reduced form and +6 at its most oxidised (Ghosh & Dam, 2009). It also consists of different states in which sulfur is transformed between organic and inorganic species (Figure 1.1). The primary abiotic source of sulfur into the sulfur cycle is from the weathering of igneous rock that contain varying levels of sulfur minerals (Hong *et al.*, 2000). Biological leaching of minerals from igneous rock also plays a major role in releasing sulfur into the environment. Pyrite (FeS_2) is a common sulfur containing mineral, from which sulfur oxyanions are released by weathering and leaching; however, many other sulfur containing minerals exist in nature (Jennings *et al.*, 2000). Gypsum (CaSO_4), chalcocite (Cu_2S) and barite (BaSO_4) are examples of an extensive group of minerals summarised in Table 1.1. Such minerals are washed into soils, streams, rivers and the oceans. Volcanic eruptions and hydrothermal vents are also significant contributors to the geochemical sulfur cycle in the form of thionates, sulfates and sulfides (Druschel *et al.*, 2003; Takano & Watanuki, 1990). Thiozonide (S_3^-) is a radical anion recently discovered to be the dominant sulfur species at the high pressures and temperatures ($> 250^\circ\text{C}$) of subduction zones in the Earth's crust. Once released by volcanic activity it is rapidly broken down to sulfide and sulfate (Chivers & Elder, 2013; Pokrovski & Dubrovinsky, 2011). When released into the environment sulfur is subject to many reactions, many of which are performed biochemically.

In soil or aqueous environments sulfur minerals can become oxidised by many different processes. This can result in disassociation of sulfur from its corresponding ion due to the natural chemistry of the environment or due to microbial activity. For example, *Acidithiobacillus ferrooxidans* (formerly “*Thiobacillus ferrooxidans*” Kelly & Wood, 2000a) is capable of oxidising pyrite to ferric iron (Fe^{3+}) and sulfate (SO_4^{2-}) (Gleisner *et al.*, 2005; Silverman, 1967), while *Leptospirillum ferrooxidans* has been shown to oxidise

chalcopyrite (CuFeS_2) in a similar fashion and in addition also leaches the copper ion (Watling, 2006). These reactions are utilised metabolically by many microorganisms in their own right but the products of their metabolism enable sulfur to become available to many other species, particularly species of the *Bacteria*.

A major source of organosulfur in the environment is from the decomposition of biological material. During this process biological molecules such as amino acids are released and used as energy sources by other organisms. This releases a significant amount of sulfide and organosulfur compounds such as dimethylsulfide (Roy & Trudinger, 1970). Although distinct from the release of sulfur in the form of minerals from weathered rock, the conversion of sulfur from its organic and inorganic forms takes place from chemical and biochemical activity (Boden *et al.*, 2010).

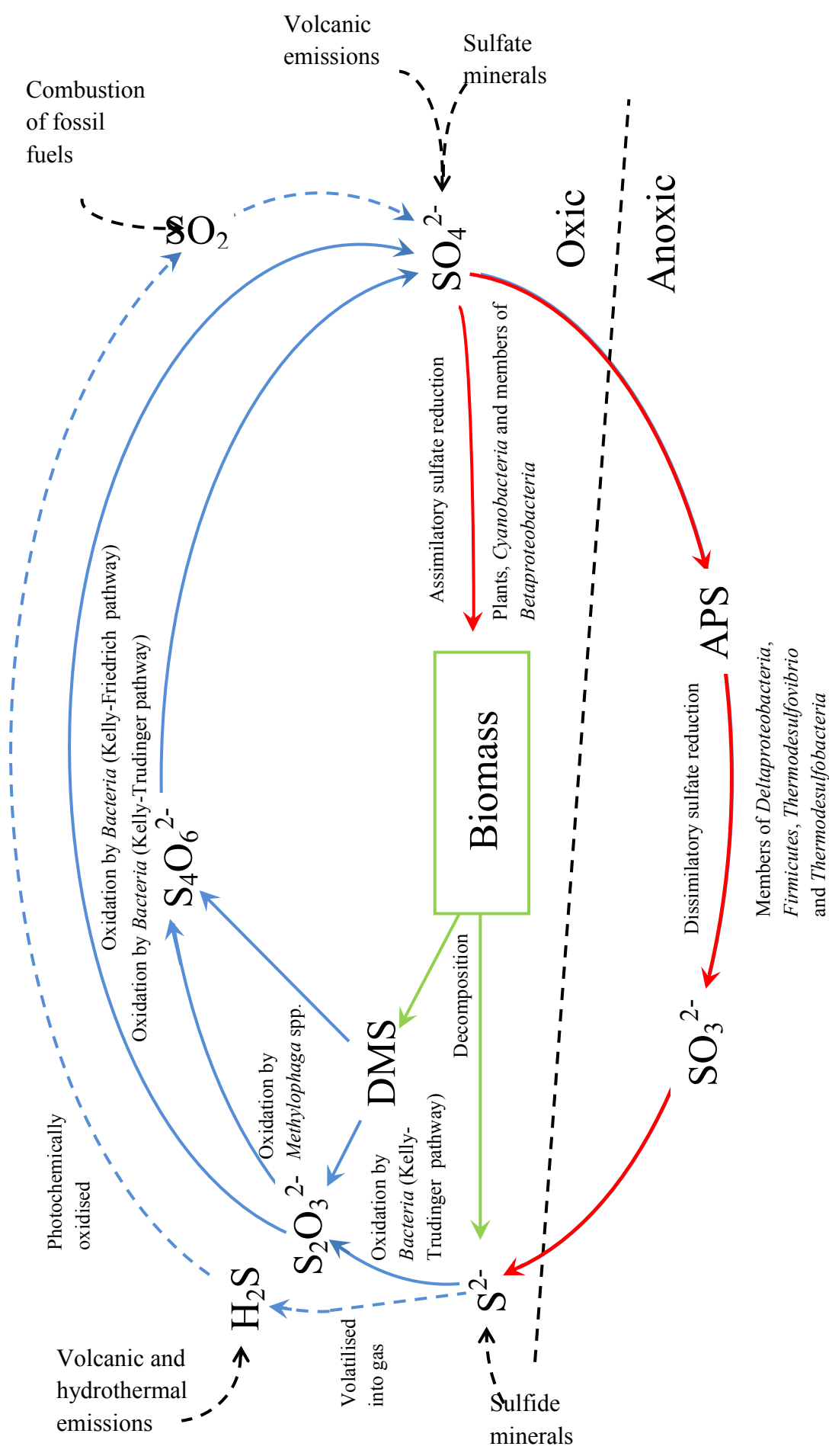


Figure 1.1. Simplified diagram of the biogeochemical sulfur cycle. Biological aspects of the sulfur cycle are indicated with solid lines and abiotic by dashed lines. The reduction of sulfur (red lines) is achieved by anaerobic *Bacteria* by dissimilatory sulfate reduction in environments such as marine sediments and by plants and bacteria in oxygenated environments by assimilatory sulfate reduction. The oxidation of sulfur (blue lines) happens mostly in aerobic environments either chemically or from the metabolism of *Bacteria* and *Archaea*. An exception to this is anaerobic metabolism by organisms like *Thiobacillus denitrificans* that utilise the S_4I pathway by using nitrate as a terminal electron acceptor. Image produced from information from Ghosh & Dam (2009) and Kelly (1980).

Table 1.1. Examples of common sulfur containing minerals released into the environment and contribute to sulfur entering the sulfur cycle. Modified from Jennings *et al.* (2000).

| Mineral name | Formula | Comments |
|---------------------|--|--|
| Barite | BaSO ₄ | |
| Anhydrite | CaSO ₄ | |
| Gypsum | CaSO ₄ .2H ₂ O | |
| Covellite | CuS | |
| Chalcocite | Cu ₂ S | Occurs from leaching of large copper deposits |
| Chalcopyrite | CuFeS ₂ | |
| Pyrite | FeS ₂ | The most abundant sulfur mineral |
| Arsenopyrite | FeAsS | |
| Jarosite | KFe ₃ (SO ₄) ₂ (OH) ₆ | Can be produced from pyrite oxidation in mining environments |
| Vaesite | NiS ₂ | |
| Galena | PbS | |
| Anglesite | PbSO ₄ | |
| Cinnabar | HgS | |

The biochemical aspect of the sulfur cycle involves the constant conversion of sulfur from its most reduced form as sulfide (with an oxidation state of -2) to its most oxidised form as sulfate (with an oxidation state of +6) before its subsequent reduction by varying amounts. The reduction of sulfate is a common form of metabolism that takes place in anoxic environments and replaces the need for oxygen. Sulfite (SO_3^{2-}) and sulfate are washed into the oceans mainly from river and coastal runoff. This maintains the oceanic sulfate concentration at approximately 28 mM (Canfield & Farquhar, 2009), of which a significant amount ends up settling into the anoxic benthic sediments of the continental shelf. These sulfur species are utilised by sulfate-reducing *Bacteria* as a terminal electron acceptor in anaerobic respiration *via* dissimilatory sulfate reduction. Sulfate itself is activated first *via* the action of ATP sulfurylase (EC 2.7.7.4) by reacting it with ATP to form adenosine 5'-phosphosulfate (APS). APS is reduced by APS reductase (EC 1.8.99.2) to sulfite and AMP (Kelly, 1980; Schiffer *et al.*, 2006). It is sulfite that is used as an electron acceptor by the action of dissimilatory sulfite reductase (EC 1.8.99.3) (Klein *et al.*, 2001). More than 200 species of sulfate-reducing *Bacteria* are currently known and form a group that span the class *Deltaproteobacter* but also a number of Gram positive genera of the *Firmicutes* (Barton & Fauque, 2009). Possibly the best studied are the *Desulfovibrio* spp. that can grow on a wide selection of different substrates heterotrophically. Such growth can utilise organic acids such as acetate, ethanol and lactate, and even sugars like fructose. Alternatively, *Desulfovibrio* spp. can grow autotrophically on molecular hydrogen (Brandis & Thauer, 1981; Ollivier *et al.*, 1988; Widdel & Pfennig, 1981). The end product of this form of metabolism is sulfur in its most reduced form, sulfide. Sulfate reducing *Bacteria* have been shown to be extremely sensitive to oxygen with cell viability of many genera being severely compromised after being exposed to oxygen (Cypionka *et al.*, 1985). This appears to be due to the

inactivation of dissimilatory sulfite reductase by molecular oxygen, although other enzymes involved in *Desulfovibrio* metabolism (NAD-dependent alcohol dehydrogenase and aldehyde dehydrogenase) are also oxygen-labile (Hensgens *et al.*, 1993; Zellner & Jargon, 1997). For these reasons sulfate reducing *Bacteria* are restricted to strictly anoxic environments.

Another form of sulfur metabolism commonly found in anoxic sediments is a process called sulfur disproportionation (Bak & Cypionka, 1987). In general terms disproportionation is the splitting of one compound in an intermediate state of oxidation into two compounds, one at lower and one at higher oxidation states. In the case of inorganic sulfur, it was first discovered that thiosulfate (+2) was split in to sulfide (S^{2-}) (-2) and SO_4^{2-} (+6) stoichiometry (Equation 1.1). The sulfide produced is oxidised as an energy source and the SO_4^{2-} is reduced as a terminal electron acceptor.



It was later discovered that elementary sulfur could also become split (Equation 1.2) to yield an energy source in the form of sulfide and an electron acceptor in the form of sulfate (Finster *et al.*, 1998; Kramer & Cypionka, 1989)



This reaction is a form of inorganic fermentation as the overall substrate oxidation state is not changed but the two sulfur atoms that are at different oxidation states are separated. Additionally, it also adds to the amount of sulfide being produced in such environments. It is estimated that the Gibb's free energy change of Equation 1.1 is $-21.9 \text{ kJ mol}^{-1}$ (Thauer *et al.*, 1977), which is relatively low but it does also produce additional amounts of sulfate to act as electron acceptors. Known organisms capable of sulfur disproportionation belong to the *Deltaproteobacteria* and include the genera *Desulfovibrio*, *Desulfocapsa* and *Desulfotomaculum* (Bak & Cypionka, 1987; Bak & Pfennig, 1987; Jackson & McInerney, 2000) and more recent studies have indicated that such activity in sediments is a common microbial process (Hardisty *et al.*, 2013).

Sulfide produced in sediments can have a number of different fates in the environment. It has low solubility in water, and moves through the water column and enters the atmosphere as hydrogen sulfide gas (H_2S). In the lower atmosphere hydrogen sulfide gas becomes photochemically oxidised to sulfate (Charlson *et al.*, 1987). Sulfate is in turn removed from the atmosphere *via* precipitation into marine and terrestrial environments. This abiotic process eventually leads to sulfate being returned to marine sediments and made available for use in dissimilatory sulfate reduction. In contrast to the reduction of sulfur in anoxic environments, sulfide is a common energy source for chemolithotrophic metabolism in *Bacteria* that oxidise sulfide to sulfate. Many other intermediate inorganic sulfur compounds can be utilised in chemolithotrophic metabolism. These include thiosulfate ($\text{S}_2\text{O}_3^{2-}$), trithionate ($\text{S}_3\text{O}_6^{2-}$), tetrathionate ($\text{S}_4\text{O}_6^{2-}$) and many other polythionates ($\text{S}_n\text{O}_6^{2-}$) where n is ≥ 3 (Schmidt, 1957). Other compounds include dithionate ($\text{S}_2\text{O}_6^{2-}$) and thiocyanate (SCN^-) (Roy & Trudinger, 1970). This process is aerobic in most *Bacteria* capable of lithotrophic growth with the sulfur becoming fully oxidised to sulfate and is usually coupled to autotrophic growth. However a number of

methylotrophic organisms have also shown to oxidise reduced or methylated sulfur compounds as auxiliary sources (De Zwart *et al.*, 1997; Friedrich *et al.*, 2001a; Boden *et al.*, 2010; Boden & Murrell, 2011).

Sulfur-oxidising *Bacteria* are ubiquitous in nature and are found in terrestrial, fresh and marine waters (Le Faou *et al.*, 1990). Initially the first species of sulfur-oxidising *Bacteria* were discovered by Nathanson (1902) who studied two organisms that are now believed to be *Thiobacillus thioparus* and *Halothiobacillus neapolitanus* found the organism grew autotrophically by oxidising thiosulfate. However it was later work by Beijerinck (1904) that isolated the type species of *Thiobacillus* and validly published the name *T. thioparus*. It was shown that *T. thioparus* grew on both thiosulfate and tetrathionate by producing sulfate and elemental sulfur. Over the following decades many other species were assigned to the genus *Thiobacillus* and by 1998 it included at least 32 different species depending on morphology and the physiological characteristic of being able to oxidise inorganic sulfur (Boden *et al.*, 2012). However, with more modern and accurate techniques such as 16S rRNA gene sequencing many species have been transferred into different, often new, genera spanning the *Alpha*, *Beta* and *Gammaproteobacteria* (Boden *et al.*, 2012; Kelly & Wood, 2000a). A number of species were recently reassigned to a new *Class* within the phylum *Proteobacteria*, the *Acidithiobacillia* (Williams & Kelly, 2013; Hudson *et al.* 2014; Kelly & Wood, 2014). At present this *Class* incorporates only the genera *Acidithiobacillus* and *Thermithiobacillus*. In recent years many other species of sulfur oxidising *Bacteria* have been reclassified from the genus *Thiobacillus*; these include members of *Paracoccus* (Kelly & Tuovinen, 1975) and *Starkeya* (Kelly, 2000) that are facultatively heterotrophic as well as being able to grow autotrophically by oxidising inorganic sulfur compounds.

The oxidation of inorganic sulfur compounds is an aerobic process in most species. There are a few notable exceptions however; for example *Thiobacillus denitrificans* can grow on inorganic sulfur compounds anaerobically using oxidised nitrogen such as nitrate and nitrite as a terminal electron acceptor (Kelly & Wood, 2000b). *Paracoccus denitrificans* is a versatile organism that can grow heterotrophically but can also oxidise inorganic sulfur while reducing nitrate (Friedrich & Mitrenga, 1981). Since the 1920s there have been many discoveries of chemolithoheterotrophic *Bacteria* that grow on organic substrates as both energy and carbon sources but additionally oxidise inorganic compounds such as thiosulfate either aerobically (Trautwein, 1921; Starkey, 1934a, b; Sijderius, 1946; Trudinger, 1967; Tuttle, 1980; Mason & Kelly, 1988; S; Boden *et al.* 2010) or anaerobically (Sorokin *et al.* 1999).

The oxidation of organic sulfur compounds plays a key role in moving organosulfur into an inorganic form. Many *Bacteria* are able to utilise organic compounds such as dimethylsulfide (DMS, $(\text{CH}_3)_2\text{S}$) as a carbon source and for ATP synthesis (Kiene *et al.*, 1986). This is possible in a number of genera including *Methylophaga* (Boden, 2012) and often leads to the formation of sulfate (Borodina *et al.*, 2000; Kanagawa & Kelly, 1986; Smith & Kelly, 1988). Boden *et al.* (2010) studied and characterised *M. thiooxydans*, originally isolated by Schäfer *et al.* (2010), which metabolised DMS to thiosulfate in order to assimilate carbon into biomass and then oxidised thiosulfate to tetrathionate for additional energy production. *M. sulfidovorans* had been previously shown to produce thiosulfate from DMS but did not go on to oxidise it to tetrathionate (Zwart *et al.*, 1997). In either case, in terrestrial or marine environments, thiosulfate and tetrathionate produced by DMS metabolism can act as an energy source for additional inorganic sulfur oxidising autotrophic *Bacteria*. It might well be the case that other organosulfur compounds have a similar inorganic fate in the environment (Boden *et al.*, 2010).

Ultimately, by whatever means reduced sulfur compounds become oxidised, the end product is sulfate, ultimately adding to the environmental pool. Sulfate can be used by sulfate-reducing *Bacteria* and the cycle of sulfur starts again. Another aspect of the production of sulfate that is of extreme biological importance is the assimilation of sulfate into biomass (Schiff & Fankhauser, 1981). Many plants and *Bacteria* convert sulfate into biomass in the form of cysteine *via* assimilatory sulfate reduction. Cysteine can be converted to methionine or go directly into protein synthesis. Two pathways of assimilatory sulfate reduction are currently known (Schiff, 1979).

The first takes place in plants and members of the *Cyanobacteria* and starts with the activation of sulfate by ATP *via* the action of ATP sulfurylase (EC 2.7.7.4) to produce adenosine 5'-phosphosulfate (APS). APS is reduced by glutathione to yield adenosine monophosphate (AMP) and sulfite. This is further reduced to sulfide by six molecules of ferredoxin. The final step involves a reaction of serine with acetyl-CoA to form *O*-acetylserine. A reaction catalysed by *O*-acetylserine lyase produces cysteine from sulfide and *O*-acetylserine (Koprivova *et al.*, 2001; Leustek & Saito, 1999). The second pathway is found extensively in *Betaproteobacteria* and is probably best studied in *Salmonella* spp. (Iwanicka-Nowicka *et al.*, 2007). As with the plant associated pathway, APS is produced from sulfate. However, APS kinase adds an additional phosphate to APS to produce adenosine 3'-phosphate 5'-phosphosulphate (PAPS) (Lochowska *et al.*, 2011; Schiff, 1979). From this, cysteine is produced, which can be fed into protein, glutathione and other essential sulfur containing compounds.

Plants and *Bacteria* act as the basis for food webs in all environments and when consumed the sulfur they have assimilated is incorporated into more complex organisms, including humans. As sulfate is the basis for sulfur assimilation, it is clear that anything that adds to the sulfate pool in the environment is of extreme biological importance.

1.2. Inorganic sulfur compounds

Many different sulfur oxyanions exist in nature. These are formed by many chemical and biological processes and range from its most reduced form of -2 to its most oxidised form of +6 (Ghosh & Dam, 2009).

1.2.1 Thiosulfate

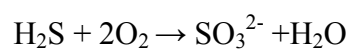
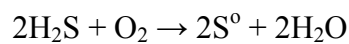
Thiosulfate ($S_2O_3^{2-}$) is an intermediate in the oxidation range of sulfur. The prefix thio- indicates that it is an ion of sulfate (SO_4^{2-}) and consists of an “outer” sulfane and an inner sulfonate (SO_3^{2-}) with oxidation states of -2 and +6 respectively. Thiosulfate can be produced in a number of chemical and biochemical processes and is found ubiquitously in terrestrial, fresh and marine waters (Vetter *et al.* 1989; Ghosh & Dam, 2009).

Thiosulfate can arise from a number of different abiotic mechanisms. It has been found to be released by volcanic and hydrothermal activities and by the weathering of rocks. However, most of the thiosulfate in the environment is not released directly but is formed chemically from elemental sulfur and sulfite via the Suzuki & Silver reaction (Suzuki & Silver 1966) (Equation 1.3) and is particularly stable at higher than neutral pH.

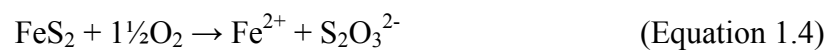


Oxidation of elemental sulfur produces sulfur dioxide (SO_2), which is common when minerals of sulfur are oxidised either spontaneously or by the direct leaching of sulfides by certain bacteria, releasing S^0 (Lizama & Suzuki, 1989). Further oxidation of sulfur

dioxide forms sulfite, which can further react with sulfur to form thiosulfate (Equation 1.3) (Suzuki, 1999; Suzuki & Silver, 1966). In marine environments thiosulfate is formed by the oxidation of sulfide being released from anoxic sediments to form both elemental sulfur and sulfite (Equation 1.4) (Nedwell 1982).



Thiosulfate is also be spontaneously produced when pyrite (FeS_2) reacts with oxygen to form thiosulfate and ferrous iron (Fe^{2+}) (Luther, 1987).

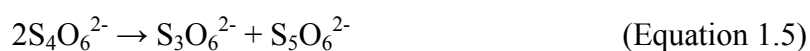


Biological production of thiosulfate has been shown by sulfate-reducing *Bacteria* that produce thiosulfate as a waste product in anaerobic respiration of molecular hydrogen and formate (Fitz & Cypionka, 1990). Thiosulfate is also produced by *M. sulfidovorans* when DMS is available as a growth substrate (De Zwart *et al.*, 1997).

1.2.2 Polythionates

The polythionates are a homologous series of inorganic sulfur oxyanions with the general formula $S_nO_6^{2-}$, with the n being equal to or greater than 3. The structure of the polythionates consists of two terminal sulfonate moieties joined by a chain of sulfane sulfurs. The shortest polythionate is trithionate ($S_3O_6^{2-}$) which contains a single sulfane sulfur not associated with oxygen atoms. Trithionate is relatively unstable when compared to thiosulfate and other higher polythionates and will easily hydrolyse into thiosulfate and sulfate in acidic or highly basic conditions (Figure 1.2). Tetrathionate ($S_4O_6^{2-}$) is considerably more stable at low pH compared to trithionate but as the sulfur chain of polythionates increases in length, they become more unstable. The highest polythionate that has been purified is hexacotathionate ($S_{24}O_6^{2-}$) although higher polythionates are believed to exist (Mandalasi, 2002).

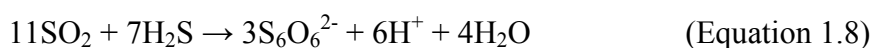
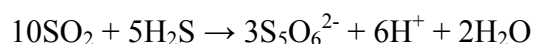
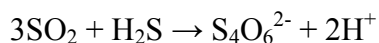
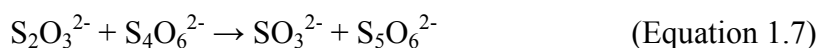
The formation of trithionate can occur chemically by the breakdown of tetrathionate (Equation 1.5). Some sulfur-oxidising *Bacteria* have shown to produce trithionate as an intermediate product of their metabolism when oxidising thiosulfate (Pankhurst, 1964). Trithionate and thiosulfate production was shown by *D. desulfuricans* and *D. vulgaris* as intermediate products of sulfite reduction in anaerobic conditions (Kobayashi *et al.*, 1974; Fitz & Cypionka, 1990).



Tetrathionate is the product of thiosulfate oxidation (Equation 1.6), particularly in acidic solutions and in the presence of oxidants such as ferric iron and peroxy radicals

(Williamson & Rimstidt, 1993). It has been shown that the surface of pyrite catalyses the formation of tetrathionate when thiosulfate acts as an electron conductor between pyrite and the electron acceptor oxygen (Xu & Schoonen, 1995). The stabilities of thiosulfate, trithionate and tetrathionate can be generalised and are highlighted in Figure 1.2.

Pentathionate is produced by the breakdown of tetrathionate (Equation 1.5) and by the reaction of tetrathionate with thiosulfate (Equation 1.7). Production of pentathionate and higher polythionates has also been detected in the media of growing bacteria utilising the Kelly-Trudinger pathway. Many polythionates arise in strongly-acidic waters by the reactions of SO₂ and H₂S and this was highlighted in studies of volcanic crater lakes where these sulfur species are released by fumaroles (Takano *et al.* 1994). The chemistry of these reactions can be seen in Equation 1.8.



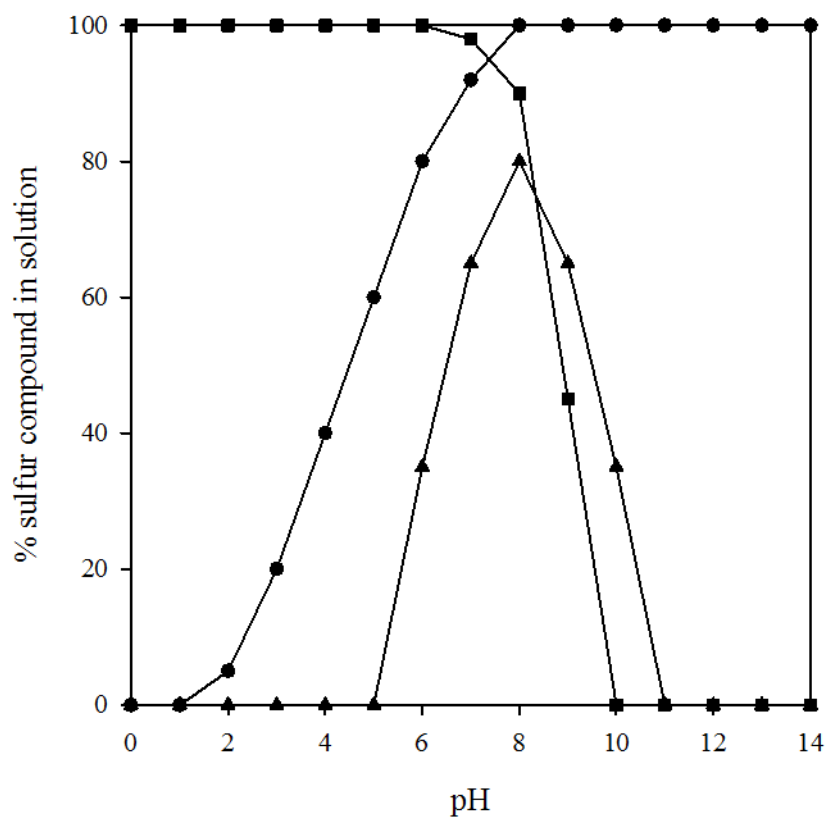


Figure 1.2. Stability of thiosulfate (●), trithionate (▲) and tetrathionate (■) in solution at a range of pH values. y-axis represents percentage of compound left in a solution after 24 hours. Modified from Xu & Schoonen, 1995.

1.2.3 Sulfide and elemental sulfur

Sulfide (S^{2-}) represents sulfur in its most reduced form and so can only be oxidised (Morse *et al.*, 1987). In aqueous solutions sulfide can shift to its alternative anion form (SH) and in acidic conditions is rapidly converted into hydrogen sulfide (H_2S) and easily evaporates from aqueous solutions (Roy & Trudinger, 1970). Sulfide and hydrogen sulfide oxidise in air and when exposed to light to sulfur dioxide and sulfate. In alkaline solutions, where polythionates are present, thiosulfate and elemental sulfur can be formed, as with the example where tetrathionate is present (Equation 1.8) (Roy & Trudinger, 1970).



Elemental sulfur (S^0) is an intermediate within the oxidation range of sulfur and exists in over 40 different allotropes (Meyer, 1975) but is most commonly found as the yellow orthorhombic α -sulfur. Its structure is a staggered eight member ring (S_8) and is the most stable under normal atmospheric temperature and pressure (Meyer, 1964). Despite the fact that this form of sulfur is insoluble in water it can be utilised as an energy source by chemolithotrophic *Bacteria*, feeding into both the Kelly-Friedrich and Kelly-Trudinger pathways, although this is not observed in all *Bacteria* that utilise either of these metabolic pathways (Ghosh & Dam, 2009).

1.2.4 Sulfate

Sulfate (SO_4^{2-}) represents sulfur in its most oxidised form (+6) and is ultimately the end product of the oxidation of other sulfur species. Sulfite (SO_3^{2-}) is rapidly autooxidised

into sulfate at neutral pH and a wide range of temperatures and is catalysed by many metal ions (Roy & Trudinger, 1970). Sulfate can arise in the atmosphere from oxidation of compounds such as sulfur dioxide before returning to terrestrial, marine and fresh water environments *via* precipitation (Hegg & Hobbs, 1986). Bacterial formation of sulfate takes place in both Kelly-Friedrich and Kelly-Trudinger pathway utilising species. Sulfate is also the substrate for both assimilatory and dissimilatory sulfate reduction (Ghosh & Dam, 2009).

1.3 Inorganic sulfur compound metabolism

1.3.1 Chemolithoautotrophy

1.3.1.1 Kelly-Friedrich (Sox) pathway

The Kelly-Friedrich or “Sox” pathway is typically found in chemolithotrophic and phototrophic *Alphaproteobacteria* (Ghosh & Dam, 2009). In this pathway inorganic sulfur compounds are fully oxidised to sulfate without any detectable intermediates (tetrathionate and other polythionates) being formed during the process. In the initial research conducted into sulfur oxidation when some species were grown on thiosulfate, the thiosulfate was found to be stoichiometrically converted to sulfate (Starkey, 1935; Peck, 1960; Peck and Fisher, 1962). Oh and Suzuki (1977) in a later study used whole cell and cell-free extracts of *Starkeya novella* (reclassified from “*Thiobacillus novellus*” by Kelly (2000)) concluded that a single, membrane-bound enzyme or system of enzymes were catalysing the oxidation of thiosulfate. It was suggested that the enzyme system worked in the following way. Firstly, thiosulfate was cleaved into sulfite and elemental sulfur. The latter atom is then oxidised to sulfite in a reaction involving oxidised glutathione. The two resulting sulfite molecules were oxidised further to sulfate by

cytochrome-*c* reductase. Later work by Lu and Kelly (1983*a*) on *Paracoccus versutus* (reclassified from “*T. versutus*” (Katayama, 1995)) found thiosulfate oxidation to be independent of the cell membrane and that it took place in the periplasm. Further research by the same research group on *P. versutus* and its metabolism found that the pathway was catalysed by an enzyme complex consisting of “enzyme A” and “enzyme B” as well as cytochromes *c*₅₅₁ and *c*_{552.5}. This was named the thiosulfate-oxidizing multienzyme system (TOMES) (Lu & Kelly, 1983*a, b, c*)

Later research into the TOMES focused on trying to identify the genes involved. TOMES is now known to be encoded by the conserved operon *soxTRS-VW-XYZABCDEFGH*. The gene encoding the ‘enzyme B’ (*soxB*) was successfully identified and sequenced in mutagenic studies of *Paracoccus pantotrophus* (Wodara *et al.*, 1994) (formerly *Thiosphaera pantotropha* Ludwig *et al.*, 1993; Rainey *et al.*, 1999). The protein product of this gene was shown to be identical to the enzyme found in *P. versutus*. This was followed by the further discoveries in *P. pantotrophus* of *soxCD* that encodes an additional sulfite dehydrogenase enzyme (Wodara *et al.* 1997), *soxYZ* that encodes the ‘enzyme A’ protein (Friedrich *et al.*, 2000) and *soxXA* that encodes for the cytochromes *c*₅₅₁ and *c*_{552.5} (Rother & Friedrich, 2002). A number of *sox* structural genes (*soxE*, *soxF*, *soxG* and *soxH* that produce a cytochrome *c*, flavocytochrome *c* sulfide dehydrogenase and two esterase enzymes, respectively) have received contradictory reports on whether they encode proteins that are essential for sulfur species oxidation in these organisms (Friedrich *et al.*, 2001; Rother *et al.*, 2001; Quentmeier *et al.*, 2004; Bardischewsky *et al.*, 2006).

The mechanism by which the proteins of the Sox pathway work commences when a thiosulfate ion binds *via* its sulfane sulfur to the two cytochromes of the TOMES (Figure 1.3). This enables the thiosulfate to bind with the “enzyme A” and release the

cytochromes and two electrons in the process (Friedrich *et al.*, 2001). The sulfane group is cleaved and oxidised from sulfite to sulfate by the action of “enzyme B” (Quentmeier *et al.*, 2003). The remaining sulfur atom is still bound to “enzyme A” and is oxidised to sulfite by the sulfur dehydrogenase enzyme using water as the donor of the oxygen atoms. This process releases an additional 6 electrons. The final step involves the oxidation of the sulfite to sulfate and its subsequent release *via* “enzyme B” again. This allows “enzyme A” to react with a new molecule of thiosulfate (Friedrich *et al.*, 2001). The whole pathway yields 8 electrons per molecule of thiosulfate oxidised which are fed to the electron transport chain by a *c*-type cytochrome.

Genetic analysis of genome sequences has revealed that Sox genes are present in many species of the *Bacteria* but not in the *Archaea*. Although not identical, a number of species have genes homologous to the Sox genes found in *P. pantotrophus*. Examples include *Chlorobaculum tepidum*, a thermophilic green sulfur bacterium (Wahlund *et al.*, 1991) and *Starkeya novella* (Kappler *et al.*, 2001). So far however, all species identified as using the Sox pathway metabolise thiosulfate and other inorganic sulfur compounds (but not tetrathionate) as facultative autotrophs (Ghosh & Dam, 2009).

1.3.1.2 The Kelly-Trudinger pathway

In contrast to the Sox pathway, the Kelly-Trudinger pathway (often referred to as the S₄I pathway) involves the oxidation of thiosulfate with the intermediate production of tetrathionate (S₄O₆²⁻) and other polythionates (Trudinger, 1961*a, b*, 1964; Kelly & Syrett, 1966; Kelly & Tuovinen, 1975). This is followed by the further oxidation of tetrathionate to the waste product, sulfate (Figure 1.4). The Kelly-Trudinger pathway is mainly observed in obligate chemolithotrophic species of the *Betaproteobacteria* and

Gammaproteobacteria as well as the newly created *Acidithiobacillia* (Williams & Kelly, 2013; Hudson *et al.* 2014; Kelly & Wood, 2014); although a number of facultative species have been found in *Alphaproteobacteria* and *Betaproteobacteria* (Meulenber *et al.* 1993; Ghosh *et al.* 2005; Dam *et al.*, 2007). Examples include species from *Thiobacillus*, *Thermithiobacillus*, *Halothiobacillus* and *Acidithiobacillus* (Kelly & Wood, 2000*a, b*; Boden *et al.* 2011). Originally all belonging to the genus *Thiobacillus*, Kelly-Trudinger organisms have been found to be far more diverse than previously thought and many have been reassigned to new genera based on their 16S rRNA gene sequence (Kelly & Wood, 2000*a, b*; Boden *et al.* 2011). The production of acid stable intermediates such as tetrathionate might be preferable in acidic environments that are dominated by *Acidithiobacillus* species, although many Kelly-Trudinger organisms are neutrophilic. Although many studies have tried to identify the key components of the Kelly-Trudinger pathway, many of the proteins and genes involved remain poorly understood.



The first step of the Kelly-Trudinger pathway (thiosulfate dehydrogenase EC 1.8.2.2) was demonstrated in *Halothiobacillus neapolitanus* (formerly *Thiobacillus* X) (Trudinger, 1961*a, b*) and has been well studied in *Thermithiobacillus tepidarius* (Lu & Kelly, 1988*a, b*) and *Acidiphilium acidophilum* (Meulenber *et al.*, 1993*b*). With cellular fractionation experiments it was proposed that in the reaction two molecules of thiosulfate became oxidised into a single molecule of tetrathionate within the periplasm of the cells (Figure 1.4). This activity has since been found to take place in *Acidithiobacillus caldus* (formerly *Thiobacillus caldus* (Kelly & Wood, 2000*a*) and other species that belong to, or previously belonged to *Thiobacillus* (Hallberg *et al.*, 1996; Visser *et al.*, 1996; Kelly, 1997). Two electrons are liberated from this oxidation and are passed *via* a cytochrome *c* to the electron transport chain. The precise details of these proteins are rather diverse in

terms of the enzyme and the cytochromes involved. Proteins with thiosulfate dehydrogenase activity were purified from *Ttb. tepidarius*, *Thiobacillus thioparus* and marine heterotroph 16B were 138, 115 and 132 kDa in size, respectively, none of which contained any haem groups (Lyric & Suzuki, 1970; Whited & Tuttle, 1983; Lu & Kelly, 1988b). In contrast Meulenburg *et al.* (1993b) purified a protein of 102 kDa that contained two subunits, each of which had cytochrome *c*₅₅₃ present. A far smaller protein of ~25 kDa was found in *A. ferrooxidans* that again contained no haem group (Silver and Lundgren, 1968; Kikumoto *et al.*, 2013) while a more recent study found thiosulfate dehydrogenase to be a 26 kDa dihaem cytochrome *c* in the phototrophic *Allochromatium vinosum* (Denkmann *et al.* 2012). *A. vinosum* contains an incomplete Sox pathway that produces sulfate and intracellular sulfur globules (Hensen *et al.* 2006) but under acidic conditions *A. vinosum* can also oxidise thiosulfate to tetrathionate although it is unable to further oxidise the tetrathionate molecule. Whether the enzyme is the same seen in Kelly-Trudinger organisms is questionable as the gene identified by Denkmann *et al.* (2012) has not been found in all genome sequences of known Kelly-Trudinger organisms. These data show that at least two distinct thiosulfate dehydrogenase enzymes might be present in the *Bacteria*. It is important to note that this enzyme is not the same as the quinone-linked thiosulfate dehydrogenase (EC 1.8.5.2) found in the *Archaea*.

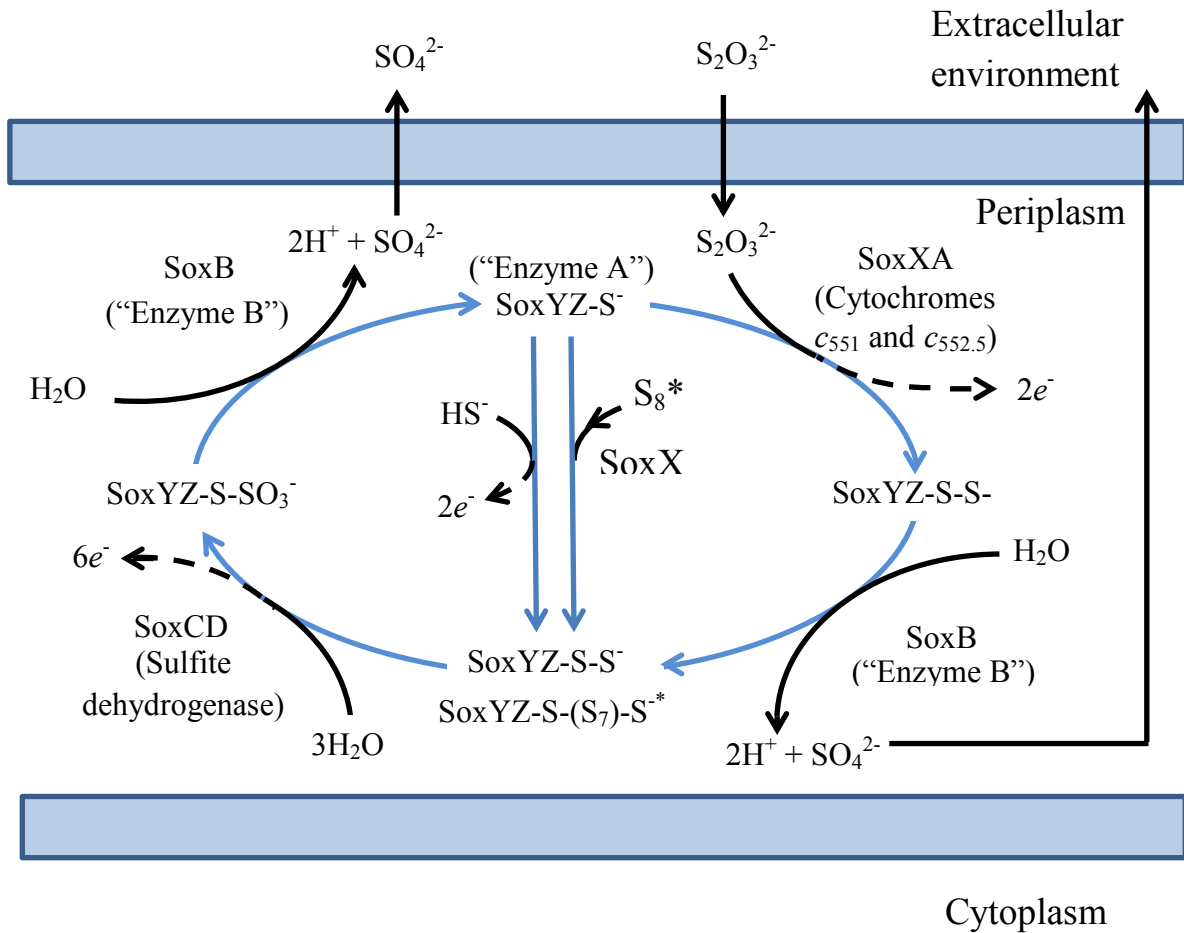


Figure 1.3. Oxidation of thiosulfate, sulfide and elementary sulfur by the TOMES (also known as the Sox complex) of the Kelly-Friedrich pathway in *Alphaproteobacteria*. First identified in *Paracoccus versutus* by Lu & Kelly (1983a, b, c) and later in *P. pantotrophus* (Wodara *et al.*, 1994; Wodara *et al.*, 1997; Friedrich *et al.*, 2000; Rother & Friedrich., 2002) the TOMES is now known to be encoded by the conserved operon *soxTRS-VW-XYZABCDEFGH* (Ghosh & Dam, 2009; Lahiri *et al.*, 2006).

1.3.1.2 The Kelly-Trudinger pathway

In contrast to the Sox pathway, the Kelly-Trudinger pathway (often referred to as the S₄I pathway) involves the oxidation of thiosulfate with the intermediate production of tetrathionate (S₄O₆²⁻) and other polythionates (Trudinger, 1961*a, b*, 1964; Kelly & Syrett, 1966; Kelly & Tuovinen, 1975). This is followed by the further oxidation of tetrathionate to the waste product, sulfate (Figure 1.4). The Kelly-Trudinger pathway is mainly observed in obligate chemolithotrophic species of the *Betaproteobacteria* and *Gammaproteobacteria* as well as the newly created *Acidithiobacillia* (Williams & Kelly, 2013; Hudson *et al.* 2014; Kelly & Wood, 2014); although a number of facultative species have been found in *Alphaproteobacteria* and *Betaproteobacteria* (Meulenberg *et al.* 1993; Ghosh *et al.* 2005; Dam *et al.*, 2007). Examples include species from *Thiobacillus*, *Thermithiobacillus*, *Halothiobacillus* and *Acidithiobacillus* (Kelly & Wood, 2000*a, b*; Boden *et al.* 2011). Originally all belonging to the genus *Thiobacillus*, Kelly-Trudinger organisms have been found to be far more diverse than previously thought and many have been reassigned to new genera based on their 16S rRNA gene sequence (Kelly & Wood, 2000*a, b*; Boden *et al.* 2011). The production of acid stable intermediates such as tetrathionate might be preferable in acidic environments that are dominated by *Acidithiobacillus* species, although many Kelly-Trudinger organisms are neutrophilic. Although many studies have tried to identify the key components of the Kelly-Trudinger pathway, many of the proteins and genes involved remain poorly understood.



The first step of the Kelly-Trudinger pathway (thiosulfate dehydrogenase EC 1.8.2.2) was demonstrated in *Halothiobacillus neapolitanus* (formerly *Thiobacillus X*) (Trudinger, 1961*a, b*) and has been well studied in *Thermithiobacillus tepidarius* (Lu & Kelly, 1988*a, b*) and *Acidiphilium acidophilum* (Meulenberg *et al.*, 1993*b*). With cellular fractionation

experiments it was proposed that in the reaction of two molecules of thiosulfate became oxidised into a single molecule of tetrathionate within the periplasm of the cells (Figure 1.4). This activity has since been found to take place in *Acidithiobacillus caldus* (formerly *Thiobacillus caldus* (Kelly & Wood, 2000a) and other species that belong to, or previously belonged to *Thiobacillus* (Hallberg *et al.*, 1996; Visser *et al.*, 1996; Kelly, 1997). Two electrons are liberated from this oxidation and are passed to a cytochrome *c* which enter the electron transport chain. The precise details of these proteins are rather diverse in terms of the enzyme and the cytochromes involved. Proteins with thiosulfate dehydrogenase activity were purified from *Ttb. tepidarius*, *Thiobacillus thioparus* and marine heterotroph 16B were 138, 115 and 132 kDa in size, respectively, none of which contained any haem groups (Lyric & Suzuki, 1970; Whited & Tuttle, 1983; Lu & Kelly, 1988b). In contrast Meulenburg *et al.* (1993b) purified a protein of 102 kDa that contained two subunits, each of which had cytochrome *c*₅₅₃ present. A far smaller protein of ~25 kDa was found in *A. ferrooxidans* that again contained no haem group (Silver and Lundgren, 1968; Kikumoto *et al.*, 2013) while a more recent study found thiosulfate dehydrogenase to be a 26 kDa dihaem cytochrome *c* in the phototrophic *Allochromatium vinosum* (Denkman *et al.* 2012). *A. vinosum* contains an incomplete Sox pathway that produces sulfate and intracellular sulfur globules (Hensen *et al.* 2006) but under acidic conditions *A. vinosum* can also oxidise thiosulfate to tetrathionate although it is unable to further oxidise the tetrathionate molecule. If the enzyme is the same seen in Kelly-Trudinger organisms is questionable as the gene identified by Denkman *et al.* (2012) has not been found in all genome sequences of known Kelly-Trudinger organisms. These data show that at least two distinct thiosulfate dehydrogenase enzymes maybe present in the *Bacteria*. It is important to note that this enzyme is not the same as the quinone-linked thiosulfate dehydrogenase (EC 1.8.5.2) found in the *Archaea*.

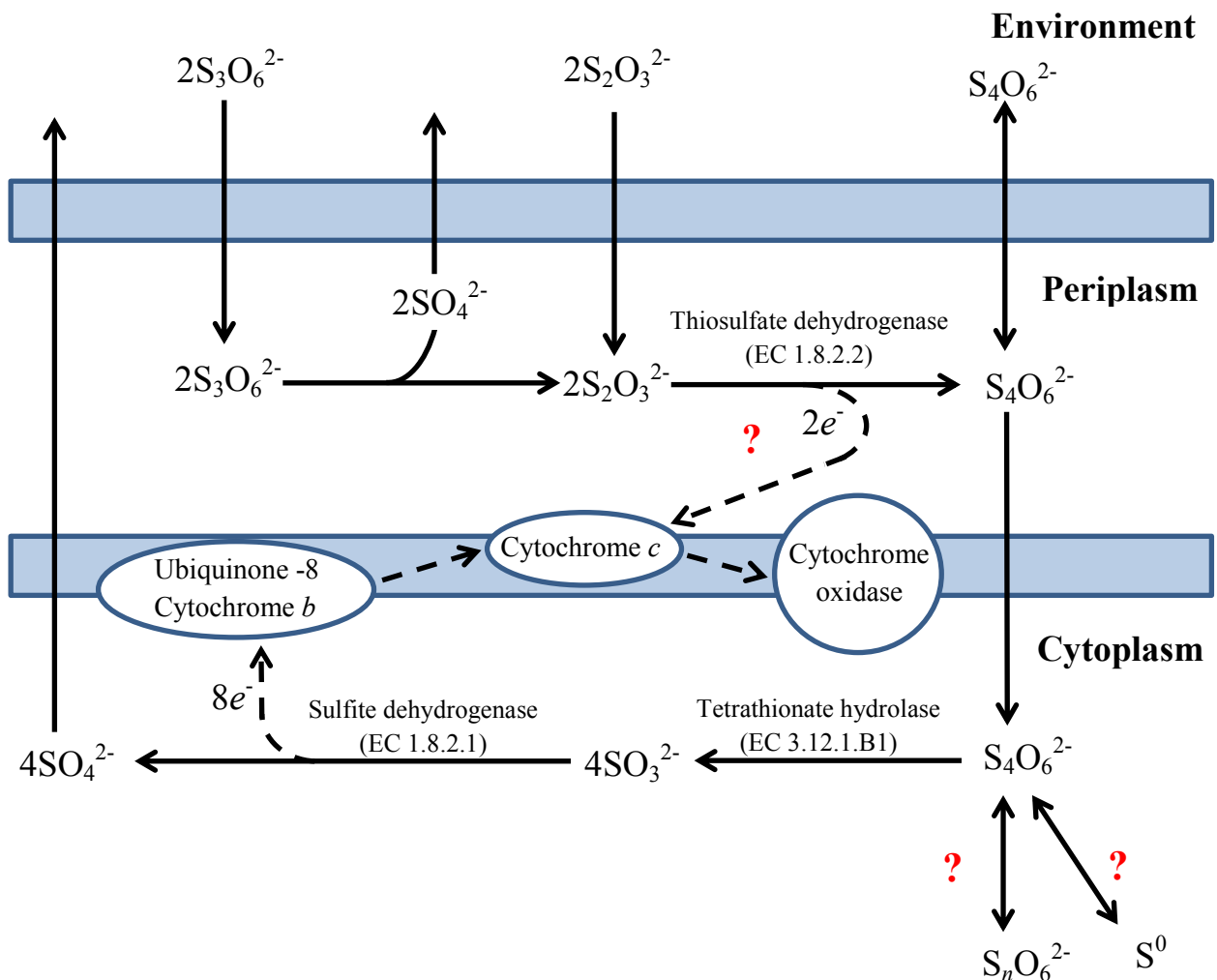


Figure 1.4. Oxidation of reduced inorganic sulfur compounds by the Kelly-Trudinger (S4I) pathway. The steps in this pathway were originally identified in *Thermithiobacillus tepidarius* by Lu & Kelly (1988a, b) and later supported in *Acidithiobacillus caldus* (Hallberg *et al.* 1996) and *Advenella kashmirensis* (Dam *et al.* 2007). The later study found that sulfate inhibited the oxidation of tetrathionate but it was not known how many enzymes were involved in sulfate production from tetrathionate, as did the previous studies on Kelly-Trudinger organisms.

Trithionate was shown to be oxidised by growing cells of *Thiobacillus thioparus* although the fate of trithionate was not understood (Vishniac, 1952). Studies by Trudinger (1964) on *Halothiobacillus neapolitanus* showed that whole cells seemed to hydrolyse trithionate stoichiometrically to thiosulfate and sulfate. Lu and Kelly (1988b) demonstrated activity of a trithionate hydrolase in the periplasm of *Thermithiobacillus tepidarius* and Meulenberg *et al.* (1992) also demonstrated this in *Acidiphilium acidophilum*. This enzyme has since been shown to be present in the genera *Dyella*, *Halothiobacillus*, *Leifsonia*, *Methylobacterium* and *Pandoraea* (Anandham *et al.*, 2008). The periplasmic location makes it possible for the thiosulfate product to become further oxidised by the activity of thiosulfate dehydrogenase and entry into the Kelly-Trudinger pathway although the reaction itself was not believed to yield available energy (Lu & Kelly, 1988b). The enzyme was partially purified in *Acidiphilium acidophilum* and was found to be ~99 kDa in size (Meulenberg *et al.*, 1992). Despite these the gene encoding this protein has never been identified.

The next step in the Kelly-Trudinger pathway involves the hydrolysis of tetrathionate by the action of tetrathionate hydrolase (EC 3.12.1.B1). There has been conflicting reports regarding the cellular location of this enzyme and the products of the reaction it catalyses. Tetrathionate hydrolase activity was observed in the cytoplasm of *Ttb. tepidarius* after tetrathionate had been transported from the periplasm (Lu & Kelly, 1988a). Similar results were obtained in studies of *Atb. caldus* and *Advenella kashmirensis* (formerly *Tetrathiobacter kashmirensis* (Gibello *et al.*, 2009) in which the use of inhibitors showed that tetrathionate was hydrolysed in the cytoplasm (Dam *et al.*, 2007; Hallberg *et al.*, 1996). However, studies of acidophilic species such as *A. acidophilum*, *Atb ferroxidans* and *Atb. thiooxidans* indicated tetrathionate hydrolase activity in the periplasm of the cell. In

contrast to the study by Hallberg *et al.* (1996), the majority of tetrathionate hydrolase activity was found within the periplasm of *Atb. caldus* (Bugaytsova & Lindstrom, 2004).

The gene (*tthH*) that encodes tetrathionate hydrolase was identified by Kanao *et al.* (2007) in cells of *A. ferrooxidans* grown on tetrathionate. The products from one molecule of tetrathionate from this enzyme were shown to be one molecule of thiosulfate, one molecule of sulfate and another atom of elemental sulfur. This was in agreement with De Jong *et al.* (1997) and was also the conclusion for tetrathionate hydrolase activity in *A. acidophilum* (Meulenberg *et al.* 1993*a*). Bugaytsova & Lindstrom (2004) also purified a tetrathionate hydrolase of ~103 kDa but found the products of this enzyme were thiosulfate and pentathionate, with no detection of any elemental sulfur. Contradictory studies into *Ttb. tepidarius* and *Atb. caldus* found that tetrathionate oxidation produced sulfite and possibly elemental sulfur which in turn was oxidised into additional sulfite (Lu & Kelly 1998*a, b*; Hallberg *et al.* 1996; Dam *et al.* 2007). Dam *et al.* (2007) also highlighted the strong inhibitory effect of sulfite had on tetrathionate oxidation and possibly its role as a regulator of the Kelly-Trudinger pathway. These data indicate once again that the Kelly-Trudinger pathway may not be identical in all species and in the case of tetrathionate hydrolase might vary in nature due to the acidic or neutral environments that they naturally inhabit.

The final reaction in the Kelly-Trudinger pathway is the oxidation to sulfate. For each molecule of tetrathionate produced by thiosulfate oxidation, four molecules of sulfate are produced. In the case of *Ttb. tepidarius*, *A. kashmirensis* and *Atb. caldus* this is catalysed *via* sulfite by the enzyme sulfite dehydrogenase (Lu & Kelly 1998*a, b*; Hallberg *et al.* 1996; Dam *et al.* 2007). This enzyme has had an extensive background of research regarding its activity in Sox organisms. The gene (*soxB*) was first identified in *Paracoccus denitrificans* with later work identifying the gene for a functional sulfite

dehydrogenase to consist of five open reading frames (*soxBCDEF*), three of which encode two *c*-type cytochromes and a flavoprotein (Wodara *et al.*, 1994; Wodara *et al.*, 1997). However, no gene has been identified in any Kelly-Trudinger organism to date and whether it is the same functional enzyme is unknown. Ultimately, sulfate (four molecules per molecule of tetrathionate) is removed from the cell adding to extracellular concentrations of this compound (Ghosh & Dam, 2009; Kelly, 1982; Vishniac & Santer, 1957). Two electrons are released per molecule of sulfite and evidence has shown that it passes to ubiquinone-8/cytochrome *b* where it is passed through the respiratory chain. With a yield of six electrons being released by tetrathionate hydrolase per molecule of tetrathionate and two electrons from the initial formation of tetrathionate, a total of 16 electrons enter the respiratory chain. The energetics of ATP production in S₄I organisms, however, remain poorly understood (Kelly, 1985; Kelly *et al.*, 1997; Lu & Kelly, 1988b).

The Kelly-Trudinger pathway and its impact to the environment and the global sulfur cycle was investigated by Anandham *et al.* (2008) who identified five additional genera of root colonizing *Bacteria* that produced polythionate intermediates during the oxidation of thiosulfate to sulfate. The gene that encodes thiosulfate dehydrogenase (*tsdA*) identified by Denkmann *et al.* (2012) was found by database searches to be present in a large number of different autotrophic and heterotrophic *Bacteria*. What was once thought to be confined to one genus of the *Betaproteobacteria* (*Thiobacillus*) could possibly be found in many other genera of the *Bacteria*, although whether this gene is actively expressed in these organisms has yet to be established.

1.3.1.3 Other pathways

A third but what seems like a far less common pathway of inorganic sulfur metabolism is termed the branched thiosulfate oxidation pathway (Ghosh & Dam, 2009). This is found in chemotrophic as well as phototrophic *Bacteria*. Early studies into chemolithotrophic *Bacteria* such as *Beggiatoa* and *Thiothrix* (now known to be *Paracoccus* (Kelly *et al.*, 2006) found that elemental sulfur accumulated in the periplasm of thiosulfate grown cells (Dahl, 1999). A number of studies indicated that thiosulfate oxidation takes place *via* rhodanese with the formation of elemental sulfur (Odintsova *et al.*, 1993; Jordon *et al.*, 1997). Later work, however, pointed towards a partial Sox system which lacks the SoxCD (sulfite dehydrogenase) protein. As a result, the sulfane sulfur of thiosulfate is left unoxidised by this system. It remains uncertain the mechanism by which this acuminated sulfur becomes fully oxidised to sulfate but it is believed to involve its transport into the cytoplasm. Elemental sulfur is then oxidised to sulfite by a cytochrome dependent reaction or sulfur oxygenase. Sulfite is then oxidised and released as sulfate by reacting with adenosine 5'-monophosphate *via* the action of adenylyl sulfate reductase to form APS which finally reacts with ATP sulfurylase to form ATP and sulfate (Meyer *et al.*, 2007; Grimm *et al.*, 2008). In photosynthetic *Bacteria* the electrons released in the pathway are fed into a photosynthetic electron transport system whereas chemolithotrophic *Bacteria* they are fed into a respiratory chain.

1.3.2 Chemolithoheterotrophy and mixotrophy

The evolution of chemolithotrophic metabolism, in particular the oxidation of inorganic sulfur appears to be a very early form of metabolism in the *Bacteria* and the *Archaea* (Matantseva & Skarlato, 2012). Whether chemolithotrophy or chemolithoheterotrophy evolved first is a controversial issue (Ghosh & Dam, 2009). One side of the argument is

phylogenetic evidence that enzymes of dissimilatory sulfite reduction in the *Bacteria* are homologues to those found in the *Archaea*, suggesting their common ancestor had this ability. This pathway often involves the oxidation of organic acids and so points towards an early form of heterotrophy in an anaerobic manner, typical of early Earth history (Hipp *et al.*, 1997). However, it is well accepted that deep sea vents were one of the first environments to support life, habitats that are rich in extremophilic *Bacteria* and *Archaea* chemolithotrophs (Fuchs *et al.*, 1996). Many of the deepest branches of the *Bacteria* also contain chemolithotrophic species, including organisms that utilise electron acceptors other than oxygen (Fe^{3+} and SO_4^{2-} for example). Regardless of whether lithoautotrophy or heterotrophy was the original microbial metabolism, many organisms have the ability to grow in one of the two strategies. Many other species have been the subject of research due to their ability to grow by both forms of metabolism, using organic and inorganic molecules as energy and/or organic molecules and CO_2 as a carbon source. Possibly this is due to the species in question never losing the ability of whichever strategy is more ancient. In theory, the ability to use both gives an organism an ecological advantage.

Mixotrophy in the *Bacteria* involves not only the catabolism of organic and inorganic compounds (or the use of light in phototrophy) but also the ability of heterotrophic and autotrophic growth (Eiler, 2006). It is often stated in the literature by the author(s) in question what the definition of a mixotroph is with regards to their research interests. For the purpose of this study the correct definition of a mixotroph is an organism that can use these different metabolic strategies simultaneously. A good example of a mixotroph with regards to inorganic sulfur is *Paracoccus versutus* (Davidson, 1985). In contrast; chemolithoheterotrophic *Bacteria* can oxidise inorganic compounds to produce ATP but lack the ability to fix inorganic carbon and so require organic compounds for growth. Many examples of inorganic sulfur oxidising chemolithoheterotrophs are known, most

notably “*Thiobacillus trautweinii*” strains (Trautwein, 1921; Starkey, 1934a). The field of mixotrophy has received far more interest than that of chemolithoheterotrophy and this is reflected in the literature (Matentseva & Skarlato, 2012; Moore, 2013). In particular, mixotrophy has been studied extensively in ocean processes influenced by planktonic blooms and ciliated phagocytes that have retained chloroplasts (Stoecker *et al.*, 1987; Stickney *et al.* 1999; Eiler, 2006; Moore, 2013). Historically far less research has been conducted on chemolithoheterotrophs but recent studies have highlighted their importance in the environment (Boden *et al.*, 2010).

Mixotrophic growth in terms of thiosulfate has been well studied on many species, including previous members of *Thiobacillus*. *Acidophilum acidophilus*, (formally *T. acidophilus* (Guay & Silver, 1975)), can grow on both glucose and tetrathionate in a heterotrophic and autotrophic manner simultaneously and gain a boost in yield greater than the sum of yields seen when grown on glucose or tetrathionate individually (Mason & Kelly, 1988a). Other fully mixotrophic strains are *Paracoccus versutus*, *Starkeya novella* and *Thiomonas intermedia* (London & Rittenberg, 1966; Perez & Martin, 1980; Wood & Kelly, 1977) which grew heterotrophically and autotrophically simultaneously. A number of studies indicated that inorganic sulfur chemolithoautotrophic systems were “turned off” in the mixotrophic organisms *Starkeya novellus* and *Paracoccus versutus* when organic nutrients were introduced into cultures maintained on inorganic sulfur (Kelly, 1971; Whittenbury & Kelly, 1977). *Thiomonas intermedius* is an example of a very versatile organism able to grow mixotrophically simultaneously on glucose and thiosulfate but the presence of glucose seemed to suppress ribulose-bisphosphate carboxylase activity and decreased CO₂ fixation, possibly from it being less energetically less favourable (London, 1963; London & Rittenberg, 1966; Martin, 1978). Later studies on *P. versutus* found that in both batch and continuous culture the ability to utilise

thiosulfate and fix CO₂ decreased when cells had been maintained on glucose. Conversely the same was also true when cells had been maintained lithotrophically becoming unable to metabolise glucose (Smith *et al.*, 1980). When grown initially on both glucose and thiosulfate, however, *P. versutus* grew as a “true” mixotroph by metabolising both compounds completely and fixing the organic carbon and CO₂. Radiolabelled [¹⁴C]-glucose confirmed that more carbon was assimilated heterotrophically at the expense of thiosulfate produced energy. An increase in glucose carbon being fixed as biomass in a glucose/tetrathionate chemostat can be seen in *Acidiphilium acidophilum* (Mason & Kelly, 1988a). A boost in yield was observed when grown as a mixotroph although the presence of glucose did result in a decrease in CO₂ fixation and decreased the rate at which tetrathionate was oxidised when compared to strictly chemolithoautotrophic growth.

Early studies into chemolithoheterotrophic organisms have shown that a number of typically heterotrophic species are capable of oxidising thiosulfate to tetrathionate. These included members of *Pseudomonas*, *Aeromonas* and *Bacillus* (Mason & Kelly, 1988b; Nelson & Castenholz, 1981; Trudinger, 1967; Sorokin *et al.*, 1999) and a heterotrophic member of *Thiobacillus* “*T. trautweinii*” (Trautwein, 1921). In most cases tetrathionate was the final product of sulfur oxidation although there are some examples of sulfate being the final product (Pepper & Miller, 1978; Schook & Berk, 1978). From the relatively minimal literature on the physiology of heterotrophic oxidation of inorganic sulfur these *Bacteria* can be split into two groups, species that gain additional energy (“fortuitous oxidation”) when grown with thiosulfate (chemolithoheterotrophy) and species that appear to oxidise thiosulfate but with no apparent benefit (“gratuitous oxidation”). Some examples of inorganic electron donors in chemolithoheterotrophy include NH₄⁺, NO₂⁻, Fe²⁺, H₂, H₂S and S⁰, which are oxidised to NO₂⁻, NO₃⁻, Fe³⁺, 2H⁺, S⁰ and SO₄²⁻, respectively (Fuchs *et al.*, 1996). It is very poorly understood whether

organisms that are true chemolithoheterotrophs and lack the ability to fix CO₂ gain a real benefit both physiologically and ecologically by oxidising thiosulfate in conjunction with heterotrophic metabolism.

An example of a heterotrophic organism that seems to benefit from addition of thiosulfate is *Methylobacterium oryzae*. Anandham *et al.* (2007) demonstrated that in batch culture the yield of dry biomass and protein significantly increased when grown in mineral media with succinate and thiosulfate compared with succinate alone. In contrast to this was a number of *Pseudomonas* spp. which oxidised up to 100% of thiosulfate when grown with glucose without an increase in the yield of the organism (Mason & Kelly, 1988b). The same pattern was seen in "*T. trautweinii*" with thiosulfate oxidation having no apparent beneficial effect (Starkey, 1934b; Trautwein, 1921). However, when Mason and Kelly (1988b) grew a strain of *P. aeruginosa* in a chemostat limited by glucose plus auxilliary thiosulfate the amount of biomass per mol of glucose increased by as much as 23%. This only happened when thiosulfate was at higher concentrations and not all thiosulfate was necessarily oxidised to tetrathionate. Chemostat cultures of *Methylophaga thiooxydans* grown on the organosulfur dimethylsulfide (DMS) also showed an increase in maximum yield co-efficient of 9% when 2 mM thiosulfate was present (Boden *et al.*, 2010). In batch cultures of "*Thiobacillus Q*" (now *Dechloromonas* sp. Q), Gommers and Kuenen (1988) found no increase in yield when thiosulfate was added to a culture growing heterotrophically on acetate. There was however a small increase in yield when grown in a chemostat up to a concentration of 7 mM of thiosulfate but when thiosulfate was increased beyond this concentration the bacterium seemed to decrease in both its ability to oxidise thiosulfate and in biomass yield. Clearly chemolithoheterotrophy with regards to inorganic sulfur metabolism differs greatly among organisms that are able to oxidise

thiosulfate and may only have a beneficial effect under certain conditions or in certain organisms.

One hypothesis to why thiosulfate is oxidised is that in some organisms thiosulfate becomes oxidised to sulfur, which is stored intracellularly and possibly used as electron acceptors if their environment becomes anaerobic. This appeared to be the case in a number of *Beggiatoa* spp. (Nelson & Castenholz, 1981). In organisms that show an increase in yield when grown chemolithoheterotrophy it is possible that the proportion of organic substrate utilised in anabolic metabolism for production of ATP is partially replaced by the anabolic breakdown of molecules like thiosulfate. This shift in ATP production allows more of the organic substrate to be used in anabolic production of biomass. However the kinetic physiology of this mixed form of metabolism has had very little study and is poorly understood. Ecologically organisms that exploit both organic and inorganic compounds may have a significant advantage in the environment. An important question to answer is whether a heterotroph that can use thiosulfate to “top up” energy production could out compete an obligate chemolithoautotroph. So far this appears not to have been the topic of any major research. An alternative advantage that has been briefly studied in the past is the toxicity of a thiosulfate/tetrathionate mixture on growing cells of various *Gammaproteobacteria*, such as *Escherichia coli* (Palumbo & Alford, 1970). The production of tetrathionate in combination with any environmental thiosulfate already present could be a strategy used to inhibit any competition.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals used throughout were of analytical grade unless stated otherwise. Chemicals were obtained from Sigma-Aldrich Corporation (MO, USA) unless stated otherwise. Sodium thiosulfate was from Fisher Scientific and all sodium polythionates were provided by Dr. Rich Boden (University of Plymouth, UK) that were prepared according to Boden *et al.* (2010). Water used in all experiments was glass-distilled de-ionised water and all glass ware was class A and acid-washed. Media were autoclaved at 121 °C for 15 minutes unless stated otherwise.

2.2 Culture media

2.2.1. Basal media

E-basal salts (EBS) medium was used for all batch and continuous growth experiments.

Table 2.1 Composition of E-basal salts medium.

| | g L ⁻¹ |
|---------------------------------|-------------------|
| Potassium dihydrogen phosphate | 3.2 |
| Dipotassium phosphate | 3.2 |
| Ammonium chloride | 0.4 |
| Magnesium chloride heptahydrate | 0.8 |
| Trace metal solution | 10 mL |
| Vitamin solution | 0.1 mL |

Growth substrates were also added to the desired concentration prior to autoclaving or were added from a filter sterilised stock solution when autoclaving was not appropriate. Phosphates were sterilised separately to prevent precipitation. Solid media were prepared with addition of 10 g granulated agar/L (Melford, Suffolk, UK).

2.2.2. Trace metal solution T

This was prepared according to Tuovinen and Kelly (1973).

Table 2.2 Composition of trace metal solution.

| | g/L |
|-------------------------------------|------|
| Sodium hydroxide | 11.0 |
| Disodium EDTAate | 50.0 |
| Ammonium molybdate tetrahydrate | 0.5 |
| Calcium chloride dihydrate | 7.34 |
| Copper(II) sulfate pentahydrate | 0.2 |
| Cobalt(II) chloride hexahydrate | 0.5 |
| Iron(II) sulfate heptahydrate | 5.0 |
| Manganese(II) chloride tetrahydrate | 2.5 |
| Zinc sulfate | 11.0 |

The pH of the solution was adjusted to 6.0 using 1 M HCl and diluted to 1 litre in a volumetric flask with ddH₂O. The final solution was stored non-actinic glass ware.

2.2.3. Vitamin solution

This was prepared according to Kanagawa *et al.* (1982).

Table 2.3 Composition of vitamin solution

| | mg L ⁻¹ |
|--|--------------------|
| Thiamine (B ₁) | 10.0 |
| Nicotinic acid (B ₃) | 20.0 |
| Pyridoxine (B ₆) | 20.0 |
| Sodium <i>p</i> -aminobenzoic acid (“B10”) | 10.0 |
| Riboflavin (B ₂) | 20.0 |
| Biotin (B ₇) | 1.0 |
| Cyanocobalamin (B ₁₂) | 1.0 |

Once dissolved, the solution was filter sterilised and stored in a glass bottle at 4 °C.

2.2.4 Nutrient broth

This was prepared using Oxoid No. 2 CM0067 premix (Thermo Scientific) according to the manufacturer's instructions. The final composition of the medium was as follows.

Table 2.4 Composition of nutrient broth

| | g L ⁻¹ |
|--------------------|-------------------|
| 'Lab-Lemco' powder | 10.0 |
| Peptone | 10.0 |
| Sodium chloride | 5.0 |

The final solution (pH 7.2) was dispensed into flasks and autoclaved.

2.3 Maintenance of bacterial strains

2.3.1. *Pseudomonas* spp.

Strains were maintained on EBS agar slopes supplemented with 10 mM glucose. *Pseudomonas* spp. were sub-cultured every two weeks and incubated at 30 °C for 24 hours to grow and stored at 4 °C. Every four weeks strains were streaked onto EBS agar plates supplemented with 10 mM glucose in order to check purity.

2.3.2. *Achromobacter* spp.

Strains were maintained on EBS agar slopes supplemented with 10 mM succinate. *Achromobacter* spp. were sub-cultured every two weeks and incubated at 30 °C for 24 hours and stored at 4 °C. Every four weeks strains were streaked onto EBS agar plates supplemented with 10 mM succinate in order to check purity.

2.3.3. *Thermithiobacillus* spp.

Strains were maintained on EBS agar slopes supplemented with 20 mM thiosulfate. *Thermithiobacillus* spp. were sub-cultured every two weeks and incubated at 42 °C for 24-48 hours to allow growth and then stored at 4 °C. Every four weeks strains were streaked onto EBS agar plates supplemented with 20 mM thiosulfate in order to check purity.

2.4. Batch culture

2.4.1 Heterotrophic batch culture

Aerobic batch growth experiments were carried out using 50 mL culture volumes in 250-mL Erlenmeyer flasks stopped with foam bungs. Cultures were inoculated with 10% (v/v) of cell suspension from washed agar slopes. The slopes were washed with sterile media identical to the growth media to ensure accurate analytical work. Growth was monitored by removing culture and measuring optical density (*OD*) in a Jenway 7315 spectrophotometer (Jenway, Staffordshire, UK) and pH was monitored with an Orion Star™ (3 Star) pH meter fitted with a Ag/AgCl₂ combination pH electrode (Nijkerk, Netherlands). When larger sample sizes were needed for analytical work, experiments were carried out using 500 mL culture volume in 2,500-mL glass Erlenmeyer flasks. For assaying of growth substrates and other compounds, culture was removed in to 1.5 mL Eppendorf tubes or 15 mL falcon tubes (depending on amount needed) and centrifuged at $14,000 \times g$ at 4 °C for 20 minutes in a Eppendorf Centrifuge 5418 (Eppendorf, Hertfordshire, UK) or a Harrier 18/80 refrigerated centrifuge (MSB, London, UK) in

order to pellet cells. The clear supernant was then removed into fresh tubes and stored at -20 °C until required.

2.4.2 Autotrophic batch culture

Conditions for autotrophic batch growth were almost identical to heterotrophic batch growth except when flasks were removed from incubation they were left for 5 minutes for elemental sulfur to settle before samples were taken for *OD*, pH and analytical measurements. This was most apparent when *Thermithiobacillus* sp. ParkerM and *Thermithiobacillus tepidarius* were grown on thiosulfate as high levels of elemental sulfur were produced during early stages of growth.

2.4.3 Optimal growth temperature determination

Batch cultures in 250-mL Erlenmeyer flasks were initiated as previously described (2.4.1) and incubated at a range of different temperatures using a Model G25 Incubator Shaker (New Brunswick Scientific, NJ, U.S.A.). *OD*₄₄₀ was monitored and used to calculate specific growth rates (2.6.1.2).

2.4.4 Optimal growth pH

Experiments involving heterotrophic strains were carried out by adjusting the pH of nutrient broth with the addition of 1 M hydrochloric acid or 1 M sodium hydroxide to nutrient broth prior to autoclaving and the pH aseptically checked when cooled. In the case of autotrophic strains, the pH was adjusted in EBS medium by altering the ratio of potassium dihydrogen phosphate and dipotassium phosphate. As a result the pH range achieved with EBS was more limiting. Cultures were incubated in a Model G25 Incubator Shaker (New Brunswick Scientific, NJ, U.S.A.) and *OD*₄₄₀ was monitored and used to calculate specific growth rates (2.6.1.2).

2.5. Continuous growth experiments

Aerobic heterotrophic growth in continuous culture was carried out in a Fermac 360 Chemostat (Electrolab, Tewkesbury, UK) with a vessel volume (V) of 491.5 ± 0.5 mL. Temperature, pH and dissolved oxygen were monitored using the Fermac 360 control panel and stored electronically using Fermentation Manager software and in a log book. EBS supplemented with an appropriate growth substrate was pumped into the chemostat vessel by a MP-3 Micro Tube pump (Eyela, Tokyo, Japan) at varying flow rates from a 10 L sterilised medium vessel and the subsequent waste overflow collected in a sterile 10 L carboy. Desired pH was monitored using a 200 mm F695 pH probe (Broadley-James Ltd, Bedford, UK) and maintained by automatic titration of 1 M sulfuric acid and 1 M sodium hydroxide. To prevent the build-up of foam on the surface of the culture 0.5% Anti-foam 289 (Sigma-Aldrich) was titrated into the vessel when any foam was detected. Temperature was maintained by a heated jacket and cooled by a water fed cooling rod. Air flow into the vessel was achieved by a Rena Air 400 aquarium air pump (Rena, France). Dissolved oxygen (DO) levels were monitored with a D340 Oxyprobe dissolved oxygen sensor (Broadley-James Ltd, Bedford, UK). Stirring rates (100-350 r.p.m.) and air flow ($600-1000 \text{ mL min}^{-1}$) rates were adjusted according to the level of DO present within the culture medium.

Continuous culture was started by inoculating with 50 mL of batch culture cells pumped into the vessel from a sampling port. The chemostat was left in batch culture until late exponential phase and continuous culture started by switching on of the media pump and pH control. OD was monitored by the removal of culture using the sampling port and measured using a Jenway 7315 spectrophotometer (Jenway, Staffordshire, UK). Samples for analytical work were collected in the same manner and processed as batch culture

samples (2.4.1.). The chemostat was checked for purity daily by streaking samples on to EBS agar supplemented with 10 mM glucose (*Pseudomonas* spp.) or 10 mM succinate (*Achromobacter* spp.). A Gram stain was also performed daily and checked microscopically. A culture was determined to be in a steady-state when *OD* readings were stable for the time period in which five *V* had passed through the chemostat.

Aerobic growth in continuous culture of autotrophs (*Thermithiobacillus* spp.) was performed under the same conditions as heterotrophs with some exceptions. The base solution used in pH control was 1 M sodium carbonate. This not only altered the pH but maintained a suitable supply of CO₂ to ensure it was not the limiting component. Culture purity was checked using the Gram stain and by streaking samples on EBS agar supplemented with 20 mM thiosulfate.

2.6 Growth kinetics (Pirt 1965, 1975, 1982)

2.6.1 Batch growth kinetics

2.6.1.1 Determination of yields (*Y*)

Yields in the form of amount of dry biomass formed (2.7.1) per unit substrate were calculated according to equation 2.1.

$$Y = \frac{(X_f - X_0)}{S} \quad (\text{Equation 2.1})$$

Where X_f and X_0 represent final amount of biomass and initial amount of biomass respectively and S represents amount of growth substrate.

2.6.1.2 Determination of specific growth rate (μ)

OD_{440} measurements were taken at known time points and the specific growth rate with regards to dry biomass was calculated by equation 2.2 and expressed h^{-1} .

$$\mu = \frac{\Delta X}{\Delta t} \quad (\text{Equation 2.2})$$

Where $\ln A$ and $\ln B$ refer to natural logarithm of amounts of biomass at two progressive time points.

2.6.1.3 Determination of specific rate of substrate uptake (q)

Media samples were taken through-out batch culture experiments and assayed for the growth substrate present and q estimated by equation

$$q = \frac{\Delta S}{\Delta t} \quad (\text{Equation 2.3})$$

Where S represents amount of substrate and t represents time.

2.6.2. Chemostat kinetics

2.6.2.1. Determination of Steady State yields (Y)

Steady-state yields were calculated according to equation 2.4 after 5 vessel volumes worth of media had been pumped through the chemostat and the OD had not varied by more than 1% during this period.

$$Y = \frac{X}{S} \quad (\text{Equation 2.4})$$

Where X refers to amount of biomass formed and S refers to amount of substrate consumed.

2.6.2.2. Determination of Maximum Theoretical Growth yield (Y_{MAX})

Calculation of Y_{MAX} was achieved using a hyperbolic model of D and Y of multiple chemostat steady states.

2.6.2.3. Determination of Maintenance Energy Coefficient (m_s)

Calculation of m_s was achieved using the slope of a hyperbolic model (2.6.2.2) of multiple chemostat steady state cultures.

2.6.3.4. Determination of maximum specific growth rate (μ_{MAX})

A steady state was reached at the highest dilution rate used during continuous growth. Preceding this, the dilution rate was increased dramatically to a known amount to induce “washout” and OD_{440} measured at timed intervals. D_{crit} was calculated and used as an estimation of μ_{MAX} according to equation 2.5.

$$D_{crit} = \frac{\Delta X}{\Delta t} - D \quad (\text{Equation 2.5})$$

Where $\ln A$ and $\ln B$ refer to the natural logarithm of amount of biomass at two progressive time points, t refers to this time in hours and D to washout dilution rate (h^{-1}).

2.7. Analytical methods

Unless stated otherwise, all analytical techniques were carried out in acid-washed Class A volumetric glassware. Determination of ATP and ADP concentrations were carried out in

glass scintillation vials that had been previously soaked in sodium hypochlorite solution ($\geq 8\%$ available chlorine) for 24 hours before rinsing with deionised distilled water and drying at 60 °C. Unless otherwise stated, all spectrophotometric assays were performed in polycarbonate cuvettes with a path length of 1 cm and 1 mL volume or when a wavelength of < 320 nm was used 1cm path length 1 mL optical quartz cuvettes were used. A Jenway 3715 spectrophotometer (Bibby Scientific Limited, Staffordshire, UK) was used for all optical density and absorbance measurements, unless stated otherwise. Unless stated otherwise all measurements were conducted in triplicate and error bars represent the standard error of the mean (SEM).

2.7.1. Determination of biomass

Chemostat grown cells were harvested on ice overnight and centrifuged in a Harrier 18/80 refrigerated centrifuge (MSB, London, UK) at $5000 \times g$ for 20 minutes at 4 °C and the supernant removed. Pellets were re-suspended in ice-cold PBS and re-centrifuged. This was repeated twice and the final pellet was re-suspended in ice-cold PBS to produce a thick suspension. Measured amounts of cell suspensions were added into pre-weighed scintillation vials and PBS used to dilute samples to create a series of dilutions to a finished volume of 10 mL. Optical densities at 440, 500 and 600 nm were taken from each vial before being placed back into the vial from which it was removed. Suspensions were placed into an MINO/6 oven at 70 °C (GenLab Limited, UK) and the vials were weighed daily until a constant weight was achieved. A set of three vials filled with 10 mL sterile PBS that lacked cells was treated in the same way and the dry weight of these were deducted from cell-containing vials. Calibration curves of biomass concentrations versus *OD* were produced from each organism, the results of which were used throughout this project. It was decided that an OD_{440} would be used due to it being the most sensitive to

increases in biomass; this is highlighted in Figure 2.1. The values used for each organism are presented in Table 2.5.

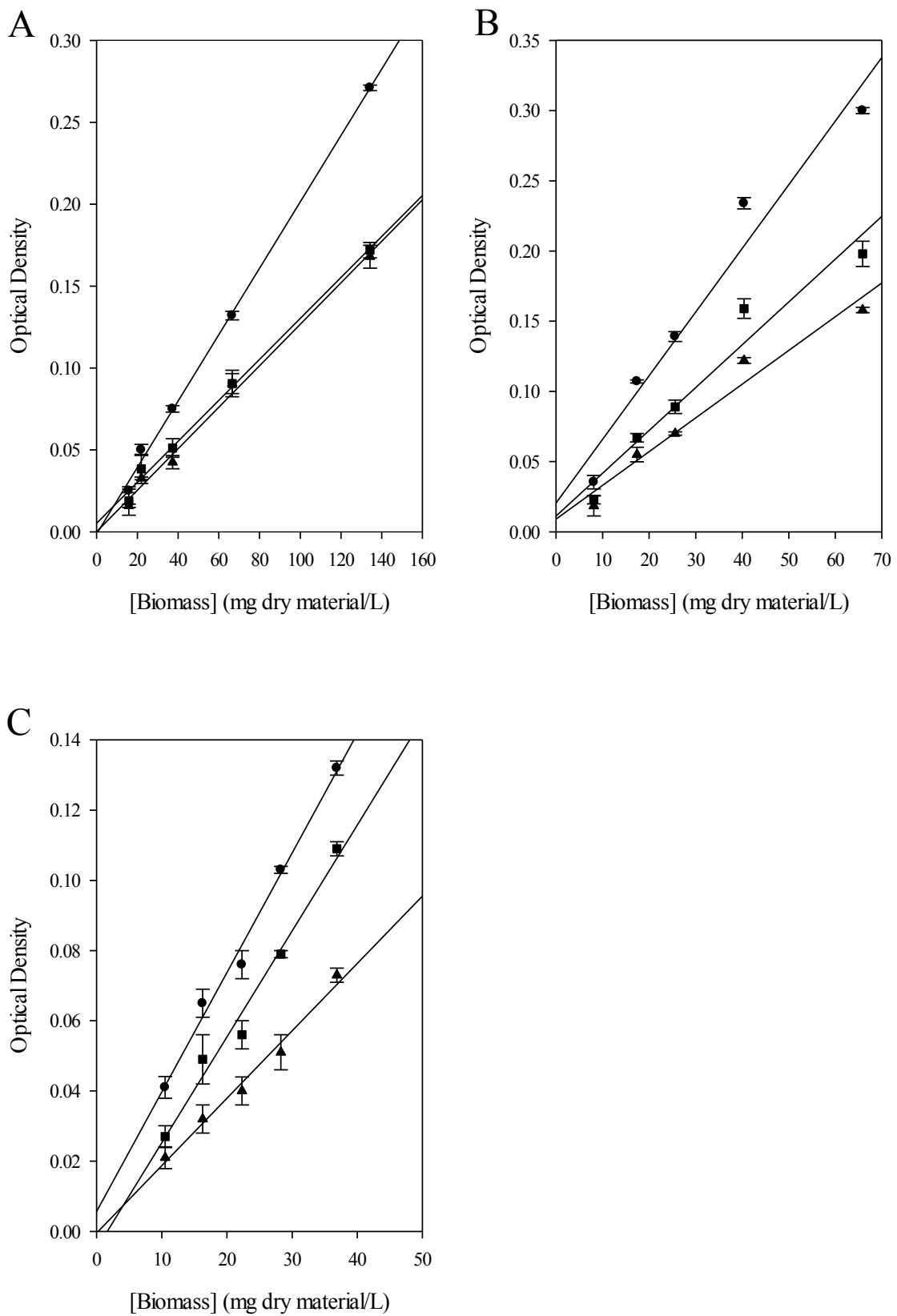


Figure 2.1 Calibration of culture optical densities at 440 nm (●), 540 nm (■) and 600 nm (▲) versus concentration of dry biomass (mg/L) of *Pseudomonas* sp. Strain T (A), *Achromobacter* sp. Strain B (B) and *Thermithiobacillus* sp. ParkerM (C).

Table 2.5 Biomass concentrations at OD_{440} for bacterial strains used in this project. * value obtained from Wood & Kelly (1986).

| Organism | Biomass concentration/ OD_{440} 0.1 (mg dry material/L) |
|---------------------------------------|--|
| <i>Pseudomonas</i> sp. Strain T | 49.5 |
| <i>Achromobacter</i> sp. Strain B | 18 |
| <i>Thermithiobacillus</i> sp. ParkerM | 28 |
| <i>Thermithiobacillus tepidarius</i> | 23* |

2.7.2. Determination of Glucose

Glucose concentrations were measured using the Glucose assay kit GAGO-20 (Sigma-Aldrich) according to the manufactures instructions and with reference to a calibration curve of known glucose standards. Samples containing thiosulfate where assayed alongside glucose standards that also contained the same concentration of thiosulfate as it was found to interfere. When assaying for glucose at concentrations higher than 0.5 mM, 10-fold or 100-fold dilutions were made in order to get accurate data.

2.7.3. Determination of succinate

This was achieved with the Succinic Acid assay kit K-SUCC (Megazyme International, Ireland) following the microplate protocol. Sample absorbance at 340 nm was read in a Versa Max plate reader (Molecular Devices, USA) and with reference to a calibration curve the concentration of succinate was determined. Samples were diluted 1/10 in order to get linearity.

2.7.4. Determination of thiosulfate, tetrathionate and trithionate.

Amounts of thiosulfate, tetrathionate and trithionate were determined using the colorimetric methods developed by Kelly *et al.* (1969).

2.7.5 Determination of ammonium

This was determined according to Solórzano (1969) in acid washed glass test tubes with a final volume of 6.0 mL. Concentrations were determined with reference to a calibration curve of ammonium standards (Fisher Scientific).

2.7.6 Determination of phosphates

This was achieved by the Malachite Green Phosphate Detection Kit (R & D Systems, Abingdon, UK) following the microplate protocol. Sample absorbance were read at 620 nm in a Versa Max plate reader (Molecular Devices, California, USA) and with reference to a calibration curve concentrations of phosphates were determined.

2.7.7. Determination of guanine plus cytosine genomic DNA content

Guanine plus cytosine content was determined as described by Fredericq *et al.* 1961. 900 μ L of 0.11 M acetic acid, pH 3 was placed into a 1 mL cuvette to which 100 μ L of purified genomic DNA was added. The solution was mixed by inversion and the absorbance was measured at 260 nm and 280 nm. The mol % G + C was calculated by equation 2.9.

$$Ratio (R) = 260/280$$

$$mol \% G + C = \frac{22400 - 9300 R}{5100 + 11200 R} \times 100 \quad (\text{equation 2.9.})$$

2.7.8. Respiratory quinone extraction and detection

Quinones were extracted according to DiSpirito *et al.* (1983) but with a few modifications. Six grams of wet biomass was suspended in 15 mL PBS and sonicated using a Vibra-Cell VCX 130PB fitted with a model CV188 probe sonicator set to 60% amplitude for 3

minutes at 4 °C. The sonicates were poured into 250 mL Erlenmeyer flasks with QuickFit necks and 180 mL acetone added. Glass stoppers were fitted immediately after addition of acetone and flasks were shaken at 60 r.p.m. at 4 °C for 12 hours. Samples were centrifuged at $9,000 \times g$ for 20 minutes at 4 °C. The yellow supernants were passed through Whatman No 1 filter paper into 500 mL glass round-bottomed QuickFit flasks and rotary evaporated. Ubiquinones were then dissolved in 180 mL *n*-hexane in the same round-bottomed flasks. This was then transferred to a 500 mL separatory funnel and mixed with 90 mL ddH₂O. Funnel was left upright to allow water and hexane to separate, the *n*-hexane was collected and the water phase discarded. This procedure of “washing” was repeated 5 times. The final *n*-hexane phase was collected in a glass beaker and 5g of anhydrous sodium sulfate added until all ddH₂O had been absorbed before being filtered through Whatman No 1 filter paper into a fresh 500 mL glass round-bottomed QuickFit flask and rotary evaporated until only yellow oil was left. The yellow oil was then dissolved in 2 mL spectral grade ethanol. Quinone extract was stored in amber cryovials at -20 °C until required.

Quinone extracts from *Escherichia coli* NCTC 9001 (UQ-8) and *Pseudomonas aeruginosa* 17B (UQ-9) were spotted (0.1 mL) using capillary tubes onto TLC (Silica Gel 60 F₂₅₄). Hexane:diethyl ether (20:80) was used as the mobile phase in order to obtain an *R_f* value from quinone bands which were detected under UV light.

2.7.9. Determination of cellular fatty acid methyl esters (FAME)

FAME analyses was carried out by the Identification Service of the DSMZ, Braunschweig, Germany. *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B had previously grown on nutrient agar for 48 h while *Thermithiobacillus* spp. were grown on EBS agar supplemented with 10 mM tetrathionate for 72 h.

2.7.10 Determination of total protein

Protein content of samples were estimated using the Bicinchoninic Acid Protein Assay Kit (BCA1) (Sigma-Aldrich) according to the manufacturer's instructions. In brief; 200 μL of BCA Working Reagent and 25 μL of protein sample were mixed in a 96-well plate and incubated for 30 minutes at 37 °C. Absorbance was read at 562 nm in a Versa Max plate reader (Molecular Devices, California, USA) against bovine serum albumin standards (P0914, Sigma-Aldrich).

2.7.11 Oxygen uptake by whole cells

Oxygen uptake rates were measured in a water-jacketed Perspex® chamber equipped with a Clarke oxygen electrode (Rank Brothers, Cambridge, UK) under a 0.1 mm PTFE membrane. The chamber was kept at a constant temperature by using a Grant TC120 heated circulating water bath (Grant Instruments, Cambridge, UK). The electrode was regularly calibrated by adding sodium dithionite. During oxygen uptake experiments cells in 0.1 M potassium phosphate buffer (pH 7.2) and left to equilibrate. Oxygen saturation was recorded for three minutes followed by addition of a substrate under investigation to give a final volume of 3 mL and oxygen saturation recorded for ≥ 5 minutes. It was assumed that saturated buffer at 30, 35 and 45 °C contained 230, 209 and 192 nmol O₂ mL⁻¹, respectively (Lu & Kelly, 1988*a*; Gnaiger, 2001).

2.7.12 Cytochrome spectra

Spectra were obtained from CFEs at room temperature (22 °C) unless stated otherwise. Cytochromes were oxidised by pumping air through the sample, the value of which were then deducted from substrate reduced cytochrome spectra in order to obtain difference spectra. To fully reduce any cytochromes present an excess of sodium dithionite was added.

2.7.13 Determination of cellular ATP and ADP

Experiments were carried out in 3 mL suspensions of whole cells in 0.1 M potassium phosphate buffer (pH 7.2) stirred in a heated oxygen electrode chamber set to the appropriate temperature. After addition of substrate, 250 μ L of cell suspension was removed at 15 s intervals into 1.45 M PCA in pre-bleach washed scint vials. Solutions were left on ice for 10 minutes and neutralised with 1.9 mL 0.6 M KOH solution to a final pH of 7.2. This was left for another 10 minutes for the supernant to clear, which was carefully removed into fresh scintillation vials (Kelly & Syrett, 1966).

ATP and ADP content of supernants were measured with the MAK135 ADP/ATP Ratio Assay Kit (Sigma-Aldrich) according to manufactures instructions. Light emission was read in a Pi-102 luminometer (Hygiena International) to give relative light units (RLU). By referring to an ATP calibration curve the absolute ATP/ADP content was estimated.

2.8. Preparation of cell-free extracts

2.8.1. Harvesting cells

Cells were harvested from chemostat cultures and centrifuged for 20 minutes at $6,000 \times g$ in order to pellet cells before being washed in ice cold PBS and re-centrifuged twice. The final cell pellet was re-suspended in ice-cold PBS to form a thick paste before being frozen in liquid nitrogen as small beads and stored at $-20\text{ }^{\circ}\text{C}$.

2.8.2 Disruption of cells

Desired amounts of frozen cell beads were defrosted rapidly at $37\text{ }^{\circ}\text{C}$ and then placed on ice. Sonication of cell paste was performed in a $4\text{ }^{\circ}\text{C}$ temperature controlled room using a

Vibra-Cell VCX 130PB fitted with a model CV188 probe sonicator set to 60% amplitude. Eight cycles of 1 minute sonication followed by 2 minutes of a cooling down period were performed on cell suspensions. Resulting suspensions were referred to as crude extracts. These were centrifuged at $14,000 \times g$ for 20 min in order to pellet unlysed cells and cellular debris. The supernants were removed and were referred to as cell-free extracts (CFE). CFEs that were not being used within 2 h were frozen in 1 mL aliquots by submerging in liquid nitrogen.

2.9. Enzyme assays

All enzyme activities were measured in polycarbonate cuvettes with a path length of 1 cm and absorbance's read in a Jenway 3715 spectrophotometer (Bibby Scientific Limited, Staffordshire, UK) fitted with a 73 series Peltier. Each assay was performed in triplicate at the temperature the organism was grown, unless stated otherwise.

2.9.1. Inorganic sulfur metabolism (Kelly & Wood, 1994)

2.9.1.1. Thiosulfate dehydrogenase (EC 1.8.2.2)

Activities were measured based on Trudinger (1964*a*). The assay was conducted in 50 mM citrate buffer (pH 5.0 or 5.5) with final concentrations of 3 mM ferricyanide and 10 mM thiosulfate. The reaction was started by addition of 100 μ L CFE and the reduction of ferricyanide to ferrocyanide measured at 420 nm for three minutes. Reactions were allowed to equilibrate for 5 min before addition of CFE. An endogenous rate of ferricyanide reduction was taken with each experiment by substituting thiosulfate with citrate buffer. A blank was also performed for quality control purposes by substituting CFE with PBS. Using the extinction coefficient of $1.0 \text{ mM}^{-1} \text{ cm}^{-1}$ the activity was

estimated and the endogenous rate subtracted. Rates of activity were normalised against protein concentrations of CFEs. Activities were expressed as amount of ferricyanide reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.1.2 Sulfite dehydrogenase (EC 1.8.2.1), thiocyanate dehydrogenase and trithionate hydrolase (EC 3.12.1.1)

Activities of sulfite dehydrogenase, thiocyanate dehydrogenase and trithionate hydrolase were measured according to Kelly & Wood (1994). The methods were similar to that of thiosulfate dehydrogenase but with the respective substrates used instead of thiosulfate. The activity of trithionate hydrolase activity was estimated indirectly by the 1:1 ratio formation of thiosulfate from trithionate. The thiosulfate molecules then became substrate for thiosulfate dehydrogenase which in turn reduced ferricyanide.

2.9.1.3 Tetrathionate hydrolase

Activity of this enzyme was measured with a discontinuous method. A series of test tubes were set up that contained 2.2 mL 0.1 M potassium phosphate buffer and 300 μL of CFE prepared in the same buffer. After incubating in a water bath set to the desired temperature for 10 minutes the reaction was started by addition of 300 μL of 0.1 M tetrathionate prepared in the same phosphate buffer. Reactions were stopped by addition of 500 μL 12 M formaldehyde solution (Sigma-aldrich). Solutions were then assayed for tetrathionate *via* cyanolysis (2.7.4) and specific activity expressed as nmol tetrathionate oxidised $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.1.4 Sulfur oxygenase (EC 1.13.11.55)

Sulfur oxygenase activity was measured by oxygen uptake described by Kelly & Wood (1994). Assays were performed in a water-jacketed Perspex chamber equipped with a Clarke oxygen electrode (Rank Brothers, Cambridge, UK). A wetted sulfur suspension

was prepared by mixing finely ground flowers of sulfur in 60 mL ddH₂O containing 0.05% (v/v) Tween® 80. The mixture was mixed briskly in order to form a hydrophilic suspension (Suzuki *et al.* 1999). 300 µL of the wetted sulfur suspension was added to 2.4 mL 0.1M potassium phosphate buffer pH 7.2 within the oxygen electrode and allowed equilibrate to the desired temperature and to become fully oxygenated. The reaction was started by addition of 300 µL of whole cells suspensions in the same potassium phosphate buffer and the percentage oxygen saturation monitored every 30 s. CFEs were found to have no effect on oxygen saturation in any of the strains examined. A negative control comprised of the same method except the 0.05% (v/v) Tween® 80 contained no sulfur. Specific activity was expressed as nmol O₂ consumed min⁻¹ (mg dry biomass)⁻¹.

2.9.1.5 Sulfur dioxygenase (EC 1.13.11.18)

This assay was identical to the method for sulfur oxygenase (2.9.1.4) bar the addition of 1 mM reduced glutathione final concentration to the assay mixture. Any previously detected sulfur oxygenase activity was deducted from this rate in order to get a specific rate of sulfur dioxygenase activity. Specific activity was expressed as nmol O₂ consumed min⁻¹ (mg dry biomass)⁻¹.

2.9.2 TCA cycle enzymes

2.9.2.1 Citrate synthase (EC 4.1.3.7)

Citrate synthase activity was assayed according to Weitzman (1969). 100 µL 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 30 µL Coenzyme A solution (10 mg in 1,000 µL water containing 100 mM KHCO₃ and 13 µL acetic anhydride), 770 µL ddH₂O and 50 µL CFE were mixed by inversion in a cuvette and absorbance read at 412 nm every 5 seconds for 3 min in order to measure any possible acetyl-CoA hydrolase activity. The

reaction was started by addition of 50 μL 10 mM oxaloacetate and the increase in absorbance read for another 3 minutes. Any acetyl-CoA hydrolase activity detected was deducted from the citrate synthase activity and using the extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for 2-nitro-5-thiobenzoate (TNB) (the product of DTNB cleavage) the specific activity was calculated. Activity was expressed as amount of TNB formed $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.2.2 Aconitase (EC 4.2.1.3)

Aconitase activity was assayed according to Fanbler & Lowenstein (1969). 200 μL 0.1 M Tris-HCl, 200 μL of 0.5 M NaCl, 100 μL 1 mM cis-aconitate and 400 μL ddH₂O were mixed in a quartz cuvette with a path length of 1 cm. The reaction was started by the addition of 100 μL of CFE and the decrease in absorbance at 240 nm read for three minutes. An endogenous rate (containing no CFE) was subtracted from all sample rates and using the extinction coefficient of $4.88 \text{ mM}^{-1} \text{ cm}^{-1}$ for cis-aconitate enzyme activities were calculated. Specific enzyme activity was expressed as amount of cis-aconitate consumed $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.2.3 Isocitrate dehydrogenase (EC 1.1.1.41)

Isocitrate dehydrogenase activity was assayed according to Cook & Sanwal (1969). 100 μL 0.5 M Tris-HCl buffer (pH 7.4), 100 μL 10 mM disodium EDTA, 100 μL 200 mM MnSO₄, 100 μL 10 mM NAD⁺, 100 μL 10 mM potassium *D-threo*-isocitrate monobasic and 400 μL ddH₂O were mixed in a 1 mL silica cuvette. The reaction was started by the addition of 100 μL CFE and the increase in absorbance at 340 nm read every 5 seconds for three minutes. The endogenous rate (no CFE) was deducted from all samples and using the extinction $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ for NADH the activities were calculated. Specific activities were expressed as amount of NADH formed $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.2.4 α -ketoglutarate dehydrogenase (EC 1.2.4.2)

α -ketoglutarate dehydrogenase activity was assayed according to Reed & Mukherjee (1969). 100 μ L 1 M Tris-H₂SO₄ buffer (pH 7.4), 100 μ L 0.1 M KCN (made fresh in Tris-H₂SO₄), 100 μ L 6 mM ferricyanide and 100 μ L CFE were mixed by inversion and absorbance read at 420 nm every 5 seconds in order to obtain an endogenous rate. Reactions were started by addition of 100 μ L 100 mM α -ketoglutarate and the decrease in absorbance read every 5 seconds for an additional 3 min. The endogenous rate was subtracted and the extinction coefficient of 1.0 mM⁻¹ cm⁻¹ for ferricyanide was used to calculate activity. Specific activities were expressed as amount ferricyanide reduced min⁻¹ (mg protein)⁻¹.

2.9.2.5 Succinyl-CoA synthetase (EC 6.2.1.5)

Succinyl-CoA synthetase activity was assayed according to Bridger *et al.* (1969). An assay mixture was made containing 50 mM Tris-HCl buffer (pH 7.2), 100 mM KCl, 10 mM MgCl₂, 10 mM sodium succinate and 0.4 mM Na₂ATP. 890 μ L of the assay mixture and 10 μ L of 10 mM CoA were mixed in a quartz cuvette and the reaction started by addition of 100 μ L of CFE and the absorbance read at 230 nm every 5 seconds for 3 minutes. An endogenous rate (no CFE) was deducted from all samples and using the extinction coefficient of 4.5 mM⁻¹ cm⁻¹ for the formation of succinyl-CoA the activities were calculated. Specific activities were expressed as amount of succinyl-CoA formed min⁻¹ (mg protein)⁻¹.

2.9.2.6 Succinate dehydrogenase (EC 1.3.99.1)

Succinate dehydrogenase activity was assayed according to Veeger *et al.* (1969). 100 μ L 1 M potassium phosphate buffer (pH 6), 100 μ L 0.1 M KCN, 100 μ L 1 mM 2,6-

dichlorophenolindophenol (DCPIP), 500 μL ddH₂O and 100 μL CFE were mixed by inversion and absorbance read at 600 nm every 5 seconds in order to obtain an endogenous rate. Reactions were started by addition of 100 μL 400 mM succinate and the decrease in absorbance read every 5 s for an additional 3 minutes. Using the extinction coefficient of 16.9 $\text{mM}^{-1} \text{cm}^{-1}$ and by deducting the endogenous rate the specific activity was calculated and expressed as amount of DCPIP reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.2.7 Fumarase (EC 4.2.1.2)

Fumarase activity was measured in reverse according to Hill & Bradshaw (1969) by adding 500 μL 0.1 M potassium phosphate buffer (pH 7.2), 300 μL ddH₂O and 100 μL CFE to a cuvette and mixed by inversion and absorbance read at 250 nm to detect the formation of fumarate every 5 seconds to obtain an endogenous rate. Reactions were started by addition of 100 μL 0.5 M L-malate and the increase in absorbance read every 5 seconds for an additional 3 min. Using the extinction coefficient of 1.45 $\text{mM}^{-1} \text{cm}^{-1}$ and by deducting the endogenous rate the specific activity was calculated and expressed as amount of fumarate formed $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.2.8 Malate dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase activity was measured according Murphey & Kitto (1969). 100 μL 1 M Tris-H₂SO₄ buffer (pH 7.4), 100 μL 1 M KCl, 100 μL 0.1 M KCN (made fresh in Tris-H₂SO₄), 100 μL 6 mM ferricyanide, 580 μL ddH₂O and 100 μL CFE were mixed in a cuvette by inversion and absorbance read at 420 nm at 5 second intervals to obtain an endogenous rate. The reaction was started by addition of 20 μL 0.5 M L-malate (made in Tris-H₂SO₄ buffer) and the decrease in absorbance read for an additional 3 minutes. The extinction coefficient of 1.0 $\text{mM}^{-1} \text{cm}^{-1}$ for ferricyanide was used to calculate specific activity and were expressed as amount ferricyanide reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.3 Carbohydrate metabolism enzymes

2.9.3.1 Glucose dehydrogenase (EC 1.1.1.47)

Activities were estimated according to Haugue (1966) by adding 100 μL CFE to 0.1 mM DCPIP in 0.1 M potassium phosphate buffer (pH 6.0) and the reduction of DCPIP measured at 600 nm which gave an endogenous rate. Glucose was added to give a final concentration of 20 mM and the apparent rate measured; from this the endogenous rate was deducted. Using the extinction coefficient of $16.9 \text{ mM}^{-1} \text{ cm}^{-1}$ the enzyme activity was calculated and normalised against protein content in the CFEs. Specific activities were expressed as amount of DCPIP reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.3.2 Glucokinase (EC 2.7.1.2)

Activities were measured by mixing 0.1 mL 0.15 M glucose solution, 0.1 mL 0.2 M magnesium chloride solution, 0.1 mL 0.2 M Tris-NCl buffer (pH 7.6), 0.1 mL 0.1 mM Na_2EDTA , 0.1 mL 1.3 mM NADP and 0.1 mL 2 U/mL glucose-6-phosphate dehydrogenase, 0.1 mL CFE and 0.23 mL ddH_2O in a 1 mL cuvette. The A_{340} of this mixture was monitored every 5 seconds for three minutes to obtain an endogenous rate. Reactions were started by addition of 0.033 mL 0.3 M Na_2ATP and A_{340} monitored every 5 seconds for an additional three minutes. The endogenous rate was deducted and specific activities expressed as amount of NADP reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.9.3.2 Glucose-6-phosphate dehydrogenase (EC1.1.1.49)

Activities were measured by mixing 500 μL 200 mM Tris-HCl buffer (pH 8.0), 100 μL 3 mM glucose-6-phosphate, 100 μL 1 mM NADP^+ , 100 μL 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 100 μL ddH_2O were mixed in a silica cuvette and the reaction started by the addition of 100 μL CFE. The increases in absorbance at 340 nm were read every 5 s for three minutes

after an initial incubation at room temperature for 1 minute. An endogenous rate was deducted from all samples and using the extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ for NADPH the activities were calculated. Specific activities were expressed as amount of NADPH formed $\text{min}^{-1} (\text{mg protein})^{-1}$.

2.10 Microscopy

2.10.1 Light microscopy

All staining procedures were carried out on ethanol washed glass microscope slides. Cells in liquid medium were spread on to a slide with use of another clean slide and allowed to dry to form a smear. When preparing cells from a solid media a small amount of sterile PBS was spread onto a washed slide and used to “wash” cells from an inoculating loop onto the slide and allowed to dry. In both cases slides were then quickly passed through a Bunsen flame (cell side up) in order to fix cells. Post staining, cells were viewed with an Olympus light microscope at $\times 1000$ magnification with oil immersion.

2.10.1.1 Staining

The Gram, endospore and capsule stains were performed according to Harley & Prescott (1993) and polyphosphate and polysaccharide stains according to Doetsch (1981).

2.10.1.2 Ryu staining for flagella

Flagella were stained using the method of Kodaka *et al.* (1982). Only smears produced from liquid cultures and that were gently prepared were used in order to ensure flagella production by the strain under investigation.

2.10.2 Transmission electron microscopy (negative staining)

Cells were grown in liquid EBS supplemented with appropriate growth substrate. Once the culture had reached stationary phase a small drop was placed on a Formvar® and carbon coated copper grid and left for five minutes for cells to adhere. The grid was washed in 0.9% saline for 10 seconds and stained with 2.0% uranyl acetate for five minutes. Stained grids were washed in 0.9% saline for 10 seconds and inserted in to a Jeol (California, USA) JEM-1400 Transmission Electron Microscope for visualisation.

2.11 Chemotaxonomic tests

2.11.1 Hydrolysis of macromolecules

Hydrolysis of casein, DNA, starch and lipids were tested on all strains according to Harley & Prescott (1993); esculin hydrolysis according to Lennette *et al.* (1974) and Tween® hydrolysis according to Barrow & Feltham (1993). Sucrose hydrolysis (levan production) was tested according to Ceska (1971).

2.11.2 Element oxidation/reduction

The reduction of selenite and tellurite was tested in strains according to Rathgeber *et al.* (2002). It was found that thiosulfate as a growth substrate caused spontaneous reduction of selenite and so filter sterilised tetrathionate was used instead for autotrophs. The oxidation of manganese was tested for according to van Veen (1973).

2.11.3 Other media based chemotaxonomic tests

2.11.3.1 Antibiotic resistance

Mastrings® (Mast, Merseyside, UK) were used to test for antibiotic resistance according to manufacture instructions and using nutrient agar for heterotrophs and EBS agar supplemented with 20 mM thiosulfate for autotrophs.

2.11.3.2 IMViC test

The IMViC test was performed according to Simmons (1926) and Harley & Prescott (1993).

2.11.3.3 Haemolysis

Haemolysis was tested with heterotrophic strains using Columbia Blood agar plates (Oxoid) supplemented with sheep red blood cells according to Ryan & Ray (2004).

2.11.3.8 Pigment production

Pigment production was tested using slopes of *Pseudomonas* A medium (Oxoid) according to King *et al.* (1954). Slopes were checked daily for the production for pigments such as pyocyanine (blue colouration of medium), phenazine-2-carboxylate (yellow/orange crystals), oxychlororaphin (pale yellow colouration of medium), chlororaphin (green crystals) and fluorescein (yellow/green fluorescence under UV).

2.11.3.9 Urease activity

Urease activity was tested on slopes of urea agar (Oxoid) according to Christensen (1946).

2.11.4 Chemical detection of enzyme activities

2.11.4.1 Catalase and cytochrome *c* oxidase activity

Activities of catalase and cytochrome *c* oxidase were tested for according to Harley & Prescott (1993).

2.11.4.2 API[®] chemotaxonomic kits

A number of chemotaxonomic traits were identified with use of the Analytical Profile Index (API) test kits (Biomérieux, Lyon, France). Specifically the API-ZYM kit was used for the detection of certain enzyme activities, API-50CH kits were used for the detection of acid production from carbohydrates and API-20E kits were used for a multitude of different traits. All kits were used according to manufacture instructions.

2.12 Molecular methods

2.12.1 Genomic DNA extraction

Two methods were used to extract and purify DNA. The first was by using the FastDNA[®] SPIN Kit for Soil (MP Biomedicals, UK) according to the manufactures instructions. When liquid medium was used, 500 μ L of liquid culture was used for extraction instead of 500 μ g of soil.

The second method was by using a modification of the CTAB method in Current Protocols in Molecular Biology. In brief; cells were suspended in 14.8 mL TE buffer (10 mM Tris; 1 mM EDTA , pH 8.0) to $OD_{600} \approx 1.0$ and 400 μ L lysozyme stock (100mg/mL) was added and incubated at 37 °C for 30 minutes. 800 μ L 10% SDS and 160 μ L Proteinase K (10 mg/mL) was added, mixed and incubated for 2 h at 56 °C. Lysed

cells were confirmed by a “clearing of solution with increased viscosity. 2 mL 5 M NaCl and 2 mL CTAB/NaCl solution was added and mixed followed by a 10 minute incubation time at 65 °C. 10 mL of chloroform:isoamyl alcohol (24:1) was mixed with the solution and centrifuged immediately at $6000 \times g$ for 10 minutes. The aqueous phase was transferred to a clean centrifuge tube and 10 mL phenol:chloroform:isoamyl alcohol added, mixed and centrifuged immediately at $6000 \times g$ for 10 minutes. The aqueous phase was once again transferred to a clean centrifuge tube and 0.6 volume isopropanol added and incubated at -20 °C overnight. Solution was centrifuged at $6000 \times g$ for 15 minutes at 4 °C. Supernant was discarded and the pellet washed in 70% ice cold ethanol before centrifuging at $6000 \times g$ for 5 minutes at 4 °C. Supernant was discarded and the pellet allowed to dry at room temperature before being re-dissolved in 200 μ L DNase free water.

Purified DNA was run in a 1% agarose gel for 40 minutes at 90 volts and 120 mA with a 1kB DNA ladder. DNA quantity and purity were checked with a NanoDrop 2000 (Thermo Scientific)

2.12.2 Polymerase chain reaction and gene sequencing

All gene sequences were amplified using DreamTaq® (Thermo Scientific). The 16S rRNA (*rrs*) gene was amplified with the forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse primer 5'-AAGGAGGTGATCCANCCRCA-3' according to Lane (1991). The DNA gyrase subunit B gene (*gyrB*) was amplified with the forward primer 5'-GAAGTCATCATGACCGTTCTGCA YGCNGGNGGNAARTTYGA-3' and the reverse primer 5'-AGCAGGGTACGGATGTGCGAGCCRTC NACRTCNGCRTCNGTCAT-3' according to Fukushima *et al.* (2002). The recombinase A gene (*recA*) was amplified with

the forward primer 5'-TGTGCITTTATWGATGCIGAGCATGC-3' and the reverse primer 5'- CCCATGTCICCTTCKATTTTCIGCTTT-3' according to Holmes *et al.* (2004). Primers and excess nucleotides were removed from PCR products using Diffinity RapidTip[®]2 (Sigma-Aldrich) and sent to GATC Biotech (Cologne, Germany) for Sanger sequencing. Phylogenetic analysis and was performed using CLUSTALW alignments (Thompson *et al.* 1994) with MEGA 6 software (Tamura *et al.* 2007).

Chapter 3

Taxonomy of the heterotrophic sulfur-oxidising
strains of “*Thiobacillus trautweinii*”

3.1 Introduction

A number of heterotrophic *Bacteria* capable of oxidising thiosulfate to tetrathionate have been isolated and studied since 1921 from both marine and terrestrial environments (Trautwein, 1921; Starkey, 1934*a, b*; Sijderius, 1946; Trudinger, 1967; Tuttle *et al.* 1972; Tuttle *et al.* 1974; Tuttle, 1980; Kuenen & Beudeker, 1982; Mason & Kelly, 1988*a*; Sorokin, 1999; Sorokin *et al.*, 2006; Boden *et al.*, 2010). These *Bacteria* have no autotrophic capability and cannot grow on thiosulfate as the sole energy source as organic carbon is required. Most reports have stated that tetrathionate and other polythionates were the end products of thiosulfate oxidation, although other studies have indicated the full oxidation to sulfate (Pepper & Miller, 1978; Schook & Berk, 1978). Unlike mixotrophy, which has received extensive research in the past, this area of bacterial metabolism has received very little research and the benefits of chemolithoheterotrophy are still poorly understood.

One group of sulfur-oxidising heterotrophs that oxidise thiosulfate to tetrathionate has historically been designated as strains of “*Thiobacillus trautweinii*” (Bergey 1925), a species that has never been validly or effectively published. Of the main strains dubbed as “*T. trautweinii*”, the original strain was isolated between 1918-1920 by Trautwein from the sediments of the River Tauber, Germany (Strain T) and was placed in the *Genus Thiobacillus* due to its ability to oxidise thiosulfate (Trautwein, 1921). At the time this was thought to be a unique ability of the genus but in the decades that followed thiosulfate oxidation has shown to be a far more diverse trait that belongs to many different classes (Kelly & Wood, 2000*a*). The initial study into this organism found that thiosulfate was oxidised to tetrathionate and other polythionates but no further utilisation of inorganic sulfur was observed, *i.e.* thiosulfate was not fully oxidised to sulfate, as

observed in true *Thiobacillus* sp.. Starkey (1934b) isolated a second strain of “*T. trautweinii*” (“Strain B”) from soil in New Jersey, USA. It was found that in batch culture thiosulfate was indeed oxidised to tetrathionate and other polythionates by Strain T and Strain B, although this was apparently at a slow rate with *c.*90% of thiosulfate having “decomposed” over a 19 day period (Starkey, 1934b; Starkey, 1934c). A third unpublished “*T. trautweinii*” strain (Strain VO) was isolated from sphagnum peat in a dyke near Wijster, Netherlands by Trijntje van Hof in 1932, it was later characterised by Miss A van Oven and was named as such due to similarities with the original strain (Trautwein, 1921).

The taxonomy of the “*T. trautweinii*” strains has been a controversial issue ever since their inclusion into *Thiobacillus*. Chemotaxonomic work by Sijderius (1946), Parker & Temple (1957) and Hutchinson (1965) on “*T. trautweinii*” strains concluded they were closely related to *Pseudomonas* spp. while a physiological study by Parker and Prisk (1964) also highlighted differences between these strains and the genus *Thiobacillus*. The fatty acid profiles obtained by Agate and Vishniac (1973) of “*T. trautweinii*” strains, other strains of *Thiobacillus* and species that would later be placed into *Acidithiobacillus* and *Halothiobacillus* found that there were significant differences and some of the fatty acids (C_{6:0} and C_{8:0}) were found only in “*T. trautweinii*” strains. Further examination of their fatty acids and quinones by Katayama-Fujumura *et al.* (1982) suggested that Strain T and Strain B were possibly a *Pseudomonas* sp. and an *Alcaligenes* sp., respectively. Jackson *et al.* (1968), however, decided there was an insufficient difference in DNA G+C content (*c.*66 mol%) when compared to other *Thiobacillus* spp. to excluded “*T. trautweinii*” strains from the genus. Since their isolation there have also been conflicting accounts on whether “*T. trautweinii*” strains were facultative autotrophs or strictly heterotrophic. Parker and Prisk (1953) reported these strains as capable of growth with no

organic carbon present, however, Starkey (1934c) concluded that they were strictly heterotrophic species. Such results suggest that in some studies these strains may have been mixed cultures or contaminants. The history of the strains used in the present study is given in Table 2.1.

With the confusion of conflicting reports on strains of “*T. trautweinii*” the aim of this study was to assess three strains of “*T. trautweinii*” (Strain T, Strain B and Strain VO) for their taxonomy using modern gene sequencing and phylogenetic analysis as well as traditional chemotaxonomic techniques. Their ability to oxidise inorganic sulfur compounds was also examined.

Table 3.1. History of “*T. trautweinii*” strains from their original isolation to the present study.

| Strain | Culture Collection | History |
|---------------|---------------------------|--|
| T | NCIMB 9549 | < NCIMB < NCIB < White < Hutchinson < Wikén < LMG < Starkey < Trautwein |
| B | NCCB 38021 | < NCCB < CBS < LMG < Starkey |
| VO | NCCB 33030 | < NCCB < CBS < LMG < A. van Oven < van Hoff |

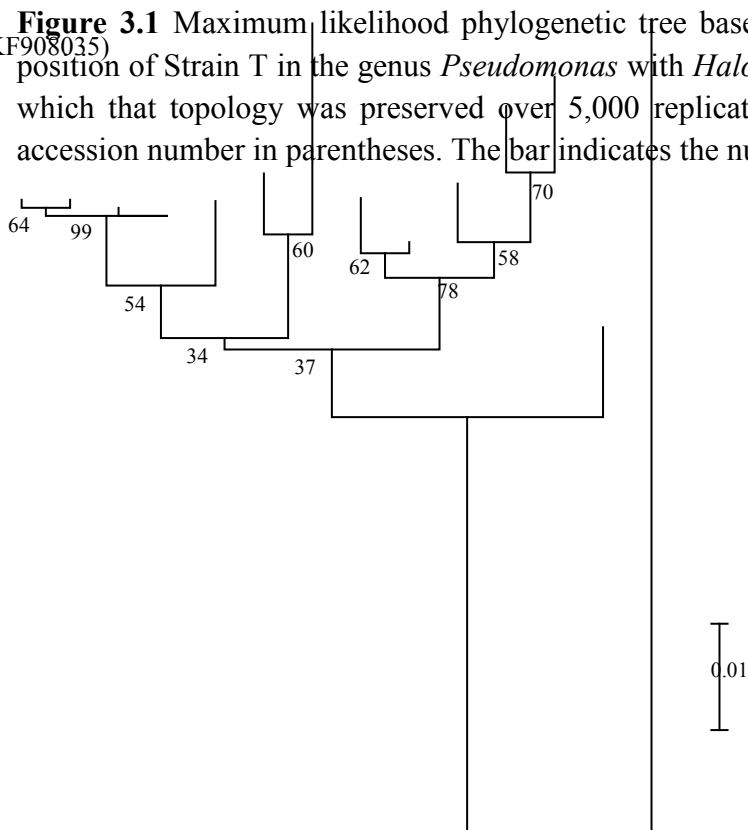
3.2 Results

3.2.1 16S rRNA gene sequences

Based on 16S rRNA (*rrs*) gene sequencing the three strains of “*T. trautweinii*” belongs to two different genera of the *Bacteria*. Strain T and Strain VO both belong to the genus *Pseudomonas*. Using the BLAST 2 (Tatusova & Madden, 1999) algorithm the *rrs* sequence of Strain T shared most identity to that of the *rrs* sequences of *P. chengduensis* MBR^T (99.9%), *P. toyotomiensis* HT-3^T (99.7%), *P. alcalophila* AL15-21^T (99.7%) and *P. mendocina* NCIMB 10541^T (98.4%), which is confirmed by their phylogenetic position in Maximum likelihood (Figure 3.1) and Neighbour-joining (Figure 3.2) phylogenetic trees. Strain VO *rrs* sequence shared closest similarity with that of *P. protegens* CHA0 6595^T (99.8%), *P. caricapapayae* ATCC 33615^T (98.4%), *P. syringae* ATCC 19310^T (98.3%) and *P. fluorescens* RH 818^T (97.4%), which is also confirmed by its phylogenetic position in Maximum likelihood (Figure 3.3) and Neighbour-joining (Figure 3.4) phylogenetic trees. Strain B of “*T. trautweinii*” belongs to the genus *Achromobacter* with a *rrs* sequence most similar to the *rrs* sequence of *A. aegrifaciens* LMG 26852^T (99.4%), *A. insuavis* LMG 26845^T (99.1%), *A. spanius* LMG 5911^T (98.9%) and *A. piechaudii* ATCC 43552^T (98.2%). The phylogenetic position of Strain B can be seen in maximum likelihood (Figure 3.5) and neighbour-joining (Figure 3.6) phylogenetic trees. The type species of *Thiobacillus*, *T. thioparus* Starkey^T shared only 83.7% *rrs* sequence identity with that of Strain T and shared 84.1% similarity with that of Strain VO. Strain B shared the most *rrs* sequence identity to the *Thiobacillus* type species but was still a relatively small value of 90.2% identity.

Pseudomonas chlororaphis subsp. *chlororaphis* DSM 50083^T (Z76)
Pseudomonas aeruginosa DSM 50071^T (HE978271)
Halomonas elongata DSM 2581^T (FN869)
Pseudomonas jessenii CIP 105274^T (AF068259)
Pseudomonas alcaligenes ATCC 33835^T (AB021397)
Pseudomonas fluorescens Pf-0 NRIC 01801 (AB060136)
Pseudomonas fluorescens Pf-5 ATCC 49619 (AB060136)
Pseudomonas monteilii CIP 104883^T (AF064458)
Pseudomonas straminea IAM1598 (D84023)

Figure 3.1 Maximum likelihood phylogenetic tree based on CLUSTALW alignment of 16S rDNA sequences. The position of Strain T in the genus *Pseudomonas* with *Halomonas elongate* as an outgroup. Bootstrap values are shown at the nodes, which that topology was preserved over 5,000 replicates. For each species the type strain accession number in parentheses. The bar indicates the number of changes per nucleotide position.



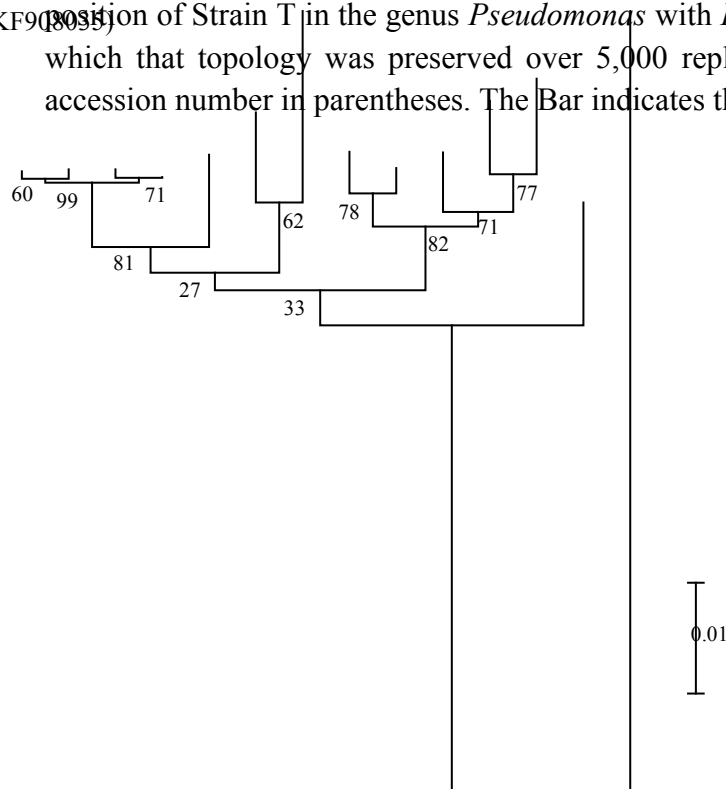
Pseudomonas chlororaphis subsp. *chlororaphis* DSM 50083^T (Z)
Pseudomonas aeruginosa DSM 50071^T (HE978271)
Halomonas elongata DSM 2581^T (FN86956)

Pseudomonas alcaligenes IAM12411^T (D84906)
Pseudomonas jesseni CIP 105274^T (AF068259)

Pseudomonas chengduensis MBB^T (FJ307411)
Pseudomonas sp. NBIC D189^T (AB069139)
Pseudomonas sp. AB021397^T (AB021397)
Pseudomonas alcaliphila AL15-24^T (AB030583)
Pseudomonas sp. 104883^T (AF064438)

Pseudomonas straminea IAM1598^T

Figure 3.2 Neighbour-joining phylogenetic tree based on CLUSTALW alignment of 16S rDNA sequences of *Pseudomonas* species. The position of Strain T in the genus *Pseudomonas* with *Halomonas elongate* as an outgroup. Bootstrap values are shown at the nodes, which that topology was preserved over 5,000 replicates. For each species the type strain accession number in parentheses. The Bar indicates the number of changes per nucleotide position.



Pseudomonas syzyathii IAM 1236^T (D84025)
Pseudomonas fluorescens IAM 1653^T (D84027)
Pseudomonas taetrolens IAM 1653^T (D84027)
Pseudomonas caricapapuae ATCC 33856^T (D84020)
Pseudomonas protegens ATCC 19049^T (D84020)
Pseudomonas syringae ATCC 19619^T (D84020)
Pseudomonas putida IAM 1236^T (D84020)
Pseudomonas parafulva DSM 17004^T (AB060132)
Pseudomonas putida IAM 1236^T (D84020)

Strain VO

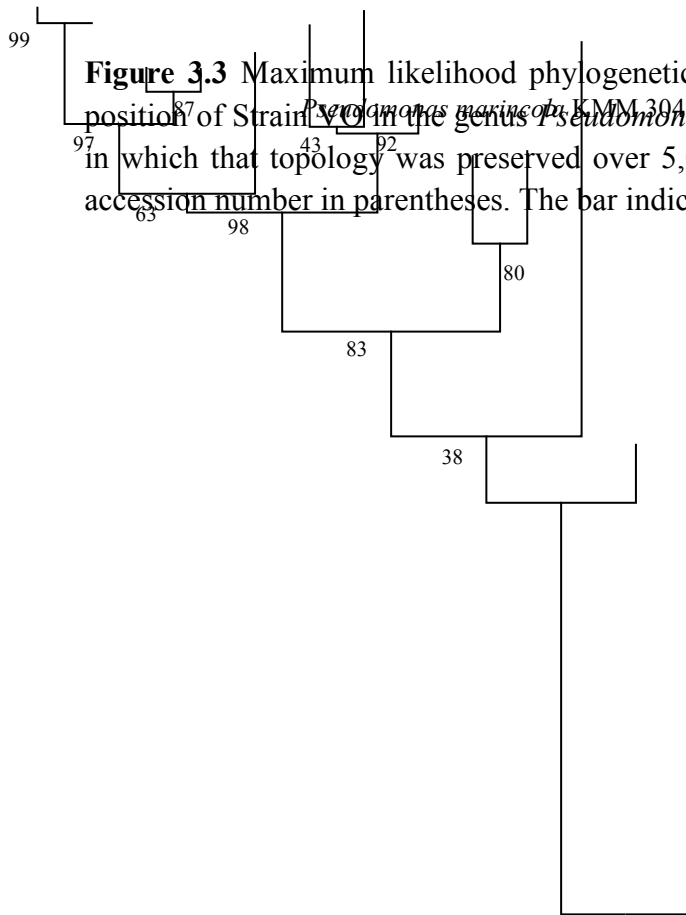


Figure 3.3 Maximum likelihood phylogenetic tree based on CLUSTALW alignment of 16S rDNA sequences of *Pseudomonas* species with *Halomonas elongate* as an outgroup. Bootstrap values are shown at the nodes. The position of Strain VO in the genus *Pseudomonas* with *Halomonas elongate* as an outgroup. Bootstrap values are shown at the nodes in which that topology was preserved over 5,000 replicates. For each species the type strain accession number in parentheses. The bar indicates the number of changes per nucleotide position.

Halomonas elongata DSM 2581^T (FN869568)

Pseudomonas caeni HY-14^T (EU620679)

Pseudomonas fluorescens IAM 12033^T (D84013)

Pseudomonas fluorescens Pf-0^T (D84025)

Pseudomonas fluorescens Pf-5^T (D84010)

Pseudomonas protegens Pf-6^T (D84019)

Pseudomonas veronii NA06^T (AB058120)

Pseudomonas putida IAM 1236^T (D84020)

Pseudomonas parafulva DSM 17004^T (AB060132)

Strain VO *Pseudomonas marincola* KMM 3042^T (AB301071)

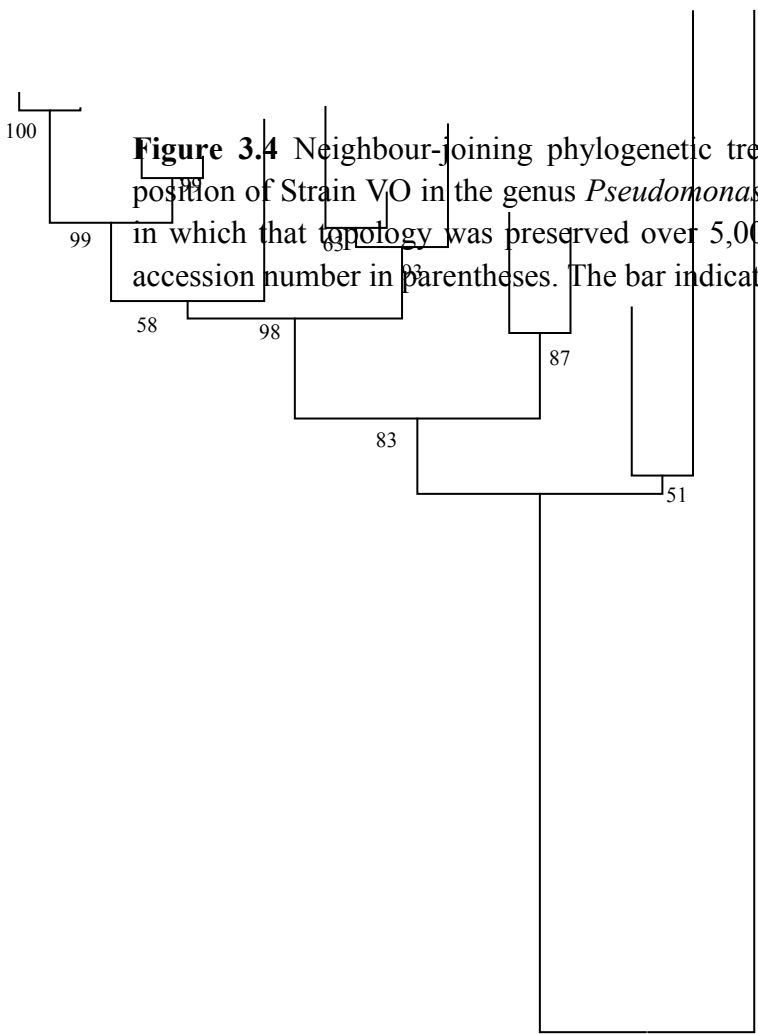
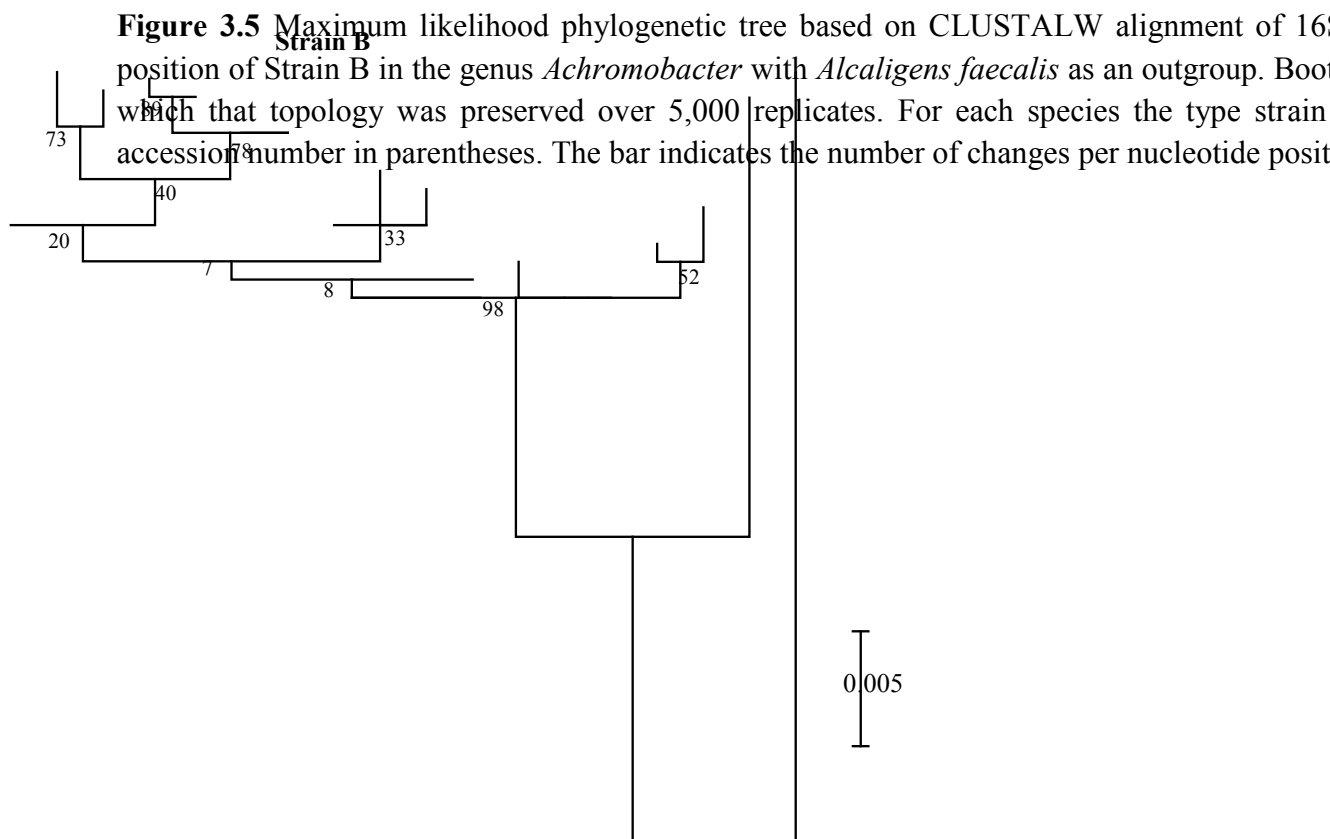


Figure 3.4 Neighbour-joining phylogenetic tree based on CLUSTALW alignment of 16S rDNA sequences of *Pseudomonas* species and *Halomonas elongata* as an outgroup. The tree shows the position of Strain VO in the genus *Pseudomonas* with *Halomonas elongata* as an outgroup. The bootstrap values are shown at the nodes. The tree was constructed in which that topology was preserved over 5,000 replicates. For each species the type strain accession number in parentheses. The bar indicates the number of changes per nucleotide position.

Achromobacter mucicolens LMG 26685^T (HF613446)
Achromobacter anitensis LMG 26698^T (HE613447)
Achromobacter marplatensis BCC 43530^T (AB0110841)
Achromobacter piechmannii BCC 43531^T (AB0110841)
Achromobacter spanius LMG 5911^T (AY170848) *Alcaligenes faecalis* ATCC 8750^T (F0110841)
Achromobacter agrocaryi LMG 26850^T (HF586509) *Achromobacter denitrificans* DSM 30026^T (AJ278451)
Achromobacter insolitus LMG 6003^T (AY170847) *Achromobacter pulmois* LMG 26696^T (HE798552)
Achromobacter ruhlandii ATCC 15749^T (AB010840)
Achromobacter anstifer LMG 26857^T (HF586308)
Achromobacter dolens LMG 26840^T (HF586509)

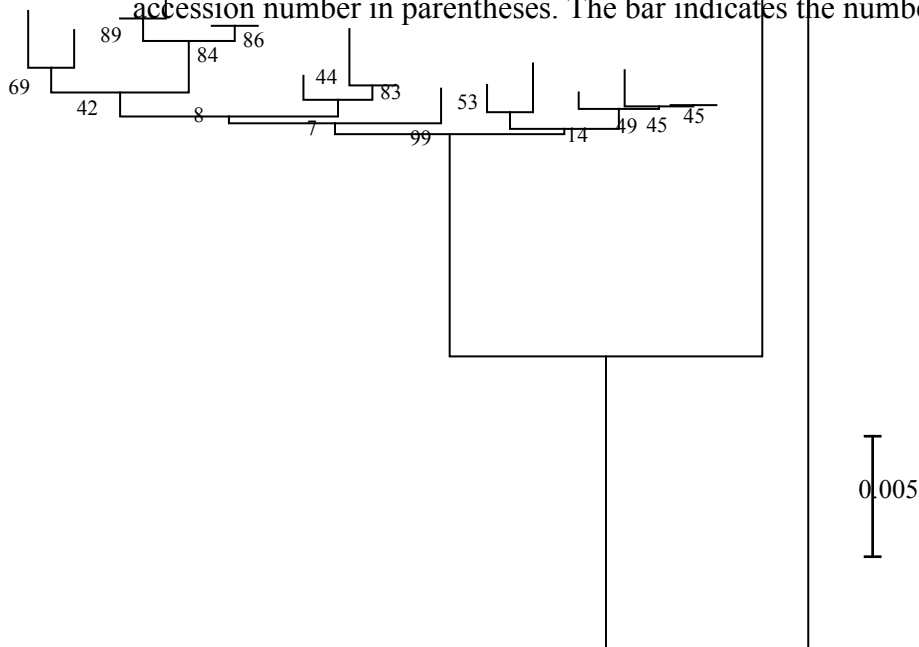


Achromobacter sediminum XH089^T (KC986)
Alcaligenes faecalis IAM12369^T (D88)

Achromobacter spirillum LMG 26623^T (HF613447) (848)
Achromobacter cummingsii LMG 26870^T (HF613436) (847)
Achromobacter xylosoxidans DSM 10346^T (Y14908)
Achromobacter gasii LMG 26851^T (HF613435) (846)
Achromobacter anisodermis LMG 26850^T (HF613434) (845)
Achromobacter palmensis LMG 26849^T (HF613433) (844)
Achromobacter anxiifer LMG 26857^T (HF586508)
Achromobacter dolens LMG 26840^T (HF586509)

Strain B

Figure 3.6 Neighbour-joining phylogenetic tree based on CLUSTALW alignment of 16S rDNA sequence of Strain B in the genus *Achromobacter* with *Alcaligenes faecalis* as an outgroup. Bootstrap values are shown at the nodes, which that topology was preserved over 5,000 replicates. For each species the type strain accession number in parentheses. The bar indicates the number of changes per nucleotide position.



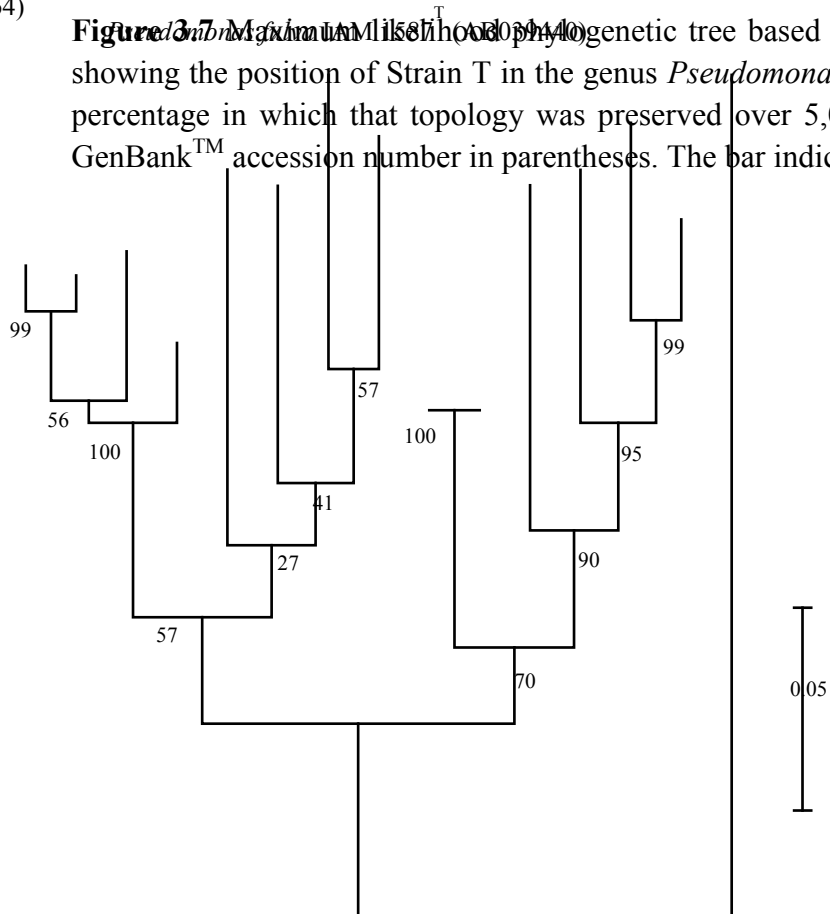
3.2.2 Gyrase subunit B gene sequences

Based on DNA gyrase subunit B gene (*gyrB*) sequencing, Strain T belongs in the genus *Pseudomonas* while Strain B has been shown to belong to the genus *Achromobacter*. Using BLAST 2 algorithm Strain T shared most identity with that of *gyrB* sequences of *P. chengduensis* MBR^T (97.4%), *P. toyotomiensis* HT-3^T (94.5%), *P. alcaliphila* AL15-21^T (93.8%), *P. mendocina* NCIB 10541^T (89.6%). The phylogenetic position of Strain T can be seen in Maximum likelihood (Figure 3.7) and Neighbour-joining (Figure 3.8) phylogenetic trees. The *gyrB* sequence of Strain B shared most identity with that of *gyrB* sequences of *A. denitrificans* DSM 30026^T (96.3%), *A. ruhlandii* ATCC 15749^T (95.4%) and *A. pulmonis* LMG 26696^T (94.5%). The phylogenetic position of Strain B can be seen in Maximum likelihood (Figure 3.9) and Neighbour-joining (Figure 3.10) phylogenetic trees. However, at the time of writing the *gyrB* sequences for *A. aegrifaciens* LMG 26852^T, *A. anxifer* LMG 26857^T, *A. dolens* LMG 26840^T, *A. insuavis* LMG 26845^T and *A. sediminum* XH089^T were available.

Pseudomonas mendocina ATCC 25411^T (AJ633103)
Pseudomonas aeruginosa DSM 16219^T (F01949566)
Pseudomonas fluorescens DSM 17150^T (AM293562)
Pseudomonas asplenii NCPPB 959^T (AB039473)
Pseudomonas tuomuerensis JCM 14085^T (AB571150)
Pseudomonas alcaliphila JCM 10620^T (AB494447)
Pseudomonas monteilii BCRC 17520^T (FJ418641)
Pseudomonas macleodii DSM 14159^T (AB039388)
Pseudomonas toyotomiensis HT-3^T (AB494447)
Pseudomonas chengdeensis MBR-11X042316^T
Pseudomonas straminea IAM 1598^T (AB03910)

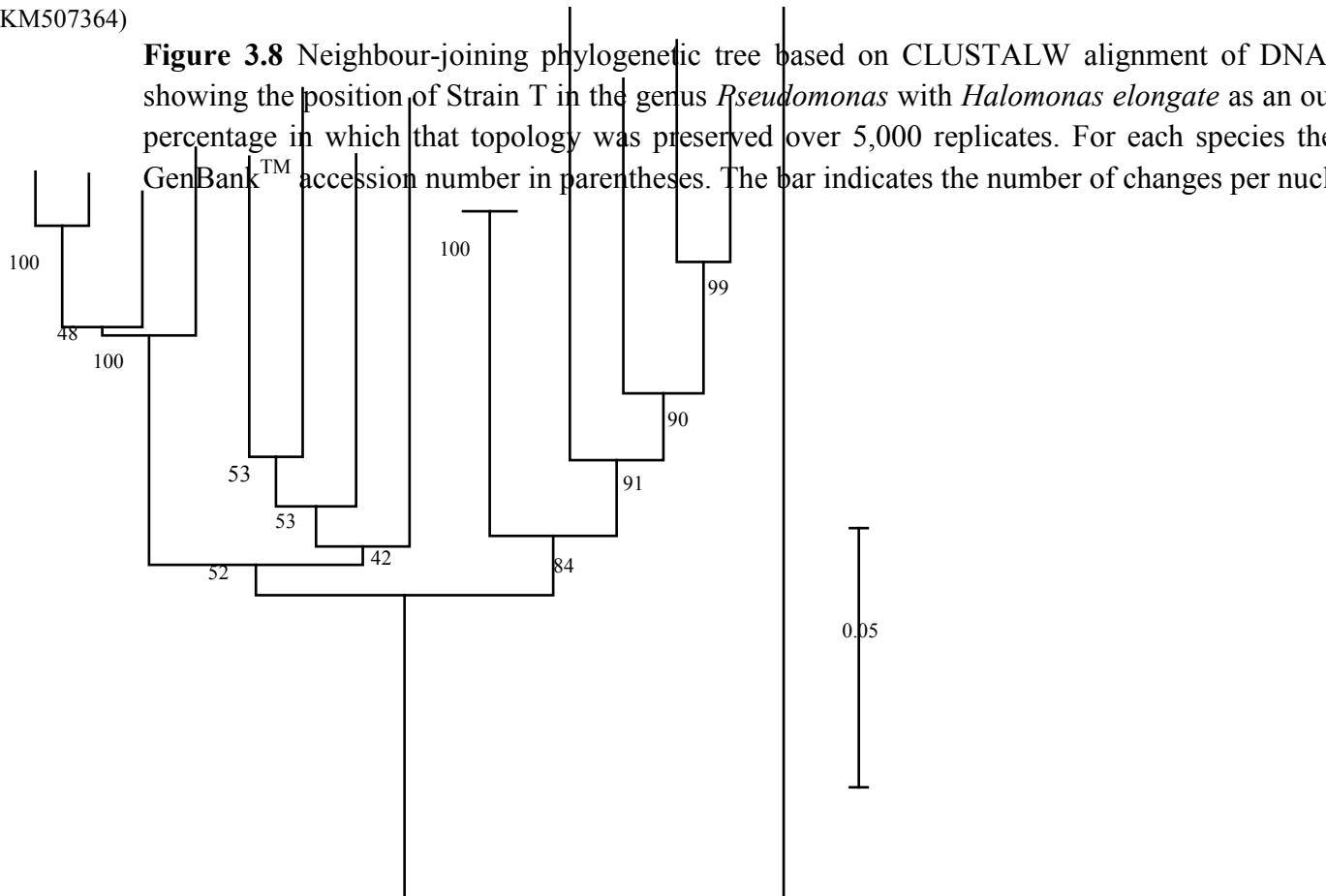
Strain T (KM507364)

Figure 3.7 Maximum likelihood phylogenetic tree based on CLUSTALW alignment of *subunit III* showing the position of Strain T in the genus *Pseudomonas* with *Halomonas elongate* as a percentage in which that topology was preserved over 5,000 replicates. For each species GenBankTM accession number in parentheses. The bar indicates the number of changes per



Pseudomonas chlororaphis ATCC 9446^T (FJ652718)
Pseudomonas monteilii BCRC 17520^T (FJ418641)
Pseudomonas fluorescens Pf0-1^T (AF035622)
Pseudomonas aspleii NCPPB 959^T (AB039473)
Pseudomonas aeruginosa ATCC 25411^T (AF035106)
Pseudomonas fluorescens ATCC 49619^T (AB039388)
Pseudomonas fluorescens Pf0-1^T (AF035622)
Pseudomonas fluorescens Pf0-1^T (AF035622)
Pseudomonas straminea IAM 1598^T (AB03910)
Pseudomonas chengduensis MBR^T (AB039440)
Pseudomonas fulva IAM 1587^T (AB039440)
Pseudomonas toyotomiensis HT-3^T (AB494447)

Strain T (KM507364)



Achromobacter ~~*denitrificans*~~ LMG 26693^T (HG454849)
Achromobacter ~~*denitrificans*~~ LMG 5911^T (HG454851)
Achromobacter ~~*denitrificans*~~ LMG 26690^T (HG454856) *Alcaligenes faecalis* ATCC 8750^T (AB9723)
Achromobacter ~~*denitrificans*~~ LMG 26692^T (HG454857)
Achromobacter ~~*denitrificans*~~ LMG 6003^T (HG454850)
Achromobacter ~~*denitrificans*~~ DSM 90026^T (HG454848)

Achromobacter ~~*denitrificans*~~ DSM 10346^T (HG454853)
Achromobacter ~~*denitrificans*~~ ATCC 15749^T (HG454854)
Achromobacter ~~*denitrificans*~~ LMG 26696^T (HG454858)

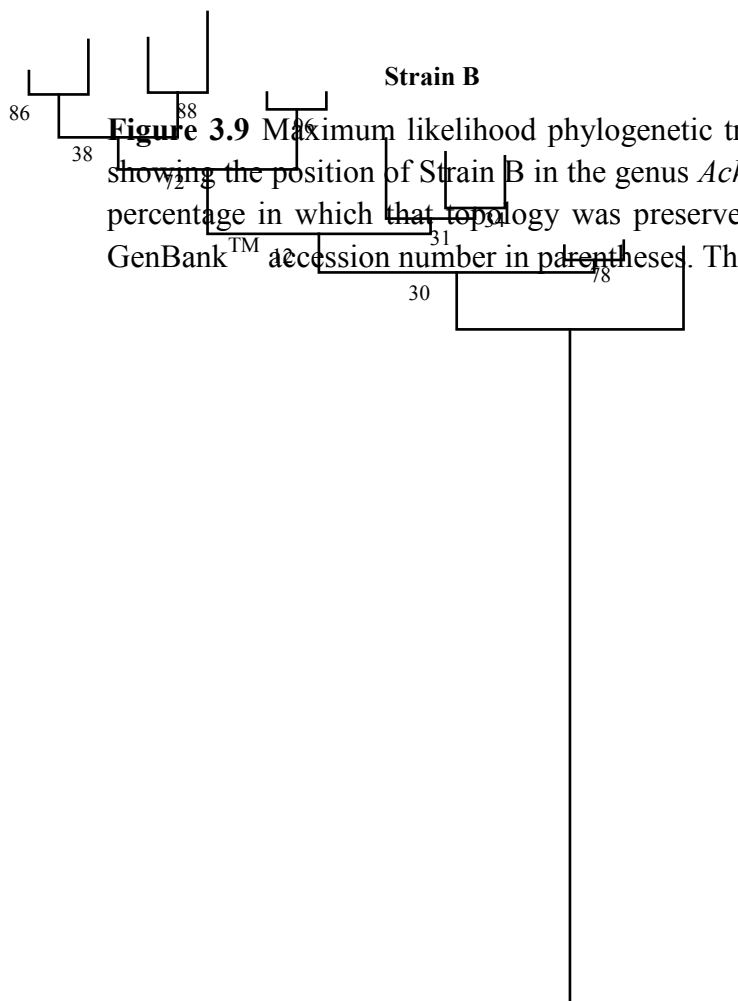
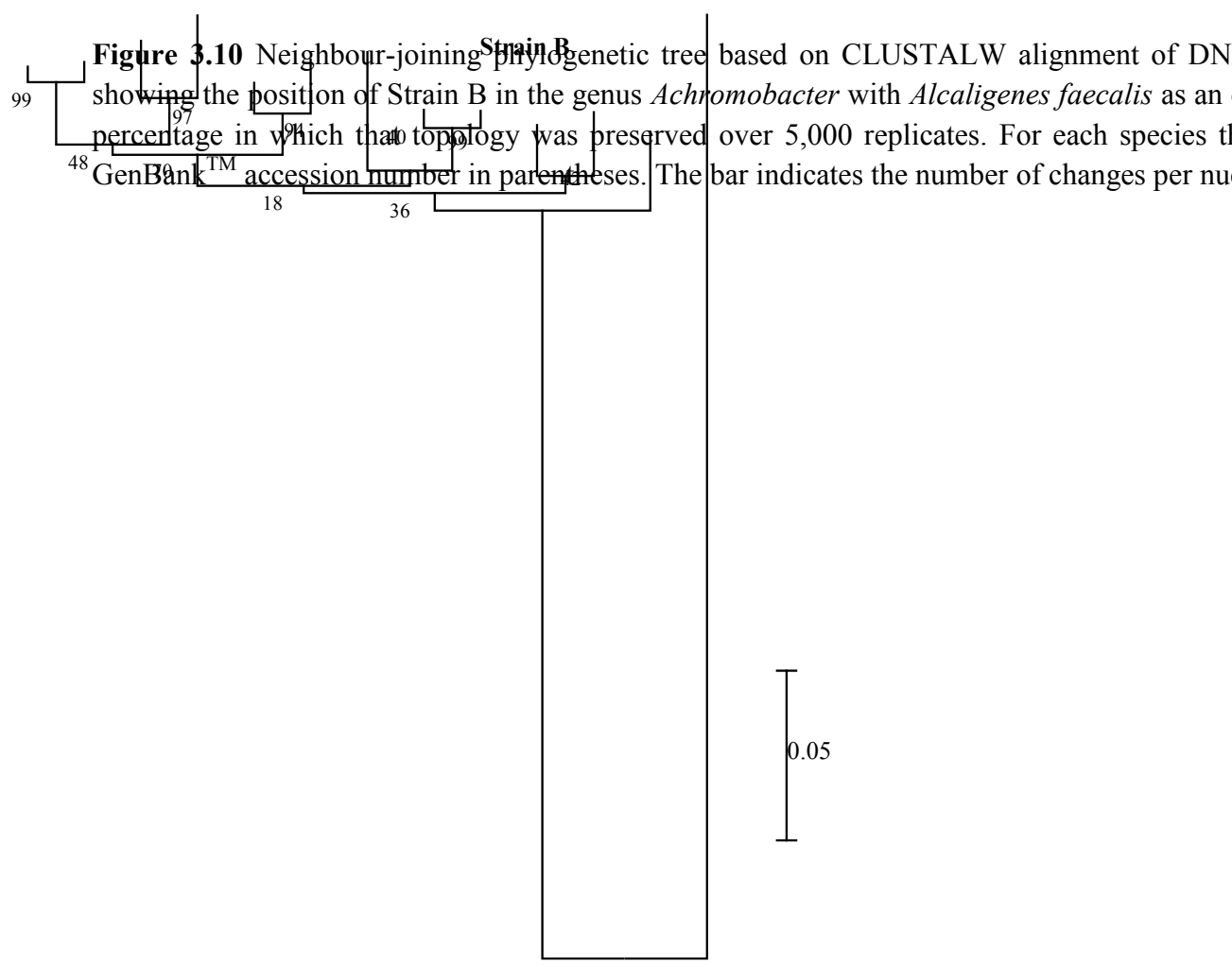


Figure 3.9 Maximum likelihood phylogenetic tree based on CLUSTALW alignment of showing the position of Strain B in the genus *Achromobacter* with *Alcaligenes faecalis* percentage in which that topology was preserved over 5,000 replicates. For each specie GenBankTM accession number in parentheses. The bar indicates the number of changes per

Achromobacter spania LMG 552^T (HG454851), *Alcaligenes faecalis* ATCC 8750^T (AB97237)
Achromobacter ananin ATCC 4952^T (HG454851), *Achromobacter mucicollis* LMG 2668^T (HG454851)
Achromobacter denitrificans DSM 30026^T (HG454848)
Achromobacter sp. LMG 6003^T (HG454850), *Achromobacter* sp. LMG 6003^T (HG454850)
Achromobacter sp. LMG 6003^T (HG454850), *Achromobacter* sp. LMG 6003^T (HG454850)



3.2.3 Chemotaxonomic characteristics

The chemotaxonomic properties of Strain T, Strain B and Strain VO were examined and compared with other closely related species, differential results of which are summarised in Tables 3.2-3.4. The dominant respiratory quinone found in Strain T and Strain VO was ubiquinone-9 (UQ-9), consistent with the *Pseudomonas* genus (Moore *et al.* 2006) and in Strain B was ubiquinone-8 (UQ-8), consistent with the genus *Achromobacter* (Vandamme *et al.* 2013a). There were a number of characteristics that distinguished Strain T from the closely related species *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T. There was a higher DNA G+C content of 68.0 mol%; a difference of 6.1 mol% and 2.9 mol% compared to *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T, respectively. It was observed that thiosulfate and manganese were oxidised by Strain T but this trait was not shared by its nearest neighbours. *P. chengduensis* MBR^T also lacked any flagella and was non-motile, in contrast to Strain T and other members of this group. There were notably more carbohydrates that Strain T produced acid from when compared to other *Pseudomonas* spp.. Strain VO also had a higher DNA G+C content of 67.2 mol% compared to 60.1-63.3 mol% for the closest relative *P. protegens* CHA0^T. However, the chemotaxonomic traits of the closely related *Pseudomonas* spp. highlighted in Table 3.3 are relatively scarce in the literature currently available and the DNA G+C content as well as other significant traits of *P. caricapapayae* ATCC 33615^T could not be obtained. Strain B shared many traits with that of its closest relative *A. aegrifaciens* LMG 26852^T and had only a fractionally higher DNA G+C content of 66.2 mol% compared to 66 mol% of *A. aegrifaciens* LMG 26852^T. There were a few notable exceptions highlighted in Table 3.4. In contrast the other *Achromobacter* spp., Strain B, was unable to grow in medium containing higher than 2% sodium chloride, and had urease activity and the ability to hydrolyse Tween® 80, but was unable to hydrolyse esculin.

Cellular fatty acids of Strain T were compared to data obtained by Tao *et al.* (2014) of closely related *Pseudomonas* spp. (Table 3.5). The dominant fatty acids in Strain T were C_{16:0}, C_{16:1} ω6c + C_{16:1} ω7c and C_{18:1} ω6c + C_{18:1} ω7c, similar to those found in the other closely related species, but the levels of C_{16:1} ω6c + C_{16:1} ω7c were 9.2% higher than that of *P. chengduensis* MBR^T while levels of C_{16:0} were 4.6% lower. Strain T also contained some fatty acids (C_{15:0}, C_{9:0} 3-OH, C_{15:1} ω8c and C_{17:1} ω6c) at various levels that were absent in the other species while it lacked other fatty acids (C_{15:0} 3-OH, C_{18:0} ISO, C_{14:1} ω5c, C_{17:0} cyclo and C_{19:0} cyclo ω8c) that were found in its nearest relatives. Cellular fatty acids of Strain B were compared to data obtained by Vandamme *et al.* (2013a) and Vandamme *et al.* (2013b) of closely related *Achromobacter* spp. (Table 2.6). The dominant fatty acids found in Strain B were C_{16:0} and C_{15:0} ISO + C_{16:1} ω7c which were of similar levels to that found in the other species. However, Strain B lacked C_{16:1} ISO + C_{14:0} 2-OH which were present in the other species at levels between 9.06-14.13%.

Table 3.2. Differential chemotaxonomic characteristics of *Pseudomonas* sp. strains T (1) and the closely related *P. chengduensis* MBR^T (2), *P. toyotomiensis* HT-3^T (3), *P. alcalophila* AL15-21^T (4) and *P. mendocina* NCIMB 10541^T within the genus *Pseudomonas*. *N.D.*; not determined.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|--|--------------|----------------------|----------------------|----------------------|------------------------|
| Cell size (µm) | 0.5 × 2.6 | 0.4-0.5 × 1.1-1.8 | 0.4-0.6 × 1.0-2.5 | 0.3-0.5 × 1.5-3.0 | 0.7-0.8 × 1.4-2.8 |
| Flagella | Monotrichous | None | Monotrichous | Monotrichous | Mono-/ peritrichous |
| DNA G+C content (mol%) | 68 | 61.9 | 65.1 | 62.3-63.2 | 62.9-64.3 |
| Hydrolysis of: | | | | | |
| <i>casein</i> | - | - | - | + | + |
| <i>esculin</i> | + | - | + | - | - |
| <i>gelatin</i> | - | + | - | + | - |
| <i>starch</i> | ± | - | - | - | - |
| Oxidation of: | | | | | |
| <i>manganese</i> | + | - | - | <i>N.D.</i> | - |
| <i>thiosulfate</i> | + | - | - | <i>N.D.</i> | <i>N.D.</i> |
| Reduction of: | | | | | |
| <i>nitrate</i> | - | + | + | + | + |
| <i>tellurite</i> | + | + | - | <i>N.D.</i> | + |
| Production of: | | | | | |
| <i>N₂ from nitrate</i> | - | + | - | - | + |
| <i>indole from tryptophan</i> | - | + | + | <i>N.D.</i> | - |
| Nitrogen sources: | | | | | |
| <i>cyanate</i> | - | - | + | <i>N.D.</i> | + |
| <i>thiocyanate</i> | - | - | + | <i>N.D.</i> | + |
| Pigmentation | - | - | - | + | + |
| Growth at: | | | | | |
| 4 °C | + | + | + | + | - |
| 42 °C | + | + | + | - | + |
| 6% (w/v) NaCl | - | + | + | + | - |
| Acid produced from: | | | | | |
| <i>L-arabinose</i> | + | <i>N.D.</i> | - | - | - |
| <i>D-ribose</i> | + | <i>N.D.</i> | - | + | - |
| <i>D-xylose</i> | - | <i>N.D.</i> | + | + | + |
| <i>D-galactose</i> | - | - | + | - | + |
| <i>D-fructose</i> | - | + | + | + | + |
| <i>D-mannose</i> | + | - | + | - | + |
| <i>D-mannitol</i> | ± | - | - | + | - |
| <i>arbutin</i> | + | <i>N.D.</i> | - | - | - |
| <i>salicin</i> | + | + | - | - | - |
| <i>D-cellobiose</i> | + | - | - | - | - |
| <i>D-maltose</i> | + | - | - | - | - |
| <i>D-melibiose</i> | - | - | + | + | + |
| <i>D-sucrose</i> | + | - | - | - | - |
| <i>D-trehalose</i> | + | - | - | - | - |
| <i>starch</i> | + | - | - | - | - |
| <i>glycogen</i> | + | <i>N.D.</i> | - | - | - |
| <i>gentiobiose</i> | - | - | - | - | + |
| <i>D-fucose</i> | - | + | + | + | + |
| <i>L-fucose</i> | - | <i>N.D.</i> | + | + | + |
| Enzyme activities: | | | | | |
| <i>tryptophan deaminase</i> | ± | + | + | + | - |
| <i>alkaline phosphatase</i> | ± | <i>N.D.</i> | - | - | - |
| <i>esterase lipase (C₈)</i> | ± | <i>N.D.</i> | + | ± | - |

| | | | | | |
|--------------------------------|---|-------------|---|-------------|---|
| <i>lipase (C₁₄)</i> | - | <i>N.D.</i> | - | - | + |
| Growth on: | | | | | |
| <i>aspartate</i> | - | + | + | <i>N.D.</i> | + |
| <i>benzoate</i> | - | - | - | <i>N.D.</i> | + |
| <i>glycine</i> | + | + | - | ± | - |
| <i>lactose</i> | + | - | - | - | - |
| <i>lysine</i> | + | + | - | <i>N.D.</i> | - |
| <i>phenylalanine</i> | - | + | + | <i>N.D.</i> | + |
| <i>propionate</i> | - | + | + | <i>N.D.</i> | + |
| <i>threonine</i> | - | - | + | <i>N.D.</i> | - |
| Antibiotic resistance: | | | | | |
| <i>sulphatrial</i> | + | + | + | <i>N.D.</i> | - |
| <i>cotrimoxazole</i> | + | + | + | <i>N.D.</i> | - |

Data were obtained from this study, Tao *et al.* 2014, Hirota *et al.* 2011 and Yumoto *et al.* 2001.

Table 3.3 Differential chemotaxonomic characteristics of *Pseudomonas* sp. Strain VO (1) and the closely related species *P. protegens* CHA0^T (2), *P. syringae* ATCC 19310^T (3) and *P. fluorescens* IAM 12022^T (4) within the genus *Pseudomonas*. *N.D.*; not determined. *P. caricapapayae* ATCC 33615^T.

| Characteristic | 1 | 2 | 3 | 4 |
|---|-----------|---------------|-------------|-------------|
| Cell size (μm) | 0.5 × 2.2 | 0.3-0.5 × 1-4 | <i>N.D.</i> | <i>N.D.</i> |
| Hydrolysis of: | | | | |
| Casein | - | <i>N.D.</i> | + | + |
| Starch | - | <i>N.D.</i> | - | - |
| Aesculin | - | - | + | - |
| Tween® 80 | + | + | <i>N.D.</i> | <i>N.D.</i> |
| Oxidation of: | | | | |
| Manganese | - | ± | <i>N.D.</i> | ± |
| Pigment production | + | + | - | + |
| Growth at 42°C | + | - | <i>N.D.</i> | + |
| DNA G + C content (mol%) | 67.2 | 60.1-63.3 | 58.3 | 63.3 |
| Acid production from: | | | | |
| mannitol | + | + | + | <i>N.D.</i> |
| sorbitol | - | - | + | <i>N.D.</i> |
| erythritol | - | - | + | <i>N.D.</i> |
| Activity of: | | | | |
| Oxidase | + | + | - | + |
| Arginine hydrolase | + | + | - | + |
| Ornithine decarboxylase | + | - | - | - |
| Gelatinase | - | + | + | + |
| Urease | - | + | + | <i>N.D.</i> |
| Gelatinase | - | + | + | + |
| Urease | - | + | + | <i>N.D.</i> |

Data were obtained from this study, Elomari *et al.* (1997), Behrendt *et al.* (2007) and Ramette *et al.* (2011).

Table 3.4 Differential chemotaxonomic characteristics of *Achromobacter* sp. Strain B (1) and the closely related *A. aegrifaciens* LMG 26852^T (2), *A. insuavis* LMG 26845^T (3), *A. spanius* LMG 5911^T (4) and *A. piechaudii* ATCC 43552^T (5) within the genus *Achromobacter*. ND; not determined.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|--|------|----|----|------|-------|
| Flagellae | + | + | - | + | + |
| Hydrolysis of: | | | | | |
| <i>DNA</i> | - | - | ± | - | - |
| <i>Aesculin</i> | - | + | + | - | - |
| <i>Tween 20</i> ® | - | + | + | + | - |
| <i>Tween 80</i> ® | + | - | - | - | - |
| Reduction of: | | | | | |
| <i>Selenite</i> | + | ± | + | + | - |
| <i>Tellurite</i> | + | + | - | - | - |
| Nitrate reduction to: | | | | | |
| <i>Nitrite</i> | + | + | + | + | + |
| <i>N₂</i> | - | + | + | - | - |
| IMViC test: | | | | | |
| <i>Indole production</i> | - | - | - | - | - |
| <i>Methyl red</i> | - | - | - | - | - |
| <i>Voges-Proskauer</i> | - | - | - | - | - |
| <i>Citrate utilization</i> | + | + | - | - | - |
| Pigment production | - | - | - | N.D. | - |
| Growth at: | | | | | |
| 42°C | - | - | + | + | - |
| 2% NaCl | + | + | + | + | + |
| 4% NaCl | - | + | + | + | + |
| 8% NaCl | - | - | + | - | - |
| DNA G+C content (mol%) | 66.2 | 66 | 68 | 64.9 | 64-65 |
| Acid produced from: | | | | | |
| <i>Glucose</i> | + | - | + | - | - |
| Enzyme activity: | | | | | |
| <i>Tryptophan deaminase</i> | - | - | - | - | + |
| <i>Leucine arylamidase</i> | + | + | + | + | + |
| <i>Alkaline phosphatase</i> | - | + | + | - | - |
| <i>Acid phosphatase</i> | ± | + | + | + | - |
| <i>Esterase (C₄)</i> | + | + | - | + | + |
| <i>Esterase lipase (C₈)</i> | - | - | - | - | - |
| <i>Urease</i> | + | - | - | - | - |
| Sources of Nitrogen: | | | | | |
| <i>Cyanate</i> | - | + | + | + | + |
| <i>Thiocyanate</i> | - | + | + | + | + |
| <i>Urea</i> | + | - | - | - | + |
| Hemolysis | α | - | - | - | - |

Data were obtained from this study, Vandamme *et al.* (2013a) and Vandamme *et al.* (2013b).

Table 3.5 Percentage fatty acid methyl esters of *Pseudomonas* sp. Strain T (1) and the closely related *P. chengduensis* MBR^T (2), *P. toyotomiensis* HT-3^T (3), *P. alcaliphila* AL15-21^T (4) and the type species of the genus *P. aeruginosa* JCM 5962^T (5) grown on nutrient agar.

| Fatty acid | 1 | 2 ^a | 3 ^a | 4 ^a | 5 ^b |
|---|-------|----------------|----------------|----------------|----------------|
| Saturated | | | | | |
| C _{10:0} | 0.2 | < 1.0 | 0.0 | < 1.0 | 0.0 |
| C _{11:0} | 0.43 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{12:0} | 7.53 | 7.46 | 5.16 | 6.02 | 3.3 |
| C _{13:0} | 0.33 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{14:0} | 0.27 | < 1.0 | < 1.0 | < 1.0 | 0.8 |
| C _{15:0} | 2.14 | 0.0 | 0.0 | 0.0 | 0.7 |
| C _{16:0} | 13.83 | 18.39 | 21.21 | 15.61 | 24.5 |
| C _{17:0} | 1.84 | 1.05 | 1.3 | < 1.0 | < 1.0 |
| C _{18:0} | 0.53 | 2.42 | 2.52 | 2.19 | < 1.0 |
| Hydroxylated | | | | | |
| C _{9:0} 3-OH | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| C _{10:0} 3-OH | 3.13 | 3.13 | 3.4 | 2.8 | < 1.0 |
| C _{11:0} 3-OH | 0.54 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{12:0} 2-OH | 0.12 | < 1.0 | < 1.0 | < 1.0 | < 1.0 |
| C _{12:0} 3-OH | 3.83 | 3.81 | 3.96 | 3.17 | < 1.0 |
| C _{15:0} 3-OH | 0.0 | 2.19 | 2.24 | 1.24 | 0.0 |
| C _{11:0} ISO 3-OH | 0.1 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{15:0} ISO 3-OH | 0.0 | 0.0 | 0.0 | < 1.0 | 0.0 |
| Unsaturated | | | | | |
| C _{14:1} ω5c | 0.0 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{15:1} ω6c | 0.34 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{15:1} ω8c | 0.13 | 0.0 | 0.0 | 0.0 | 0.0 |
| C _{16:1} ω5c | 0.34 | < 1.0 | < 1.0 | 0.0 | 0.0 |
| C _{16:1} ω9c | 0.0 | 0.0 | 0.0 | 0.0 | 15.4 |
| C _{17:1} ω6c | 1.45 | 0.0 | 0.0 | 0.0 | 0.0 |
| C _{17:1} ω8c | 3.49 | < 1.0 | 1.47 | 1.32 | 0.0 |
| C _{18:1} ω9c | 0.0 | 0.0 | < 1.0 | 0.0 | 46.0 |
| Branched | | | | | |
| C _{13:0} ISO | 0.14 | < 1.0 | 0.0 | < 1.0 | 0.0 |
| C _{16:0} ISO | 0.18 | < 1.0 | < 1.0 | 1.84 | 0.0 |
| C _{17:0} ISO | 0.34 | < 1.0 | < 1.0 | < 1.0 | 0.0 |
| C _{18:0} ISO | 0.0 | 1.31 | 1.58 | 1.86 | 0.0 |
| Cyclopropane | | | | | |
| C _{17:0} cyclo | 0.0 | 7.33 | 6.16 | 2.05 | 1.3 |
| C _{19:0} cyclo ω8c | 0.0 | < 1.0 | < 1.0 | 0.0 | 0.2 |
| Summed Features | | | | | |
| C _{16:1} ω6c + C _{16:1} ω7c | 19.68 | 10.47 | 13.43 | 18.11 | 1.7 |
| C _{18:1} ω6c + C _{18:1} ω7c | 38.87 | 36.18 | 33.41 | 33.45 | 1.5 |

Data obtained from this study and ^a Tao *et al.* (2014); ^b Hirota *et al.* (2011). Data for *P. mendocina* NCIMB 10541^T were not available at the time of writing.

Table 3.6 Percentage fatty acid methyl esters of *Achromobacter* sp. Strain B (1) and the closely related *A. aegrifaciens* LMG 26852^T (2), *A. insuavis* LMG 26845^T (3), *A. spanius* LMG 5911^T (4) and *A. piechaudii* ATCC 43552^T (5) within the genus *Achromobacter*.

| Fatty acid | 1 | 2^a | 3^a | 4^b | 5^b |
|--|----------|----------------------|----------------------|----------------------|----------------------|
| Saturated | | | | | |
| C _{12:0} | 1.3 | 1.18 | <1.0 | 2.3 | 1.11 |
| C _{14:0} | 4.77 | 5.52 | 2.95 | 2.92 | 4.76 |
| C _{16:0} | 38.7 | 32.08 | 32.19 | 24.41 | 29.5 |
| C _{18:0} | 1.85 | 1.22 | 3.15 | 1.12 | 1.21 |
| Hydroxylated | | | | | |
| C _{12:0} 2-OH | 1.89 | 2.94 | 3.42 | 4.44 | 3.25 |
| C _{14:0} 2-OH | 0.0 | 0.0 | 1.86 | 0.0 | 0.0 |
| Unsaturated | | | | | |
| C _{14:1} ω5c | 0.0 | <1 | 0.0 | 0.0 | 0.0 |
| C _{18:1} ω7c | 4.77 | 6.16 | 8.47 | 8.77 | 9.97 |
| Cyclopropane | | | | | |
| C _{17:0} cyclo | 6.71 | 6.75 | 8.12 | 3.43 | 11.34 |
| Summed Features | | | | | |
| C _{15:0} ISO + C _{16:1} ω7c | 32.62 | 31.69 | 28.02 | 36.3 | 28.12 |
| C _{16:1} ISO + C _{14:0} 2-OH | 0 | 9.94 | 9.73 | 14.13 | 9.06 |
| Unknown | 5.35 | 0.0 | 0.0 | 0.0 | 0.0 |

Data obtained from this study and ^a Vandamme *et al.* (2013a) and ^b Vandamme *et al.* (2013b). Fatty acids that had levels of < 1% in all species were excluded.

3.2.4 Optimal growth parameters

The optimal growth parameters with regards to temperature, pH and sodium chloride concentrations were ascertained by measuring specific growth rates. The ranges from which growth was observed with *Pseudomonas* sp. Strain T (Figure 3.10) were 4-42 °C, pH 6.0-10.0 and 0-4% (w/v) sodium chloride, with optimal growth at 36 °C, pH 7.0 and 0% (w/v) sodium chloride. Ranges from which growth was observed with *Achromobacter* sp. Strain B (Figure 3.11) were 10-40 °C, pH 7 and 0-2% (w/v) sodium chloride, with optimal growth at 30 °C, pH 7.0 and 0% (w/v) sodium chloride.

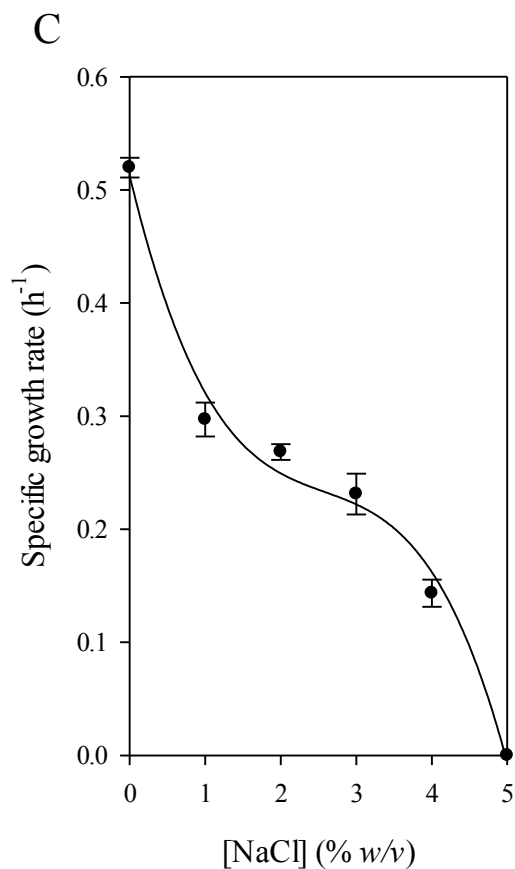
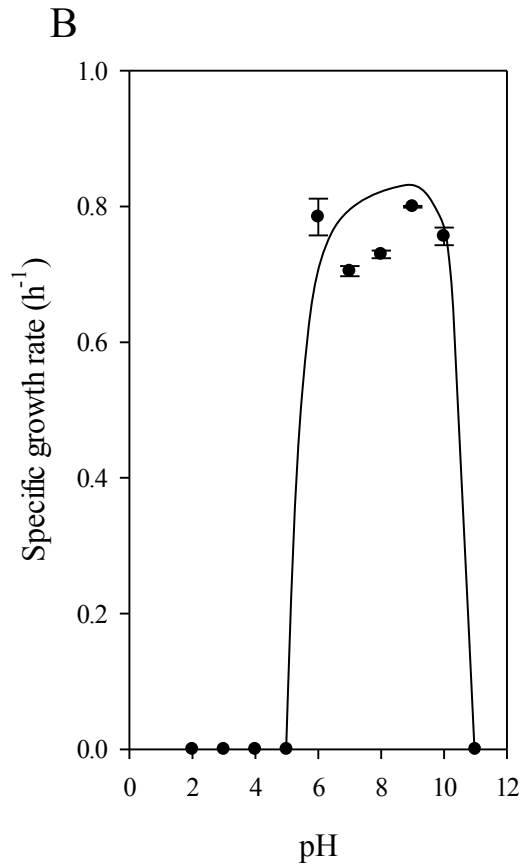
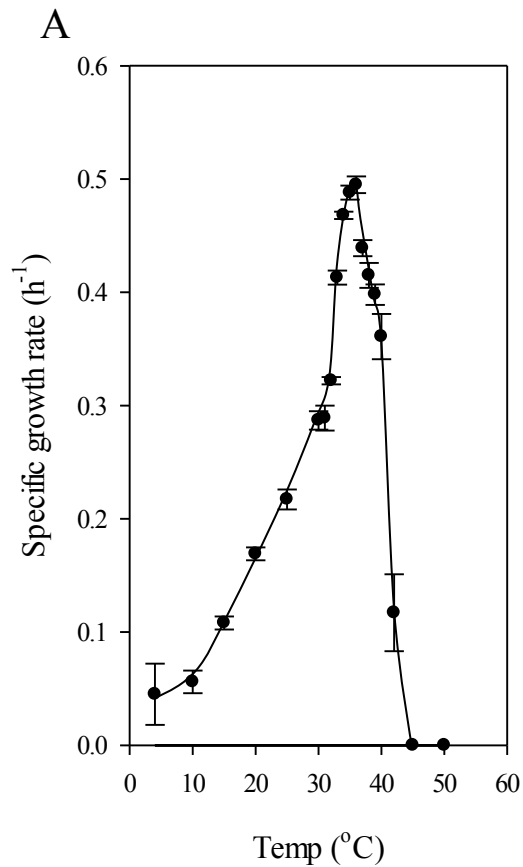


Figure 3.11 Growth of *Pseudomonas* sp. Strain T on EBS with glucose (10 mM) as growth substrate at a wide range of different temperatures (A) and shaken at 100 r.p.m.. Growth rates of Strain T in nutrient broth at a range of different pH (B) and different NaCl concentrations (C) at 30 °C for 12 hours shaken at 100 r.p.m.. Data are mean \pm SEM ($n = 3$).

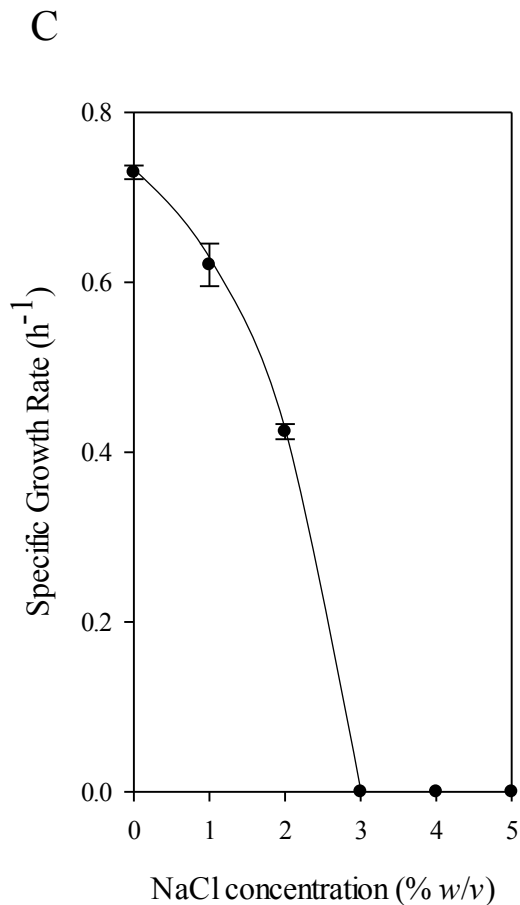
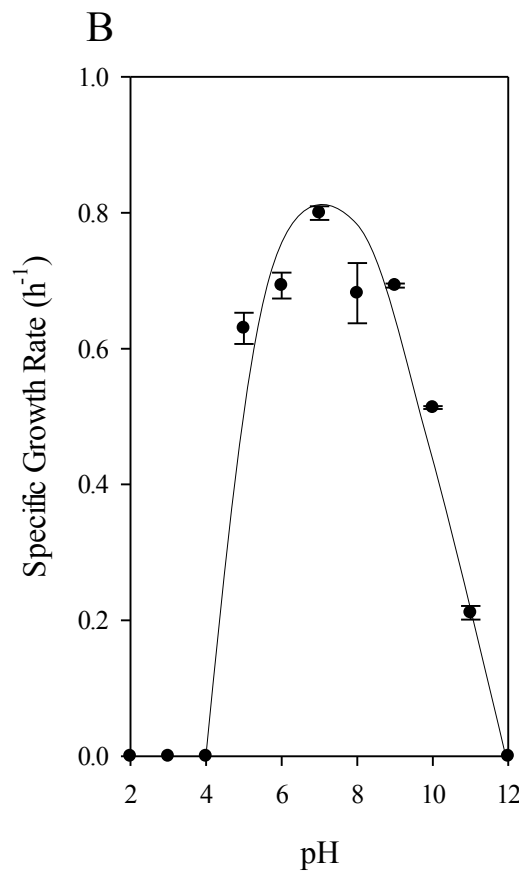
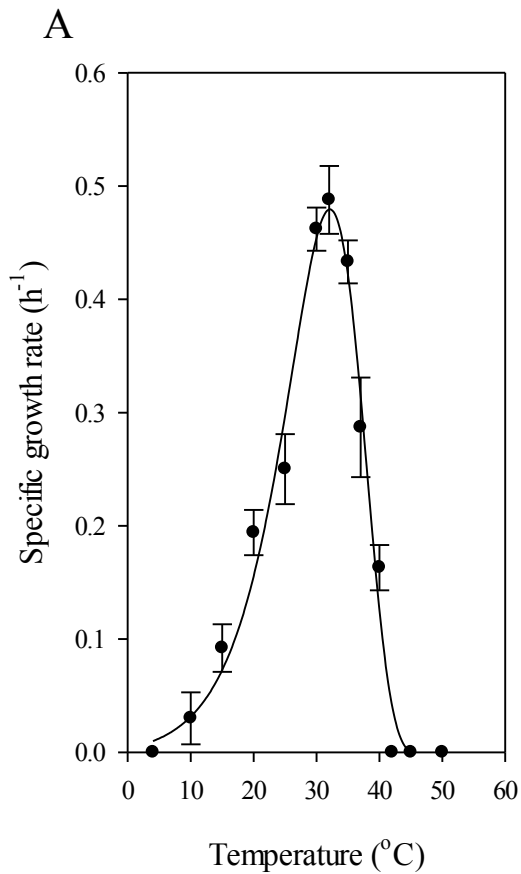


Figure 3.12 Growth of *Achromobacter* sp. Strain B on EBS with succinate (10 mM) as growth substrate at a wide range of different temperatures (A) and shaken at 100 r.p.m.. Growth rates of Strain B in nutrient broth at a range of different pH (B) and different NaCl concentrations (C) at 30 °C for 12 hours shaken at 100 r.p.m.. Data are mean \pm SEM ($n = 3$).

3.2.5 Oxidation of inorganic sulfur compounds

All three strains of this study were found to oxidise thiosulfate stoichiometrically to tetrathionate. There was no further oxidation of tetrathionate to sulfate in any of the strains tested. There was also no evidence of other polythionates (trithionate, pentathionate or hexathionate) being oxidised. No autotrophic growth was observed with inorganic sulfur compounds although small amounts (< 10%) of thiosulfate were oxidised, possibly from chemical oxidation rather than biological oxidation.

3.2.6 Transmission electron microscopy

Cells of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B were negatively stained with 2% uranyl acetate as described previously (Section 2.10.2) during late exponential stages of growth in EBS supplemented with 10 mM glucose and 10 mM succinate respectively. Figure 3.12A and 3.12B show typical cells of *Pseudomonas* sp. Strain T with a single polar flagellum approximately three times the length of the cell. Figure 3.12C and Figure 3.12D appear to show cells during the last stages of binary fission prior to the formation of two daughter cells. Figure 3.13A and 3.13B show cells of *Achromobacter* sp. Strain B which typically had peritrichous flagella of which there were between 4 and 8 per cell. The lengths of these flagella were approximately 4-5 times the length of the cells. Figure 3.13C appears to show two cells in early stages of binary fission while Figure 3.13D shows a cell developing a septum prior to complete cellular division.

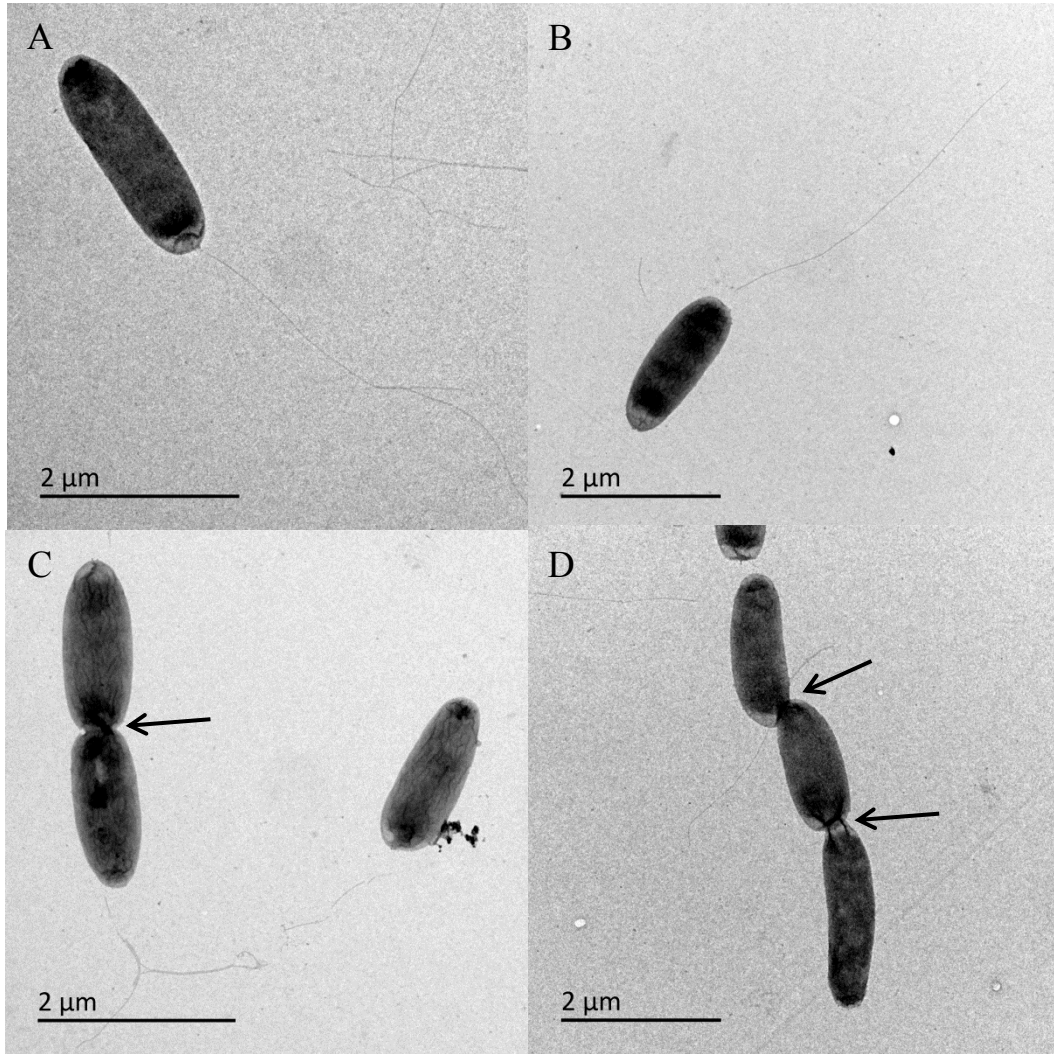


Figure 3.13 Transmission electron micrographs of *Pseudomonas* sp. Strain T during late exponential stage of growth on EBS supplemented with 10 mM glucose. Arrows indicate what appear to be the formations of a septum during the process of binary fission.

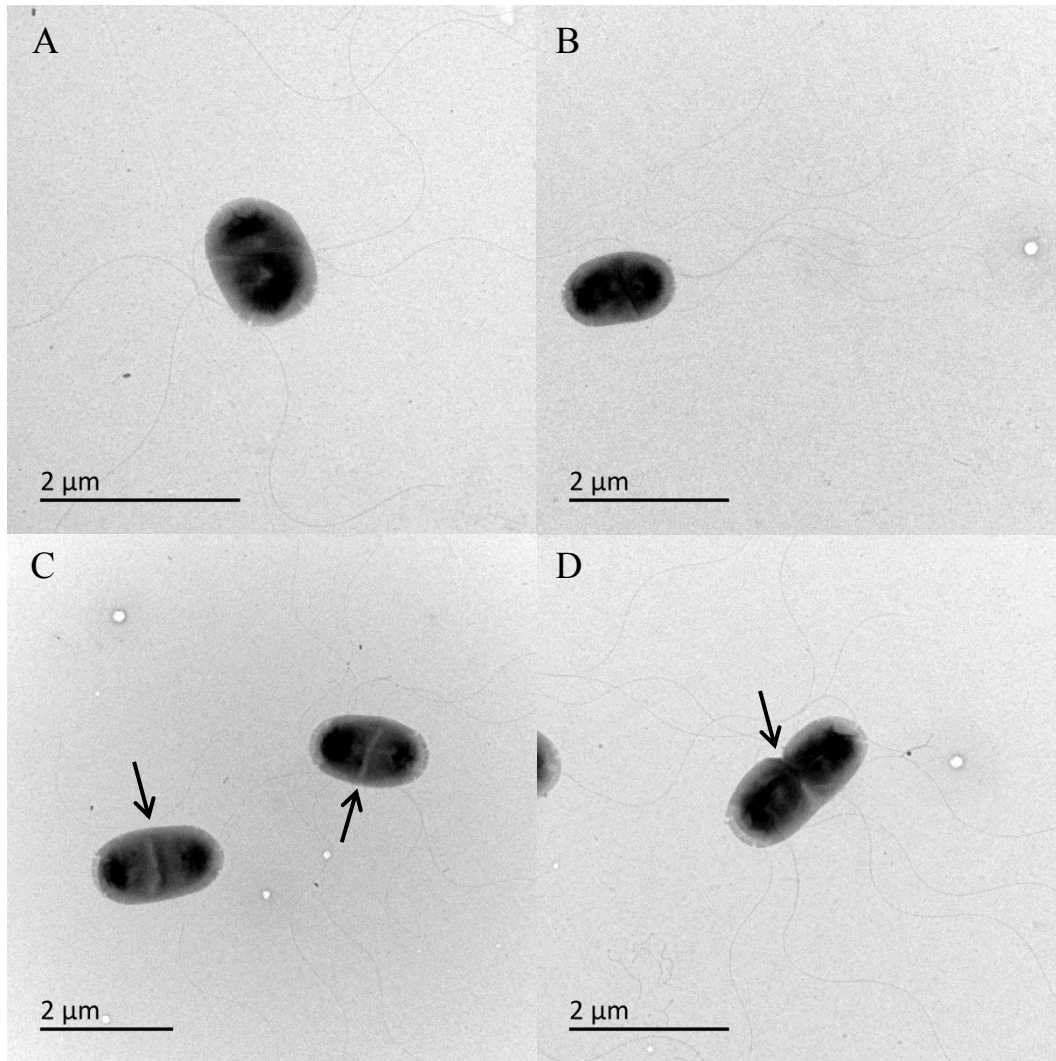


Figure 3.14 Transmission electron micrographs of *Achromobacter* sp. Strain B during late exponential stage of growth on EBS supplemented with 10 mM succinate. Arrows indicate the formation of what appear to be a septum during the process of binary fission.

3.3 Discussion

The current study confirms that three strains that were grouped historically as “*T. trautweinii*” actually belong to different genera and should be excluded from the genus *Thiobacillus*. Based on their 16S rRNA (*rrs*) and DNA gyrase subunit B (*gyrB*) gene sequences and in agreement with Sijderius (1946), Parker & Temple (1957), Hutchinson (1965) and Katayama-Fujumura *et al.* (1982) Strain T is a member of the genus *Pseudomonas*. *Pseudomonas* sp. Strain T shares most *rrs* and *gyrB* sequence identity with that of *P. chengduensis* MBR^T, *P. toyotomiensis* NCIMB 14511^T, *P. alcalophila* AL15-21^T and *P. mendocina* NCIMB 10541^T (Figure 3.1, 3.2 and 3.7, 3.8). In comparison, *Pseudomonas* sp. Strain T shares a relatively small amount of *rrs* sequence identity (84.1%) with that of the type species of *Thiobacillus* (*T. thioparus* Starkey^T) to which it was previously allied. Analyses of the *rrs* sequence of Strain VO found it shared most similarity with *P. protegens* CFBP 6595^T, *P. caricapapayae* ATCC 33615^T, *P. syringae* ATCC 19310^T and *P. fluorescens* RH 818^T (Figure 3.3 and 3.4).

In contrast to *Pseudomonas* sp. Strain T and *Pseudomonas* sp. Strain VO, however, the *rrs* sequence of Strain B placed the strain within the genus *Achromobacter* sharing the highest identity with that of *A. aegrifaciens* LMG 26852^T, *A. insuavis* LMG 26845^T, *A. spanius* LMG 5911^T and *A. piechaudii* ATCC 43552^T (Figure 3.5 and 3.6). The *gyrB* sequence put Strain B within a different cluster within *Achromobacter* occupied by *A. denitrificans* DSM 30026^T, *A. ruhlandii* ATCC 15749^T and *A. pulmonis* LMG 26696^T (Figure 3.9 and 3.10).

Many of the taxonomic tests performed on these three strains of “*T. trautweinii*” support the evidence shown by *rrs* and *gyrB* gene sequencing that *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B are novel species. A number of chemotaxonomic

comparisons between *Pseudomonas* sp. Strain T and its closest phylogenetic relations of *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T show some important differences (Table 3.2). *Pseudomonas* sp. Strain T was the only strain tested capable of oxidising thiosulfate and manganese while not being able to produce nitrogen gas from nitrate and was unable to produce indole from tryptophan, in contrast to *P. chengduensis* MBR^T. *Pseudomonas* sp. Strain T was also unable to grow in nutrient broth containing 6% (w/v) sodium chloride unlike the other closely related strains highlighted in Table 3.2. The hydrolysis of esculin and starch and the presence of single polar flagella were also differential traits observed with *Pseudomonas* sp. Strain T but not were detected or observed with *P. chengduensis* MBR^T. The differential characteristics of *Achromobacter* sp. Strain B were fewer in number when compared to its nearest phylogenetic relation in terms of *rrs* sequencing of *A. aegrifaciens* LMG 26852^T. *Achromobacter* sp. Strain B was unable to hydrolyse aesculin and Tween 20® but was able to hydrolyse Tween 80® and was able to lyse sheep red blood cells. Notable differences in enzyme activities between *Achromobacter* sp. Strain B and *A. aegrifaciens* LMG 26852^T were a lack of alkaline phosphatase activity and the presence of urease activity in *Achromobacter* sp. Strain B. The nitrogen sources utilised by these two strains for growth were markedly different as *Achromobacter* sp. Strain B was unable to use cyanate and thiocyanate but was able to utilise urea.

The whole cell fatty acid profiles of *Pseudomonas* sp. Strain T and other closely related *Pseudomonas* (Table 3.5) show that the major fatty acids to be C_{16:0}, C_{16:1} ω6c + C_{16:1} ω7c and C_{18:1} ω6c + C_{18:1} ω7c in Strain T as well as in both *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T (Tao *et al.* 2014), although levels of C_{16:0} were c.32.9% lower than its closest relation while C_{16:1} ω6c + C_{16:1} ω7c was c.53.2% higher in *Pseudomonas* sp. Strain T. *Pseudomonas* sp. Strain T also contained some fatty acids (C_{15:0}, C_{15:1} ω8c and

C_{17:1} ω6c) that were absent in the other *Pseudomonas* spp., while completely lacking C_{15:0} 3-OH, C_{18:0} ISO and C_{17:0} cyclo. Fatty acid contents of *Achromobacter* sp. Strain B and other closely related *Achromobacter* spp. (Table 3.6) were more homogeneity than that of *Pseudomonas* sp. Strain T but notable differences were a complete lack of C_{16:1} ISO + C_{14:0} 2-OH in *Achromobacter* sp. Strain B while other closely related species contained between 9.06-14.13% total content.

Optimal growth conditions of *Pseudomonas* sp. Strain T highlighted a number of contrasts to that reported of *P. chengduensis* MBR^T, *P. toyotomiensis* NCIMB 14511^T, *P. alcaliphila* AL15-21^T and *P. mendocina* NCIMB 10541^T (Palleroni *et al.*, 1970; Yumoto *et al.*, 2001; Hirota *et al.* 2011; Tao *et al.* 2014). *Pseudomonas* sp. Strain T shared similar temperature ranges but with a slightly higher optimal growth temperature of 36 °C compared to that of *P. toyotomeinsis* HT-3^T (35°C). At the time of writing the optimal growth temperature of *P. chengduensis* MBR^T, *P. alcaliphila* AL15-21^T and *P. mendocina* NCIMB 10541^T had not been reported. *Pseudomonas* sp. Strain T, *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T grew in an alkaliphilic range with growth being observed between pH 6-10. *Pseudomonas* sp. Strain T grew in sodium chloride concentration between 0-4% (w/v) with optimal growth being observed without the presence of sodium chloride. However, the other *Pseudomonas* spp. examined all tolerated sodium chloride concentrations as high as 8% (w/v) with optimal growth of both *P. toyotomiensis* HT-3^T and the marine species *P. alcaliphila* AL15-21^T requiring the presence of sodium chloride at >1% (w/v) (Yumoto *et al.* 2001; Hirota *et al.* 2011).

Many of the chemotaxonomic traits for *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B line up with many results published previously by Trautwein (1920, 1924), Sijderius (1948) and Parker and Temple (1957). In the study by Parker & Temple (1957) it was noted that the strain had 6-8 peritrichous flagella, in contradiction to the current

study. This supports the hypothesis that historically *Pseudomonas* sp. Strain T may have been a mixed culture. Regarding *Achromobacter* sp. Strain B there were few chemotaxonomic traits to set it aside from other *Achromobacter* species; as noted by Vandamme *et al.* (2013a) the genus has little intraspecies variability. Some differences however between Strain B and other species examined (Table 3.4) included hydrolysis of Tween® 80, its α -hemolytic ability and its intolerance of sodium chloride concentration of 4%.

There is sufficient data in this study to exclude three strains of “*T. trautweinii*” from the genus *Thiobacillus* and reassign them to *Pseudomonas* (Strain T and Strain VO) and *Achromobacter* (Strain B). Strain T has been shown from phylogenetic variation and from numerous chemotaxonomic traits to be adequately different from any currently validly or effectively published species to be a novel species. Although Strain B has shown less variation in terms of chemotaxonomy there was significant variation between Strain B and other *Achromobacter* spp. in terms of phylogeny also to pronounce it as a novel species. Further research on *Pseudomonas* sp. Strain VO would need to be carried out in order to identify its true position in *Pseudomonas*; particularly on its fatty acid content and by further investigation of the chemotaxonomy of its closest genetic relations. In order to conclusively confirm both *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B as novel species the DNA-DNA hybridisation of these two strains with their closest relations would need to be performed but almost certainly would confirm the results already obtained from phylogenetic and chemotaxonomic data.

3.3.1 Description of *Pseudomonas trautweiniana* sp. nov.

Pseudomonas trautweinii (traut.wei'ni.i) N. L. fem. adj. *trautweiniana*, belonging to Trautwein, named after Konrad 'Kurt' Trautwein (1881 - 1958), Bavarian bacteriologist

and botanist and originator of Strain T, later dubbed '*Thiobacillus trautweinii*' by Bergey (1925).

Cells are Gram negative rods of $0.5 \times 2.6 \mu\text{m}$ and motile by means of a single polar flagellum. Does not form rosettes, palisades or pellicles. Sometimes forms pairs or short chains in late stationary phase. Grows at 4-42 °C with an optimum of 36 °C and at pH 6-10 with an optimum of pH 7. Can grow with a sodium chloride concentration of up to 4% (w/v) but grows best when sodium chloride is absent. Reduces selenite and tellurite and can oxidise Mn (II) to Mn (IV) and thiosulfate to tetrathionate. Does not reduce nitrate or nitrite and cannot grow anaerobically with denitrification. Does not grow autotrophically. Does not produce pigment when grown on King's Medium A or B. Does not form intracellular polyphosphate, polysaccharide or β -polyhydroxybutyrate granules and does not form spores. Colonies are flat to convex and off white on nutrient agar and basal agar supplemented with glucose.

Oxidase, catalase, tryptophan deaminase, alkaline phosphatase, acid phosphatase, C₄ esterase, C₈ esterase/lipase, leucine arylamidase and arginine dihydrolase activities present. Urease, C₁₄ lipase, trypsin, α -chymotrypsin and α - and β -galactosidase, α -glucosidase, β -glucosidase, valine arylamidase, cysteine arylamidase, lysine decarboxylase, ornithine decarboxylase, α -mannosidase and α -fucosidase activities absent. Cells show the activity of thiosulfate dehydrogenase when grown in the presence of thiosulfate.

Hydrolyses starch and Tween® 20, 40, 60 and 80 but does not hydrolyse esculin, DNA, rape seed oil, gelatin or casein. Is α -hemolytic. Does not produce indole, acetoin, levan, acid from glucose or hydrogen sulfide from thiosulfate. Ferments L-arabinose, D-mannitol and D-glucose. Produces acid from glycerol, L-arabinose, D-ribose, D-mannose,

D-mannitol, D-cellobiose, D-maltose, D-sucrose, D-trehalose, starch and glycogen but does not produce acid from erythritol, D-arabinose, D-xylose, L-xylose, adonitol, β -methylxyloside, D-glucose, D-fructose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α -methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, lactose, inulin, melezitose, D-raffinose, xylitol, β -gentobiose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate.

Can utilise as sole energy and carbon source (mM): acetate (10), L-alanine (5), L-arginine (5), citrate (5), D-fructose (5), fumarate (5), D-glucose (5, 10, 20), L-glycine (5), L-histidine (5), lactose (5), L-leucine (5), L-lysine (5), L-proline (5), pyruvate (5), L-serine (5) and succinate (5, 10). Does not utilise (mM): acetamide (2.5), L-arabinose (5), L-aspartate (5), benzoate (5), L-citrulline (5), L-cysteine (5), ethanol (20), formate (10), D-galactose (5), D-malate (5), D-maltose (5), methanesulfonate (25), methanol (50), methionine (5), L-phenylalanine (5), propan-2-ol (5), propionate (10), D-sorbitol (5), D-ribose (5), D-sucrose (5), thiophene-2- acetate (5), thiophene-3-acetate (5), thiophene-2-carboxylate (5), thiophene-3-carboxylate (5), threonine (5), L-tryptophan (5), D-xylose (5). As sole source of nitrogen can use (2 mM): ammonium, glycine, urea, nitrate and nitrite but not elementary nitrogen, cyanate or thiocyanate.

The dominant respiratory quinone is UQ-9. Dominant fatty acids when grown on nutrient agar are C18:1 ω 7c, C16:1 ω 7c and C16:0. GC content of genomic DNA is 66.2 mol%. Resistant to the antibiotics ampicillin, cephalothin, chloramphenicol, clindamycin, cotrimoxazole, erythromycin, fusidic acid, penicillin G, sulphamethoxazole, sulfatriad and trimethoprim but sensitive to colistin sulfate, gentamycin, streptomycin and tetracycline.

Isolated from the River Tauber, Germany 1918 by K. Trautwein. Type strain is Strain T (NCIMB 9549^T = NCCB 38020^T = JCM 20427^T = NBRC 102364^T).

3.3.2 Description of *Achromobacter starkeyanus* sp. nov.

Achromobacter starkeyi (star.key'i) N. L. masc. adj. *starkeyi*, belonging to Starkey, named after Robert Lyman Starkey (1899 - ?), American soil microbiologist and originator of Strain B.

Cells are Gram negative rods of $0.8 \times 1.2 \mu\text{m}$ and motile by means of peritrichous flagella. Does not form rosettes, palisades or pellicles. Grows at 10-40 °C with an optimum of 35 °C and at pH 6-10 with an optimum of pH 7. Can grow with a NaCl concentration of up to 2% (w/v) but grows best with 0%. Reduces selenite and tellurite and oxidises thiosulfate to tetrathionate but does not oxidise Mn (II) to Mn (IV). Does reduce nitrate to nitrite but cannot grow anaerobically with denitrification and does not grow autotrophically. Does not produce pigment when grown on King's Medium A or B. Does not form intracellular polyphosphate, polysaccharide or β -polyhydroxybutyrate and does not form spores. Colonies are convex and white on nutrient agar and basal agar supplemented with succinate

Oxidase, catalase, urease, acid phosphatase, C₄ esterase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities present. Alkaline phosphatase, C₈ esterase/lipase, C₁₄ lipase, trypsin, α -chymotrypsin and α - and β -galactosidase, α -glucosidase, β -glucosidase, valine arylamidase, cysteine arylamidase, lysine decarboxylase, ornithine decarboxylase, α -mannosidase and α -fucosidase activities absent. Cells show the activity of thiosulfate dehydrogenase with and without the presence of thiosulfate.

Hydrolyses starch and Tween® 80 but does not hydrolyse Tween® 20, 40, 60, esculin, DNA, rape seed oil, gelatin or casein. Is α -hemolytic. Does not produce indole, acetoin, levan, acid from glucose or hydrogen sulfide from thiosulfate. Produces acid from glycerol, D-arabinose L-arabinose, D-ribose, D-xylose, adonitol, D-galactose, D-glucose, D-fructose, D-mannose, D-fucose, D-arabitol but not from erythritol, L-xylose, D-cellobiose, D-maltose, D-sucrose β -methylxyloside, L-sorbose, rhamnase, dulcitol, inositol, sorbitol, α -methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, lactose, inulin, melezitose, D-raffinose, xylitol, β -gentobiose, D-tagatose, D-fucose, , D-arabitol, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, starch and glycogen.

Can utilise as sole energy and carbon source (mM): acetate (5), citrate (5), formate (5) fumarate (5), and succinate (5, 10). Does not utilise (mM), L-arabinose (5), D-fructose (5), D-glucose (5, 10), lactose (5), D-malate (5), D-maltose (5), D-mannose (5), D-raffinose (5), D-ribose (5), D-sucrose (5), D-xylose (5). As sole source of nitrogen can use (2 mM): ammonium, glycine, urea, nitrate and nitrite but not elementary nitrogen, cyanate or thiocyanate.

The dominant respiratory quinone is UQ-8. Dominant fatty acids when grown on nutrient agar are C_{15:0} ISO + C_{16:1} ω 7c and C16:0. GC content of genomic DNA is 68.0 mol%. Resistant to the antibiotics cephalothin, chloramphenicol, clindamycin, cotrimoxazole, erythromycin, fusidic acid, gentamycin, penicillin G, streptomycin, sulphamethoxazole, sulfatriad, tetracycline and trimethoprim but sensitive to ampicillin and colistin sulfate.

Isolated from soil in New Jersey, U.S.A 1934 by R. L. Starkey, R. L.. Type strain is Strain B (NCCB 38021^T = IAM 12564^T = NCIB 9550^T).

Chapter 4

Physiology and biochemistry of the
chemolithoheterotrophs *Pseudomonas* sp. Strain T
and *Achromobacter* sp. Strain B

4.1 Introduction

Bacterial chemolithoheterotrophy is a form of mixed metabolism in which an organic carbon substrate is essential for both carbon source and energy production but the oxidation of inorganic compounds also yields additional ATP (Kuenen & Beudeker, 1982; Hipp *et al.* 1997; Ghosh & Dam, 2009). Such organisms are unable to fix CO₂, making an organic carbon source vital for growth. This distinguishes chemolithoheterotrophic metabolism from true mixotrophy where cells are able to grow solely as an autotroph under certain conditions in conjunction with heterotrophy. Some examples of inorganic electron donors in chemolithoheterotrophy include NH₄⁺, NO₂⁻, Mn²⁺, Fe²⁺, U⁴⁺, H₂, H₂S and S⁰ which are oxidised to NO₂⁻, NO₃⁻, Mn⁴⁺, Fe³⁺, U⁶⁺, H₂O, S⁰ and SO₄²⁻, respectively (Kelly, 1971; Fuchs *et al.*, 1996; Beller, 2006; Nealson, 2006).

Heterotrophic *Bacteria* that are capable of oxidising thiosulfate (S₂O₃²⁻) have been shown to do so in two manners. The first is the complete oxidation of thiosulfate to sulfate (SO₄²⁻) *via* the Kelly-Trudinger (S₄I) or Kelly-Freidrich (Sox) pathways; the second is the oxidation of thiosulfate to tetrathionate (S₄O₆²⁻) by a partial Kelly-Trudinger pathway (Equation 1). Such species have been isolated and studied since 1921 (Trautwein, 1921) from both marine and terrestrial environments (Starkey, 1934*a, b*; Sijderius, 1946; London & Rittenberg, 1967; Trudinger, 1967; Tuttle *et al.*, 1972; Tuttle *et al.*, 1974; Tuttle, 1980; Mason & Kelly, 1988*a*; Sorokin, 1999; Sorokin *et al.*, 2006; Boden *et al.*, 2010) including a number of strains of “*Thiobacillus trautweinii*” (Trautwein, 1921) which are the subject of this study. From the few studies conducted on these organisms, they appear to be split into two main groups: species that gain additional biomass when grown in the presence of reduced inorganic sulfur (“fortuitous oxidation”) and species that oxidise sulfur but gain no apparent benefit from it (“gratuitous oxidation”). Most reports have stated that tetrathionate and other polythionates were the end products of

thiosulfate oxidation, although some studies have indicated the full oxidation to sulfate (Pepper & Miller, 1978; Schook & Berk, 1978). Unlike mixotrophy, which has received extensive research in the past, this area of bacterial metabolism has received very little research and the benefits of chemolithoheterotrophy are very poorly understood.



Oxidation of thiosulfate to tetrathionate precedes in the full or partial Kelly-Trudinger pathway *via* thiosulfate dehydrogenase (EC 1.8.2.2), which catalyses the reaction in Equation 1 and is believed to involve a *c* type cytochrome. The details of this enzyme remain uncertain as at least two discrete groups of thiosulfate dehydrogenase enzymes have been studied in the *Bacteria*. Denkmann *et al.* (2012) found that thiosulfate dehydrogenase in *Allochromatium vinosum* is a cytochrome *c* of *c.*25 kDa. A similar size protein has also been found in cells of *Acidithiobacillus ferrooxidans*, although no haem groups were detected (Silver and Lundgren, 1968; Kikumoto *et al.*, 2013), ruling out it being a cytochrome *c*. In contradiction to this are the much larger proteins associated with thiosulfate oxidation activity in *Thermithiobacillus tepidarius*, *Thiobacillus thioparus*, *Acidiphilium acidophilum* and the marine heterotroph 16B that range between 100 – 140 kDa (Trudinger, 1961; Lyric and Suzuki, 1970; Whited and Tuttle, 1983; Lu and Kelly, 1988). This is a strong indication that more than one type of thiosulfate dehydrogenase may be present in the *Bacteria*.

A group of sulfur-oxidising heterotrophs that oxidise thiosulfate to tetrathionate has historically been classified as “*Thiobacillus trautweinii*”. Of the main strains dubbed as “*T. trautweinii*”, the original strain was isolated in the 1920s by Trautweinii from the River Tauber, Germany (Strain T) and was placed in the genus *Thiobacillus* due to its ability to oxidise thiosulfate (Trautwein, 1921). At the time this was thought to be a

unique ability of this genus but in the decades that followed thiosulfate oxidation has shown to be a far more diverse trait belonging to many genera. The initial study into this organism found that thiosulfate was oxidised to tetrathionate and other polythionates but no further utilisation of inorganic sulfur was observed. That is, thiosulfate was not fully oxidised to sulfate like that observed in true *Thiobacillus* spp.. Later work found that in batch culture thiosulfate was indeed oxidised to tetrathionate and other polythionates although this was apparently at a relatively slow rate with *c.*90% of thiosulfate having decomposed over a 19 day period under the conditions used (Starkey, 1934*b*; Starkey, 1934*c*). Starkey also isolated a second strain of “*T. trautweini*” (Strain B) from soil in New Jersey, U.S.A that similarly oxidised thiosulfate to polythionates and it was concluded that Strain T and Strain B were in fact obligate heterotrophs. More recently, it has been shown that Strain T and Strain B are members of *Pseudomonas* and *Achromobacter*, respectively (Chapter 3). However, whether *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B were oxidising thiosulfate in a “fortuitous” or “gratuitous” manner was left unresolved.

The purpose of this study was to examine *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B for their chemolithoheterotrophic ability and to compare any benefits these organisms gain when grown with thiosulfate as a chemolithotroph compared to heterotroph.

4.2 Results

4.2.1 The growth of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B in batch culture

4.2.1.1 *Pseudomonas* sp. Strain T growth in batch culture

Pseudomonas sp. Strain T was grown in 500 mL EBS medium supplemented with 10 mM glucose, 20 mM thiosulfate or 10 mM glucose plus 20 mM thiosulfate together in 2,500-mL Erlenmeyer flasks at 30 °C (Figure 4.1). Growth kinetics of *Pseudomonas* sp. Strain T on these different substrates are summarised in Table 4.1. No glucose was detected at or after 10 and 11 h for medium supplemented with glucose and glucose plus thiosulfate, respectively. This coincided with a drop in specific growth rate in glucose plus thiosulfate batch cultures, although this was not statistically significant. Batch growth of *Pseudomonas* sp. Strain T when grown on glucose plus thiosulfate did show a significant increase in yield with 86.5% of the thiosulfate having been oxidised to tetrathionate. When *Pseudomonas* sp. Strain T was grown in batch culture with 20 mM thiosulfate alone there was no evidence of growth and only a small proportion of thiosulfate had been oxidised to tetrathionate (0.37 ± 0.08 mM).

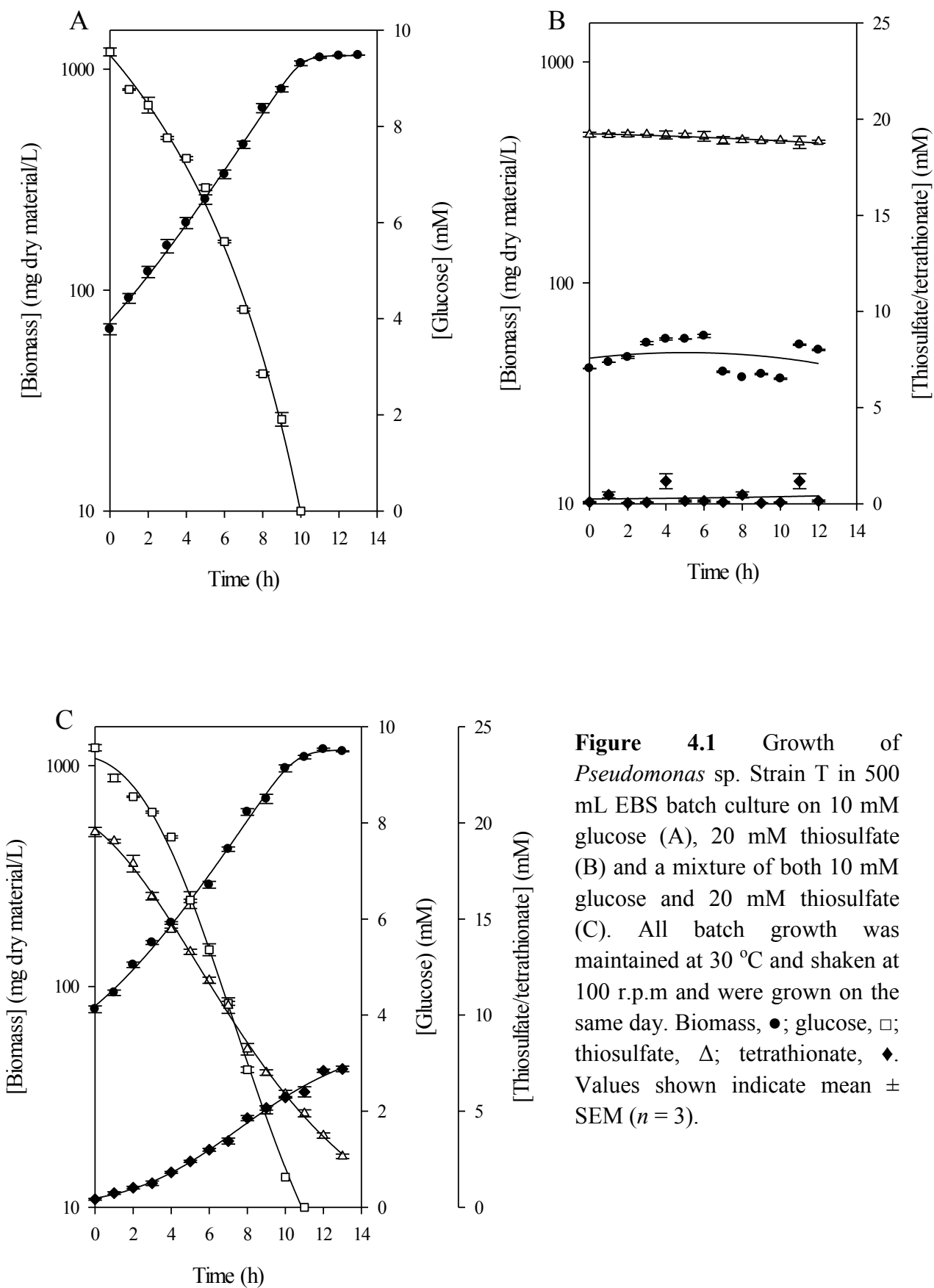


Figure 4.1 Growth of *Pseudomonas* sp. Strain T in 500 mL EBS batch culture on 10 mM glucose (A), 20 mM thiosulfate (B) and a mixture of both 10 mM glucose and 20 mM thiosulfate (C). All batch growth was maintained at 30 °C and shaken at 100 r.p.m and were grown on the same day. Biomass, ●; glucose, □; thiosulfate, Δ; tetrathionate, ◆. Values shown indicate mean ± SEM ($n = 3$).

Table 4.1 Growth kinetics of *Pseudomonas* sp. Strain T grown in batch culture on glucose, glucose plus thiosulfate and thiosulfate only. * Statistically significant difference (Student's t-test, $p = 0.049$). Parameters are maximum biomass formed (X_{\max} - mg dry biomass/L), yield (Y - mg dry biomass/mM glucose), specific growth rate (μ - per hour) and specific rate of substrate uptake (q - mmol/h). Values shown are mean \pm SEM ($n = 3$).

| Kinetic Parameter | Growth substrate: | | |
|-------------------|-------------------|-------------------------------------|-------------------|
| | 10 mM glucose | 10 mM glucose/ 20 mM thiosulfate | 20 mM thiosulfate |
| X_{\max} | 1040 \pm 13.2 | 1084 \pm 4.6 | 0 |
| Y | 109 \pm 0.8* | 113 \pm 0.8* | 0 |
| μ | 0.297 \pm 0.005 | 0.277 \pm 0.008 | 0 |
| q | 0.955 \pm 0.008 | 0.869 \pm 0.006 | 0 |

4.2.1.2 *Achromobacter* sp. Strain B growth in batch culture

Achromobacter sp. Strain B was grown in 500 mL EBS medium supplemented with either 10 mM succinate, 20 mM thiosulfate or 10 mM succinate plus 20 mM thiosulfate together in 2,500-mL Erlenmeyer flasks at 30 °C (Figure 4.3). Growth kinetics of *Achromobacter* sp. Strain B on these different substrates are summarised in Table 4.2. No succinate was detected at or after 9 and 11 hours for medium supplemented with succinate and succinate plus thiosulfate, respectively. This coincided with a drop in specific growth rate in the succinate plus thiosulfate batch cultures, although this was not statistically significant. Batch growth of *Achromobacter* sp. Strain B grown on succinate plus thiosulfate showed a decrease in yield with 98.5% of the thiosulfate having been oxidised to tetrathionate although this decrease was not statistically significant. When *Achromobacter* sp. Strain B was grown in batch culture with 20 mM thiosulfate alone there was no evidence of growth, however 22.8% of thiosulfate had been oxidised to tetrathionate after 36 h (4.58 ± 1.20 mM).

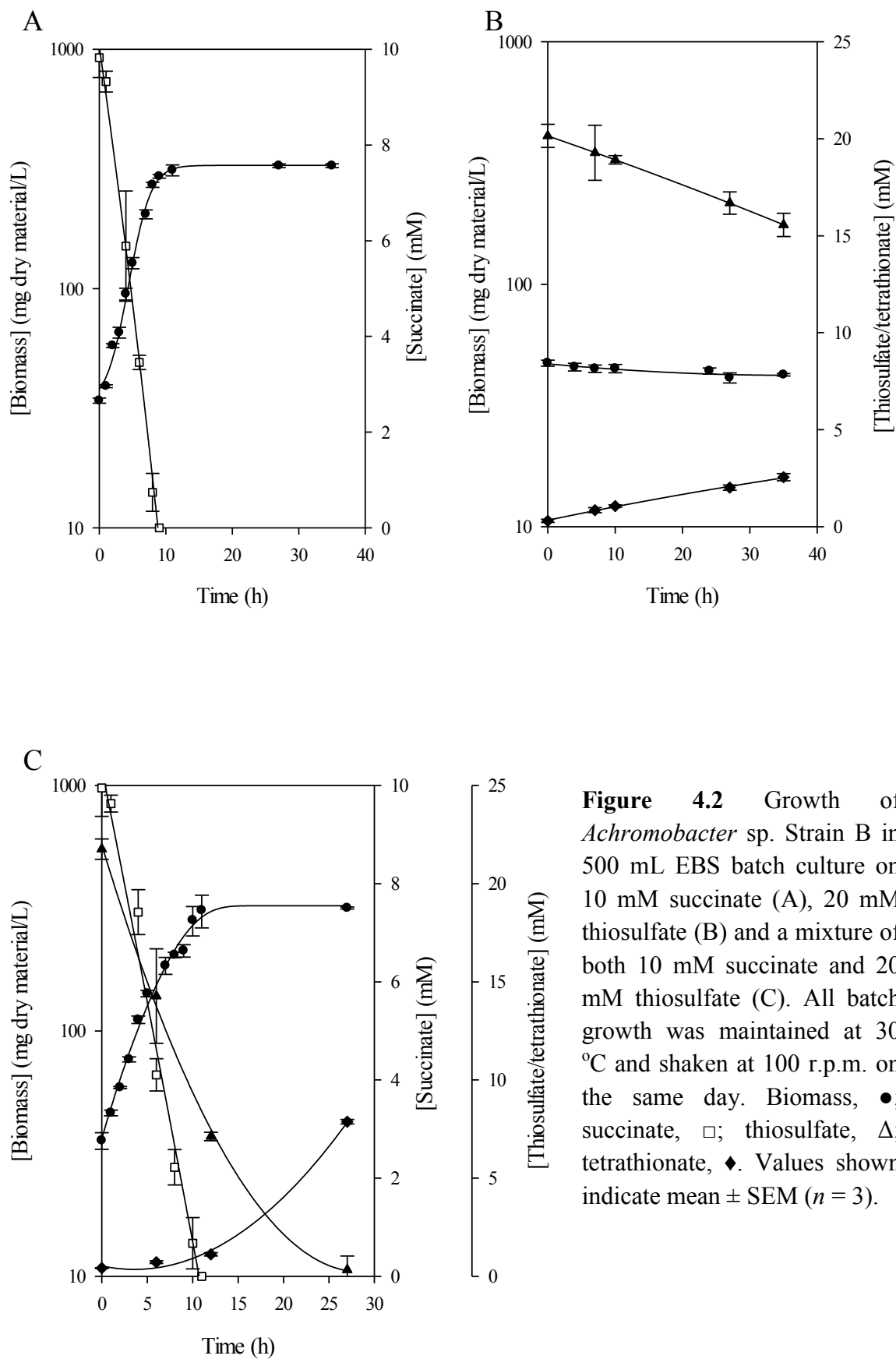


Figure 4.2 Growth of *Achromobacter* sp. Strain B in 500 mL EBS batch culture on 10 mM succinate (A), 20 mM thiosulfate (B) and a mixture of both 10 mM succinate and 20 mM thiosulfate (C). All batch growth was maintained at 30 °C and shaken at 100 r.p.m. on the same day. Biomass, ●; succinate, □; thiosulfate, Δ; tetrathionate, ◆. Values shown indicate mean ± SEM ($n = 3$).

Table 4.2. Growth kinetics of *Achromobacter* sp. Strain B grown in batch culture on succinate, thiosulfate or succinate plus thiosulfate. Parameters are maximum biomass formed (X_{\max} - mg dry biomass/L), yield (Y - g dry biomass/mole succinate), specific growth rate (μ - per hour) and specific rate of substrate uptake (q - mmol/h). Values shown are mean \pm SEM ($n = 3$).

| Kinetic Parameter | Growth substrates: | | |
|-------------------|--------------------|---------------------------------------|-------------------|
| | 10 mM succinate | 10 mM succinate/ 20 mM thiosulfate | 20 mM thiosulfate |
| X_{\max} | 292 \pm 7 | 281 \pm 1 | 0 |
| Y | 30 \pm 0.6 | 28 \pm 1.3 | 0 |
| μ | 0.293 \pm 0.014 | 0.273 \pm 0.018 | 0 |
| q | 0.983 \pm 0.07 | 0.905 \pm 0.05 | 0 |

4.2.1.3 Comparisons in growth of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B growth in batch culture

The percentage increases and decreases of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B grown chemolithoheterotrophically compared to heterotrophic growth were compared, the results of which can be seen in Table 4.2. There were similar decreases in specific growth rates (μ) and specific rate of substrate uptake (q) for both strains. However, notable differences were the significant increase in Y observed in *Pseudomonas* sp. Strain T and the non-significant decrease in Y observed in *Achromobacter* sp. Strain B.

4.2.2 The growth of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B in continuous culture

4.2.2.1 Growth of *Pseudomonas* sp. Strain T on glucose (carbon limitation)

Steady-state cultures of *Pseudomonas* sp. Strain T were established with 10 mM glucose as the sole limiting substrate at dilution rates (D) = 0.032 – 0.178 h⁻¹. Between these D the growth yield (Y) increased from 92.77 to 117.83 g dry biomass per mol glucose (equivalent to 15.46 to 19.64 g dry biomass per mol substrate carbon). By producing hyperbolic models (Figure 4.3) the maximum theoretical growth yield (Y_{MAX}) was determined as 129.69 g dry biomass per mol of glucose (21.62 g dry biomass per mol of substrate carbon). From washout kinetics, the maximum specific growth rate (μ_{MAX}) was found to be 0.361 h⁻¹. The maintenance energy coefficient with respect to carbon source (m_s) was calculated from the hyperbolic curve (Figure 4.3) and was estimated as 0.107 mmol glucose (g•h)⁻¹.

4.2.2.2 Growth of *Pseudomonas* sp. Strain T on glucose plus thiosulfate (carbon limitation)

Steady-state cultures of *Pseudomonas* sp. Strain T were established with 10 mM glucose as the limiting substrate with the addition of 50 mM thiosulfate $D = 0.033 - 0.178 \text{ h}^{-1}$. Between these D the growth Y increased from 97.81 to 131.02 g dry biomass per mol glucose (equivalent to 16.3 to 21.84 g dry biomass per mol substrate carbon). By producing a hyperbolic model (Figure 4.3) the Y_{MAX} was determined as 159.0 g dry biomass per mol of glucose (26.5 g dry biomass per mol of substrate carbon). From washout kinetics, the μ_{MAX} was found to be 0.41 h^{-1} . The m_s with respect to carbon source was calculated from the hyperbolic curve (Figure 4.3) and was estimated as $0.121 \text{ mmol glucose (g}\cdot\text{h)}^{-1}$.

Table 4.3. Percentage increases and decreases of chemolithoheterotrophic batch growth of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B compared to heterotrophic batch growth. Parameters are yield (Y - g dry biomass/mole succinate), specific growth rate (μ - per hour) and specific rate of substrate uptake (q - mmol/h). Values shown are mean \pm SEM ($n = 3$).

| Kinetic Parameter | <i>Pseudomonas</i> sp. Strain T | <i>Achromobacter</i> sp. Strain B |
|--------------------------|--|--|
| Y | + 4.1 \pm 0.8* | - 7.3 \pm 5 |
| μ | - 6.8 \pm 3 | - 6.7 \pm 6 |
| q | - 9.0 \pm 1 | - 8.0 \pm 3 |

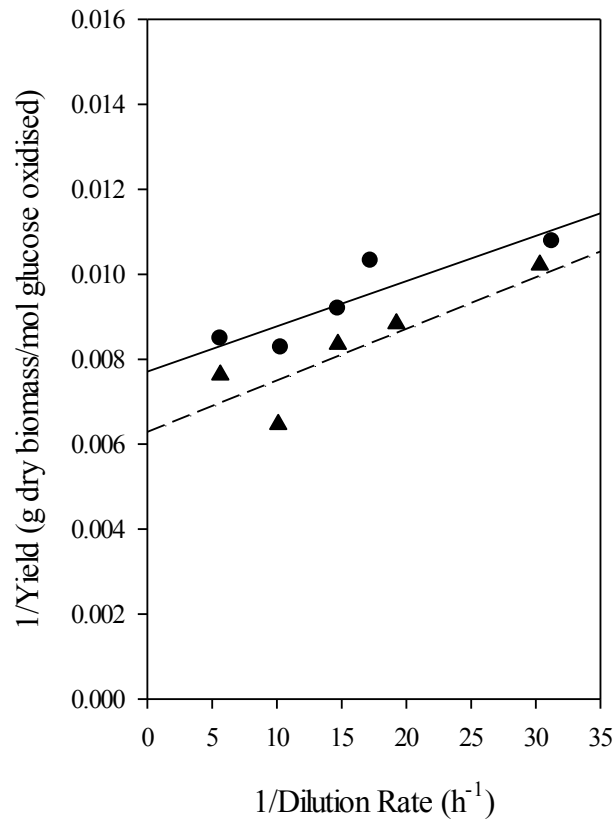


Figure 4.3 Derived double reciprocal plot yield (Y) as amount of dry biomass versus dilution rate of *Pseudomonas* sp. Strain T in a carbon limited chemostat. Regressions are based on hyperbolic fit and represent growth on 10 mM glucose as sole carbon source (solid line, circles) and growth on 10 mM glucose supplemented with 50 mM thiosulfate (dashed line, triangles). From these curves the m_s were calculated.

4.2.2.3 Growth of *Pseudomonas* sp. Strain T under oxygen limitation

A single steady-state ($D = 0.068 \text{ h}^{-1}$) was established of *Pseudomonas* sp. Strain T with 10 mM glucose as the sole substrate but with dissolved oxygen concentration maintained at 0% saturation. The growth Y was 93.05 g dry biomass per mol glucose (equivalent to 15.51 g dry biomass per mol substrate carbon). At this point there was still excess glucose left in the medium ($7.95 \pm 0.05 \text{ mM}$).

A single steady-state ($D = 0.068 \text{ h}^{-1}$) was established of *Pseudomonas* sp. Strain T with 10 mM glucose as the sole substrate supplemented with 50 mM thiosulfate but with dissolved oxygen concentration maintained at 0%. The growth Y was 96.48 g dry biomass per mol glucose (equivalent to 16.08 g dry biomass per mol substrate carbon). At this point there was still excess glucose left in the medium ($8.14 \pm 0.05 \text{ mM}$).

4.2.2.4 Growth of *Pseudomonas* sp. Strain T under phosphate limitation

A single steady-state ($D = 0.068 \text{ h}^{-1}$) was established of *Pseudomonas* sp. Strain T with 10 mM glucose as the sole substrate but with phosphate in the growth medium 1/10 of the standard concentration. The growth Y was 104.79 g dry biomass per mol glucose (equivalent to 17.47 g dry biomass per mol substrate carbon). At this point there was still excess glucose left in the medium ($6.84 \pm 0.02 \text{ mM}$).

A single steady-state ($D = 0.068 \text{ h}^{-1}$) was established of *Pseudomonas* sp. Strain T with 10 mM glucose as the sole substrate supplemented with 50 mM thiosulfate but with phosphates in the growth medium 1/10 of the standard concentration. The growth Y was 104.79 g dry biomass per mol glucose (equivalent to 17.47 g dry biomass per mol substrate carbon). At this point there was still excess glucose left in the medium ($6.73 \pm 0.09 \text{ mM}$).

4.2.2.5 Growth of *Pseudomonas* sp. Strain T supplemented with manganese

A single steady-state ($D = 0.068 \text{ h}^{-1}$) was established of *Pseudomonas* sp. Strain T with 10 mM glucose as the sole limiting substrate. The growth Y was 109.03 g dry biomass per mol glucose (equivalent to 18.17 g dry biomass per mol substrate carbon). A second steady-state was established at the same D and same concentration of glucose but with the addition of 50 mM manganese as a possible additional energy source. The growth Y of which was 105.35 g dry biomass per mol glucose (equivalent to 17.56 g dry biomass per mol substrate carbon).

4.2.2.6 Growth of *Achromobacter* sp. Strain B on succinate (carbon limitation)

Steady-state cultures of *Achromobacter* sp. Strain B were established with 10 mM succinate as the sole limiting growth substrate at D of $0.034 - 0.195 \text{ h}^{-1}$. Between these D the Y increased from 21.98 to 38.8 g dry biomass per mol succinate (equivalent to 5.50 to 9.70 g dry biomass per mol substrate carbon). By producing a hyperbolic model (Figure 4.4) the Y_{MAX} was determined as 42.29 g dry biomass per mol of succinate (10.57 g dry biomass per mol of substrate carbon). From washout kinetics, the μ_{MAX} was found to be 0.388 h^{-1} . The m_s with respect to carbon source was calculated from the hyperbolic curve (Figure 4.4) and was estimated as $0.63 \text{ mmol succinate (g}\cdot\text{h)}^{-1}$.

4.2.2.7 Growth of *Achromobacter* sp. Strain B on succinate plus thiosulfate (carbon limitation)

Steady-state cultures of *Achromobacter* sp. Strain B were established with 10 mM succinate as the limiting growth substrate with the addition of 50 mM thiosulfate at D of $0.031 - 0.163 \text{ h}^{-1}$. Between these D the growth yield (Y) increased from 21.15 to 45.18 g dry biomass per mol succinate (equivalent to 5.29 to 11.30 g dry biomass per mol substrate carbon). By producing a hyperbolic model (Figure 4.4) the Y_{MAX} was determined as 62.61 g dry biomass per mol of succinate (15.65 g dry biomass per mol of substrate

carbon). From washout kinetics, the μ_{MAX} was found to be 0.398 h^{-1} . The m_s with respect to carbon source was calculated from the hyperbolic curve (Figure 4.4 B) and was estimated as $1.16 \text{ mmol succinate (g}\cdot\text{h)}^{-1}$.

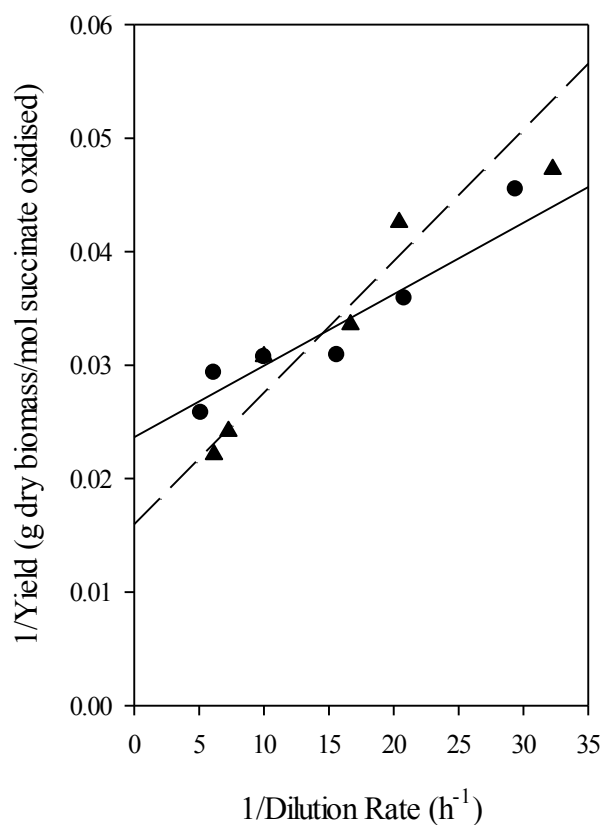
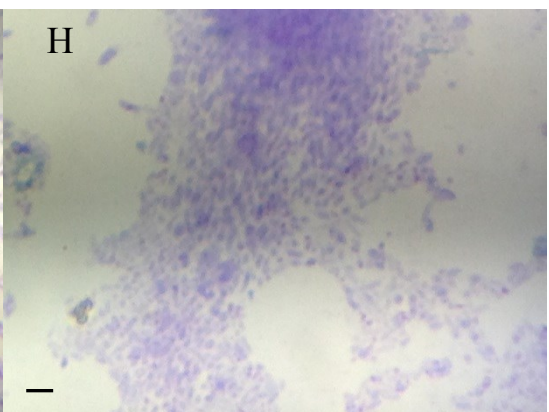
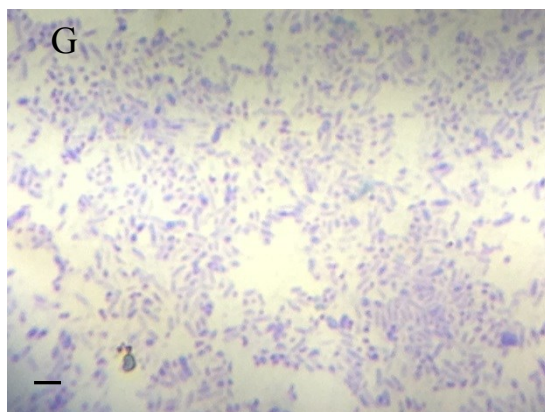
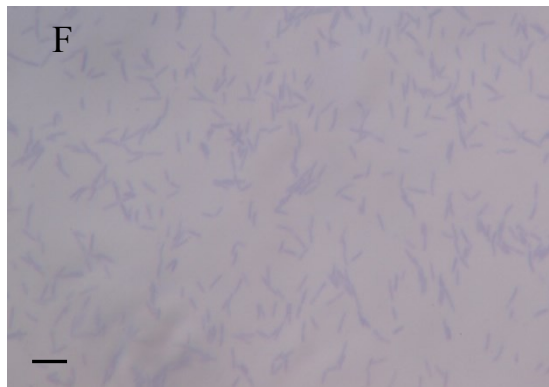
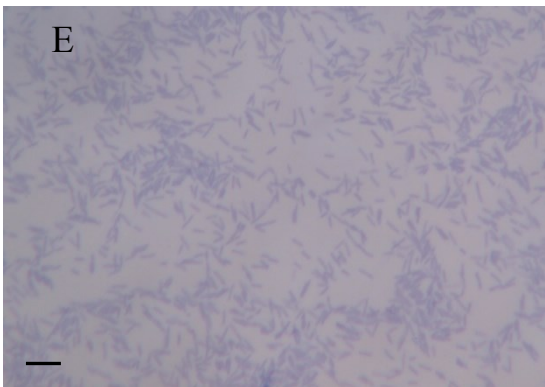
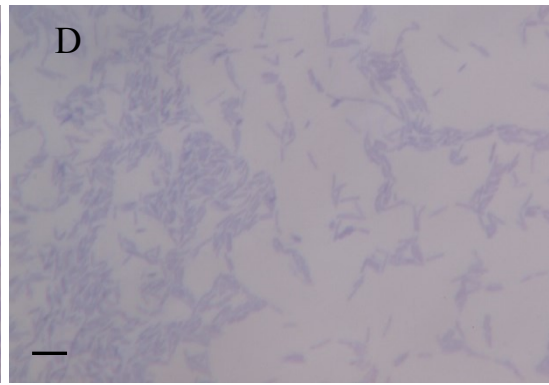
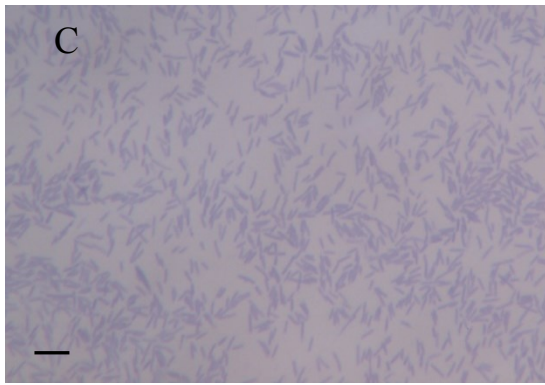
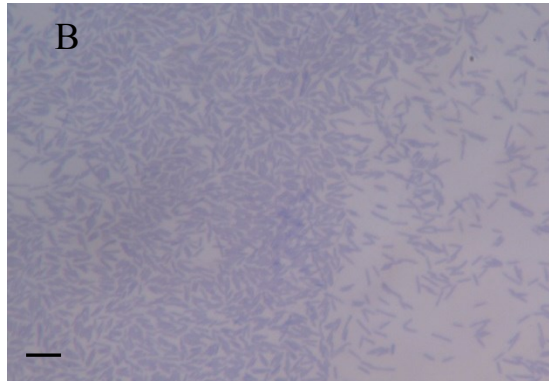
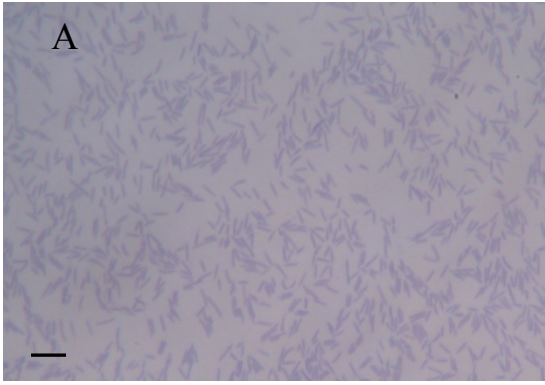


Figure 4.4 Derived double reciprocal plot of yield (Y) as amount of dry biomass versus dilution rate of *Achromobacter* sp. Strain B in a carbon limited chemostat. Regressions are based on hyperbolic fit and represent growth on 10 mM succinate as sole carbon source (solid line, circles) and growth on 10 mM succinate supplemented with 50 mM thiosulfate (dashed line). From these curves the m_s were calculated.

4.2.3 Polyphosphate production in *Pseudomonas* sp. Strain T and *Achromobacter* sp.

Strain B

Steady-state cells of *Pseudomonas* sp. Strain T ($D = 0.068 \text{ h}^{-1}$) and *Achromobacter* sp. Strain B ($D = 0.10 \text{ h}^{-1}$) were investigated for the production of intracellular polyphosphate granules with *Corynebacterium xerosis* DSM 7176 as a positive control (Figure 4.5). Cells were stained using the volutin granule stain. The production of polyphosphates and any subsequent increase in cell size were not observed under any growth regime.



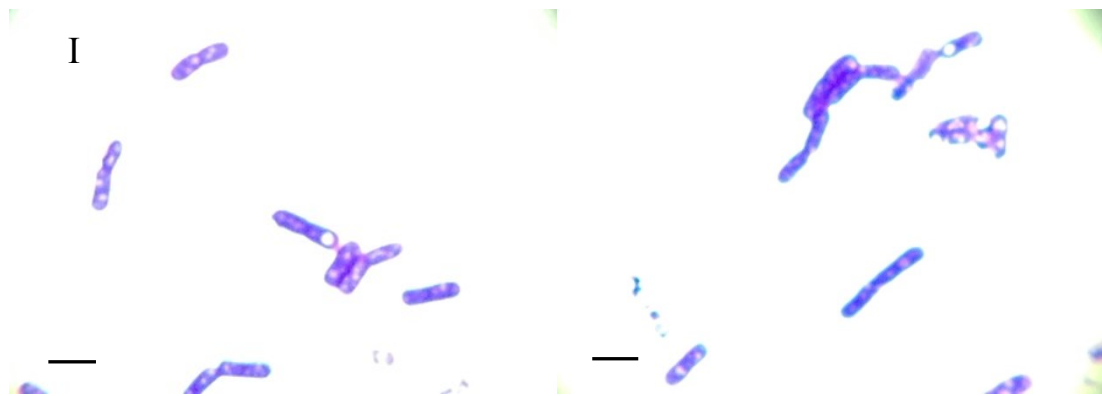


Figure 4.5 Volutin granule stains of steady-state cells of *Pseudomonas* sp. Strain T ($D = 0.068 \text{ h}^{-1}$) grown on (A) 10 mM glucose (carbon limited), (B) 10 mM glucose/50 mM thiosulfate (carbon limited), (C) 10 mM glucose (oxygen limited), (D) 10 mM glucose/50 mM thiosulfate (oxygen limited), (E) 10 mM glucose (phosphate limited), (F) 10 mM glucose/50 mM thiosulfate (phosphate limited). Also shown are steady-state cells of *Achromobacter* sp. Strain B ($D = 0.10 \text{ h}^{-1}$) grown on (G) 10 mM succinate and (H) 10 mM succinate/50 mM thiosulfate. (I) is a control species (*Corynebacterium xerosis* DSM 7176).

4.2.4 Enzymology of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B in chemostat steady-state cultures.

4.2.4.1 Specific enzyme activities of *Pseudomonas* sp. Strain T

In order to understand the biochemistry of *Pseudomonas* sp. Strain T under heterotrophic and chemolithoheterotrophic growth conditions a number of important metabolic enzymes were assayed and compared in order to highlight any key differences. Cells of *Pseudomonas* sp. Strain T were harvested from steady state chemostats ($D = 0.068 \text{ h}^{-1}$) of 10 mM glucose and 10 mM glucose plus 50 mM thiosulfate when the limiting factors were carbon, oxygen or phosphate. Cell-free extracts were prepared from these cells and used for enzyme assays summarised in Table 4.4.

Thiosulfate dehydrogenase (EC 1.8.2.2) activity was detected in cell-free extracts of cells grown in the presence of thiosulfate under carbon, oxygen and phosphate limitation but no activity was detected in heterotrophically grown cells. Other enzymes involved in inorganic sulfur metabolism were not detected under any growth regimes.

Enzymes of the tricarboxylic acid (TCA) cycle were assayed from cell-free extracts obtained from chemolithoheterotrophically and heterotrophically grown cells under carbon, oxygen and phosphate. Pairwise comparisons of chemolithoheterotrophic versus heterotrophic under the different limiting growth factors were made and no significant differences were found between the activities of citrate synthase (EC 4.1.3.7), aconitase (4.2.1.3), iso-citrate dehydrogenase (1.1.1.41), α -ketoglutarate dehydrogenase (EC 1.2.4.2), succinyl-CoA synthetase (EC 6.2.1.4) and fumarase (EC 4.2.1.2) under any limiting growth factor. There were significant increases in succinate dehydrogenase (EC 1.3.5.1) under carbon (Student's t test, $p < 0.05$) and oxygen limitation (Student's t test, $p < 0.005$) and a significant increase of malate dehydrogenase (EC 1.1.1.37) under carbon

limitation (Student's t test, $p < 0.005$). Activities of succinate dehydrogenase under phosphate limitation and malate dehydrogenase under oxygen and phosphate limitation showed no significant differences.

4.2.4.2 Specific enzyme activities of *Achromobacter* sp. Strain B

Cells of *Achromobacter* sp. Strain B were harvested from steady state chemostat cultures ($D = 0.10 \text{ h}^{-1}$) on 10 mM succinate and 10 mM succinate plus 50 mM thiosulfate when the limiting factor was carbon. Cell-free extracts were prepared from these cells and were assayed for a number of different enzymes (Table 4.5). Thiosulfate dehydrogenase (EC 1.8.2.2) activity was detected under heterotrophic and chemolithoheterotrophic growth conditions although there was significantly more activity present when cells were grown in the presence of thiosulfate (Student's t test, $p < 0.001$). Other enzymes involved in inorganic sulfur metabolism that were detected under both grow regimes but were significantly higher in the presence of thiosulfate were sulfite dehydrogenase (EC 1.8.2.1) (Student's t test, $p < 0.001$) and trithionate hydrolase (EC 3.12.1.1) (Student's t test, $p < 0.001$). No other enzymes involved with inorganic oxidation were detected.

The specific enzyme activities of the TCA cycle were detected and showed some variation between heterotrophic and chemolithoheterotrophic growth. There were no significant differences between the activities of citrate synthase (EC 4.1.3.7), aconitase (EC 4.2.1.3), iso-citrate dehydrogenase (EC 1.1.1.41), succinyl-CoA synthetase (EC 1.3.5.1) and succinate dehydrogenase (EC 1.3.5.1) between the different growth regimes. However; there was a significant drop in the activities of α -ketoglutarate dehydrogenase (EC 1.2.4.2) (Student's t test, $p < 0.001$), fumarase (EC 4.2.1.2) (Student's t test, $p = 0.005$) and malate dehydrogenase (EC 1.1.1.37) (Student's t test, $p < 0.001$) in cells grown chemolithoheterotrophically when compared to heterotrophically grown cells.

A number of other enzymes that are involved in carbohydrate metabolism were assayed (glucose dehydrogenase, glucokinase and glucose-6-phosphate dehydrogenase) but in all cases there were no significant differences between chemolithoheterotrophic and heterotrophic growth under carbon, oxygen or phosphate limitation.

Table 4.4 Specific enzyme activities in *Pseudomonas* sp. Strain T cell-free extracts from cells grown in chemostats on glucose or glucose plus thiosulfate under carbon, oxygen and phosphate limitation ($D = 0.068 \text{ h}^{-1}$). Values in bold represent significantly higher activities when pairwise comparisons were made between heterotrophic and chemolithoheterotrophic grown cells from the same limiting factor. *BDL* – below detection limit. Values represent mean \pm SEM ($n = 3$).

| Enzyme | EC number | Specific activities [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] in CFEs from cells grown on: | | | | | |
|---|-----------|--|----------------|-------------------|---------------------------------|-------------------------------|-------------------------------|
| | | 10 mM glucose | | | 10 mM glucose/50 mM thiosulfate | | |
| | | Carbon limited | Oxygen limited | Phosphate limited | Carbon limited | Oxygen limited | Phosphate limited |
| <i>Inorganic sulfur enzymes:</i> | | | | | | | |
| Thiosulfate dehydrogenase | 1.8.2.2 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | *134 \pm 6 | *96 \pm 5 | *84 \pm 6 |
| Trithionate hydrolase | 3.12.1.1 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| Tetrathionate hydrogenase | 3.12.1.B1 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| Sulfite dehydrogenase | 1.8.2.1 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| Thiocyanate dehydrogenase | - | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| <i>TCA cycle enzymes:</i> | | | | | | | |
| Citrate synthase | 4.1.3.7 | *111 \pm 7 | 63 \pm 4 | 61 \pm 2 | 89 \pm 9 | 91 \pm 11 | 55 \pm 1 |
| Aconitase | 4.2.1.3 | 18 \pm 2 | 17 \pm 1 | 10 \pm 1 | 21 \pm 1 | 16 \pm 2 | 12 \pm 2 |
| Isocitrate dehydrogenase | 1.1.1.41 | 66 \pm 2 | 69 \pm 3 | 54 \pm 3 | 74 \pm 3 | 63 \pm 4 | 59 \pm 3 |
| α -ketoglutarate dehydrogenase | 1.2.4.2 | 31 \pm 3 | 9 \pm 2 | 13 \pm 1 | 25 \pm 5 | 15 \pm 2 | 14 \pm 1 |
| Succinyl-CoA synthetase | 6.2.1.4 | 39 \pm 2 | 27 \pm 2 | 32 \pm 1 | 45 \pm 5 | 39 \pm 3 | 40 \pm 2 |
| Succinate dehydrogenase | 1.3.5.1 | 18 \pm 1 | 10 \pm 1 | 7 \pm 1 | *21 \pm 1 | *18 \pm 1 | 7 \pm 1 |
| Fumerase | 4.2.1.2 | 89 \pm 11 | 36 \pm 5 | 45 \pm 7 | 101 \pm 3 | 46 \pm 3 | 43 \pm 1 |
| Malate dehydrogenase | 1.1.1.37 | 222 \pm 2 | 180 \pm 12 | 186 \pm 4 | *264 \pm 7 | 184 \pm 33 | 204 \pm 33 |
| <i>Carbohydrate enzymes:</i> | | | | | | | |
| Glucose dehydrogenase | 1.1.1.47 | 51 \pm 1 | 48 \pm 2 | 44 \pm 3 | 49 \pm 1 | 49 \pm 2 | 47 \pm 3 |
| Glucokinase | 2.7.1.2 | 236 \pm 5 | 201 \pm 22 | 221 \pm 8 | 226 \pm 4 | 195 \pm 5 | 243 \pm 4 |
| G-6-P dehydrogenase | 1.1.1.49 | 14 \pm 1 | 5 \pm 1 | 4 \pm 1 | 9 \pm 1 | 7 \pm 1 | 4 \pm 1 |

Table 4.5 Specific enzyme activities in *Achromobacter* sp. Strain B cell-free extracts from cells grown in chemostat culture on succinate or succinate plus thiosulfate under carbon limitation ($D = 0.10 \text{ h}^{-1}$). Values in bold represent significantly higher activities when pairwise comparisons were made between heterotrophic and chemolithoheterotrophic grown cells. *BDL* – below detection limit. Values represent mean \pm SEM ($n = 3$).

| Enzyme | EC number | Specific activities of CFEs from cells grown the following substrates | |
|---|-----------|---|-----------------------------------|
| | | 10 mM succinate | 10 mM succinate/50 mM thiosulfate |
| <i>Inorganic sulfur enzymes:</i> | | | |
| Thiosulfate dehydrogenase | 1.8.2.2 | 110 \pm 4 | *1,163 \pm 14 |
| Trithionate hydrolase | 3.12.1.1 | 4 \pm 1 | *50 \pm 4 |
| Tetrathionate hydrolase | 3.12.1.B1 | <i>BDL</i> | <i>BDL</i> |
| Sulfite dehydrogenase | 1.8.2.1 | 122 \pm 14 | *881 \pm 18 |
| Thiocyanate dehydrogenase | - | <i>BDL</i> | <i>BDL</i> |
| <i>TCA cycle enzymes:</i> | | | |
| Citrate synthase | 4.1.3.7 | 39 \pm 1 | 40 \pm 3 |
| Aconitase | 4.2.1.3 | 11 \pm 1 | 7 \pm 1 |
| Iso-citrate dehydrogenase | 1.1.1.41 | 19 \pm 1 | 19 \pm 2 |
| α -ketoglutarate dehydrogenase | 1.2.4.2 | *22 \pm 1 | 13 \pm 1 |
| Succinyl-CoA synthetase | 6.2.1.4 | 33 \pm 4 | 35 \pm 3 |
| Succinate dehydrogenase | 1.3.5.1 | 48 \pm 2 | 51 \pm 2 |
| Fumarase | 4.2.1.2 | *178 \pm 16 | 83 \pm 5 |
| Malate dehydrogenase | 1.1.1.37 | *1,011 \pm 6 | 233 \pm 19 |
| <i>Carbohydrate enzymes:</i> | | | |
| Glucose dehydrogenase | 1.1.1.47 | <i>BDL</i> | <i>BDL</i> |

4.2.5 Oxygen consumption by intact cells of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B

4.2.5.1 *Pseudomonas* sp. Strain T

Intact cells of *Pseudomonas* sp. Strain T were harvested from carbon limited, glucose and glucose plus thiosulfate chemostats ($D = 0.068 \text{ h}^{-1}$) and exposed to increasing concentrations of glucose (0.5 – 10 mM) and thiosulfate (0.5 – 10 mM) from which oxygen consumption was recorded (Table 4.6). From these, the maximum substrate-dependent oxygen consumption rate (V_{\max}) and apparent Michaelis constant (K_m) were determined by non-linear regression based on estimates from Lineweaver-Burk and Haynes-Woolf plots. V_{\max} for glucose under both growth conditions were similar values of 1527 and 1343 $\text{nmol O}_2 \text{ min}^{-1} (\text{mg dry biomass})^{-1}$ for heterotrophic and chemolithoheterotrophic growth respectively; as was the K_m values of 16.5 and 16.8 mM for heterotrophic and chemolithoheterotrophic growth, respectively. In comparison, V_{\max} on thiosulfate was much lower in heterotrophic grown cells compared to chemolithoheterotrophic grown cells (9 and 119 $\text{nmol O}_2 \text{ min}^{-1} (\text{mg dry biomass})^{-1}$, respectively) but with comparable K_m values (20.8 and 22.5 mM, respectively).

2.2.5.2 *Achromobacter* sp. Strain B

Intact cells of *Achromobacter* sp. Strain B were harvested from carbon limited, succinate and succinate plus thiosulfate chemostats ($D = 0.10 \text{ h}^{-1}$) and exposed to increasing concentrations of succinate (0.5 – 10 mM) and thiosulfate (0.5 – 10 mM) from which oxygen consumption was recorded (Table 4.7). V_{\max} for succinate under both growth conditions were similar (628 and 602 $\text{nmol O}_2 \text{ min}^{-1} (\text{mg dry biomass})^{-1}$ for heterotrophic and chemolithoheterotrophic growth, respectively) as were K_m values (7.2 and 7.8 mM for heterotrophic and chemolithoheterotrophic growth, respectively). In comparison, V_{\max}

on thiosulfate was much lower in heterotrophic grown cells compared to chemolithoheterotrophic grown cells (26 and 209 nmol O₂ min⁻¹ (mg dry biomass)⁻¹, respectively). There were also significantly higher K_m values in heterotrophic cells compared to those of chemolithoheterotrophic cells (30.0 and 14.5 mM, respectively).

Table 4.6. Maximum rates of substrate-dependent oxygen uptake (V_{\max}) and Michaelis constant (K_m) of whole cells of *Pseudomonas* sp. Strain T grown in carbon limited chemostats on glucose or glucose plus thiosulfate ($D = 0.068 \text{ h}^{-1}$). Values shown are mean \pm SEM ($n = 3$).

| Cells grown on: | glucose | | thiosulfate | |
|-----------------------|--|------------|--|------------|
| | V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹] | K_m (mM) | V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹] | K_m (mM) |
| Glucose | 1527 \pm 115 | 16.5 | 9 \pm 2 | 20.8 |
| Glucose + thiosulfate | 1343 \pm 124 | 16.8 | 119 \pm 12 | 22.5 |

Table 4.7. Maximum rates of substrate-dependent oxygen uptake (V_{\max}) and Michaelis constant (K_m) of whole cells of *Achromobacter* sp. Strain B grown in carbon limited chemostats on succinate or succinate plus thiosulfate ($D = 0.10 \text{ h}^{-1}$). Values shown are mean \pm SEM ($n = 3$).

| Cells grown on: | succinate | | thiosulfate | |
|-------------------------|--|------------|--|------------|
| | V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹] | K_m (mM) | V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹] | K_m (mM) |
| Succinate | 628 \pm 88 | 7.2 | 26 \pm 1 | 30.0 |
| Succinate + thiosulfate | 602 \pm 46 | 7.8 | 209 \pm 6 | 14.5 |

4.2.6 Reduction of cytochromes from cell-free extracts of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B by thiosulfate

Thiosulfate-reduced absolute spectra of cell-free extracts from cells grown in a chemostat chemolithoheterotrophic culture for *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B can be seen in Figure 4.6A and Figure 4.6B, respectively. These spectra show absorbance maxima in nm in *Pseudomonas* sp. Strain T of 416 (Soret), 523 (β) and 552 (α) when dithionite-reduced and 414 (Soret), 523 (β) and 552 (α) when thiosulfate-reduced. *Achromobacter* sp. Strain B showed absorbance spectra of 416 (Soret), 512 (β) and 550 (α) when dithionite-reduced and 412 (Soret), 514 (β) and 551(α) when thiosulfate-reduced. With difference spectra of thiosulfate reduced minus air oxidised in chemolithoheterotrophically grown *Pseudomonas* sp. Strain T the concentration of thiosulfate-reducible cytochrome c_{552} was calculated as $5.7 \text{ nmol (mg protein)}^{-1}$, whereas dithionite-reducible cytochrome c_{552} was $15 \text{ nmol (mg protein)}^{-1}$. Difference spectra of chemolithoheterotrophically grown *Achromobacter* sp. Strain B indicated the concentration of thiosulfate-reducible cytochrome c_{550} to be $1.8 \text{ nmol (mg protein)}^{-1}$ whereas dithionite-reducible cytochrome c_{551} was $8.1 \text{ nmol (mg protein)}^{-1}$. The pool of non-thiosulfate-reducible cytochrome c in these two strains suggests the concentration of thiosulfate used (1 mM) was insufficient to fully oxidise the whole pool of cytochrome c . A second explanation could be that there are two different type c cytochromes present in the cell-free extracts.

There was no evidence of thiosulfate-reducible cytochrome c_{551} in heterotrophically grown *Pseudomonas* sp. Strain T (Figure 4.6A). In contrast however, *Achromobacter* sp. Strain B had a concentration of $0.6 \text{ nmol (mg protein)}^{-1}$ of thiosulfate-reducible cytochrome c_{552} when grown heterotrophically (Figure 4.6C).

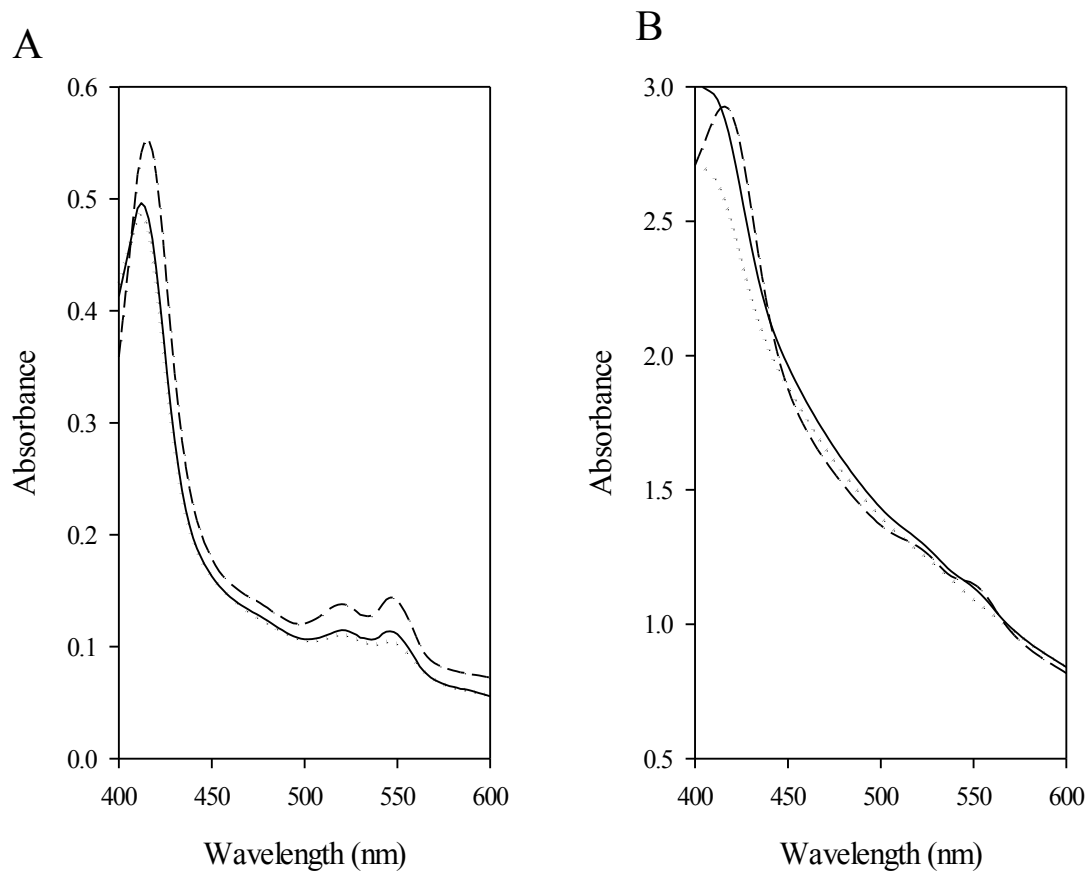


Figure 4.6 Absolute spectra of *Pseudomonas* Strain T (**A**) and *Achromobacter* Strain B (**B**) cell-free extracts in 10 mM potassium phosphate buffer (pH 7.0) previously grown in carbon limited chemostats on glucose plus thiosulfate ($D = 0.068 \text{ h}^{-1}$) and succinate plus thiosulfate ($D = 0.10 \text{ h}^{-1}$) respectively. Shown are air-oxidised (dotted line), 1 mM thiosulfate-reduced (solid line) and dithionite-reduced (dashed line).

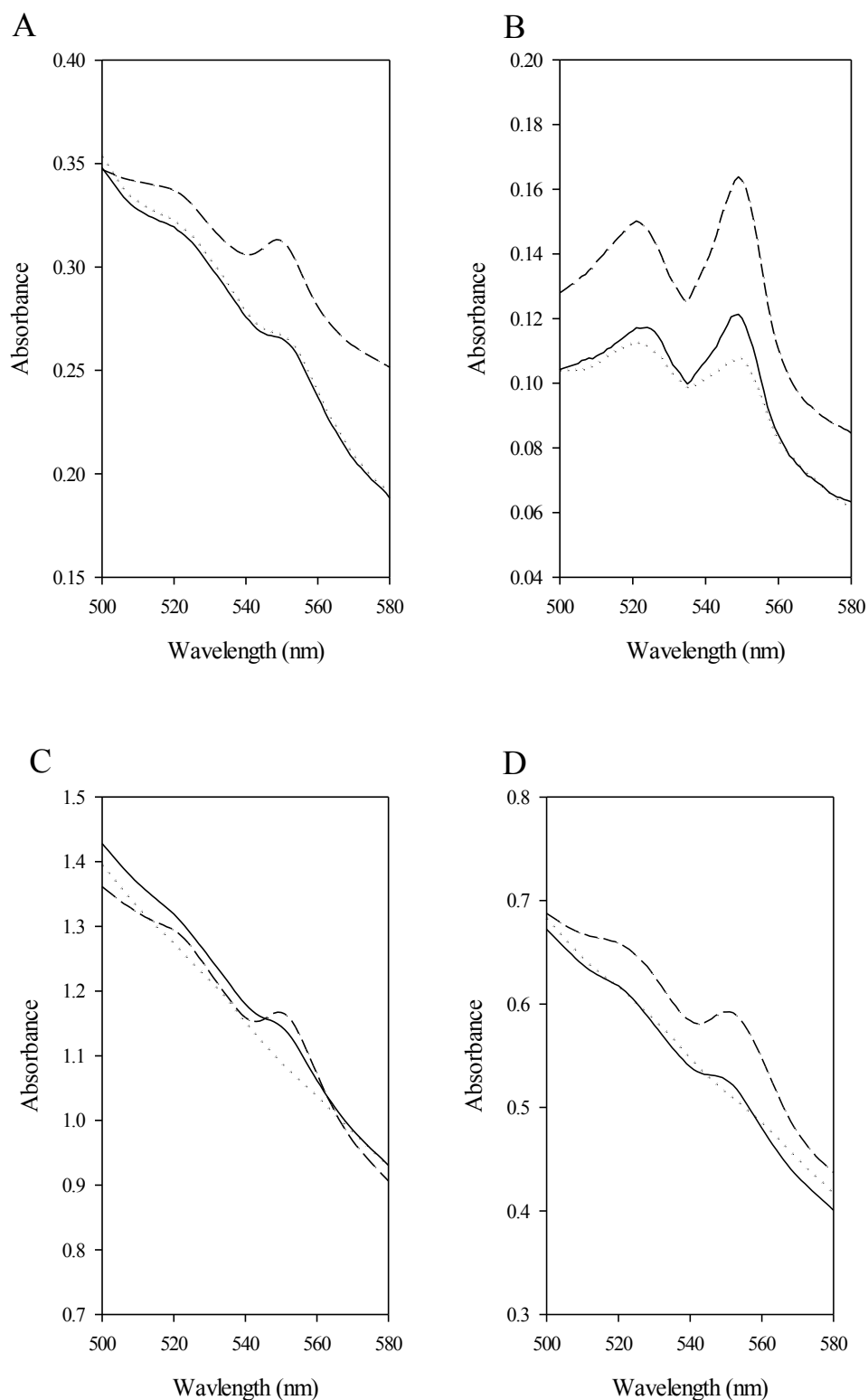


Figure 4.7 Absolute spectra of *Pseudomonas* sp. Strain T cell-free extracts previously grown in a carbon limited chemostat ($D = 0.069 \text{ h}^{-1}$) on glucose (A) and glucose plus thiosulfate (B). Also shown are absolute spectra of *Achromobacter* sp. Strain B cell-free extracts previously grown in a carbon limited chemostat ($D = 0.10 \text{ h}^{-1}$) on succinate (C) and succinate plus thiosulfate (D). Shown are air-oxidised (dotted line), 1 mM thiosulfate-reduced (solid line) and dithionite-reduced (dashed line).

4.2.7 Thiosulfate-dependent production of ATP in *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B

The production of ATP was measured from intact cells of *Pseudomonas* sp. Strain T that had been previously grown in a carbon limited chemostat on 10 mM glucose and (50 mM) thiosulfate ($D = 0.068 \text{ h}^{-1}$). When cells were exposed to 1 mM thiosulfate ATP production was seen and levels were significantly higher when compared to control cells not exposed to thiosulfate (Figure 4.7A). The rate of thiosulfate-dependent ATP production was estimated at $0.6 \text{ nmol ATP min}^{-1} (\text{mg dry biomass})^{-1}$.

The production of ATP was measured from intact cells of *Achromobacter* sp. Strain B that had been previously grown in a carbon limited chemostat on succinate and thiosulfate ($D = 0.10 \text{ h}^{-1}$). When cells were exposed to 1 mM thiosulfate ATP production was seen and levels were significantly higher when compared to control cells not exposed to thiosulfate (Figure 4.7B). The rate of thiosulfate-dependent ATP production was estimated at $0.34 \text{ nmol ATP min}^{-1} (\text{mg dry biomass})^{-1}$.

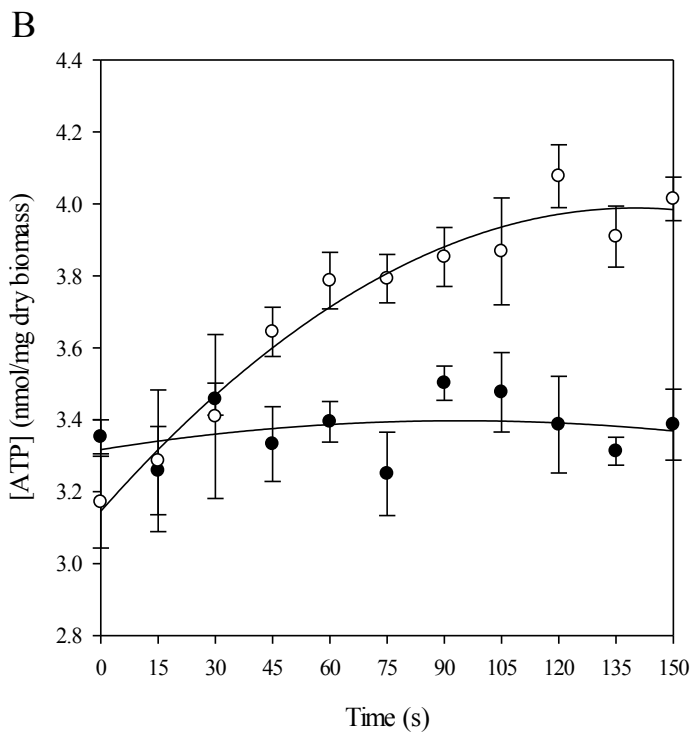
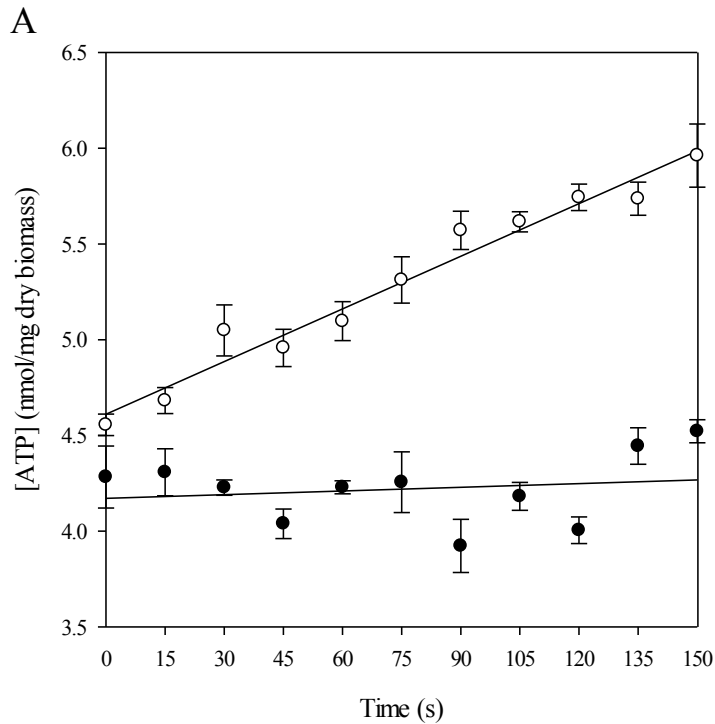


Figure 4.8 ATP production over time of whole cells of *Pseudomonas* sp. Strain T (A) and *Achromobacter* sp. Strain B (B) harvested from a carbon limited chemostat grown on glucose plus thiosulfate ($D = 0.068 \text{ h}^{-1}$). Closed circles represent control cells not exposed to thiosulfate. Open circles represent cells exposed to a final concentration of 1 mM thiosulfate at time point zero. Values shown indicate mean \pm SEM ($n = 3$).

4.2.8 Cellular ATP concentrations during batch culture of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B

The concentrations of ATP from intact cells of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B were measured during the exponential and stationary phases of batch growth (Figure 4.9). Throughout exponential growth of *Pseudomonas* sp. Strain T the concentration of ATP remained fairly constant between 12.6 – 14.4 nmol ATP (mg dry biomass)⁻¹. At early stationary phase (12 h) the ATP concentration dropped significantly to 6.2 ATP nmol (mg dry biomass)⁻¹ (Student's t test, $p < 0.05$). ATP concentration in *Achromobacter* sp. Strain B during exponential phase was between 12.9 – 14.2 nmol ATP (mg dry biomass)⁻¹ and dropped significantly to 4.3 nmol ATP (mg dry biomass)⁻¹.

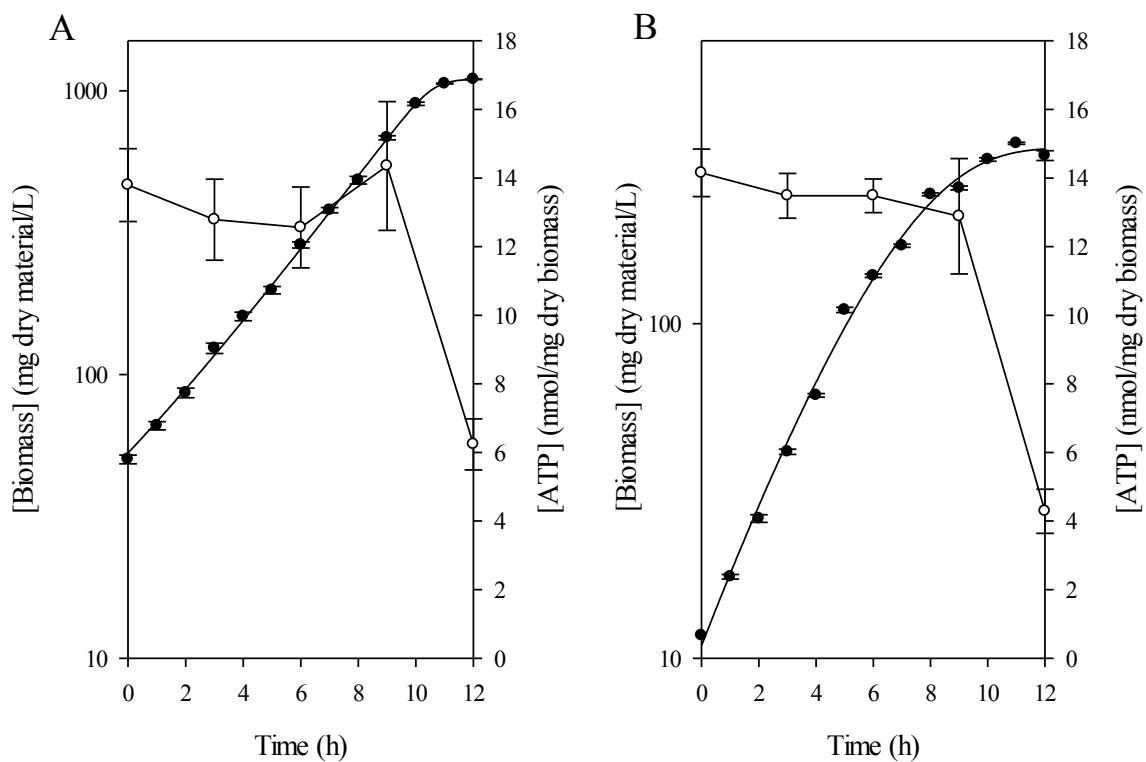


Figure 4.9 Growth (●) and intracellular concentration of ATP (○) of *Pseudomonas* sp. Strain T (A) in 500 mL EBS batch culture on 10 mM glucose and 20 mM thiosulfate and *Achromobacter* sp. Strain B (B) on 10 mM succinate and 20 mM thiosulfate. Values shown indicate mean \pm SEM ($n = 3$).

4.2.9 Cellular ATP and ADP concentrations in steady state cells of *Pseudomonas* sp.

Strain T and *Achromobacter* sp. Strain B

Cells were harvested from glucose and glucose plus thiosulfate steady-state chemostat cultures when the limiting factors were carbon, oxygen or phosphate and intracellular ATP and ADP were assayed. It can be seen in Figure 4.10 that levels of ADP were significantly higher than ATP in all growth conditions. Although there was some variation in both ATP and ADP concentrations between growth conditions, none were statistically significant.

Cells of *Achromobacter* sp. Strain B were harvested from succinate and succinate plus thiosulfate chemostat steady-state cultures when the limiting factor was carbon and intracellular ATP and ADP assayed (Figure 4.11). ADP concentrations were significantly higher than ATP in both growth conditions but pairwise concentrations of ATP and ADP were not significantly different between growth conditions.

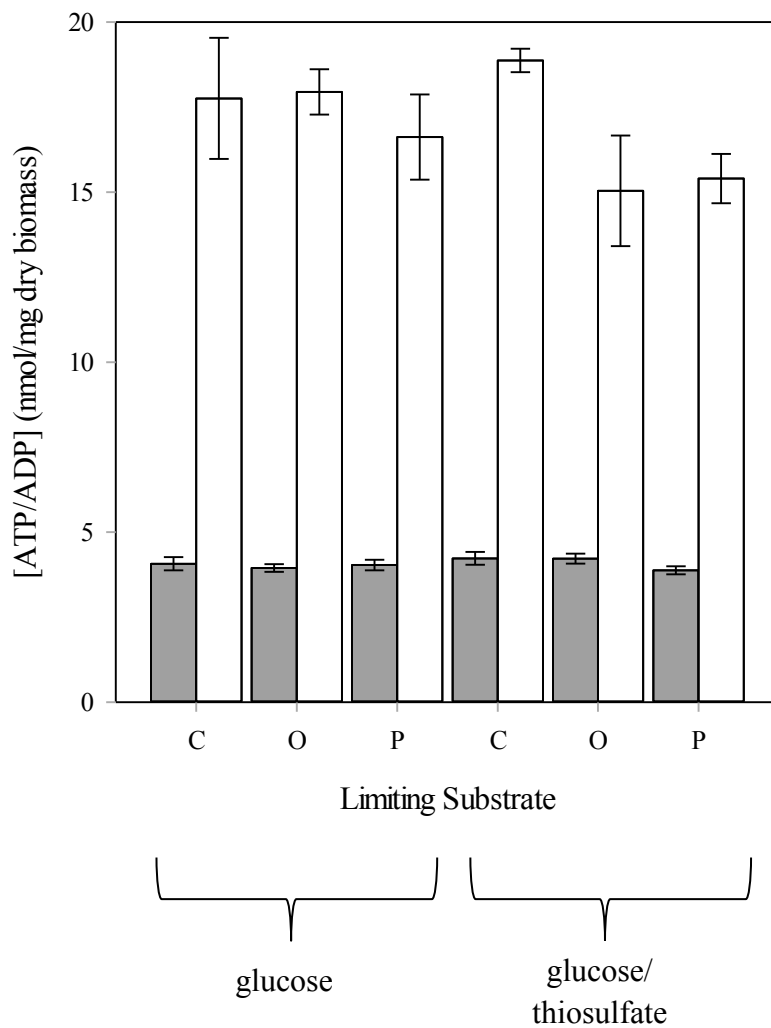


Figure 4.10 ATP and ADP concentration in *Pseudomonas* sp. Strain T cells harvested from glucose or glucose plus thiosulfate chemostats ($D = 0.068 \text{ h}^{-1}$) limited by carbon, oxygen or phosphates. ATP is shown by shaded bars, ADP by white bars. C – carbon limited, O – oxygen limited and P – phosphate limited. Values shown indicate mean \pm SEM ($n = 3$).

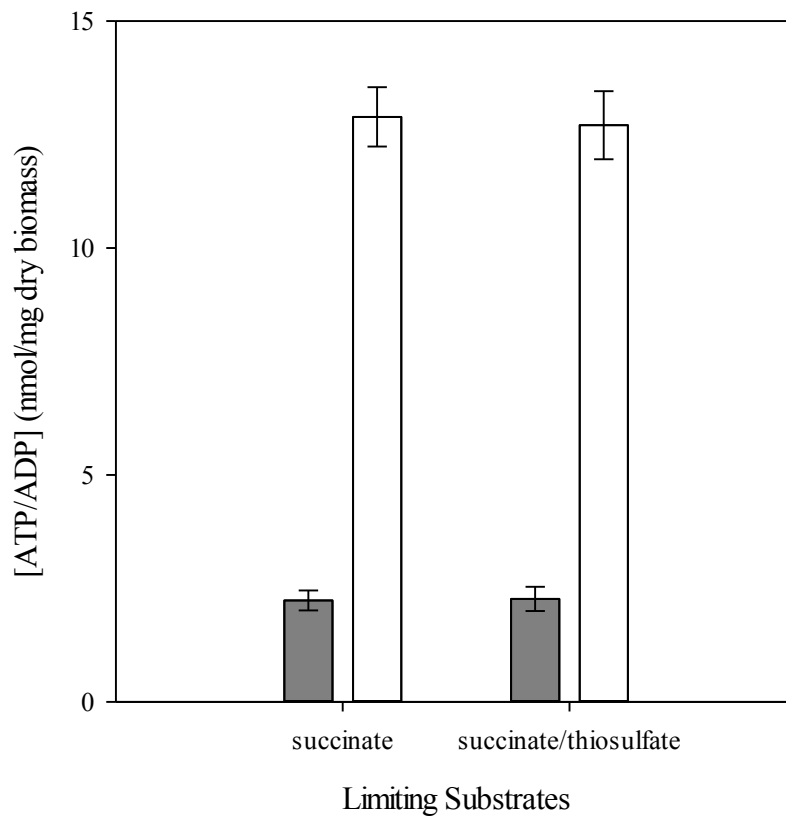


Figure 4.11 ATP and ADP concentrations in *Achromobacter* sp. Strain B cells harvested from succinate and succinate plus thiosulfate chemostats ($D = 0.10 \text{ h}^{-1}$) limited by carbon. ATP is shown by shaded bars, ADP by white bars. Values shown indicate mean \pm SEM ($n = 3$).

4.3 Discussion

The major aim of this study was to examine two strains of “*T. trautweini*” for their ability to oxidise thiosulfate as chemolithoheterotrophs. Their ability to oxidise thiosulfate has been known since their isolation and is the major reason they were placed into the genus *Thiobacillus* (Trautwein, 1921; Starkey, 1934a). However, a number of subsequent studies have contradicted each other with some stating “*T. trautweini*” Strain T was capable of autotrophic growth (Parker & Prisk, 1953; Trautwein, 1921) while Starkey (1934b) found no evidence of autotrophic growth in *Pseudomonas* sp. Strain T or in *Achromobacter* sp. Strain B and concluded that they were both strictly heterotrophic organisms. The current study supported the latter conclusion. When *Pseudomonas* sp. Strain T was grown in batch culture (Figure 4.1) there was no apparent growth when organic carbon was absent (Figure 4.1C). This trait was also observed with *Achromobacter* sp. Strain B (Figure 4.2). With regards to the oxidation of thiosulfate there was a small decrease in thiosulfate when organic carbon was absent in both Strain T and Strain B. In comparison, when organic carbon was present there was significantly more thiosulfate oxidation with most of it being oxidised to tetrathionate. There was a small yet significant increase in yield when Strain T was grown as a chemolithoheterotroph compared to heterotrophic growth (109 ± 0.8 and 113 ± 0.8 mg dry biomass/L respectively ($p < 0.05$) although the presence of thiosulfate did cause a decrease in specific growth rate (0.287 and 0.260 h⁻¹, respectively) but was not statistically significant. *Achromobacter* sp. Strain B showed a lower yield and a lower specific growth rate when grown as a chemolithoheterotroph (Table 4.2) although neither was statistically significant. There are many other examples of heterotrophic species of the *Bacteria* that have the ability to oxidise thiosulfate (Starkey, 1934a; Tuttle *et al.*, 1972; Tuttle *et al.*, 1974; Sorokin *et al.*, 2006). With many earlier studies an increase in yield or

growth rate were not always observed and the oxidation of thiosulfate to tetrathionate was seen as a “gratuitous” reaction which served no purpose. Mason and Kelly (1988b) found the specific growth rate of a strain of *P. aeruginosa* decreased while a second strain of *P. aeruginosa* showed no change when grown with thiosulfate. Trudinger (1967) also demonstrated no change in growth rates and yields in an organism isolated from soil (strain A-50). In contrast, however, *Tepidimonas ignava* showed a “fortuitous” boost in yield by oxidizing thiosulfate completely to sulfate, although this was only briefly examined and true yields were not calculated (Moreira *et al.*, 2000). The full oxidation of thiosulfate by *T. ignava* presumably took place either by the full Kelly-Trudinger pathway or the Kelly-Freidrich pathway and the release of electrons would have been far greater than through the activity of thiosulfate dehydrogenase alone. This might explain the increase observed in *T. ignava* when other strains of the *Bacteria* that were only capable of partial thiosulfate oxidation showed no such increases to yield or specific growth rates.

This is the first study in which *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B were grown in continuous culture. The addition of thiosulfate to the growth medium increased the maximum theoretical growth yield of *Pseudomonas* sp. Strain T by 22.6% and increased the maximum specific growth rate by 12%. Similar increases in maximum theoretical growth yield of 48.0% and 2.5% in maximum specific growth rate were also observed in *Achromobacter* sp. Strain B. Increased steady-state biomass of 23% has previously been shown by Manson and Kelly (1988b) with *P. aeruginosa*, although this was only tested at a single dilution rate. A comparable yet more in depth study of a marine species of *Gammaproteobacteria* (*Methylophaga thiooxydans*) by Boden *et al.* (2010) highlighted an increase of approximately 19% in the maximum theoretical growth yield when this bacterium was grown chemolithoheterotrophically on dimethyl sulfide

and thiosulfate. Gommers & Kuenen (1988) found an increase in biomass of *c.*20% with “*Thiobacillus*” Strain Q (now *Dechloromonas* sp. Q) from thiosulfate in an acetate limited chemostat. In all cases these strains proved to be unable to grow autotrophically and so were shown to be true chemolithoheterotrophs and not mixotrophs. Evidence presented in this present study also suggests that thiosulfate oxidation is coupled to reduction of the respiratory chain and the production of ATP is via *c* type cytochrome(s). In the case of *Pseudomonas* sp. Strain T it is possible that having an extra supply of ATP from the oxidation of thiosulfate would enable more carbon atoms from glucose to go towards biomass production by shifting the partitioning of carbon away from ATP and CO₂ production. This had previously been shown by Tuttle (1980) who found that more organic carbon from radio-labeled glucose was incorporated into biomass coupled with a reduction in respiration by two marine heterotrophs when thiosulfate was being partially oxidised to tetrathionate. This would suggest that thiosulfate oxidation was subsidizing ATP production and allowing carbon that would normally be released as CO₂ to become assimilated into biomass. Of course this would be an important aspect of any future work looking into increased biomass formation in *Pseudomonas* sp. Strain T and any other chemolithoheterotrophs. These data strongly indicate that in a “real world” scenario (being simulated in continuous culture) thiosulfate gives a significant physiological boost and possibly an ecological advantage over strictly heterotrophic *Bacteria*.

Thiosulfate oxidation in *Pseudomonas* sp. Strain T was coupled to synthesis of ATP in intact cells previously grown in the presence of thiosulfate at a rate of 0.6 nmol ATP min⁻¹ (mg dry biomass)⁻¹. ATP synthesis was also observed in intact cells of *Achromobacter* sp. Strain B although at a slower rate of 0.34 nmol ATP min⁻¹ (mg dry biomass)⁻¹. The levels of ATP in steady-state cells was far lower (*c.*4 nmol (mg dry biomass)⁻¹) than that of batch culture cells in exponential phase of growth (*c.*12 – 13 nmol (mg dry biomass)⁻¹)

until stationary phase in which it dropped to 3.2 nmol (mg dry biomass)⁻¹. This three-fold higher ATP concentration during exponential phase might explain the relatively small boost in *Y* in batch culture compared to continuous culture in that even though thiosulfate was being oxidised, the ATP produced simply entered the ATP pool (Cole *et al.* 1967). However, in chemostat culture growth was limited by a low concentration of ATP and the ATP produced by thiosulfate oxidation topped up the ATP pool in cells that have utilised all available energy from glucose metabolism and was used immediately. We suggest that not only is chemolithoheterotrophy driven by the presence of thiosulfate but that there is an ATP concentration threshold, below which ATP synthesis from thiosulfate oxidation relieves ATP-limitation. This then affects the fate of carbon by shifting carbon towards biomass formation instead of ATP and CO₂ production.

The enzymology of chemolithoheterotrophy has only been looked at in detail by Boden *et al.* (2010). In terms of inorganic sulfur oxidation only the inducible thiosulfate dehydrogenase enzyme was present in cell-free extracts of *Pseudomonas* sp. Strain T and only when grown chemolithoheterotrophically. No other enzymes of the Kelly-Trudinger pathway were detected. This appears to be the case with many of the chemolithoheterotrophs studied to date. However; nearly the whole Kelly-Trudinger pathway was present in *Achromobacter* sp. Strain B as activities of thiosulfate dehydrogenase, sulfite dehydrogenase and trithionate hydrolase in both heterotrophic and chemolithoheterotrophic grown cells were present, although activities were significantly higher when thiosulfate had been present in the growth medium. The lack of tetrathionate hydrolase activity would explain the build-up of tetrathionate from thiosulfate in the growth medium of *Achromobacter* sp. Strain B and the lack of further oxidation to sulfate. In order to understand the impact of chemolithoheterotrophy on central metabolism enzymes of the TCA cycle were examined and some notable changes were found. Under

carbon limitation there were significant increases in succinate dehydrogenase and malate dehydrogenase activities in *Pseudomonas* sp. Strain T. Succinate dehydrogenase contributes to both the TCA cycle and the electron transport chain. The product of malate dehydrogenase (oxaloacetate) is a precursor for many amino acids (Musrati *et al.* 1998; Bender 2011). With the demand for energy production partially fulfilled by thiosulfate oxidation, more oxaloacetate may have been converted to aspartate and other amino acids at the expense of glutamate. Alternatively the extra oxaloacetate may have been converted to α -ketoglutarate at the expense of aspartate to produce glutamate and other associated amino acids. Succinate dehydrogenase contributes to both the TCA cycle and the electron transport chain (ETC) and so the increase observed in *Pseudomonas* sp. Strain T may add another dimension. The increase may be due to an increase in the ETC activity, possible from electrons entering the ETC from thiosulfate dehydrogenase depending on where the electrons enter the chain. Oxaloacetate is a known inhibitor of succinate dehydrogenase (Rutter *et al.* 2010) and so it is likely that any increase in oxaloacetate is being rapidly metabolised. The pattern of TCA cycle enzyme activities observed in *Achromobacter* sp. Strain B differed from that of *Pseudomonas* sp. Strain T. The drop in α -ketoglutarate dehydrogenase, fumarase and malate dehydrogenase activities may indicate a preference for the biosynthesis of the amino acid glutamate from the TCA cycle intermediate α -ketoglutarate. Clearly to understand more conclusively the measuring of these reactions, particularly the action of transaminase enzymes would be important for any future work as the effect chemolithoheterotrophic growth on central metabolism may be diverse and differ between species.

This study adds to the increasing body of evidence that bacterial thiosulfate oxidation in the environment is not only carried out by autotrophs but significantly driven by heterotrophs. Since the genus *Thiobacillus* was first identified it was thought that

members of the genus were the major if only bacterial based turnover of inorganic sulfur in the environment. Many of these organisms have since been reassigned to different genera and it has become apparent that, in terms of autotrophic metabolism, it is a wide spread form of metabolism. Heterotrophic metabolism that also utilizes thiosulfate may also be a far more common process than previously thought (Vishniac & Santer, 1957). It is a real possibility that heterotrophic oxidation of thiosulfate could be a major, if not the dominate contributor of biological oxidation of thiosulfate in the terrestrial environment; further supporting what has been highlighted in marine environments (Tuttle *et al.*, 1972; Tuttle *et al.*, 1974; Boden *et al.*, 2010).

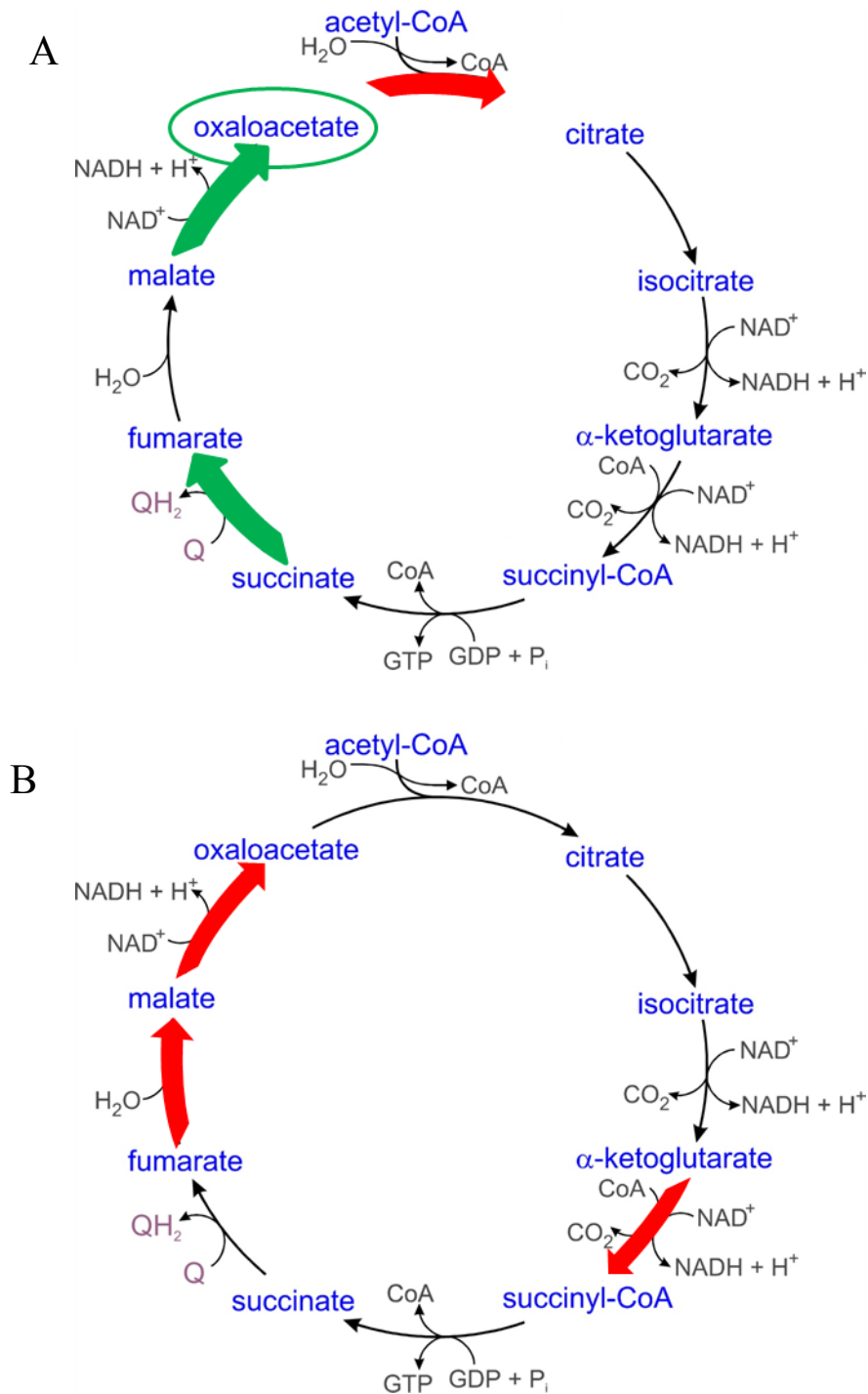


Figure 4.12 Changes in TCA cycle enzymes as a result of chemolithoheterotrophic growth (carbon limitation) with thiosulfate in *Pseudomonas* sp. Strain T (A) and *Achromobacter* sp. Strain B. Highlighted are significant increases (green arrows) and decreases (red arrows) in specific enzymes activities. In the case of *Pseudomonas* sp. Strain T the potential increase in oxaloacetate is indicated. Image modified from and supplied by Dr. John Moody (personal communication).

Chapter 5

Taxonomy, physiology and biochemistry of

Thermithiobacillus sp. ParkerM

5.1 Introduction

Inorganic sulfur-oxidising chemolithoautotrophic *Bacteria* are ubiquitous in all natural environments and can be halophilic or thermophilic, and can tolerate extremes of pH and high levels of toxic metals (Nathanson, 1902; London, 1963; Starkey, 1934a; Hutchinson, 1966; Kelly, 1971; Wood & Kelly, 1985; Rawlings, 2002). The majority of known species are strictly aerobic although a few facultative anaerobic *Bacteria* are also known (Sorokin *et al.* 2004; Kelly & Wood, 2000b). Historically all *Bacteria* that were capable of oxidising inorganic sulfur compounds were assigned to the genus *Thiobacillus*, within the *Betaproteobacteria*. Since their initial discovery by Nathanson (1902) many species that were added to *Thiobacillus* have been shown to be hugely diverse in terms of their physiology and their biochemistry. Such organisms use energy from compounds such as thiosulfate for autotrophic growth and their biochemistry and physiology have been the subject of research for many decades. In more recent years, however, most species have been reassigned to new or other existing genera within *Proteobacteria*; based on gene sequencing and other taxonomic traits (Moreira & Amils 1997; Kelly 2000; Kelly & Wood 2000a; Boden *et al.* 2011).

There are currently two known pathways of inorganic sulfur oxidation within the *Proteobacteria* (Kelly *et al.* 1997; Ghosh & Dam 2009). The first is the full oxidation of elemental sulfur, sulfide and thiosulfate to sulfate without the formation of any intermediate products (Kelly-Friedrich (Sox) pathway) that is found in the *Alphaproteobacteria*. The second pathway (Kelly-Trudinger (S₄I) pathway) produces polythionates (S_nO₆²⁻) as metabolic intermediates of thiosulfate oxidation and is found in the *Betaproteobacteria*, *Gammaproteobacteria* and *Acidithiobacillia*. The stoichiometric formation of tetrathionate (S₄O₆²⁻) from thiosulfate has been shown to be the predominant

intermediate of the Kelly-Trudinger pathway in many species (Trudinger 1961a; Kelly *et al.* 1997). The former pathway is the better understood of the two pathways and is now known to be carried out by the Sox multienzyme system encoded for by the conserved operon *soxTRS-VW-XYZABCDEFGH* (Wodara *et al.* 1994, 1997; Friedrich *et al.* 2000; Rother *et al.* 2001; Rother & Friedrich 2002). The latter pathway is still poorly understood in terms of biochemistry, physiology and genetics.

Parker (1947) isolated a number of sulfur oxidising bacteria from the sewers of Melbourne, Australia, and showed that their metabolism caused high levels of corrosion of the concrete structures within sewers. The first organism (now lost from culture collections) was named “*Thiobacillus concretivorus*” but has since shown to be a strain of *Acidithiobacillus thiooxidans*. This isolate grew on thiosulfate and quantitatively formed tetrathionate before further oxidation to sulfate. A second isolate was dubbed *T. thioparus*-like and was an obligate autotroph capable of growing on thiosulfate and polythionates with the production of sulfate. It was also confirmed in a study by Parker and Prisk (1953) that the *T. thioparus*-like species (labelled *Thiobacillus X*) produced tetrathionate and to a lesser extent pentathionate as intermediates from thiosulfate oxidation. This strain was later named as the type species of *Halothiobacillus neopolitanus* (Kelly & Wood 2000; Boden *et al.* 2011). A third group was a mixture of facultative autotrophs that could grow on nutrient media as well as on thiosulfate. In contrast to the previous strains, this group stoichiometry oxidised thiosulfate to tetrathionate but was unable to oxidise tetrathionate to sulfate. One strain of Parker’s third group (M79) was studied in more detail by Parker and Prisk (1953) who also found that there was a lack of tetrathionate oxidation but with 87% of thiosulfate being oxidised to tetrathionate and other polythionates. For these reasons this strain was compared to heterotrophic species such as “*Thiobacillus trautweini*” strains “T” and “K” (Trautwein

1921). However, strain M79 took a lengthy 56 day period to achieve this oxidation. The slow rate of oxidation possibly indicates that the growth conditions employed were not fully suitable for this strain. Later unpublished work by K.R. Butlin and J.R. Postgate indicated M79 to be a strain of *T. thioparus* and this strain was deposited into the National Collection of Industrial, Food and Marine Bacteria as NCIMB 8349.

In a recent study by Boden *et al.* (2011), in which twelve strains that had questionably been deposited into culture collections as *T. thioparus*, found that based on its 16S rRNA gene sequence strain M79 was most closely related to *Thermithiobacillus tepidarius* at 99.4% identity. *Ttb. tepidarius* had previously been assigned to *Thiobacillus* (Wood and Kelly 1985) until Kelly and Wood (2000) demonstrated through 16S rRNA gene sequencing and other taxonomic methods that this strain belonged to a different genus within the *Gammaproteobacteria*. However, this assignment has also been updated with the use of multiprotein phylogenetic analysis which showed that *Thermithiobacillus*, along with *Acidithiobacillus*, belong outside the *Gammaproteobacteria* and in a newly proposed Class of *Proteobacteria*, *Acidithiobacillia* (Williams & Kelly, 2013; Hudson *et al.* 2014; Kelly & Wood, 2014). Presently this *Class* incorporates only seven validly published species, six in the *Acidithiobacillus* and one (*Ttb. tepidarius*) in the *Thermithiobacillus*.

Thermithiobacillus tepidarius was studied extensively by Kelly and collaborators (Wood & Kelly, 1986; Lu & Kelly, 1988a; Lu & Kelly 1988b; Lu & Kelly 1988c; Kelly *et al.*, 1993) and gave the best insights into the Kelly-Trudinger pathway to date. With the use of experiments with inhibitors and cellular fractionation it was found that the oxidation of thiosulfate and the hydrolysis of trithionate occurred in the periplasm of the cell. The product of these reactions, namely tetrathionate, was further oxidised after its transportation to the cytoplasm. This finding was supported by Hallberg *et al.* (1995) in

what is now *Acidithiobacillus caldus* and by Meulenberg *et al.* (1993b) in what is now *Acidiphilium acidophilum*. In contrast to these studies, Bugaytsova and Lindstrom (2004) purified tetrathionate hydrolase from the periplasm of *Atb. caldus* indicating that tetrathionate oxidation takes place in the same cellular compartment as thiosulfate oxidation.

The energy conservation of inorganic sulfur metabolism in the *Bacteria* has been indirectly compared in many species with the use of chemostat culture kinetics. Such studies have shown a high variation in terms of maximum theoretical growth yield (Y_{MAX}), which Kelly *et al.* (1982) separated into two groups. One group consisted of what are now known to be *Halothiobacillus neapolitanus*, *Paracoccus versutus* and *Thiomicrospira pelophila* that when grown on thiosulfate showed Y_{MAX} values of approximately 7 g dry biomass per mol thiosulfate. The second group contained the facultative anaerobe *Thiobacillus denitrificans* which had a far higher Y_{MAX} of approximately 14 g dry biomass per mol thiosulfate when grown in the absence of oxygen. Subsequent studies have shown a greater range in yields with regards to thiosulfate, with Y_{MAX} values for *Thermithiobacillus tepidarius*, *Thioalkalivibrio versutus* and *Thermothrix thiopara* of 11.0, 13.5 and 22.8 dry biomass per mol thiosulfate, respectively (Wood & Kelly, 1986; Banciu *et al.* 2004; Mason *et al.* 1986). There is clearly a high level of variation in efficiency of chemolithautotrophic thiosulfate oxidation in terms of amount of biomass formed within the *Bacteria*. This is even seen between organisms that are apparently utilising the same metabolic pathway.

The purpose of this study was to confirm the taxonomic position of M79 (Parker 1947) (referred to as ParkerM in this study) within *Thermithiobacillus*. The growth physiology and biochemistry of thiosulfate, trithionate and tetrathionate oxidation was also examined

in both batch and continuous cultures. The growth kinetics of this moderate thermophile were compared on these different substrates with that of the type species *Ttb. tepidarius*.

5.2 Results

5.2.1 16S rRNA gene sequences

Based on 16S rRNA (*rrs*) gene sequencing ParkerM belongs to the genus *Thermithiobacillus*, within the Class *Acidithiobacillia*. Using the BLAST 2 (Tatusova & Madden, 1999) algorithm the *rrs* sequence of *Thermithiobacillus* sp. ParkerM shared most identity to that of the *rrs* sequence of the only other species *Thermithiobacillus tepidarius* DSM 3134^T (99.5%) (Figure 5.1 & 5.2). The *rrs* sequence of *Thermithiobacillus* sp. ParkerM shared only 85.6% sequence identity with the *rrs* sequence of the type species of *Thiobacillus*, *T. thioparus* Starkey^T, from which it had been previously allied.

5.2.2 Chemotaxonomic characteristics

The chemotaxonomic properties of *Thermithiobacillus* sp. ParkerM were examined and compared with other closely related species, differential results of which are summarised in Tables 5.1. The dominant respiratory quinone found in *Thermithiobacillus* sp. ParkerM was ubiquinone (UQ-8), consistent with the genus *Thermithiobacillus* (Kelly & Wood 2000a). There were some notable differences in taxonomic characteristics that distinguish *Thermithiobacillus* sp. ParkerM from the closely related species *Ttb. tepidarius* DSM 3134^T. There was a higher DNA G+C content of 71.5 mol%, a difference of 4.9 mol% compared to *Ttb. tepidarius* DSM 3134^T. There were also some significant differences in growth parameters, particularly in batch culture. The highest specific growth rate obtained on thiosulfate was significantly higher (0.25 h⁻¹) compared to that of

Ttb. tepidarius DSM 3134^T (0.055 h⁻¹). The lowest pH reached at the end of batch growth was significantly lower (3.6) and the pH range in which *Thermithiobacillus* sp. ParkerM was able to grow was a wider range of acidic pHs. A morphological difference between the two species was the length of flagella which, on average, were *c.*40% longer on cells of *Thermithiobacillus* sp. ParkerM.

5.2.3 Growth substrates

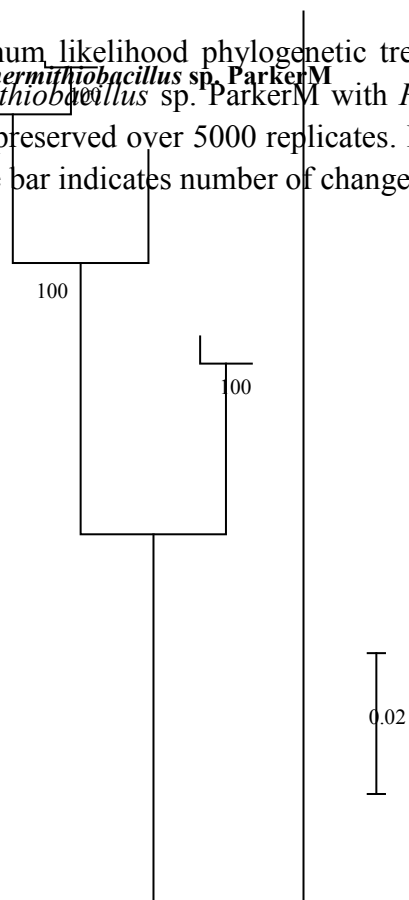
These were examined using glass universal bottles and concentrated (90% volume) EBS medium to which 10% volume sterilised growth substrates were added. Growth of *Thermithiobacillus*. sp. ParkerM and *Ttb. tepidarius* DSM 3134^T was tested on a number of different inorganic and organic compounds, which are summarised in Table 5.2. Growth was observed on a number of inorganic sulfur compounds but no growth was observed on organic compounds or any other inorganic compounds.

Acidithiobacillus ferrooxidans DSM 22755^T (AF376020)
Acidithiobacillus thiooxidans ATCC 29641^T (F02678) | *Paracoccus dehydrogenans* ATCC 17741^T (Y16927)
Acidithiobacillus caldus ATCC 51756^T (Z29975) | *Acidithiobacillus thiemeri* ATCC 53403^T (AF439804)

Acidithiobacillus caldus ATCC 51756^T (Z29975)

Thermithiobacillus tepidarius DSM 3134^T (AJ459801)

Figure 5.1 Maximum likelihood phylogenetic tree based on CLUSTALW alignment of 16S rRNA sequences. Position of *Thermithiobacillus* sp. ParkerM (43) and *Paracoccus dehydrogenans* as an outgroup. Values at nodes indicate bootstrap support that topology was preserved over 5000 replicates. For each species the type strain code is given above. The bar indicates number of changes per nucleotide position.



Acidithiobacillus ferrooxidans ATCC 23270^T (ATCC 23270)
Acidithiobacillus thiooxidans ATCC 29634^T (ATCC 29634)
Acidithiobacillus ferridurans DSM 3134^T (AJ459804)
Acidithiobacillus caldus ATCC 51756^T (Z29975)
Paracoccus denitrificans ATCC 17741^T (Y16927)

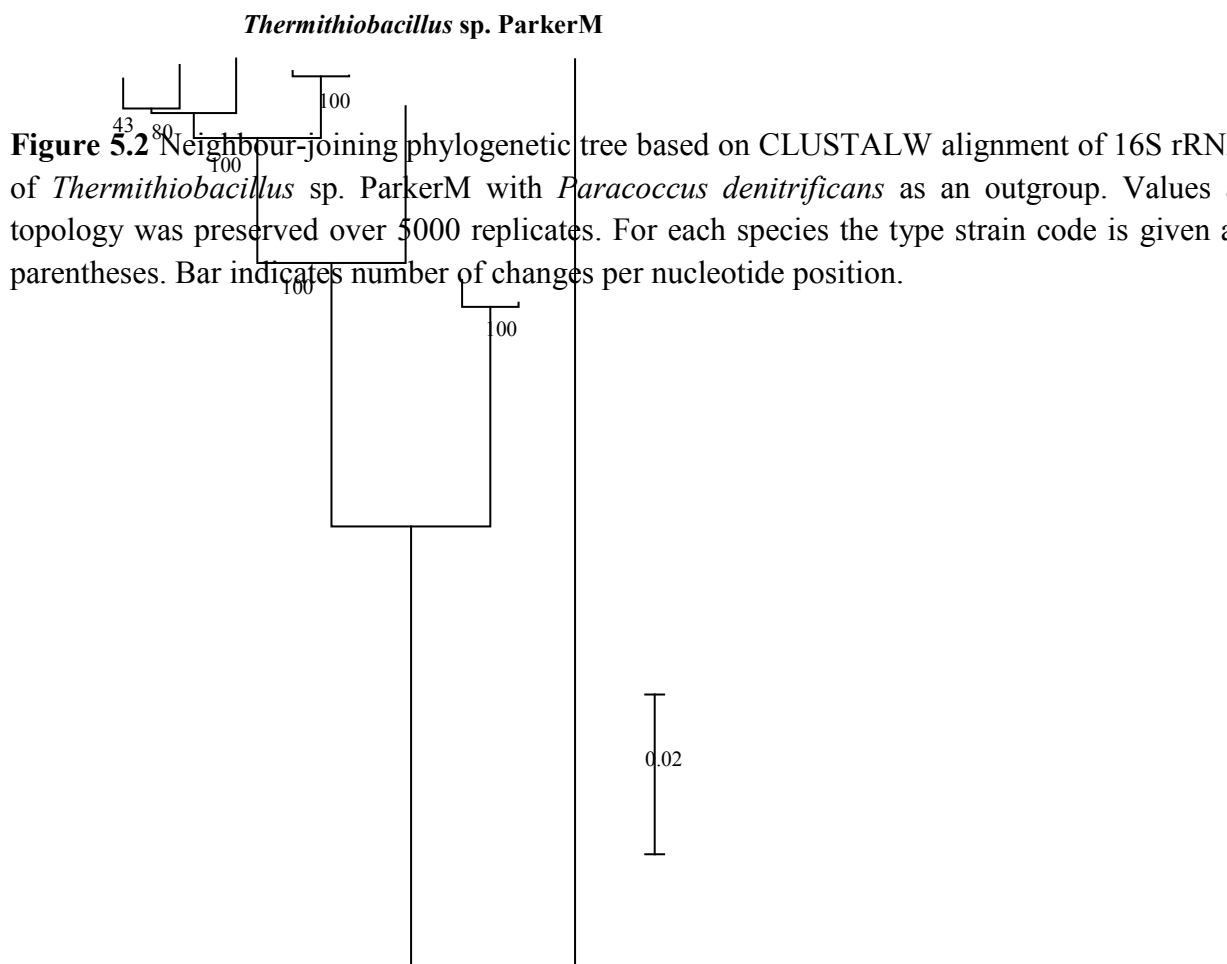


Table 5.1. Differential chemotaxonomic characteristics of *Thermithiobacillus* sp. ParkerM (1) and the closely related *Thermithiobacillus tepidarius* DSM 3134^T (2). Also shown are the type species of *Acidithiobacillus*, *Atb. thiooxidans* ATCC 19377^T (3) and *Halothiobacillus*, *Htb. neapolitanus* NCIB 8539^T (4). *N.D.*; not determined.

| Characteristic | 1 | 2 | 3 | 4 |
|---|-------------|-------------|-------------|-------------------|
| Cell size (µm) | 0.7 × 1.45 | 1 × 1.5-2.0 | 0.5 × 1.0 | 0.3-0.5 × 1.0-1.5 |
| Flagellum length (µm) | 5.2 | 3.1 | <i>N.D.</i> | <i>N.D.</i> |
| Optimum temperature | 42-45 | 43-45 | 28-30 | 28-32 |
| Temperature limits | 20-54 | 20-52 | 10-37 | 8-39 |
| Optimal pH | 6.0-7.0 | 6.0-7.5 | 2.0-3.0 | 6.5-6.9 |
| pH limits | 4.0-8.5 | 5.5-8.0 | 0.5-5.0 | 4.5-8.5 |
| Optimal NaCl (mM) | 350 | 0 | 0 | <i>N.D.</i> |
| Highest µ on thiosulfate in batch growth (44°C) | 0.25 | 0.055 | <i>N.D.</i> | <i>N.D.</i> |
| Maximum µ on tetrathionate in continuous culture(44°C) | 0.429 | 0.440 | <i>N.D.</i> | <i>N.D.</i> |
| Lowest pH after growth on sulfur compounds | 3.6 | 4.5 | 0.5 | 2.8 |
| Thiosulfate dehydrogenase activity * | 10,070 ± 76 | 4,408 ± 139 | <i>N.D.</i> | <i>N.D.</i> |
| Reduction of: | | | | |
| <i>Nitrate</i> | + | ± | + | - |
| Antibiotic resistance: | | | | |
| <i>Penicillin G</i> | + | - | <i>N.D.</i> | <i>N.D.</i> |
| <i>cephalothin</i> | - | + | <i>N.D.</i> | <i>N.D.</i> |
| Sources of nitrogen: | | | | |
| <i>EDTA</i> | - | + | <i>N.D.</i> | <i>N.D.</i> |
| DNA G + C content (mol%) | 71.5 | 66.6 | 52 | 56 |

*Activity measured from cells grown in a thiosulfate (20 mM) limited chemostat under identical growth conditions.

Table 5.2. Growth of *Thermithiobacillus* sp. ParkerM and *Thermithiobacillus tepidarius* DSM 3134^T on EBS medium supplemented with inorganic and organic compounds. Values in parentheses are the final concentrations of growth substrate used.

| <i>Growth substrate</i> | <i>Ttb. sp. ParkerM</i> | <i>Ttb. tepidarius</i> |
|----------------------------------|-------------------------|------------------------|
| <i>Inorganic sulfur:</i> | | |
| Sulfur (5g L ⁻¹) | + | + |
| Thiosulfate (20 mM) | + | + |
| Trithionate (10 mM) | + | + |
| Tetrathionate (10 mM) | + | + |
| Pentathionate (5 mM) | - | ± |
| Hexothionate (5 mM) | + | + |
| Thiocyanate (20 mM) | - | - |
| Dithionate (20 mM) | - | - |
| Sulfite (20 mM) | - | - |
| <i>Inorganic salt:</i> | | |
| Cuprous sulfate (10 mM) | - | - |
| Ferrous sulfate (10 mM) | - | - |
| <i>Mineral:</i> | | |
| Pyrite (5g L ⁻¹) | - | - |
| <i>Organic compounds:</i> | | |
| Glucose (10 mM) | - | - |
| Fructose (10 mM) | - | - |
| Xylose (10 mM) | - | - |
| Arabinose (10 mM) | - | - |
| Succinate (10 mM) | - | - |
| Citrate (10 mM) | - | - |
| Raffinose (5 mM) | - | - |
| Sucrose (5 mM) | - | - |

5.2.4 Optimal growth parameters of *Thermithiobacillus* sp. ParkerM and

Thermithiobacillus tepidarius DSM 3134^T grown on thiosulfate

Thermithiobacillus sp. ParkerM was grown on EBS medium supplemented with 20 mM thiosulfate between 4-60 °C with a starting pH of 7.0. These species were also grown between pH 4.0-8.5 with an incubation temperature of 44 °C. Specific growth rates were compared to data obtained by Wood & Kelly (1985) (Figure 5.3). Growth of *Thermithiobacillus* sp. ParkerM was observed between 20-54 °C with optimum growth rate at 44 °C. Growth was observed between pH 4.0-8.5 with optimum growth rate at 6.0-7.0.

5.2.5 Transmission electron microscopy of *Thermithiobacillus* sp.

Negatively stained transmission electron micrographs of *Thermithiobacillus* sp. ParkerM and *Thermithiobacillus tepidarius* DSM 3134^T were obtained from cells in late exponential phase of growth on EBS supplemented with 20 mM thiosulfate (12 h) (Figure 5.4). Both species were similar in terms of morphology but for the notably longer flagella present on cells of *Thermithiobacillus* sp. ParkerM compared to cells of *Ttb. tepidarius* DSM 3134^T.

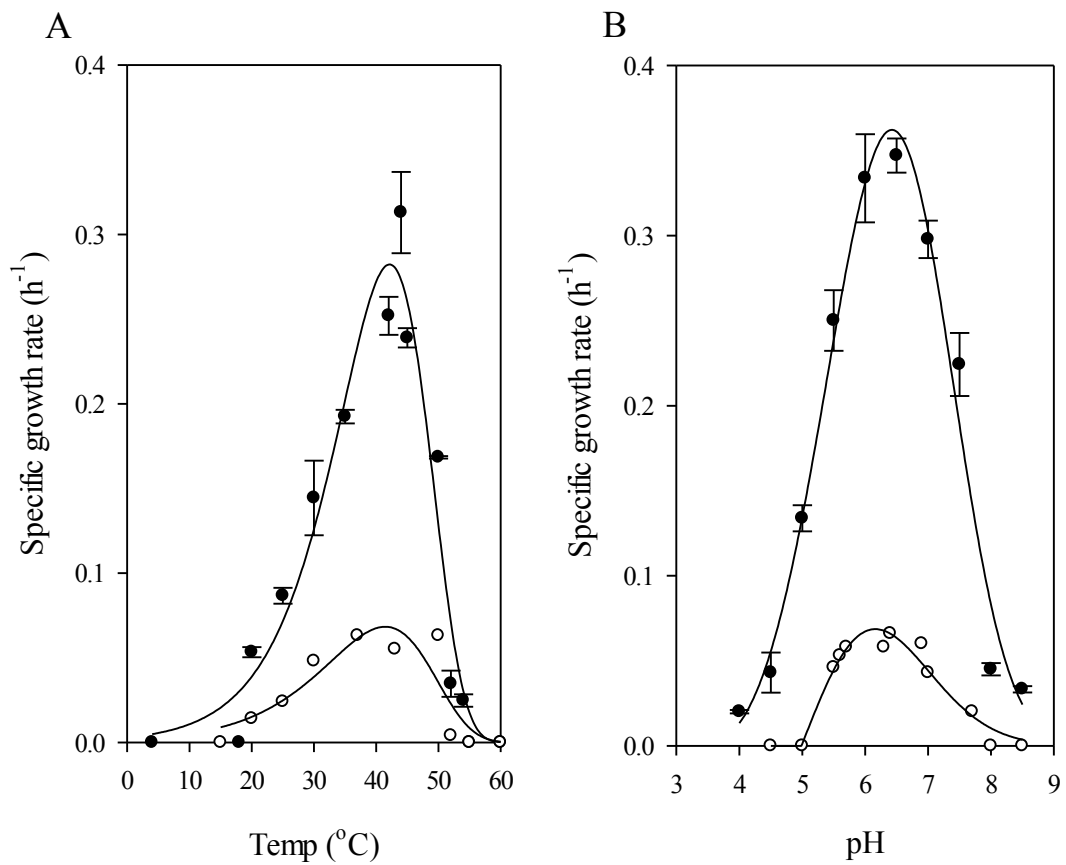


Figure 5.3 Temperature (A) and pH (B) profiles of *Thermithiobacillus sp. ParkerM* (●) and *Thermithiobacillus tepidarius* (○) grown on EBS supplemented with 20 mM thiosulfate. Values are mean \pm SEM ($n = 3$).

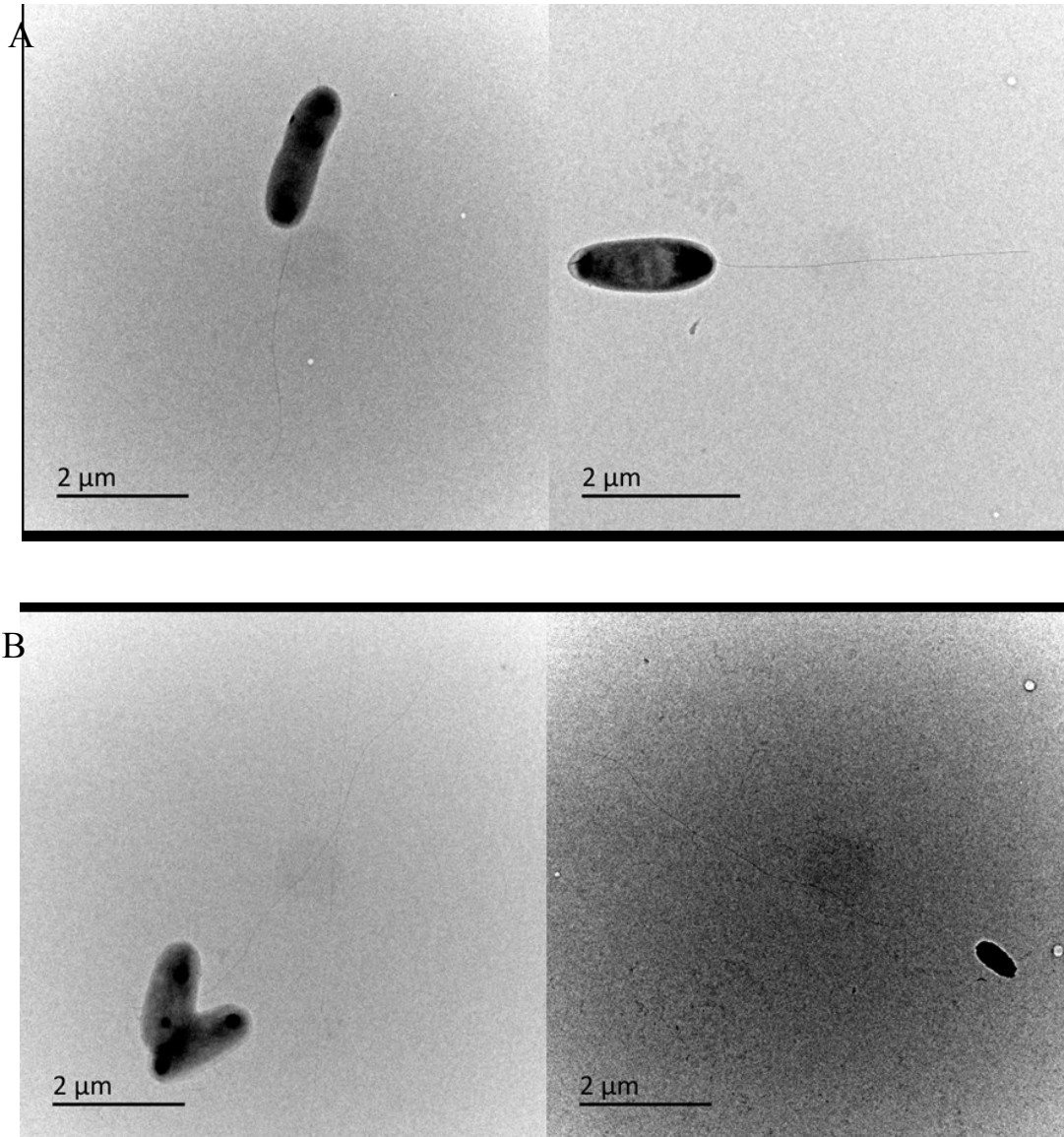


Figure 5.4 Negatively stained transmission electron micrographs of late exponential stage cells of *Thermithiobacillus tepidarius* (A) and *Thermithiobacillus* sp. ParkerM (B) previously grown on EBS supplemented with 20 mM thiosulfate.

5.2.6 Batch growth of *Thermithiobacillus* sp. ParkerM and *Thermithiobacillus tepidarius* on thiosulfate

Thermithiobacillus sp. ParkerM was grown in EBS liquid medium supplemented with 20 mM thiosulfate as sole energy substrate (Figure 5.5A, B). During the start of exponential growth thiosulfate was completely oxidised after 8 hours; trithionate was detected through this period and peaked at 4 h but had completely disappeared from the medium by 10.5 h. During this period visible amounts of extracellular sulfur were observed. Tetrathionate was present from the start of growth and peaked at 8 h at 9.7 mM from the stoichiometrical oxidation of thiosulfate. This coincided with a peak in pH of 7.49. Tetrathionate slowly decreased in concentration until none was detected after 56 h at which time the pH had dropped to 3.94. No visible extracellular sulfur was present by the end of growth.

Growth of *Ttb. tepidarius* (Figure 5.5C, D) under the same conditions as *Thermithiobacillus* sp. ParkerM produced similar results with regards to the production of trithionate and tetrathionate, but the oxidation process was slower in comparison with that of *Thermithiobacillus* sp. ParkerM. There was an initial decrease in thiosulfate concentration with increases in trithionate and a slower increase in tetrathionate. Trithionate rapidly disappeared from the growth medium and was not detected after 10 h. At the end of growth (105 h) there was still 2.3 mM of tetrathionate left in the growth medium. This coincided with a plateauing of the drop in pH. Comparison of growth kinetics of these two species can be seen in Table 5.3. There were significantly higher yields (Student's t-test, $p < 0.001$) and specific growth rates (Student's t-test, $p < 0.001$) in *Thermithiobacillus* sp. ParkerM but the specific rate of substrate uptake with regards to thiosulfate was not significantly different.

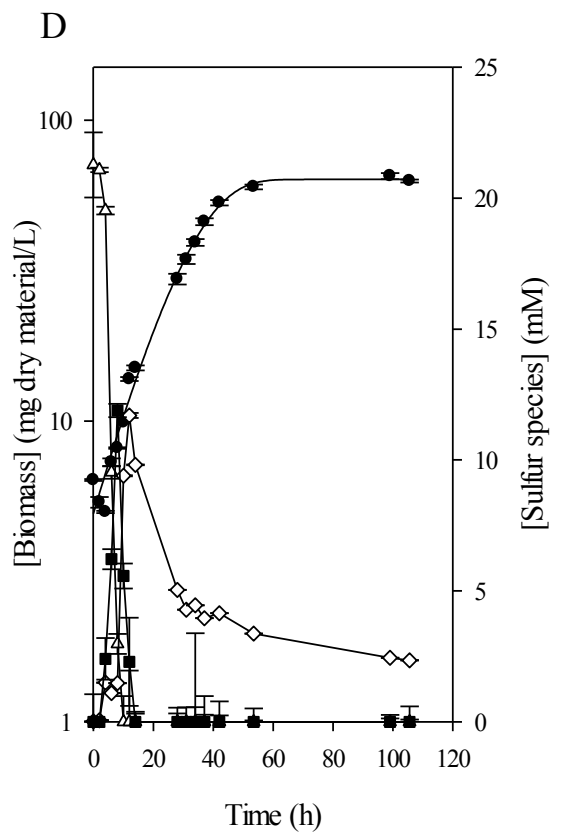
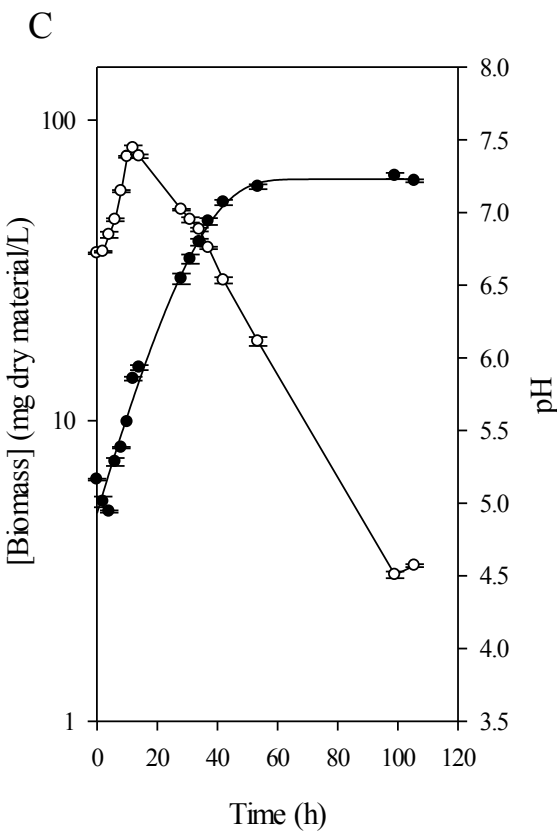
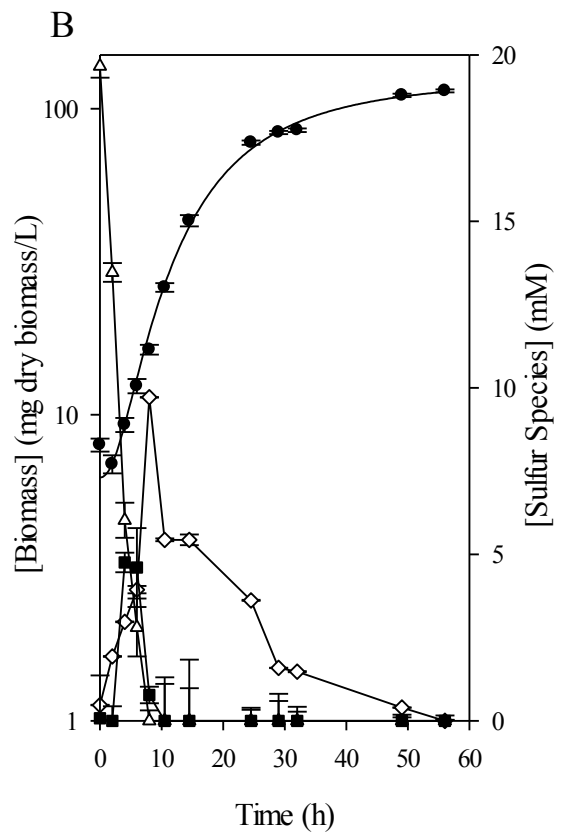
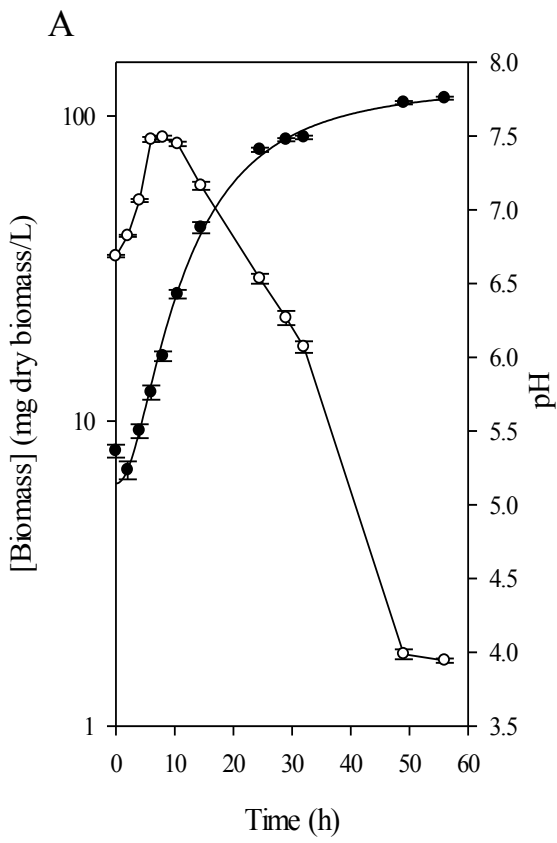


Figure 5.5 Growth (●) of *Thermithiobacillus* sp. ParkerM (A, B) and *Thermithiobacillus tepidarius* (C, D) in 500 mL batch culture on EBS supplemented with 20 mM thiosulfate at 44 °C shaken at 100 r.p.m.. (A) and (C) indicate changes of pH (○) during growth, (B) and (D) indicate changes in concentration of thiosulfate (Δ) and the subsequent formation of tetrathionate (◇) and trithionate (■). Values are means ± SEM ($n = 3$).

Table 5.3 Growth kinetics of *Thermithiobacillus* sp ParkerM and *Thermithiobacillus tepidarius* grown in batch culture on EBS supplemented with 20 mM thiosulfate. Parameters are yield (Y - mg dry biomass/mM thiosulfate), specific growth rate (μ - per hour) and specific rate of substrate uptake (q – mmol/h). * indicates significantly higher values. Values are means \pm SEM ($n = 3$).

| <i>Kinetic Parameter</i> | <i>Thermithiobacillus sp. ParkerM</i> | <i>Thermithiobacillus tepidarius</i> |
|--------------------------|---------------------------------------|--------------------------------------|
| Y | 5.4 \pm 0.1* | 2.8 \pm 0.1 |
| μ | 0.153 \pm 0.008* | 0.047 \pm 0.004 |
| q | 2.5 \pm 0.04 | 2.1 \pm 0.12 |

5.2.7 Batch growth of *Thermithiobacillus* sp. ParkerM on tetrathionate and trithionate

Thermithiobacillus sp. ParkerM was grown in EBS medium supplemented with 10 mM tetrathionate or 10 mM trithionate as sole energy substrates under the same conditions as that employed for growth on 20 mM thiosulfate (Section 5.3.6). Growth on tetrathionate (Figure 5.5) produced no intermediate products but for a small production of thiosulfate (0.7 mM) that peaked at 3 hours. No trithionate was detected at any point during growth. Tetrathionate was not detected in the growth medium at or after 24 hours. Growth of *Thermithiobacillus* sp. ParkerM on 10 mM trithionate produced both thiosulfate and tetrathionate during early stages of trithionate oxidation which peaked after 6 hours at 0.8 mM and 0.6 mM respectively. Table 5.4 summarises kinetic parameters of batch growth on thiosulfate (Section 5.3.6) as well as tetrathionate and trithionate. There were significantly higher yields and specific rates of substrate uptake when *Ttb.* sp. ParkerM when grown on 20 mM thiosulfate when compared to 10 mM tetrathionate and 10 mM trithionate (ANOVA, $p < 0.01$). The specific growth rate was significantly higher when grown on 10 mM trithionate when compared to 20 mM thiosulfate and 10 mM tetrathionate (ANOVA, $p < 0.05$).

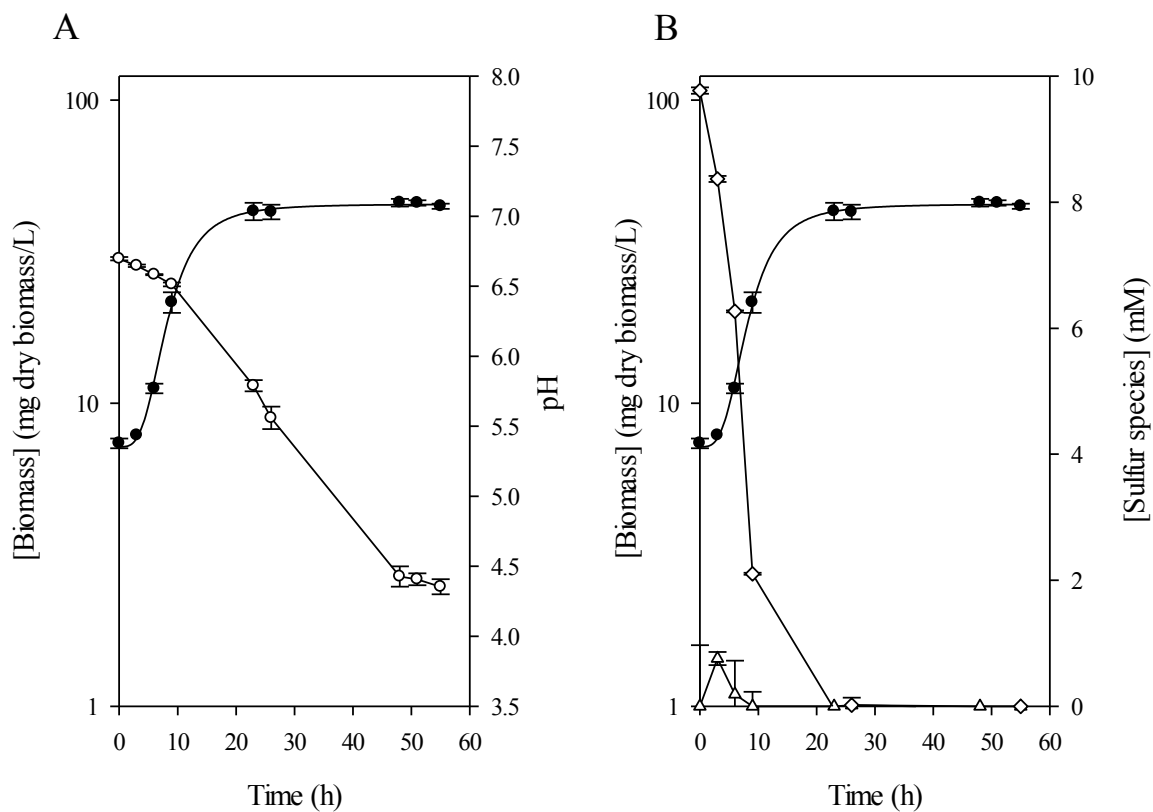


Figure 5.6 Growth (●) of *Thermithiobacillus* sp. ParkerM in 500 mL batch culture in liquid EBS medium supplemented with 10 mM tetrathionate at 44 °C shaken at 100 r.p.m.. (A) indicates change of pH (○) during growth; (B) indicates changes in concentration of tetrathionate (◇) and the production of thiosulfate (Δ). Values are mean ± SEM ($n = 3$).

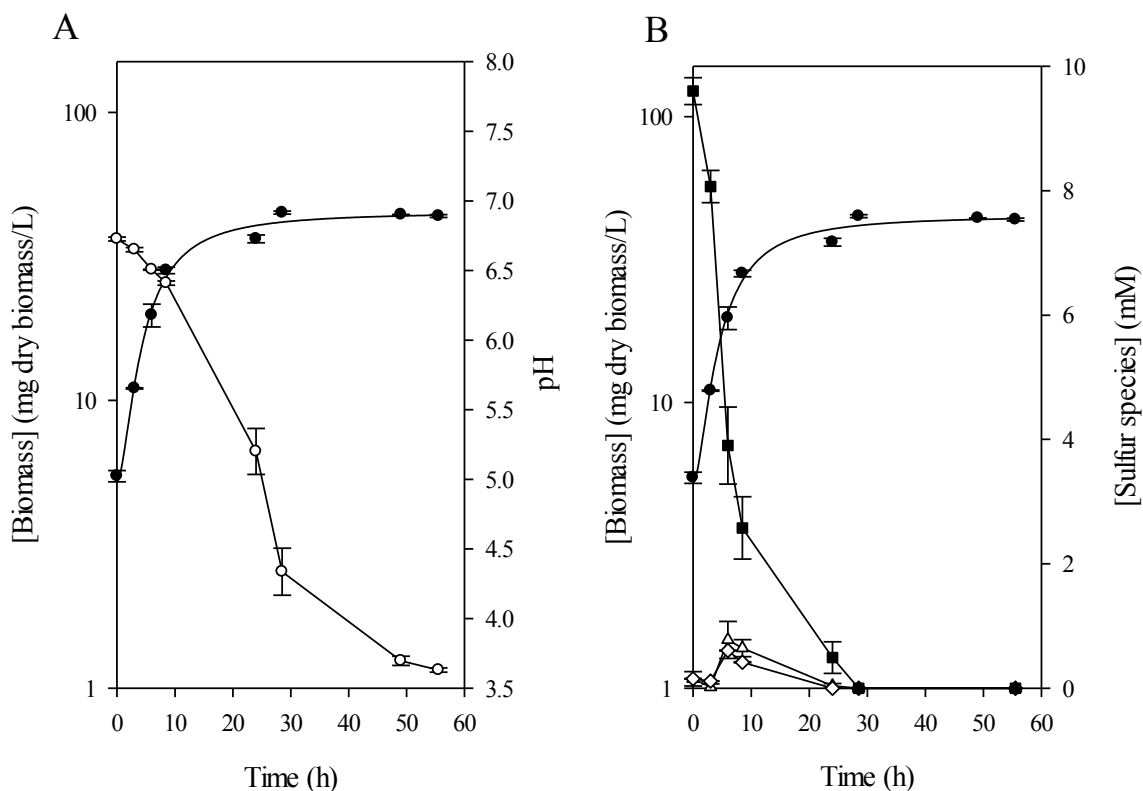


Figure 5.7 Growth (●) of *Thermithiobacillus* sp. ParkerM in 500 mL batch culture in liquid EBS supplemented with 10 mM trithionate at 44 °C shaken at 100 r.p.m.. (A) indicates change of pH (○) during growth; (B) indicates changes in concentration of trithionate (■) and the production of thiosulfate (Δ) and tetrathionate (◇) during growth. Values are means ± SEM ($n = 3$).

Table 5.4 Growth kinetics of *Thermithiobacillus* sp. ParkerM grown in batch culture on 20 mM thiosulfate, 10 mM tetrathionate and 10 mM trithionate. Parameters are yield (Y - mg dry biomass/mM growth substrate), specific growth rate (μ - per hour) and specific rate of substrate uptake (q - mmol/h). * indicates significantly higher values. Values are means \pm SEM ($n = 3$).

| Kinetic Parameter: | Growth substrate: | | |
|-------------------------------|--------------------------|----------------------|--------------------|
| | Thiosulfate | Tetrathionate | Trithionate |
| Y | $5.4 \pm 0.1^*$ | 3.9 ± 0.2 | 4.0 ± 0.1 |
| μ | 0.153 ± 0.008 | 0.167 ± 0.02 | $0.211 \pm 0.04^*$ |
| q | $2.5 \pm 0.04^*$ | 0.42 ± 0.02 | 0.4 ± 0.01 |

5.2.8 Continuous growth of *Thermothiobacillus* sp. ParkerM on thiosulfate, tetrathionate and trithionate

Steady-state cultures of *Thermothiobacillus* sp. ParkerM were established with 20 mM thiosulfate as the sole limiting energy substrate at dilution rates (D) = 0.037 – 0.183 h⁻¹, between which the growth yields (Y) increased from 6.86 to 10.1 g dry biomass per mol thiosulfate (equivalent to 3.43 to 5.05 g dry biomass per mol sulfur oxidised). Using a hyperbolic fit model the maximum theoretical growth yield (Y_{MAX}) was determined as 11.4 g dry biomass per mol thiosulfate (5.66 g dry biomass per mol of sulfur oxidised). From washout kinetics, the maximum specific growth rate (μ_{MAX}) was found to be 0.480 h⁻¹. The maintenance energy coefficient with respect to energy source (m_s) was estimated as 2.1 mmol thiosulfate (g•h)⁻¹ (4.2 mmol sulfur (g•h)⁻¹).

Steady-state cultures of *Thermothiobacillus* sp. ParkerM were established with 10 mM tetrathionate as the sole limiting energy substrate at $D = 0.033 – 0.183$ h⁻¹, between which the Y increased from 12.32 to 17.37 g dry biomass per mol tetrathionate (equivalent to 3.08 to 4.34 g dry biomass per mol sulfur oxidised). The Y_{MAX} was determined as 18.2 g dry biomass per mol of tetrathionate (4.6 g dry biomass per mol sulfur oxidised). From washout kinetics, the μ_{MAX} was found to be 0.430 h⁻¹. The m_s with respect to energy source was estimated as 0.8 mmol tetrathionate (g•h)⁻¹ (3.2 mmol sulfur (g•h)⁻¹).

Steady-state cultures of *Thermothiobacillus* sp. ParkerM were established with 10 mM trithionate as the sole limiting energy substrate at $D = 0.041 – 0.150$ h⁻¹, between which the Y increased from 6.56 to 10.0 g dry biomass per mol trithionate (equivalent to 2.19 to 3.33 g dry biomass per mol sulfur oxidised). The Y_{MAX} was determined to be 12.4 g dry biomass per mol of trithionate (4.1 g dry biomass per mol sulfur oxidised). From washout

kinetics, the μ_{MAX} was found to be 0.430 h^{-1} . The m_s with respect to energy source m_s was estimated as $3.3 \text{ mmol trithionate (g}\cdot\text{h)}^{-1}$ (mmol 9.9 of sulfur oxidised).

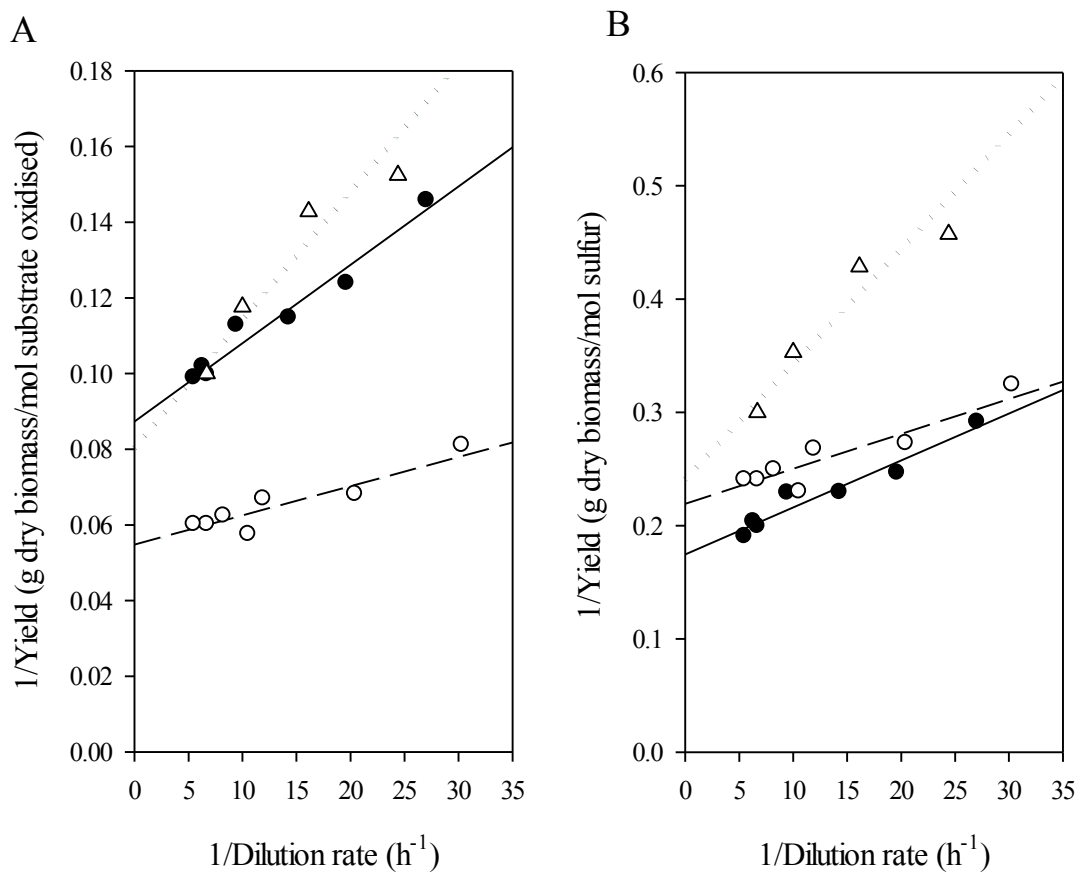


Figure 5.8 Derived double reciprocal plots based on kinetic parameters from hyperbolic fit of yield (Y) as amount of dry biomass formed per mol substrate oxidised versus dilution rate (A) and amount of biomass formed per mol sulfur oxidised versus dilution rate (B) of *Thermoithobacillus* sp. ParkerM in substrate limited chemostats. Plots represent growth on thiosulfate as sole energy source (solid line), tetrathionate as sole energy source (dashed line) and trithionate as sole energy source (dotted line).

5.2.9 Enzymology of *Thermithiobacillus* sp. ParkerM in chemostat culture

Enzymes involved in inorganic sulfur metabolism and TCA cycle metabolism were assayed and compared in cell-free extracts (CFE) obtained from thiosulfate (20 mM), tetrathionate (10 mM) and trithionate (10 mM) limited chemostats ($D = 0.150 \text{ h}^{-1}$). Specific enzyme activities of these can be seen in Table 5.5.

There were a number of variations in specific enzyme activities between different CFEs grown on different substrates (Table 5.5). There was significantly more activity of thiosulfate dehydrogenase when thiosulfate was the growth substrate compared to growth on tetrathionate and trithionate (ANOVA, $p < 0.005$). There was also significantly more thiosulfate dehydrogenase activity under trithionate limitation compared to tetrathionate limitation (Student's t-test, $p < 0.001$). If the expression of this enzyme requires the presence of thiosulfate then the conversion of one mole of trithionate to one mole of thiosulfate may explain this increase under trithionate limitation. There was significantly more trithionate hydrolase activity in trithionate grown CFE (Kruskal-Wallis, $p < 0.05$) but there was significantly less sulfite dehydrogenase activity compared to other growth conditions (ANOVA, $p < 0.05$). There was no significant variation between different substrates for activities of tetrathionate hydrolase or for the low levels of thiocyanate dehydrogenase. There were low levels of activity of sulfur oxygenase in thiosulfate and trithionate grown CFEs but sulfur oxidation appeared to be achieved *via* sulfur dioxygenase in tetrathionate grown CFE whereas sulfur dioxygenase activity was not observed in thiosulfate and trithionate grown CFEs.

Many enzymes of the TCA cycle were present in the three substrate limited conditions. However, activity of α -ketoglutarate dehydrogenase and succinyl- CoA synthetase were not detected under any growth conditions. There was also no succinate dehydrogenase

detected and significantly less activity of malate dehydrogenase (Kruskal-Wallis, $p < 0.005$) in tetrathionate grown CFE. Malate dehydrogenase was also significantly higher in trithionate grown cells when directly compared to thiosulfate grown cells (Student's t-test, $p < 0.001$). There were small levels of variation with other TCA cycle enzymes assayed but were not statistically significant.

Table 5.5 Specific enzyme activities in *Thermothiobacillus* sp. ParkerM cell-free extracts from cells grown in chemostat culture on thiosulfate, tetrathionate and trithionate under energy limitation ($D = 0.150 \text{ h}^{-1}$). *BDL* – below detection limit. Values represent mean \pm SEM ($n = 3$). Values in bold indicate statistically significant differences.

| Enzyme | EC number | Specific enzyme activities of CFEs from cells grown on the following substrates: | | |
|---|------------|--|---------------|-----------------------------------|
| | | Thiosulfate | Tetrathionate | Trithionate |
| <i>Inorganic sulfur enzymes:</i> | | | | |
| Thiosulfate dehydrogenase | 1.8.2.2 | *10,070 \pm 76 | 494 \pm 13 | *3,466 \pm 34 |
| Trithionate hydrolase | 3.12.1.1 | 8.6 \pm 6 | 7.5 \pm 5 | *52.5 \pm 4 |
| Tetrathionate hydrolase | 3.12.1.B1 | 218 \pm 4 | 221 \pm 7 | 211 \pm 8 |
| Sulfite dehydrogenase | 1.8.2.1 | 87 \pm 4 | 85 \pm 2 | *61 \pm 2 |
| Thiocyanate dehydrogenase | - | 9 \pm 2 | 0 \pm 1 | 15 \pm 10 |
| Sulfur oxygenase* | 1.13.11.55 | 4 \pm 0.3 | <i>BDL</i> | 7 \pm 0.8 |
| Sulfur dioxygenase* | 1.13.11.18 | <i>BDL</i> | 4 \pm 1 | <i>BDL</i> |
| <i>TCA Cycle Enzymes:</i> | | | | |
| Citrate synthase | 4.1.3.7 | 21 \pm 3 | 18 \pm 2 | 23 \pm 2 |
| Acoitase | 4.2.1.3 | 5 \pm 1 | 3 \pm 1 | 8 \pm 1 |
| Isocitrate dehydrogenase | 1.1.1.41 | 38 \pm 4 | 43 \pm 4 | 35 \pm 5 |
| α -ketoglutarate dehydrogenase | 1.2.4.2 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| Succinyl-CoA synthetase | 6.2.1.4 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |
| Succinate dehydrogenase | 1.3.5.1 | 19.8 \pm 1 | <i>BDL</i> | 5.8 \pm 1 |
| Fumarase | 4.2.1.2 | 108 \pm 11 | 103 \pm 9 | 168 \pm 19 |
| Malate dehydrogenase | 1.1.1.37 | *349 \pm 5 | 79 \pm 4 | *943 \pm 47 |
| <i>Carbohydrate enzymes:</i> | | | | |
| Glucose dehydrogenase | 1.1.1.47 | <i>BDL</i> | <i>BDL</i> | <i>BDL</i> |

5.2.10 Oxygen consumption by intact cells of *Thermithiobacillus* sp. ParkerM

Intact cells of *Thermithiobacillus* sp. ParkerM were harvested from thiosulfate, tetrathionate and trithionate limited chemostats ($D = 0.150 \text{ h}^{-1}$) and exposed to increasing concentrations of thiosulfate (0.5 – 10 mM), tetrathionate (0.5 – 10 mM) and trithionate (0.5 – 10 mM) from which oxygen consumption was recorded. There were significantly higher V_{max} values for thiosulfate and trithionate oxidation in cells previously grown on thiosulfate and trithionate compared to tetrathionate grown cells (Table 5.6) (ANOVA, $p < 0.05$), possibly due to the decrease in thiosulfate dehydrogenase activity previously noted in tetrathionate grown CFE (Table 5.5). There was some variation in V_{max} values for tetrathionate oxidation although this was not statistically significant. There was no evidence of sulfite oxidation under any of the different substrate limited conditions employed. K_m values showed less variation in terms of thiosulfate and tetrathionate oxidation (Table 5.6) although there was a lower value in thiosulfate grown cells. There was a notably higher K_m value for trithionate oxidation for cells previously grown on tetrathionate.

Table 5.6 Maximum rates of substrate-dependent oxygen uptake (V_{\max}) and Michaelis constants (K_m) of whole cells of *Thermothio bacillus* sp. ParkerM previously grown in substrate limited chemostats on thiosulfate, tetrathionate and trithionate ($D = 0.150 \text{ h}^{-1}$). Values are mean \pm SEM ($n = 3$).

| Substrate: | Grown on: | V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹] | | | K_m (mM) | | |
|---------------|-----------|--|---------------|--------------|-------------|---------------|-------------|
| | | thiosulfate | tetrathionate | trithionate | thiosulfate | tetrathionate | trithionate |
| Thiosulfate | | 500 \pm 28 | 187 \pm 20 | 427 \pm 47 | 12.9 | 14.0 | 14.1 |
| Tetrathionate | | 408 \pm 37 | 358 \pm 41 | 325 \pm 11 | 15.3 | 15.1 | 14.3 |
| Trithionate | | 349 \pm 18 | 87 \pm 9 | 455 \pm 9 | 18.9 | 20.5 | 17.2 |
| Sulfite | | 0 | 0 | 0 | 0 | 0 | 0 |

5.2.11 Cytochromes of inorganic sulfur metabolism in *Thermithiobacillus* sp. ParkerM

5.3.11.1 Reduction of cell-free extracts of thiosulfate, tetrathionate and trithionate grown cells of *Thermithiobacillus* sp. ParkerM by thiosulfate

Thiosulfate-reduced absolute spectra of cell-free extracts from cells grown in chemostat cultures on thiosulfate, tetrathionate and trithionate were obtained from *Ttb.* sp. ParkerM. These spectra showed absorbance maxima of 416 (Soret), 523 (β) and 551 (α) when dithionite-reduced and 414 (Soret), 523 (β) and 551 (α) nm when thiosulfate-reduced. These absorbance spectra were identical with those from cell-free extracts of cells grown on thiosulfate, tetrathionate and trithionate.

Concentrations of thiosulfate-reducible cytochrome c_{551} were estimated at 4.3 nmol (mg protein)⁻¹, 2.3 nmol (mg protein)⁻¹ and 1.4 nmol (mg protein)⁻¹ for thiosulfate, tetrathionate and trithionate grown cells, respectively. Concentrations of dithionite-reducible cytochrome c_{551} were 5.0 nmol (mg protein)⁻¹, 5.5 nmol (mg protein)⁻¹ and 4.3 nmol (mg protein)⁻¹ for thiosulfate, tetrathionate and trithionate grown cells, respectively.

5.3.11.1 Reduction of cell-free extracts of thiosulfate, tetrathionate and trithionate grown cells of *Thermithiobacillus* sp. ParkerM by sulfite

Sulfite-reduced absolute spectra of cell-free extracts from cells grown in chemostat cultures on thiosulfate, tetrathionate and trithionate were obtained from *Thermithiobacillus* sp. ParkerM. Spectra from thiosulfate grown cell-free extract showed absorbance maxima of 416 (Soret), 523 (β) and 551 (α) nm when dithionite-reduced and 411 (Soret), 524 (β) and 550 (α) nm when sulfite-reduced. In comparison, spectra from tetrathionate grown cell-free extract showed absorbance maxima of 409 (Soret), 524 (β) and 550 (α) nm when sulfite-reduced; spectra from trithionate grown cell-free extract

showed absorbance maxima of 414 (Soret), 521 (β) and 550 (α) nm when sulfite-reduced. Dithionite-reduced spectra showed identical absorbance maxima between thiosulfate, tetrathionate and trithionate grown cell-free extracts.

Concentrations of sulfite-reducible cytochrome c_{550} were estimated at 1.1 nmol (mg protein)⁻¹, 0.4 nmol (mg protein)⁻¹ and 1.0 nmol (mg protein)⁻¹ for thiosulfate, tetrathionate and trithionate grown cells respectively. Concentrations of dithionite-reducible cytochrome c_{550} were 4.8 nmol (mg protein)⁻¹, 5.8 nmol (mg protein)⁻¹ and 4.1 nmol (mg protein)⁻¹ for thiosulfate, tetrathionate and trithionate grown cells, respectively.

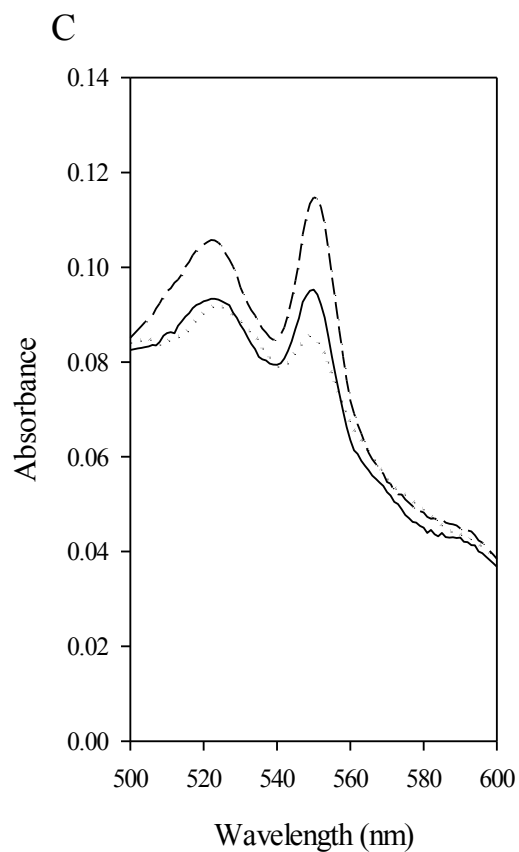
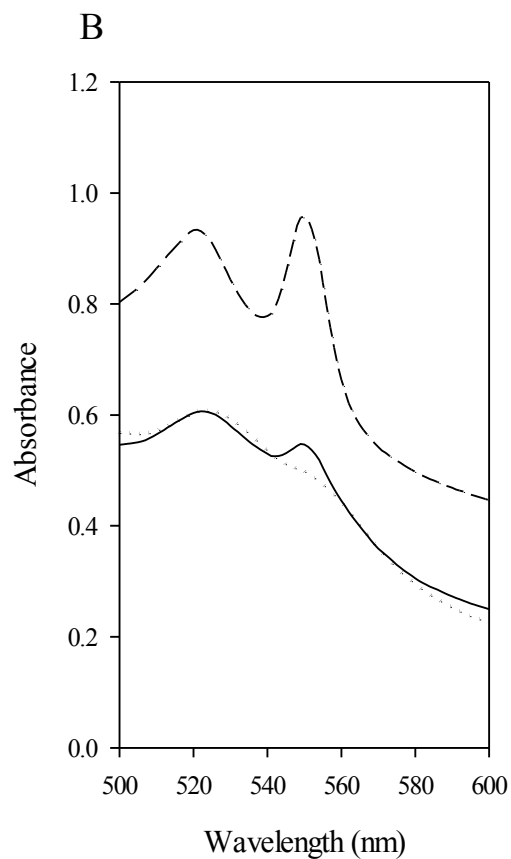
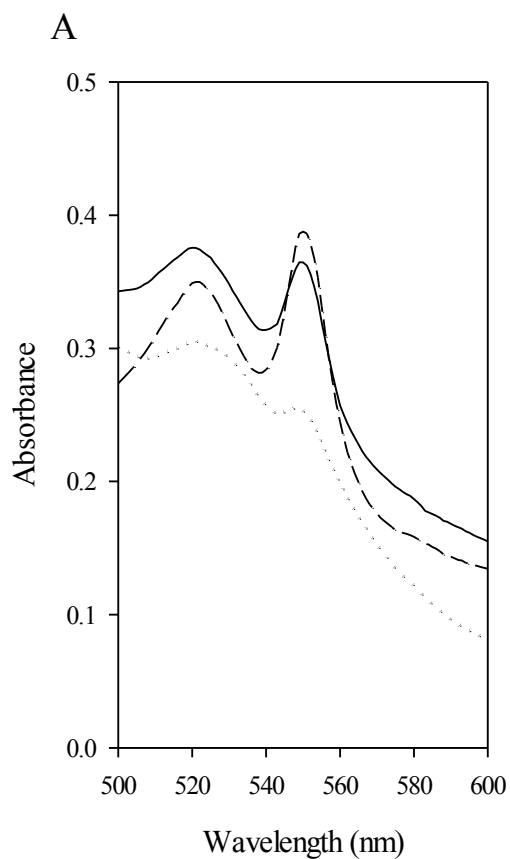


Figure 5.9 Thiosulfate-reduced absolute spectra of *Thermithiobacillus* sp. ParkerM cell-free extracts previously grown in energy limited chemostats ($D = 0.150 \text{ h}^{-1}$) on thiosulfate (**A**), tetrathionate (**B**) and trithionate (**C**). Shown are air oxidised (dotted line), 1 mM thiosulfate-reduced (solid line) and dithionite-reduced (dashed line).

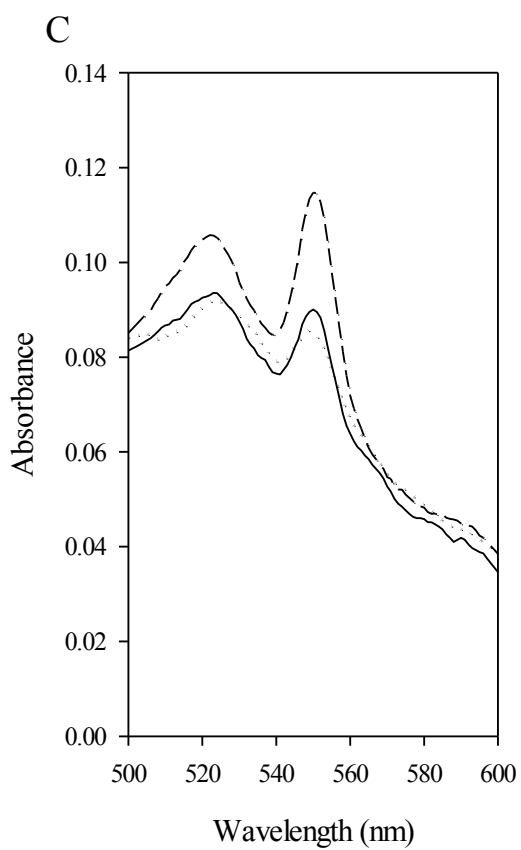
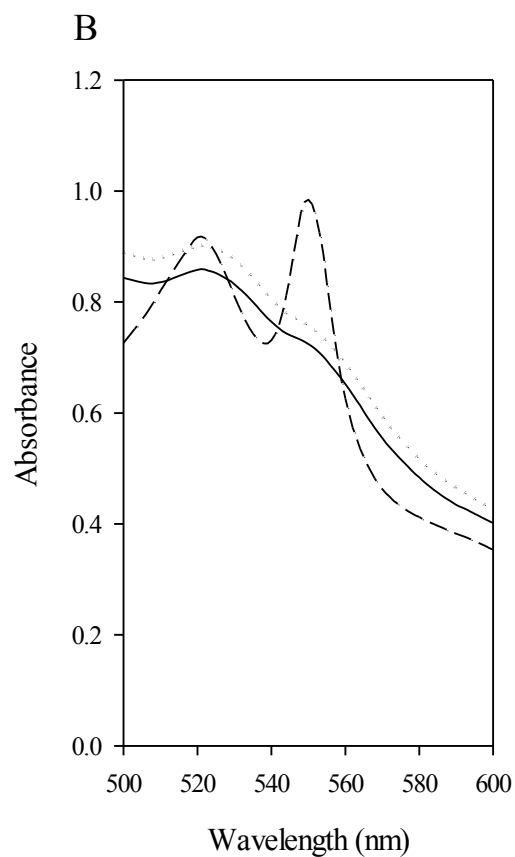
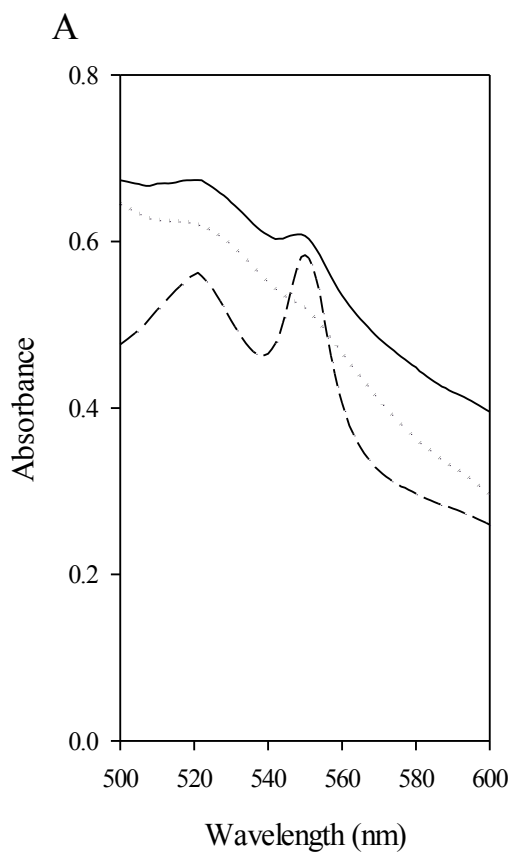


Figure 5.10 Sulfite-reduced absolute spectra of *Thermithiobacillus* sp. ParkerM cell-free extracts previously grown in energy limited chemostats ($D = 0.150 \text{ h}^{-1}$) on thiosulfate (**A**) tetrathionate (**B**) and trithionate (**C**). Shown are air oxidised (dotted line), 1 mM sulfite-reduced (solid line) and dithionite-reduced (dashed line).

5.2.12 Effect of succinate on growth of *Thermithiobacillus* sp. ParkerM

Thermithiobacillus sp. ParkerM was grown on EBS medium supplemented with 20 mM thiosulfate, 2 mM succinate and 20 mM thiosulfate plus 2 mM succinate in batch culture at 44 °C. The growth of *Thermithiobacillus* sp. ParkerM was monitored in order to identify any beneficial effects (Figure 5.10). Observed yields and specific growth rates are summarised in Table 5.7. When growth on thiosulfate with succinate was compared to growth without the presence of succinate there was a small but non-significant decrease in yield and specific growth rates. As previously examined (Table 5.2) there was no evidence of growth on succinate alone. There was no change in succinate concentration in both succinate and thiosulfate plus succinate growth media.

A single steady-state ($D = 0.10 \text{ h}^{-1}$) was established of *Thermithiobacillus* sp. ParkerM with 20 mM thiosulfate as the sole limiting substrate. The growth Y was 8.4 g dry biomass per mol thiosulfate (equivalent to 4.2 g dry biomass per mol substrate sulfur). A second steady-state was established at the same D and same concentration of thiosulfate but with the addition of 2 mM succinate. The growth Y of which was 8.2 g dry biomass per mol thiosulfate (equivalent to 4.1 g dry biomass per mol substrate sulfur). There was no change in succinate concentration in the growth medium.

Cell-free extract was obtained from the steady state of *Thermithiobacillus* sp. ParkerM grown in the presence of 2 mM succinate. Cytochrome spectra showed no evidence cytochrome reduction by 1 mM succinate (Figure 5.11).

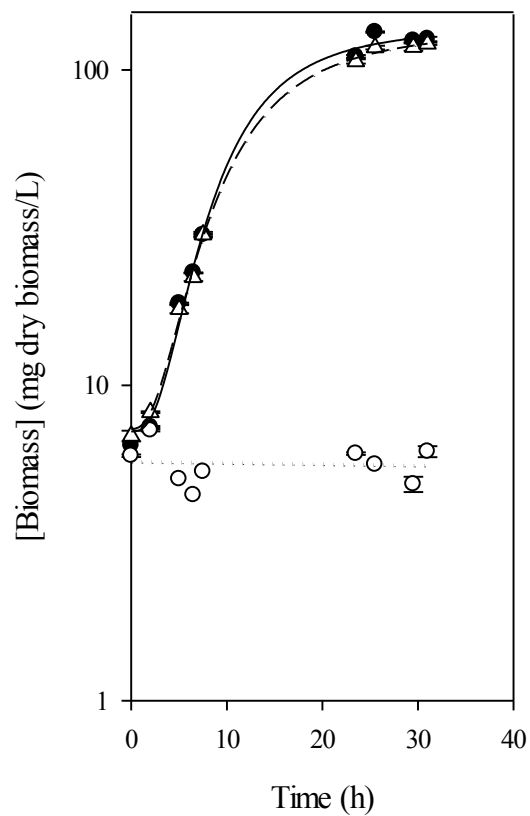


Figure 5.11 Growth of *Thermithiobacillus* sp. ParkerM in 50 mL batch culture of EBS medium supplemented with 20 mM thiosulfate (●; solid line), 2 mM succinate (○; dotted line) and 20 mM thiosulfate plus 2 mM succinate (Δ; dashed line) at 44 °C shaken at 100 r.p.m.. Values are mean ± SEM ($n = 3$).

Table 5.7 Summary of yields (Y - mg dry biomass/mM growth substrate) and specific growth rates (μ - per hour) of *Thermithiobacillus* sp. ParkerM grown on 20 mM thiosulfate, 2 mM succinate and 20 mM thiosulfate plus 2 mM succinate.

| Kinetic Parameter: | Growth substrate: | | |
|-------------------------------|--------------------------|------------------|------------------------------|
| | thiosulfate | succinate | thiosulfate/succinate |
| Y | 6.0 ± 0.2 | 0 | 5.8 ± 0.1 |
| μ | 0.247 ± 0.005 | 0 | 0.229 ± 0.004 |

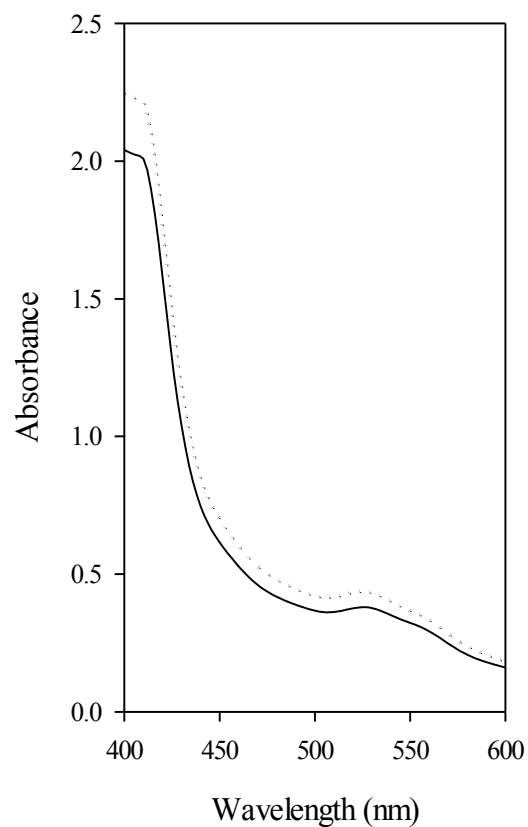


Figure 5.12 Absolute cytochrome spectra of cell-free extracts obtained from *Thermithiobacillus* sp. ParkerM grown in a thiosulfate limited chemostat in with 2 mM succinate ($D = 0.10 \text{ h}^{-1}$). Dotted line indicates air oxidised cell-free extract, solid line indicates cell-free extracts after the addition of succinate (10 mM final concentration).

5.3 Discussion

Phylogenetic analysis of the 16S rRNA gene sequence placed *Thermithiobacillus* sp. ParkerM strongly within the genus *Thermithiobacillus* along with the only other species validly published, *Ttb. tepidarius*. This is in agreement with Boden *et al.* (2011) who first showed this strain does not belong to the Genus *Thiobacillus*. With a sequence similarity of 99.5% in this study and that of Boden *et al.* (2011) it is not possible to distinguish this strain as a distinct species from *Ttb. tepidarius*. However, there are a number of chemotaxonomic differences between these two species that would agree to it being a novel species. There is a marked difference in the lowest pH reached when grown in batch culture (Table 5.1), possibly due to a higher acid tolerance in *Ttb. sp. ParkerM*. The drop in pH during batch growth is caused by the production of sulfate and might also explain the significantly higher yield and specific growth rate observed by *Ttb. sp. ParkerM* in batch culture (Table 5.3) as it was able to compensate for any cellular damage caused by the acidic conditions. When both species were grown in a pH regulated chemostat the yields and specific growth rates were similar (Wood & Kelly 1986). There was no apparent growth of *Ttb. sp. ParkerM* on pentathionate but growth on this substrate was seen of *Ttb. tepidarius* in this study. With our current understanding of the Kelly-Trudinger pathway there is no clear explanation for this, especially as both species were able to grow on hexathionate. Potentially the oxidation of higher polythionates may involve additional enzymes and in the case of pentathionate these might be lacking in *Ttb. sp. ParkerM*. Clearly this requires further investigation, possibly in continuous culture while growth parameters are being controlled while pentathionate is supplied as growth substrate.

In this study *Ttb. sp. ParkerM* has shown to exhibit a higher maximum theoretical growth yield (Y_{MAX}) in continuous culture on thiosulfate as was shown with *Ttb. tepidarius* by

Wood and Kelly (1986), 11.4 and 10.4 g dry biomass/mol thiosulfate, respectively. An almost identical Y_{MAX} in terms of thiosulfate of 11.6 was observed with another moderately thermophilic organism, *Thiobacillus aquaesulis* when grown at 43 °C (Wood & Kelly 1988). A more extreme thermophile, *Thermothrix thiopara*, achieved a Y_{MAX} of 20.3 at 70 °C (Brannan & Caldwell 1983) and 22.8 at 72 °C (Mason *et al.* 1986c). Many mesophilic species have shown more modest Y_{MAX} values, 8.6 for *Halothiobacillus neapolitanus* (Mason *et al.* 1986c), 7.5 for *Acidithiobacillus ferrooxidans* (Eccleston & Kelly 1978) and 7.0 for *Thiomicrospira pelophila* (Kuenen & Veldkamp 1973). *Thermithiobacillus* sp. ParkerM appears to support the idea of an intermediate group of thiosulfate oxidising *Bacteria* that are occupied by moderately thermophilic organisms. In terms of yield per mole sulfur atoms oxidised there was a 26% increase in Y_{MAX} when *Ttb.* sp. ParkerM was grown on thiosulfate compared to growth on tetrathionate. This is close to the theoretical increase of 25% that would be expected from the release of 2 electrons from two molecules of thiosulfate being oxidised to one molecule of tetrathionate, compared to the release of 8 electrons from the oxidation of one molecule of tetrathionate to four molecules of sulfate. In terms of yield per substrate oxidised there was a small difference between trithionate and thiosulfate (12.4 and 11.4 g dry biomass/mol, respectively). This strongly indicates that there is energy conserved (i.e. substrate-level phosphorylation) by the reaction of trithionate to thiosulfate. In contrast, identical yields on thiosulfate and trithionate were seen with *T. aquaesulis* (9.6 g dry biomass/mol) although this was only compared at a single dilution rate ($D = 0.110 \text{ h}^{-1}$) (Wood & Kelly 1988) while slightly higher trithionate Y_{MAX} values were also observed when compared to thiosulfate in *Ttb. tepidarius* (10.8 and 10.4 g dry biomass/mol respectively) and *Htb. neapolitanus* (8.4 and 8.6 g dry biomass/mol, respectively) (Kelly & Wood 1986; Mason *et al.* 1986c). Clearly this requires further investigation to see if there is true energy

conservation occurring or if increased trithionate yields are just an artefact. It would also be vital to investigate whether any possible energy conservation takes places in all trithionate oxidising *Bacteria*.

Enzymes involved in the oxidation of inorganic sulfur were found to vary considerably depending on which sulfur species were used as the energy substrate. Both thiosulfate dehydrogenase and trithionate hydrolase activities were significantly higher when the organism was grown on the respective substrates (Table 5.5), showing upregulation of the genes involved. However this was not seen with tetrathionate hydrolase as activities remained constant between the different compounds. Interestingly, activities of sulfur oxygenase were detected in thiosulfate and trithionate grown CFE but the activity of sulfur dioxygenase was detected instead in tetrathionate grown CFE. Deposits of elemental sulfur were commonly seen in batch cultures of both *Thermithiobacillus* sp. ParkerM and *Ttb. tepidarius*, but this did not occur in chemostat cultures with the exception of when the dilution rate was turned up drastically. However, sulfur would soon disappear from chemostat cultures once the growth rate increased to match the dilution rate. The oxidation of elemental sulfur is still poorly understood and the evidence that there is a shift between sulfur oxygenase and sulfur dioxygenase depending on initial energy substrate does add another dimension. It could be hypothesised that growth on thiosulfate is identical to that of tetrathionate but for the initial presence of thiosulfate and the energy conservation it yields. However, production of elemental sulfur that might take place during thiosulfate oxidation (and trithionate oxidation) might have caused an upregulation of the sulfur oxygenase gene in response. Sulfur deposits were also noted in tetrathionate batch cultures but not to the same extent and could possibly explain the presence of sulfur dioxygenase in chemostat culture. At this stage it cannot be ruled out that the production of sulfur might be chemical rather than enzymatic, especially in batch

culture where higher amounts of sulfur were seen, possibly due to the drastic changes in pH.

It has been shown in species of autotrophs (including members of *Thiobacillus*) that enzymes of the TCA cycle operate in a purely biosynthetic fashion with no energy production being observed (Johnson & Abraham 1969; Peeters *et al.* 1970). Some enzymes have shown to be missing from this set of reactions, however, and it has been described as a horseshoe rather than a cycle (Lengeler *et al.* 1998). In agreement with that found in CFE of *Thiobacillus denitrificans* and *Paracoccus versutus* previously grown on thiosulfate by Peeters *et al.* (1970), the activities of α -ketoglutarate dehydrogenase and succinyl-CoA synthetase were not detected in CFE of *Therithiobacillus* sp. ParkerM grown on any of the sulfur species tested (Table 5.5). The enzyme activities of succinate and α -ketoglutarate dehydrogenase were absent from ferrous iron grown cells of *Acidithiobacillus ferrooxidans* but were inducible when grown mixotrophically on ferrous iron and glucose (Tabita & Lundgren 1971). In this example succinyl-CoA synthetase was not assayed but presumably would be missing. α -ketoglutarate is known to be an important precursor in the biosynthesis of glutamate and subsequently other amino acids making the activity of isocitrate dehydrogenase vital for protein synthesis. With the activities of α -ketoglutarate dehydrogenase and succinyl-CoA synthetase lacking than the production of porphyrins from succinate would require the reverse reactions of malate dehydrogenase, fumarase and succinate dehydrogenase (Figure 5.13). The activities of these three enzymes were detected and it seems possible that this is how porphyrins and subsequently haems and cytochromes are being synthesised in cells of *Tib.* sp ParkerM. It can be seen in Table 5.5 that no detectable succinate dehydrogenase activity was found in tetrathionate grown CFE but it was detected in thiosulfate and trithionate grown CFE. This could be due to the upregulation of thiosulfate dehydrogenase which has been shown

to require the presence of a cytochrome *c*₅₅₂ (Trudinger 1961*a,b*; Kelly *et al.* 1993). When cells were grown on tetrathionate and by passed the initial steps of the Kelly-Trudinger pathway the need for cytochrome *c*₅₅₂ and so succinate fell drastically. This can be seen in the 48% drop in thiosulfate-reducible cytochrome observed in tetrathionate grown cells compared to that observed in thiosulfate grown cells (5.3.11.1). The significant drop in malate dehydrogenase activity in tetrathionate grown CFE may also support this hypothesis. It has already been shown by Matin & Rittenberg (1971) that enzyme profiles of the obligate autotrophs *Thiobacillus thioparius* and *Halothiobacillus neapolitanus* changed when glucose was present; this included a notable increase in isocitrate dehydrogenase activity. Gottschal & Kuenen (1980) examined *Paracoccus versutus* in a mixotrophic chemostat on varying amounts of acetate and thiosulfate, and found malate dehydrogenase activity to be higher when thiosulfate was absent. However, research into the TCA cycle in obligate autotrophic sulfur oxidising *Bacteria* appears to be lacking and the current study is the first in which TCA cycle enzymes activities were compared between cells grown on different inorganic sulfur substrates. While many mixotrophic species of inorganic sulfur oxidising *Bacteria* (*Acidiphilium acidophilum*, *Paracoccus versutus*) have been studied in terms of TCA cycle enzymes (Guay & Silver, 1975; Gottschal & Kuenen, 1980) this area of biochemistry in obligately autotrophic species requires further examination, especially as the current study would seem to indicate a significant change enzyme profiles when grown on different sulfur substrates.

It had previously been shown that acetate, succinate and a number of amino acids boosted the yields of *Thiobacillus thioparus*, *Halothiobacillus neapolitanus* and *Thiomicrospira pelophila* by as much as 24% in thiosulfate-limited chemostat cultures (Kuenen & Veldkamp, 1973). In terms of succinate, there was no evidence of succinate utilisation by *Thermithiobacillus* sp. ParkerM in this study in either batch culture or continuous culture.

In fact succinate appeared to have a slight negative impact on growth in terms of yields and specific growth rates.

In conclusion, this study has confirmed a second species of *Thermithiobacillus* although further phylogenetic analysis would be needed to validly publish this strain as *Thermithiobacillus parkeri*. Tetrathionate and trithionate have shown to be intermediates in *Ttb.* sp. ParkerM in common with other Kelly-Trudinger pathway utilising *Bacteria*. Y_{MAX} values obtained from chemostat cultures place this strain within an intermediate group, along with other moderately thermophilic species. The enzyme activities of both inorganic sulfur metabolism and of the TCA cycle vary significantly when grown on either thiosulfate, tetrathionate or trithionate. Notably thiosulfate dehydrogenase and trithionate hydrolase activities are significantly higher when the respective substrate was used as the growth substrate. Malate dehydrogenase activity was significantly lower when cells were grown tetrathionate while succinate dehydrogenase was completely absent. The reason for these different enzyme activity profiles would be the topic of future work.

5.4.1 Description of *Thermithiobacillus parkerianus* sp. nov.

Thermithiobacillus parkerianus (par.ker.i.an'us) N. L. masc. adj. *parkerianus*, belonging to Parker, named after Cecil David 'Guy' Parker (1912 - 1981), American soil microbiologist and originator of ParkerM.

Cells are Gram negative rods of $0.5 \times 1.45 \mu\text{m}$ and motile by means of a single polar flagellum. Does not form rosettes, palisades or pellicles. Grows at 20-54 °C with an optimum of 44 °C and at pH 4.0-8.5 with an optimum of 6.0-7.0. Does not reduce selenite and tellurite. Does reduce nitrate but not nitrite and cannot grow anaerobically with denitrification. Grows as an autotroph by oxidising thiosulfate, trithionate, tetrathionate, pentathionate, hexathionate and sulfur but not on sulfite, thiocyanate, or dithionate. Does

not grow heterotrophically, mixotrophically or using sulfur disproportionation. Colonies are flat to convex with entire margins and white on basal agar supplemented with thiosulfate after 48 h, colonies turn yellow after 5 days.

The dominant respiratory quinone is UQ-8. GC content of genomic DNA is 71.5 mol%. Oxidase and catalase positive. Negative for hydrolysis of casein, DNA, lipids, starch, Tween® 20, 40, 60 and 80. As sole source of nitrogen can use (2 mM) ammonium, glycine, urea and nitrate but not EDTA, nitrite, cyanate and thiocyanate. Resistant to the antibiotics cephalothin, clindamycin, colistin sulfate, cotrimoxazole, fusidic acid, penicillin G, trimethoprim, streptomycin, sulphamethoxazole and sulphatriad but sensitive to ampicillin, cephalothin, erythromycin, gentamicin and tetracycline.

Isolated from the sewers of Melbourne, Australia 1947 by C. D. Parker. Type strain is ParkerM (NCIMB 8349^T; DSM 103443^T).

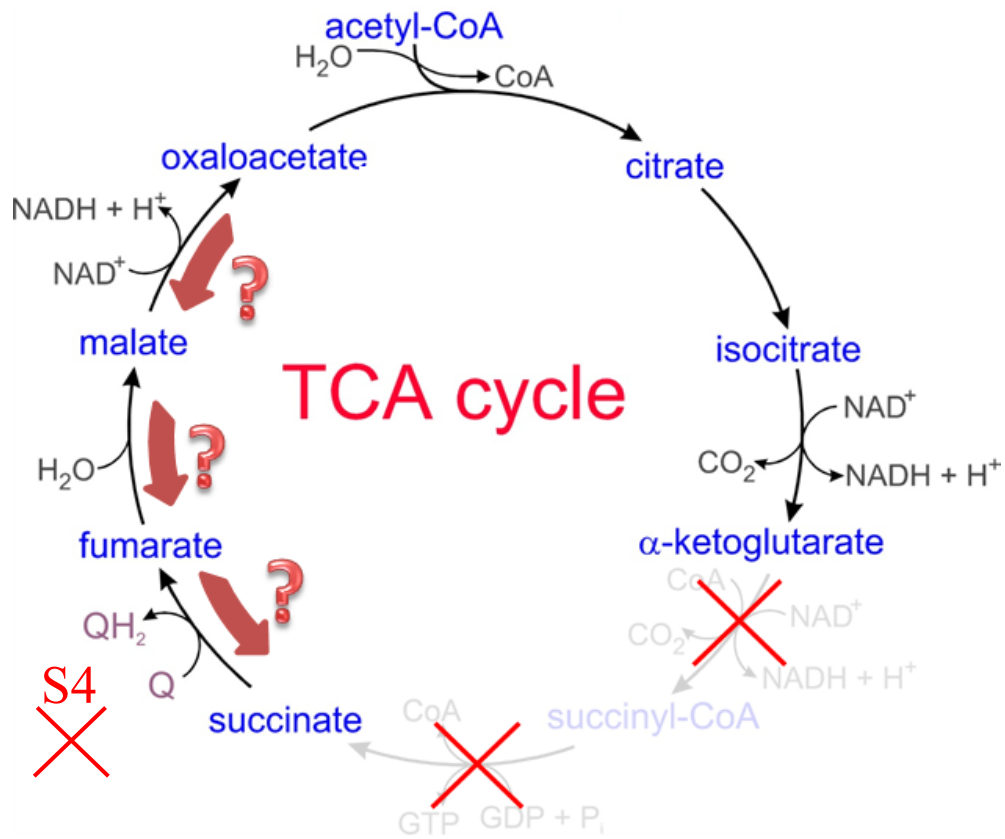


Figure 5.13 The TCA “hourseshoe” found in *Thermithiobacillus* sp. ParkerM grown in thiosulfate and trithionate limited chemostats. When tetrathionate was the limiting substrate the activity of succinate dehydrogenase was also absent (S4). Also shown are the potential reverse reaction of malate dehydrogenase, fumarase and succinate dehydrogenase. Image modified from and supplied by Dr. John Moody (personal communication).

Chapter 6

Properties of thiosulfate dehydrogenases from
Pseudomonas sp. Strain T, *Achromobacter* sp.
Strain B and *Thermithiobacillus* sp. ParkerM

6.1 Introduction

Thiosulfate dehydrogenase activity has been demonstrated in a variety of different autotrophic and heterotrophic *Bacteria* and the properties of this enzyme vary considerably between different species, even between closely related species. The specific activity of this enzyme in cell-free extracts has been found to be as high as $146 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ in *Thiobacillus thioparus* (Lyric & Suzuki 1970) but as low as $0.06 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ in *Acidithiobacillus thiooxidans* (Nakamura *et al.* 2000). Thiosulfate dehydrogenase in *Atb. ferrooxidans* has a pH optimum of 2.5 and an optimum temperature of 70 °C (Kikumoto *et al.* 2013) while the enzyme in an *Alcaligenes* sp. showed a pH optimum of 7.2, however, *Allochromatium vinosum* had a temperature optimum of 25 °C (Hall & Berk 1972; Hensen *et al.* 2006). The variation is undoubtedly due to the differing environments in which these strains were found; for example *Acidithiobacillus* spp. are commonly isolated from highly acidic drainage from mines (Valdés *et al.* 2008). There is also strong evidence of more than one thiosulfate dehydrogenase being present in the *Bacteria* (Trudinger, 1961; Silver and Lundgren, 1968; Lyric and Suzuki, 1970; Whited and Tuttle, 1983; Lu and Kelly, 1988; Denkmann *et al.* 2012; Kikumoto *et al.*, 2013).

The purpose of this study was to investigate and compare the properties of thiosulfate dehydrogenases from the chemolithoheterotrophic strains *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B and the chemolithoautotrophic strain *Thermithiobacillus* sp. ParkerM.

6.2 Results

All experiments were conducted on cell-free extracts (CFE) or whole cells obtained from steady-state chemostat cultures grown on 10 mM glucose plus 50 mM thiosulfate (*Pseudomonas* sp. Strain T), 10 mM succinate plus 50 mM thiosulfate (*Achromobacter* sp. Strain B) and 20 mM thiosulfate (*Thermithiobacillus* sp. ParkerM) at dilution rates of 0.068 h⁻¹, 0.10 h⁻¹ and 0.150 h⁻¹, respectively.

6.2.1 Thiosulfate dehydrogenase pH and temperature profiles

Thiosulfate dehydrogenase activities were assayed in CFEs of *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM in order to ascertain the optimum pH and temperatures for activity. CFEs were obtained from chemostat cultures as previously described (Section 2.8) and assayed for thiosulfate dehydrogenase using the method described previously (Section 2.9.1.1). The activities were measured at 20 °C at a range of different pH using 50 mM citrate buffer for the pH range 5.0-5.5 and 50 mM phosphate buffer for the pH range 6.0-8.0. The optimum specific activities were found to be 0.29 μmol min⁻¹ (mg protein)⁻¹ at pH 5.5 for *Pseudomonas* sp. Strain T; 1.32 μmol min⁻¹ (mg protein)⁻¹ at pH 5.0 for *Achromobacter* sp. Strain B and 8.73 μmol min⁻¹ (mg protein)⁻¹ at pH 5.0 for *Thermithiobacillus* sp. ParkerM (Figure 6.1, 6.2 & 6.3). Once optima pH was obtained, thiosulfate dehydrogenase activities were assayed at pH optima at a range of temperatures (Figure 6.1, 6.2 & 6.3). The optimum specific activities were 0.28 μmol min⁻¹ (mg protein)⁻¹ at 25 °C for *Pseudomonas* sp. Strain T; 1.34 μmol min⁻¹ (mg protein)⁻¹ at 25 °C for *Achromobacter* sp. Strain B and 8.74 μmol min⁻¹ (mg protein)⁻¹ at 25 °C for *Thermithiobacillus* sp. ParkerM.

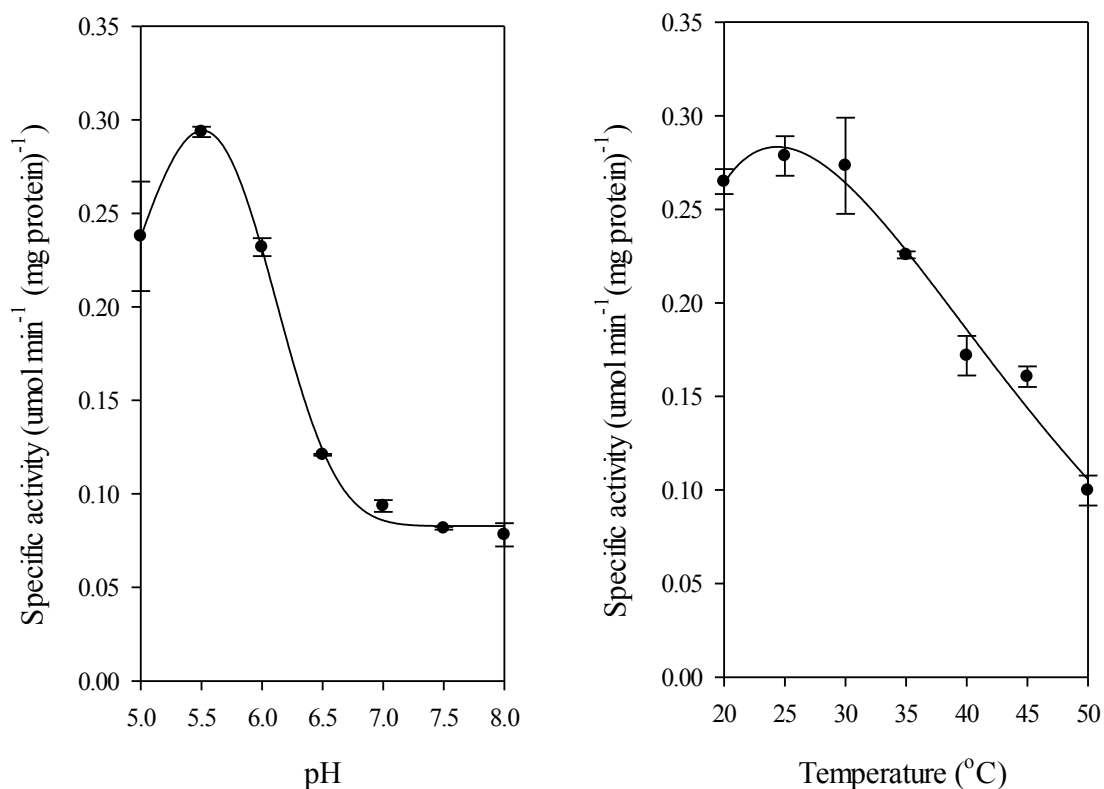


Figure 6.1 Temperature and pH profiles of thiosulfate dehydrogenase activities in *Pseudomonas* sp. Strain T cell-free extract grown in chemostat culture on 10 mM glucose and 50 mM thiosulfate ($D = 0.068 \text{ h}^{-1}$). pH activities were measured at 20 °C with either citrate or phosphate buffer and temperature activities were measured at pH 5.5. Data are mean \pm SEM ($n = 3$).

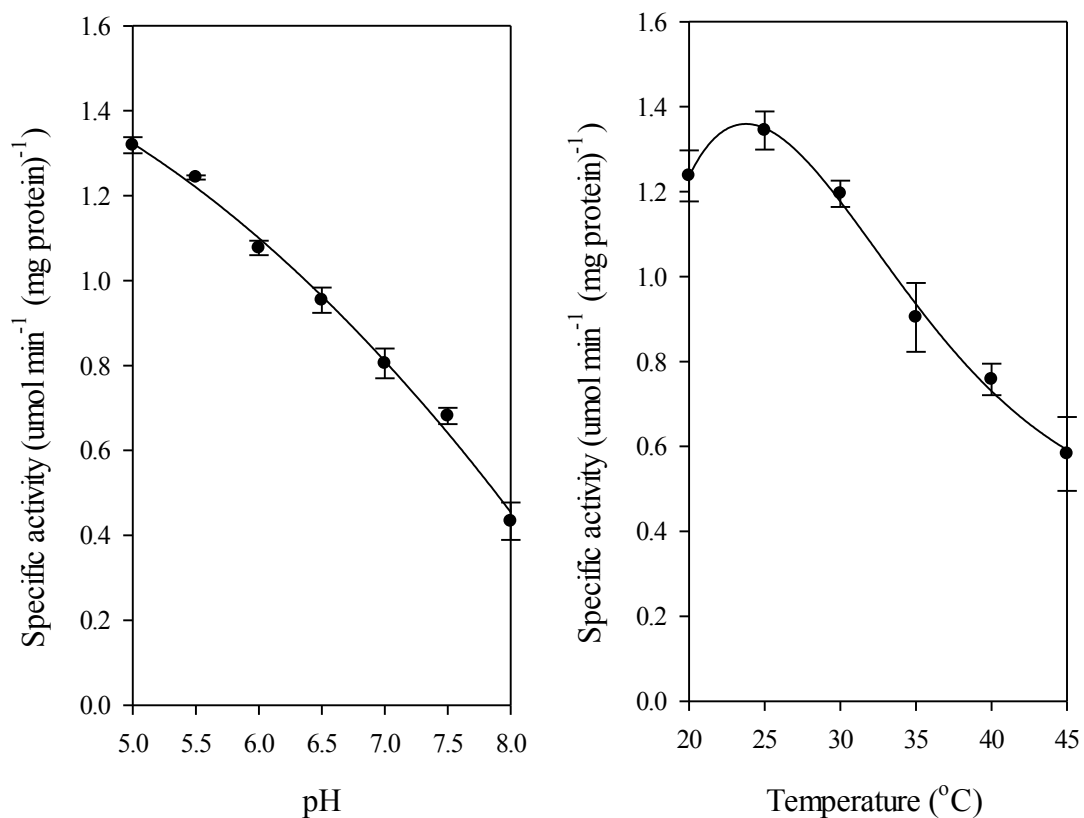


Figure 6.2 Temperature and pH profiles of thiosulfate dehydrogenase activities in *Achromobacter* sp. Strain B cell-free extract grown in chemostat culture on 10 mM succinate and 50 mM thiosulfate ($D = 0.10 \text{ h}^{-1}$). pH activities were measured at 20 °C with either citrate or phosphate buffer and temperature activities were measured at pH 5.0. Data are mean \pm SEM ($n = 3$).

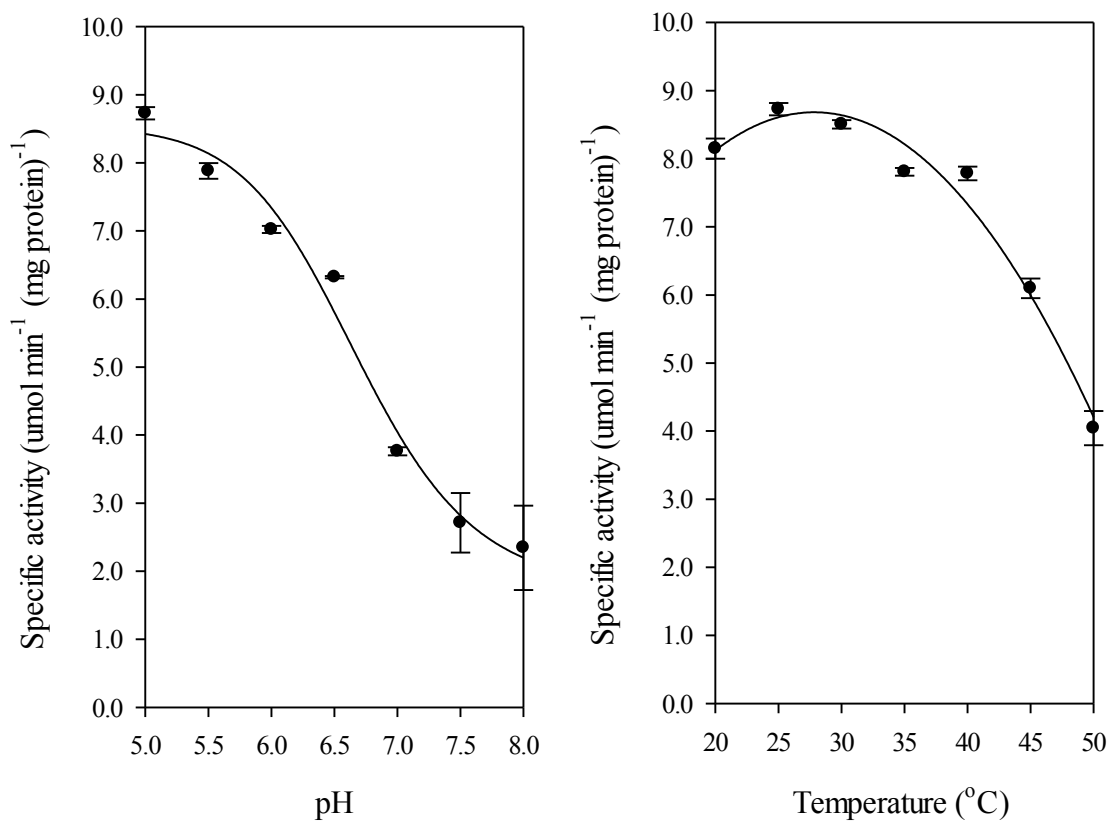


Figure 6.3 Temperature and pH profiles of thiosulfate dehydrogenase activities in *Thermithiobacillus* sp. ParkerM cell-free extract grown in chemostat culture on 20 mM thiosulfate ($D = 0.150 \text{ h}^{-1}$). pH activities were measured at 20 °C with either citrate or phosphate buffer and temperature activities were measured at pH 5.0. Data are mean \pm SEM ($n = 3$).

6.2.2 Electron acceptors of thiosulfate dehydrogenase

Thiosulfate dehydrogenase assays were carried out at the strain's optimal pH at 20 °C using a selection of different possible electron acceptors. The potential e^- acceptors 2,6 dichlorophenol-indophenol (DCPIP) (600 nm), ethyl violet (600 nm), ferricyanide (420 nm), indophenol (600 nm), phenol blue (600 nm) or Lauth's violet (600 nm) were added to the assay mixture (2.9.1.1) to give a final concentration of 3 mM. Bovine heart cytochrome *c* (550 nm) or horse heart cytochrome *c* (550 nm) was added to assay mixtures to give a final concentration of 0.15 μ M. Activities were monitored at the respective wavelengths (shown in parentheses) for the electron acceptor being used (Figure 6.1-6.3). Specific enzyme activities can be seen in Table 6.1. There was no evidence of reduction of DCPIP or phenol blue in any of the strains tested. There were trace levels ($< 0.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$) of activity in all three strains when ethyl violet and indophenol were used and trace levels detected when Lauth's violet was used with *Pseudomonas* sp. Strain T. Bovine and horse heart cytochromes proved to be moderately weak electron acceptors with CFE obtained from *Thermithiobacillus* sp. ParkerM but only trace levels were detected with the heterotrophic strains. Significantly higher activities were observed in all three strains when ferricyanide was used as the electron acceptor (ANOVA, $p < 0.005$).

Table 6.1 Specific enzyme activities of thiosulfate dehydrogenase in CFEs of *Psuedomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM obtained from chemostat cultures. Specific activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Values are means \pm SEM ($n = 3$).

| Electron acceptor: | Strain: | | |
|---------------------------|-----------------|-----------------|-----------------|
| | Strain T | Strain B | ParkerM |
| Ferricyanide | 165 \pm 15 | 1,109 \pm 28 | 11,177 \pm 50 |
| DCPIP | 0 | 0 | 0 |
| Ethyl violet | < 0.1 | < 0.1 | < 0.1 |
| Indophenol | < 0.1 | < 0.1 | < 0.1 |
| Phenol blue | 0 | 0 | 0 |
| Lauth's violet | < 0.1 | 0 | 0 |
| Bovine heart Cyt c | 0.2 \pm 0.03 | 0 | 12 \pm 2 |
| Horse heart Cyt c | < 0.1 | < 0.1 | 2 \pm 0.2 |

6.2.3 Inhibitors of thiosulfate oxidation in whole cells

Ten millilitre aliquots of EBS in 25 mL conical flasks were inoculated with cells previously grown in steady-state chemostat cultures. Batch cultures contained 0.1 mM of potential inhibitors and were incubated for 10 minutes at the optimal growth temperature for the strain being examined followed by the addition of thiosulfate to 10 mM. Cultures were incubated at optimal temperature and 0.5 mL samples removed every hour for cyanolysis. Control cultures contained no inhibitors. Whole cells of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B had completely oxidised thiosulfate to tetrathionate after 4 hours while thiosulfate was found to have completely disappeared from the culture medium of *Thermithiobacillus* sp. ParkerM after 2 hours. Incubation with mercuric chloride, *N*-ethylmaleimide (NEM), rotenone and *p*-chloromercuribenzoic acid (pCMB) inhibited thiosulfate oxidation in *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM to varying amounts (Figure 6.4, 6.5 and 6.6).

Thiosulfate oxidation by whole cells of *Pseudomonas* sp. Strain T was significantly inhibited by mercuric chloride and pCMB (Student's *t* test, $p < 0.05$) as well as Retenone ($p < 0.01$). NEM decreased the amount of thiosulfate oxidised but was not statistically significant. The same compounds were found to significantly inhibit thiosulfate oxidation in whole cells of *Achromobacter* sp. Strain B (Student's *t* test, pCMB $p < 0.05$; Retenone $p < 0.01$), although cells were more sensitive to mercuric chloride with only 11% of thiosulfate having been oxidised (Student's *t* test, $p < 0.01$). Similar significant drops were also seen in *Thermithiobacillus* sp. ParkerM but with the addition of cells exposed to NEM which oxidised only 8% of thiosulfate (Student's *t* test, $p < 0.01$) and no significant effect was seen with cells exposed to pCMB.

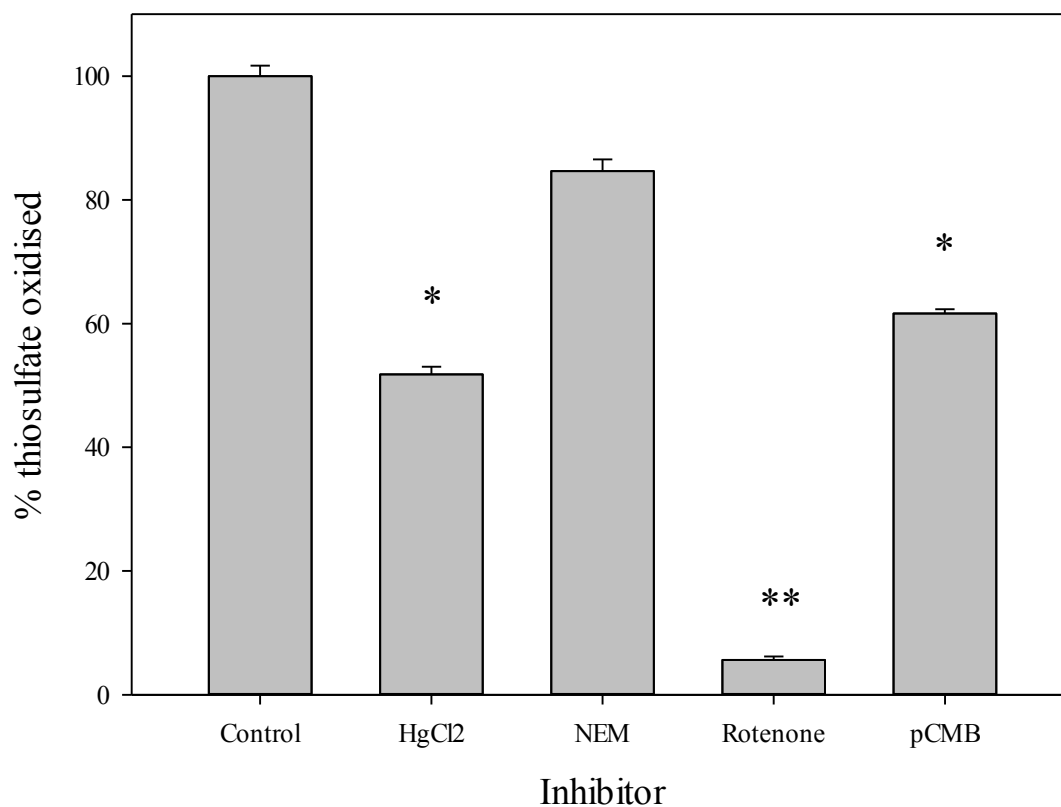


Figure 6.4 Percentage of thiosulfate oxidised in the presence of whole cells of *Pseudomonas* sp. Strain T and certain inhibitors (0.1 mM) after 4 hours of incubation at 36 °C. * significant difference in relation to control ($p < 0.05$). ** significant difference in relation to control ($p < 0.01$). Values are mean \pm SEM ($n = 3$).

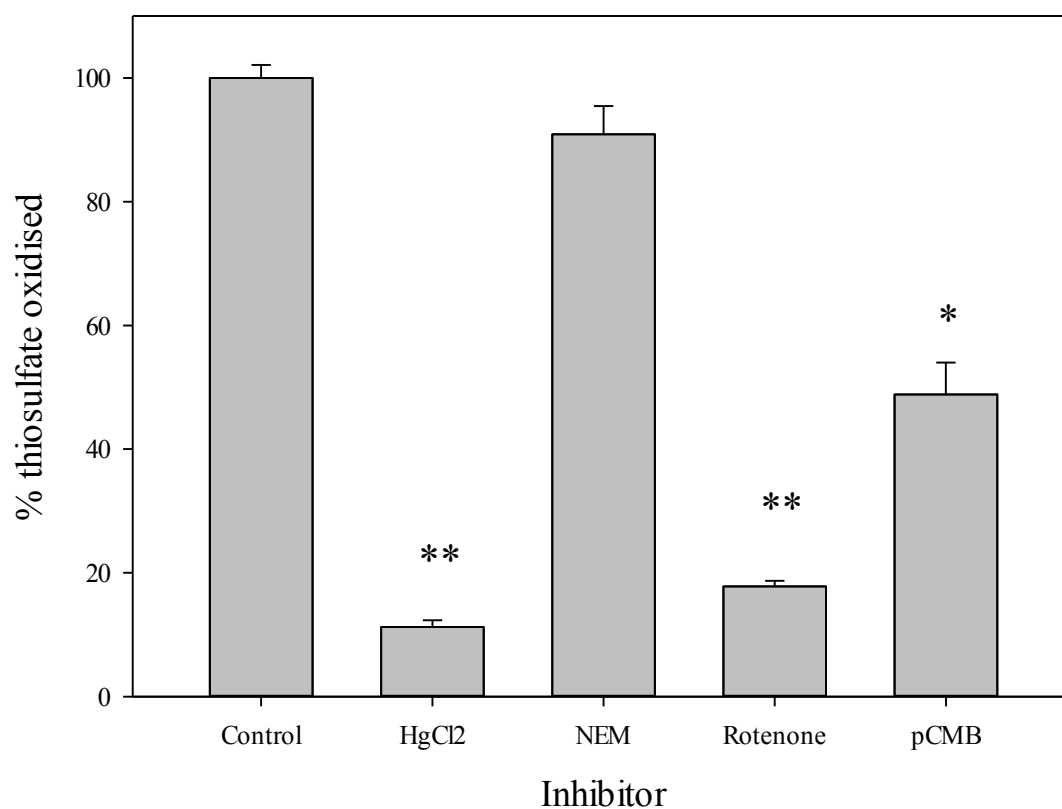


Figure 6.5 Percentage of thiosulfate oxidised in the presence of whole cells of *Achromobacter* sp. Strain B and certain inhibitors (0.1 mM) after 4 hours of incubation at 30 °C. * significant difference in relation to control ($p < 0.05$). ** significant difference in relation to control ($p < 0.01$). Values are mean \pm SEM ($n = 3$).

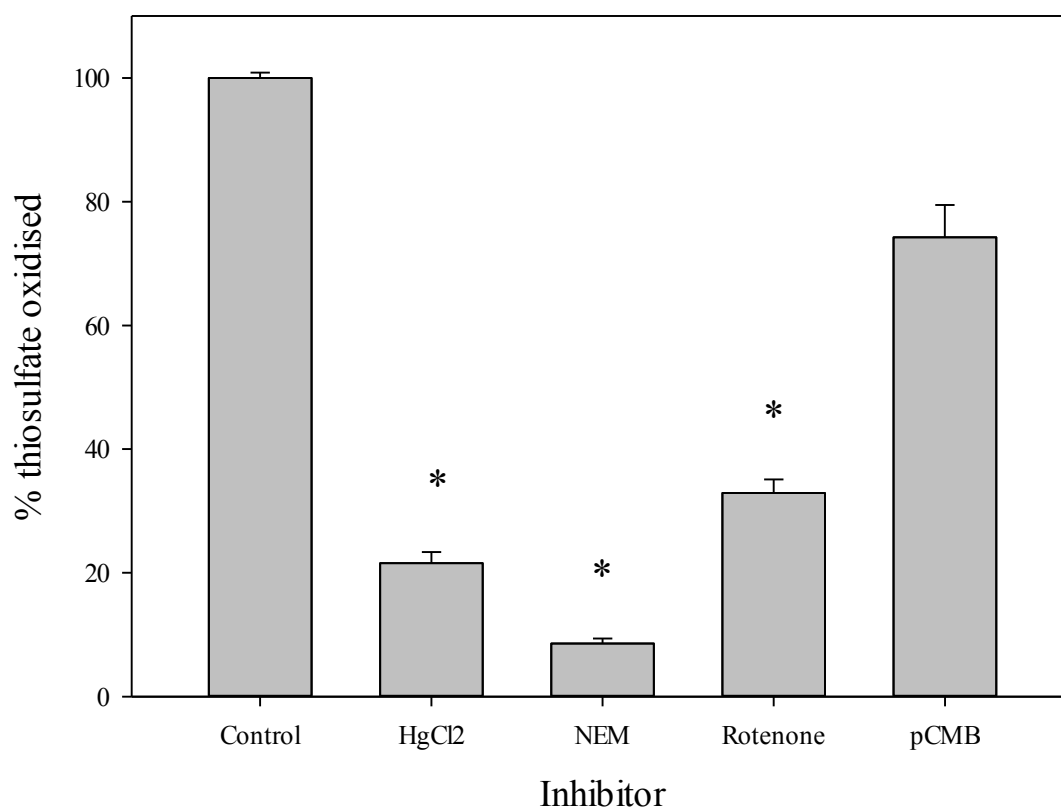


Figure 6.6 Percentage of thiosulfate oxidised in the presence of whole cells of *Thermithiobacillus* sp. ParkerM and certain inhibitors (0.1 mM) after 4 hours of incubation at 44 °C. * significant difference in relation to control ($p < 0.01$). Values are mean \pm SEM ($n = 3$).

6.2.4 Inhibitors of thiosulfate dehydrogenase in cell-free extracts

Thiosulfate dehydrogenase assays were conducted as previously described (Section 2.9.1.1) but with the addition of 1 mM final concentration of inhibitor to the CFE which was left on ice for 1 hour prior to the assay. These inhibitors were disodium EDTA, mercury chloride, NEM, pCMB and tetrathionate. Control experiments lacked any inhibitory compounds in the CFE but were also left on ice for 1 hour prior to the assay.

Specific enzyme activities were compared to a control non-inhibited rate (Figure 6.7-6.9). When activities in *Pseudomonas* sp. Strain T CFE were compared to a control specific activity of $0.14 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ activity was inhibited by all compounds tested. The strongest inhibitor was shown to be NEM with a significant drop to 23.1% activity (Student's t test, $p < 0.05$). There was also a significant drop in activity in response to pCMB (Student's t test, $p < 0.05$). The product of thiosulfate dehydrogenase, tetrathionate, was also inhibitory to this enzyme with to drop to 83.0% activity but was not statistically significant. Thiosulfate dehydrogenase activities in *Achromobacter* sp. Strain B CFE were also inhibited by all compounds tested and was significantly inhibited by NEM ($p < 0.05$) and pCMB (Student's t test, $p < 0.05$) with only 22% and 48% activities when compared to the control rate of $1.1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. There was also a significant drop in activity in response to EDTA (Student's t test, $p < 0.05$). Thiosulfate dehydrogenase activity in CFE of *Thermithiobacillus* sp. ParkerM were significantly lower as a result of mercuric chloride (Student's t test, $p < 0.05$), NEM (Student's t test, $p < 0.05$) and pCMB (Student's t test, $p < 0.05$) when compared to the control rate of $10.1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

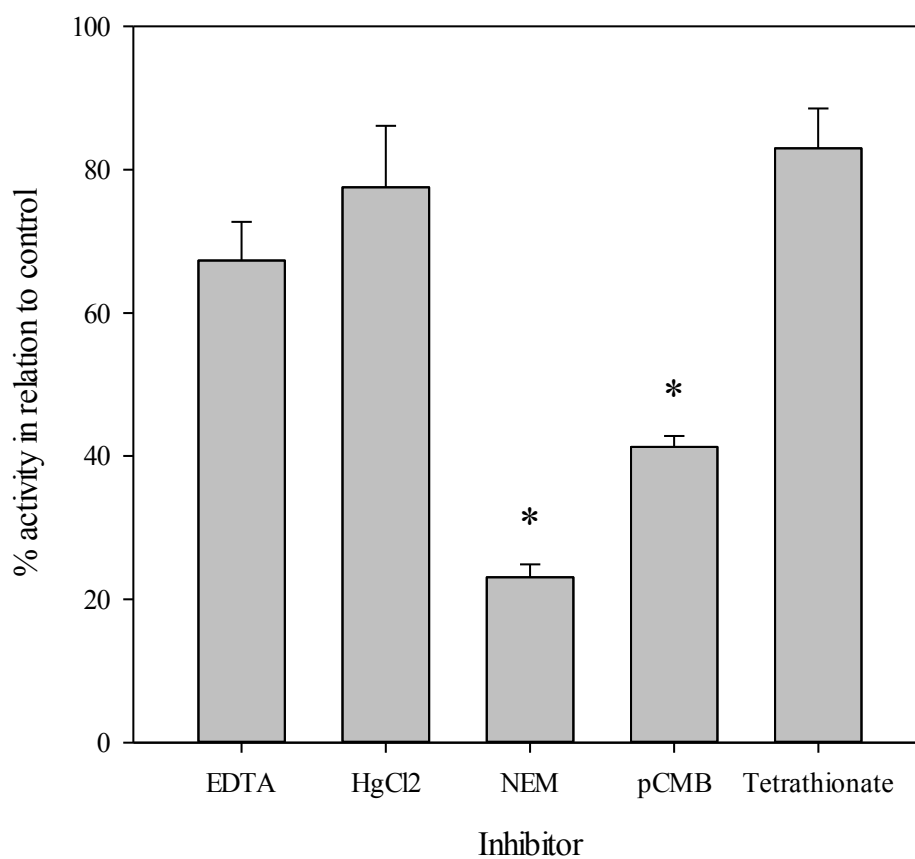


Figure 6.7 Percentage of thiosulfate dehydrogenase activity in relation to a control in cell-free extracts of *Pseudomonas* sp. Strain T pre-incubated for 1 hour with 1 mM of inhibitor. * significant difference in relation to control ($p < 0.05$). Values are mean \pm SEM ($n = 3$).

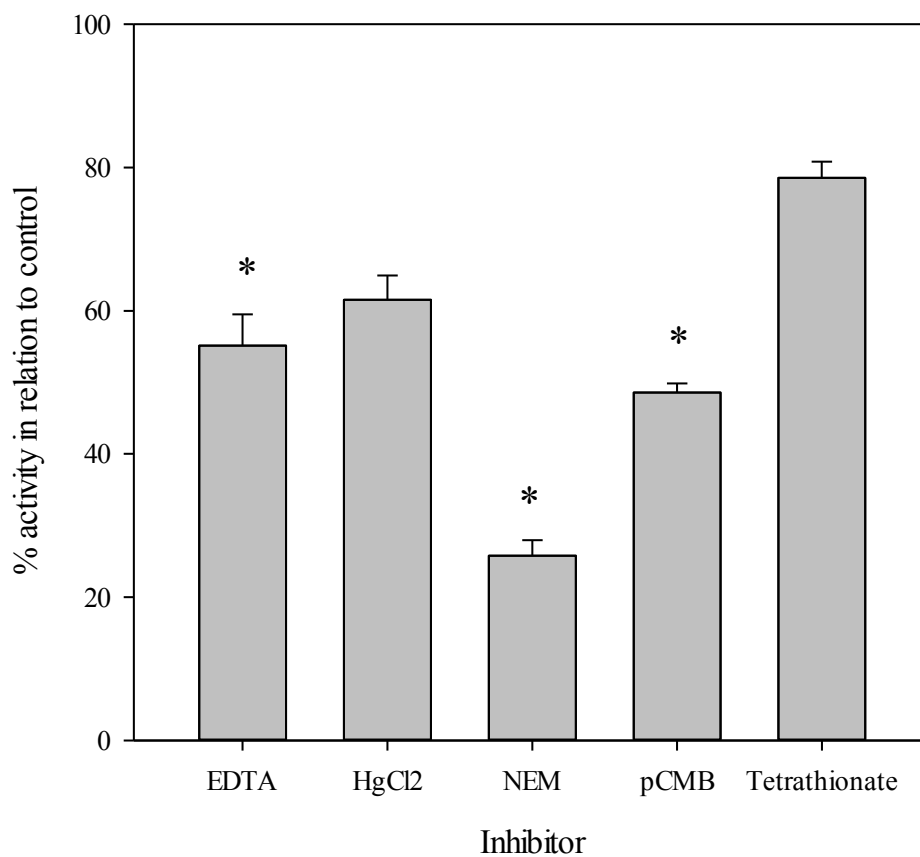


Figure 6.8 Percentage of thiosulfate dehydrogenase activity in relation to a control in cell-free extracts of *Achromobacter* sp. Strain B pre-incubated for 1 hour with 1 mM of inhibitor. * significant difference in relation to control ($p < 0.05$). Values are mean \pm SEM ($n = 3$).

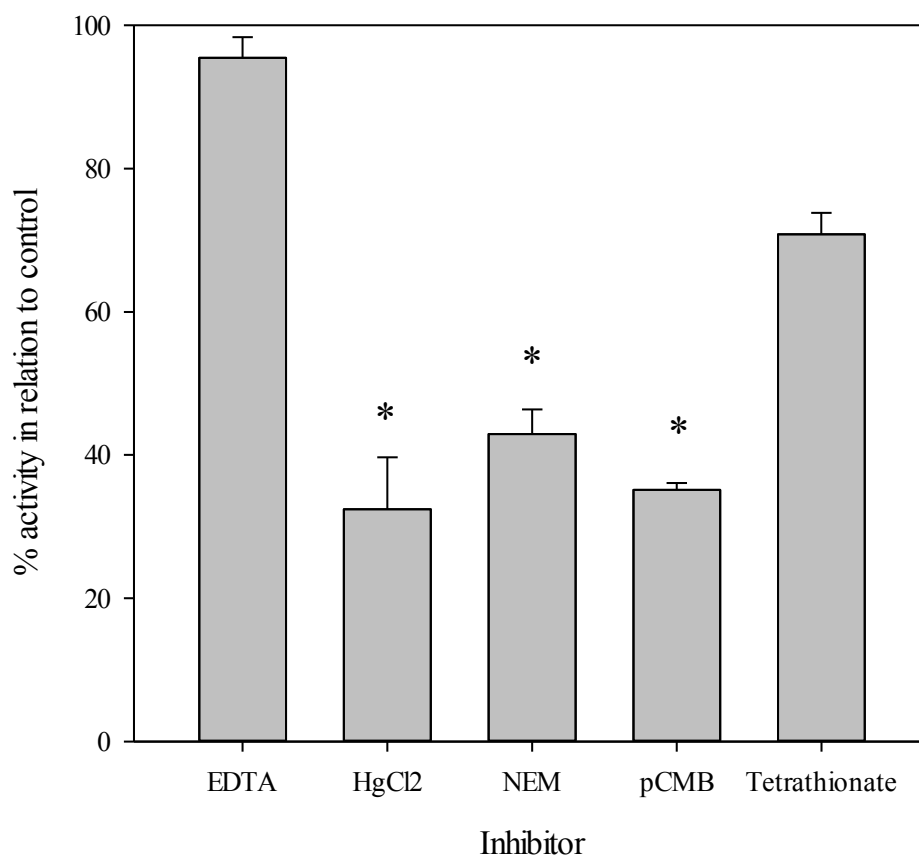


Figure 6.9 Percentage of thiosulfate dehydrogenase activity in relation to a control in cell-free extracts of *Thermithiobacillus* sp. ParkerM pre-incubated for 1 hour with 1 mM of inhibitor. * significant difference in relation to control ($p < 0.05$). Values are mean \pm SEM ($n = 3$).

6.2.5 Maximum thiosulfate dehydrogenase rates (V_{MAX}) and Michaelis constant (K_M)

Thiosulfate dehydrogenase activities were assayed in CFE of *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM with increasing concentrations of thiosulfate (0.3-20 mM) at 25 °C. These data were used to calculate maximum thiosulfate dehydrogenase rates (V_{MAX}) and Michaelis constant (K_M) with regards to thiosulfate for the three strains. These values are summarised in Table 6.2. The highest V_{MAX} but lowest K_M value was observed in CFE of *Thermithiobacillus* sp. ParkerM. In contrast, *Pseudomonas* sp. Strain T had the lowest V_{MAX} and highest K_M of the three strains with intermediate values obtained for *Achromobacter* sp. Strain B.

Table 6.2 Maximum rates of thiosulfate dehydrogenase activity (V_{\max}) and Michaelis constant (K_m) in terms of thiosulfate in CFEs of *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM. Data are mean \pm SEM ($n = 3$).

| Strain: | V_{\max} ($\mu\text{mol min}^{-1}$ (mg protein^{-1})) | K_m (mM) |
|-----------------|---|-----------------|
| Strain T | 0.321 \pm 0.02 | 0.69 \pm 0.08 |
| Strain B | 5.69 \pm 0.3 | 0.53 \pm 0.10 |
| ParkerM | 12.81 \pm 0.5 | 0.3 \pm 0.04 |

6.3 Discussion

This study has highlighted a number of differences in the properties of thiosulfate dehydrogenase between the chemolithoheterotrophic strains *Pseudomonas* sp. strain T and *Achromobacter* sp. Strain B and in the chemolithoautotrophic species *Thermithiobacillus* sp. ParkerM. pH profiles of thiosulfate dehydrogenase activities have shown there to be a preference for acidic conditions, a result that is supported by many studies into this enzyme(s) from other species (Trudinger 1961a; Kelly & Wood 1994; Meulenberg *et al.* 1992; Denkmann *et al.* 2012; Kikumoto *et al.* 2013). In terms of differences between strains, *Pseudomonas* sp. Strain T showed the highest pH optimum of 5.5. *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM thiosulfate dehydrogenase activities both peaked at pH 5.0 but due to the production of Prussian blue at pH lower than 5.0 the activities could not be measured below this pH. Optimal temperatures were approximately 25 °C for all three strains and activities were still observed at 50 °C, although these activities had dropped significantly. An identical optimal pH to that of *Pseudomonas* sp. Strain T was found in a strain of *Pseudomonas aeruginosa*, although the optimal temperature was found to be higher, between 30-42 °C (Schook & Berk 1979). A marine heterotroph believed to be a *Pseudomonas* sp. had a similar optimal temperature of 30°C although the pH optimum was not examined in the particular study (Tuttle *et al.*, 1983). A pH optimum of 5.0 was found in a member of *Betaproteobacteria*, *Alcaligenes* sp. (Hall & Berk 1972), similarly to *Achromobacter* sp. Strain B which also belongs to the Class *Betaproteobacter*. Oddly, the optimal growth temperature previously shown for *Thermithiobacillus* sp. ParkerM (5.2.4) of 44 °C was considerably higher than the optimal temperature for the thiosulfate dehydrogenase activity of 25 °C; however, although specific activity was still relatively high even at 45 °C (6.1 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$).

The pH range of thiosulfate dehydrogenase activity reported by previous studies has shown considerable variation between species. The lowest pH range of 2.5-3.5 is dominated by the genera *Acidithiobacillus* and *Acidiphilium*, *Bacteria* found in highly acidic environments such as mine effluents (Meulenburch *et al.* 1993; Nakamura *et al.* 2001; Janiczek *et al.* 2007; Kikumoto *et al.* 2013). The stability of thiosulfate falls with a decreasing pH but tetrathionate is a far more stable sulfur species at low pH. Having an enzyme that catalyses the conversion of thiosulfate to tetrathionate effectively at acidic pH may well give a physiological advantage in such environments (Pronk *et al.* 1990; Denkmann *et al.* 2012). In terms of optimal growth temperature there are fewer data available from the literature but studies have also shown a large range of between 25-42 °C. Strains in the current study sit at the lower end of this spectrum. An important factor, however, is that optimal pH and temperatures are dependent on the electron acceptor being used. For example, Hall & Berk (1972) found an optimal pH value of 5.0 when ferricyanide was used as an electron acceptor but an optimal pH value of *c.*7.4 when cytochrome *c* was used. This was also seen in *Thiobacillus thioparus* which exhibited an optimum pH of 5.0 with ferricyanide but 6.5 with cytochrome *c* (Lyric & Suzuki, 1970). With the majority of studies (including the current study) having predominantly used ferricyanide the values obtained may not truly reflect *in vivo* activity.

The inhibitors that are known to strongly bind with thiols, specifically sulfhydryl groups (NEM, mercuric chloride and pCMB) inhibited the ability of whole cells of *Pseudomonas* sp. Strain T, *Achromobacter* sp. Strain B and *Thermithiobacillus* sp. ParkerM to oxidise thiosulfate. This drop in thiosulfate oxidation was statistically significantly as a result of mercuric chloride with all three strains. The inhibitor NEM also resulted in a significant drop in thiosulfate oxidation with cells of *Thermithiobacillus* sp. ParkerM. Similar results have previously been noted in *Pseudomonas aeruginosa* and an *Alcaligenes* sp. (Schook

& Berk, 1979; Hall & Berk, 1972) although such inhibitory effects were not found in an unidentified heterotrophic species found in soil (Trudinger, 1967). The inhibition caused by these compounds indicates that the active site of thiosulfate dehydrogenase contains thiol groups needed for activity, probably in the form of cysteine. Potentially these inhibitors bind with thiosulfate causing it to become unavailable for the enzyme. NEM is also known to inhibit membrane transport proteins (Baldwin & Henderson 1989) but this appears unlikely with these heterotrophic strains as NEM was significantly inhibitory of thiosulfate dehydrogenase activity in CFEs but was not inhibitory in whole cell thiosulfate oxidation. There was an effect of whole cell thiosulfate oxidation with cells of *Thermithiobacillus* sp. ParkerM so membrane transport as well as enzyme inhibition may be taking place. Rotenone is a compound known to prevent electrons from moving through the electron transport chain (ETC), specifically it inhibits the transfer of electrons from Complex I (NADH dehydrogenase) to ubiquinone (Gutman and Singer 1970). It significantly inhibited both heterotrophic species, strongly indicating that electrons released from thiosulfate oxidation entered into the ETC at complex I. Data on the effects of these inhibitors are not available for *Thermithiobacillus tepidarius* but the effect of these inhibitors on *Acidithiobacillus* sp. and *Thiobacillus* sp. have shown to be very potent although a higher concentration of inhibitors were used in these studies (Silver & Lundgren 1968; Lyric & Suzuki 1970). To gain further insight into this work the effect of these inhibitors at a series of dilutions would highlight sensitivity of thiosulfate oxidation to such compounds. Measuring of thiosulfate oxidation *via* oxygen uptake with inhibitors would also be essential.

The inhibitory effects of certain compounds on the activity of thiosulfate dehydrogenase in CFEs of the three strains were examined. The divalent metal chelating agent ethylenediamine tetraacetic acid (EDTA) inhibited activity in the heterotrophic strains

although this was only statistically significant in *Achromobacter* sp. Strain B possibly indicating the involvement of a metal cofactor in thiosulfate dehydrogenase activity. EDTA was only 3% inhibitory in *Thermithiobacillus* sp. ParkerM specifying labile metals in the enzyme and potentially highlighted a major difference between autotrophic and heterotrophic thiosulfate dehydrogenases. Evidence of such a contrast between heterotrophic and autotrophic species can be seen in *Acidiphilium acidophilum* and a *Thiobacillus* sp. which showed no inhibitory effect from EDTA (Meulenberg *et al.* 1993; Visser *et al.* 1996) but EDTA was 13% and 18% inhibitory to activities in *Pseudomonas aeruginosa* and an *Alcaligenes* sp., respectively, when used at a concentration identical to the current study (Hall & Berk, 1972; Schook & Berk, 1979). A decrease of 44% was noted in *Thiobacillus thioparius* but only when a far higher concentration of 10 mM EDTA was employed. As with whole cell thiosulfate oxidation, the presence of NEM and pCMB caused significant inhibition of thiosulfate dehydrogenase activity and as previously stated is probably due to the binding of thiol groups and from this evidence enzyme activity inhibition eliminates the possibility of transport of thiosulfate across the cell membranes being inhibited. Mercuric chloride was also significantly inhibitory in CFE of *Thermithiobacillus* sp. ParkerM. There was evidence of product inhibition from tetrathionate in all three strains with a fall of approximately 17% and 20% activity of that of a control rate for *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B and 28% activity in *Thermithiobacillus* sp. ParkerM, although these drops in activity were not statistically significant. This appears to be the first time the effect of tetrathionate has been tested in this manner but the inhibitory effects of enzymatic products are well known. The affinity of thiosulfate dehydrogenase for its substrate was found to be highest in the autotrophic *Thermithiobacillus* sp. ParkerM (0.3 mM) and was comparable to that of *Campylobacter jejuni* (0.2 mM) (Liu *et al.* 2013). However, *Thermithiobacillus*

tepidarius had a notably lower K_m value of 0.11 mM although this was from partially purified enzyme which may account for the different values (Lu & Kelly, 1988). This does still make the K_m value obtained for *Thermithiobacillus* sp. ParkerM as one of the lowest identified with only *Thiobacillus thioparius*, a *Rhodotorula* sp. and another *Thiobacillus* sp. having lower values between 0.1-0.16 mM (Lyric & Suzuki, 1970; Kurek 1985; Visser *et al.* 1996). The K_m values for *Pseudomonas* sp. Strain T (0.69 mM) and *Achromobacter* sp. Strain B (0.53 mM) were similar to that of *Pseudomonas aeruginosa* (0.67 mM) (Schook & Berk, 1979) and *Rhodopila globiformis* (0.62 mM) (Then & Trüper, 1981). Of the species that have been examined to date it appears that heterotrophic strains exhibit lower affinity of thiosulfate dehydrogenase for thiosulfate compared to obligate autotrophic species. These results are certainly reflected in the current study. This may be explained by the facultative nature of heterotrophic thiosulfate oxidation, which is not essential for growth but is beneficial. Autotrophic growth in chemolithotrophic species such as *Thermithiobacillus* sp. ParkerM would be far more sensitive to mutations in this enzyme and natural selection would favour strains with higher affinity for the substrate. In contrast, although chemolithoheterotrophs have been shown to benefit from thiosulfate oxidation, a decrease in enzyme affinity would not necessarily have such a selective effect, while the complete loss of thiosulfate dehydrogenase activity may well have a negative impact on such strains.

The results obtained in this study add to the considerable body of evidence that there is a large variation in thiosulfate dehydrogenase properties. A number of attempts have been made to purify this enzyme but have also highlighted sizable variation. Based on molecular weight there appear to be at least two discrete groups of thiosulfate dehydrogenase enzymes present in the *Bacteria*. A protein with thiosulfate dehydrogenase activity of c.25kDa was purified in *Allochromatium vinosum* and

Acidithiobacillus ferrooxidans (Silver and Lundgren, 1968; Denkmann *et al.* 2012; Kikumoto *et al.*, 2013). In contrast, much larger proteins were purified in *Thermithiobacillus tepidarius*, *Thiobacillus thioparus*, *Acidiphilium acidophilum* and marine heterotroph 16B that were in the range of 100 – 140 KDa (Trudinger, 1961; Lyric and Suzuki, 1970; Whited & Tuttle, 1983; Lu & Kelly, 1988). It is not known whether the three strains in the present study possess differing thiosulfate dehydrogenase enzymes but this would be the focus of future research. This would of course require the purification of this enzyme from all three strains with the goal to identify the gene that encodes it.

Chapter 7

Final discussion and conclusions

It was demonstrated from phylogenetic sequences of 16S rRNA (*rrs*) and DNA gyrase subunit B (*gyrB*) genes that three strains of the *Bacteria* historically classified as “*Thiobacillus trautweinii*” are, in fact, members of two different genera. The original strain isolated in 1921 by Trautwein (Strain T) and a strain isolated later in 1932 (Strain VO) sit within the genus *Pseudomonas*, while Starkey’s Strain B isolated in 1934 belongs to the genus *Achromobacter*. All three strains oxidise thiosulfate to tetrathionate but lack any autotrophic ability and cannot grow as “true” mixotrophs. A significant amount of chemotaxonomic data indicates *Pseudomonas* sp. Strain T to be a novel species within the *P. mendocina* clade. When compared to the nearest phylogenetic relations of *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T, *Pseudomonas* sp. Strain T was the only strain tested capable of oxidising thiosulfate and manganese while not being able to produce nitrogen gas from nitrate and was unable to produce indole from tryptophan. *Pseudomonas* sp. Strain T was also unable to grow in the presence of 6% (w/v) sodium chloride, in contrast to *P. chengduensis* MBR^T and *P. toyotomiensis* HT-3^T. Likewise, *Achromobacter* sp. Strain B has shown significant variation from that of its closest relation (*A. aegrifaciens* LMG 26852^T) to also be a novel species. *Achromobacter* sp. Strain B was unable to hydrolyse aesculin and Tween 20® but interestingly was able to hydrolyse Tween 80® despite the compound having a longer polyoxyethylene chain. *Achromobacter* sp. Strain B was able to lyse sheep red blood cells (α hemolysis) but this trait was not shared by any other species of *Achromobacter* tested. Notable differences in enzyme activities between *Achromobacter* sp. Strain B and *A. aegrifaciens* LMG 26852^T were lack of alkaline phosphatase activity and presence of urease activity in *Achromobacter* sp. Strain B. The nitrogen sources utilised by these two strains for growth were markedly different as *Achromobacter* sp. Strain B was unable to use cyanate or

thiocyanate but was able to utilise urea. This study adds to the considerable body of work showing inorganic sulfur-oxidation to be a diverse trait within the *Proteobacteria*.

In order to fully conclude *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B as novel species it would require the DNA-DNA hybridization of these strains with the nearest phylogenetic relations. Sadly due to budget constraints this was not possible during this project but would be the focus of future work. This would be most beneficial with regards to *Achromobacter* sp. Strain B as the genus shows little variation between species.

The ability of *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B to grow in the presence of thiosulfate as chemolithoheterotrophs was examined and confirmed. When grown in continuous chemostat culture there were significant increases in maximum theoretical growth yields (Y_{MAX}) with both species when thiosulfate was present as an auxiliary energy source on top of an organic growth substrate. The increases observed were 22.6% with *Pseudomonas* sp. Strain T and 48.0% with *Achromobacter* sp. Strain B. There was also a significant increase in maximum specific growth rate of 12% for *Pseudomonas* sp. Strain T and a more modest increase of 1.2% for *Achromobacter* sp. Strain B with the addition of thiosulfate. However, a far lower increase in yield of 3.5% was seen in batch culture of *Pseudomonas* sp. Strain T while a 7% decrease in yield was found with *Achromobacter* sp. Strain B. It was demonstrated that thiosulfate oxidation in both strains was coupled to ATP production via a *c* type cytochrome(s). There is strong evidence that the differential results between batch and continuous culture was caused by the concentration of ATP within the cells. During batch growth intracellular ATP concentrations were relatively high (*c.*14 nmol/mg dry biomass) until the organic growth substrate was depleted and the cultures entered into stationary phase, by which the ATP levels dropped significantly to *c.*6 nmol/mg dry biomass ($p < 0.05$). In contrast, growth in

continuous culture by its very nature is constantly limited by growth substrate and so had significantly lower ATP concentration of *c.*4 nmol/mg dry biomass. The ATP produced during continuous culture *via* thiosulfate oxidation helped alleviate this ATP starvation but was being used instantly. This allowed carbon that would normally be released as CO₂ from glucose or succinate oxidation to become assimilated into biomass. During the exponential phase of batch culture the extra ATP produced from thiosulfate oxidation merely entered the ATP pool that was already high in the non-substrate limited cells. In order to explore this hypothesis further, future work would focus on the fate of substrate carbon. For example, cells of *Pseudomonas* sp. Strain T previously grown in chemostat culture chemolithoheterotrophically could be used to start batch growth on radio-labeled glucose with and without thiosulfate and monitoring glucose carbon fate. If the hypothesis is correct then more carbon would be retained by the bacterium for biosynthesis instead of released as CO₂.

The “real world” environment simulated by continuous chemostat culture has provided strong evidence that chemolithoheterotrophic metabolism in terms of thiosulfate oxidation gives a significant physiological boost in terms of yield and growth rate. Currently it is common scientific practice to sequence the genomes of *Bacteria* in order to identify the metabolic pathways. However, it is questionable to make definitive conclusions from such studies because many factors can influence whether a gene produces a fully functional enzyme. The gene may not be expressed or the protein product may be non-functional due to a mutation in the gene sequence. Such studies, although they expand our knowledge, still require traditional culture based techniques in order to give true insights into physiology and biochemistry. Continuous chemostat cultures have been employed for many decades in this respect and although perhaps not

considered “fashionable” in a world of next generation sequencing, they are still an invaluable tool for studying how a bacterium actually grows *in vivo*.

Undoubtedly the identification and purification of the proteins involved would be the focus of any future research into *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B, particularly the enzyme thiosulfate dehydrogenase (EC 1.8.2.2). From the research conducted on this enzyme there appears to be at least two distinct thiosulfate dehydrogenase enzymes present in the *Bacteria*. Proteins between 100 - 140kDa in size have been purified from *Thermithiobacillus tepidarius*, *Thiobacillus thioparus*, *Acidiphilium acidophilum* and marine heterotroph 16B (Trudinger, 1961; Lyric & Suzuki, 1970; Whited & Tuttle, 1983; Lu & Kelly, 1988). However, a much smaller protein of *c.*25kDa with thiosulfate dehydrogenase activity was purified and the gene identified in the purple sulfur bacterium *Allochromatium vinosum* and was shown to be a cytochrome *c*₅₅₄ (TsdA) (Denkman et al. 2012). A protein of *c.*25kDa was partially purified in cell extracts of tetrathionate grown *Acidithiobacillus ferrooxidans*, however no heme(s) were detected in the partially purified preparation and so ruled out the possibility of the enzyme being a cytochrome (Kikumoto *et al.*, 2013). Interestingly, homologs of the gene identified by Denkman *et al.* (2012) were not found in the genomes of several species of *Bacteria* known to have thiosulfate dehydrogenase activity. The difference in protein sizes between the closely related *Ttb. tepidarius* and *Atb. ferrooxidans* may also highlight just how diverse this enzyme(s) actually is. It would be of great interest to purify the thiosulfate dehydrogenase from both *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B and to identify the corresponding gene(s) in order to ascertain whether they express a homologous enzyme to that found in *A. vinosum* or whether the much larger enzyme is expressed. Alternatively, the enzyme may differ between these two *Proteobacteria* which are only distantly related.

There were significant differences in enzyme activity profiles between heterotrophic and chemolithoheterotrophic grown cells of both *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B. Regarding enzymes of inorganic sulfur metabolism, only thiosulfate dehydrogenase activity was present in *Pseudomonas* sp. Strain T and only in cells grown in the presence of thiosulfate. In contrast, in *Achromobacter* sp. Strain B thiosulfate dehydrogenase, trithionate hydrolase and sulfite dehydrogenase were present under both growth regimes but were significantly higher when grown with thiosulfate. The activity of tetrathionate hydrolase was completely lacking in *Achromobacter* sp. Strain B and so it does not possess the complete Kelly-Trudinger pathway. A future study could investigate the effect of trithionate on growth of *Achromobacter* sp. Strain B as the activity of trithionate hydrolase, although it is unclear as to whether the reaction conserves energy, does produce thiosulfate which could go on to be oxidised *via* thiosulfate dehydrogenase. The effect of sulfite on growth would also need investigating, although the direct sulfite oxidation has not previously been reported in heterotrophs it is worth noting that not all chemolithoautotrophs that show activity of sulfite dehydrogenase oxidise sulfite from the growth medium and in some cases sulfite has shown to be detrimental to growth.

Certain enzymes of the TCA cycle were found to be significantly different in chemolithoheterotrophic cells of *Pseudomonas* sp. Strain T when compared to heterotrophic grown cells. Specifically there were significant increases in succinate dehydrogenase and fumarase and a significant decrease in the activity of citrate synthase. This would suggest that there was a buildup of the metabolite, oxaloacetate, although this was not directly measured. Oxaloacetate is a precursor to aspartate biosynthesis and subsequently numerous other amino acids. It is a possibility that protein synthesis is being regulated by having a preference for oxaloacetate associated amino acids that might be

required for the upregulation of thiosulfate dehydrogenase. The significant changes in TCA cycle enzymes seen in *Achromobacter* sp. Strain B were the decreased activities of α -ketoglutarate dehydrogenase, fumarase and malate dehydrogenase. This suggests that whatever effect chemolithoheterotrophy is having on TCA cycle enzymes may not be identical in all heterotrophs. In cells of *Achromobacter* sp. Strain B there was also an upregulation of trithionate dehydrolase and sulfite dehydrogenase and the effects of this (if any) on amino acid biosynthesis might have masked the effects of thiosulfate dehydrogenase upregulation on amino acid production as was observed in *Pseudomonas* sp. Strain T cells. A thorough examination of amino acid levels and of key enzymes involved amino acid synthesis would hopefully clarify this.

A moderately thermophilic chemolithoautotroph originally isolated in 1945 was examined in terms of taxonomy, physiology and biochemistry. Phylogenetic and chemotaxonomic tests placed this strain within the Genus *Thermithiobacillus*; an apparently rare genus that currently incorporates just a single validly published species (*Ttb. tepidarius* DSM 3134^T, Wood & Kelly 1985). *Thermithiobacillus* sp. ParkerM was able to oxidise thiosulfate and polythionates in order to support growth, indicative of the Kelly-Trudinger pathway. When grown on thiosulfate there was a formation (*c.*5 mM) of trithionate that rapidly disappeared from the growth medium followed by the stoichiometric formation of tetrathionate with the subsequent rise in pH. This was followed by a decrease in pH as tetrathionate was fully oxidised to sulfate. This strain was considerably more tolerant of acidic conditions caused by the production of sulfate than the type species of the Genus, as yields on thiosulfate in batch culture were 93% higher while specific growth rates were over three-fold higher. However, when grown on thiosulfate in a pH controlled chemostat the Y_{MAX} was comparable to, although still slightly higher than, that of *Ttb. tepidarius* (11.4 and 10.4 g dry biomass per mol

thiosulfate for *Thermithiobacillus* sp. ParkerM and *Ttb. tepidarius* respectively). There was a higher Y_{MAX} when grown on trithionate (12.4 g dry biomass per mol trithionate) suggesting that energy is being conserved in the reaction of trithionate to thiosulfate. This was seen to a lesser extent in *Ttb. tepidarius* (10.8 g dry biomass per mol trithionate) and in *Halothiobacillus neapolitanus* (8.6 g dry biomass per mol thiosulfate and 8.8 g dry biomass per mol trithionate), showing a higher efficiency of energy conservation of trithionate metabolism in *Thermithiobacillus* sp. ParkerM, possibly from a different mechanism. However, the yield was less on tetrathionate when compared to *Ttb. tepidarius* (18.2 and 19.9 g dry biomass per mol tetrathionate) but was still considerably higher than *Acidithiobacillus* spp. and *Halothiobacillus* spp.. To date, it appears that no “true” *Thiobacillus* spp. Y_{MAX} data have been published. These data would be vital to further our understanding the potential differences in efficiency of the Kelly-Trudinger pathway. In terms of thiosulfate limited growth of *Thiobacillus* spp., yields are available for a moderately thermophilic species (*T. aquaesulis* – 11.3 g dry biomass per mol thiosulfate) and another species grown under anaerobic conditions (*T. denitrificans* – 14.7 g dry biomass per mol thiosulfate). It would be interesting to examine yields of mesophilic *Thiobacillus* spp. grown aerobically to see whether factors like temperature affect the efficiency of energy conservation pathways. Lower Y_{MAX} values of *Acidithiobacillus* spp. grown under acidic conditions do indicate a drop in efficiency, possibly due to an increase in maintenance energy and cellular repair.

The biochemistry of *Thermithiobacillus* sp. ParkerM grown in substrate limited chemostats on thiosulfate, trithionate or tetrathionate differed considerably regarding the activities of certain enzymes. The activities of thiosulfate dehydrogenase and trithionate hydrolase were significantly higher when the respective sulfur species were used as the growth substrate. In contrast, there were no differences in the activities of tetrathionate

hydrolase between different growth substrates. This was also shown by similar tetrathionate-dependent oxygen uptake rates: although the activity of sulfite dehydrogenase was detected in cell-free extracts of *Thermithiobacillus* sp. ParkerM there was no sign of sulfite oxidation by whole cells, indicating that sulfite was unable to be transported across the outer and/or inner membranes.

This was the first study in which all of the enzymes of the TCA cycle were assayed in a member of *Acidithiobacillia*. There were no differences in the activities of enzymes of the TCA cycle with the exceptions of succinate dehydrogenase, which was not detected in tetrathionate grown cells, and malate dehydrogenase, which was significantly higher in thiosulfate grown cells compared to tetrathionate grown cells and significantly higher in trithionate grown cells compared to thiosulfate grown cells. As with many autotrophic *Bacteria* the activities of α -ketoglutarate dehydrogenase and succinyl-CoA synthetase were not detected. The permanent genome of *Thermithiobacillus tepidarius* DSM 3134^T has been sequenced by the Joint Genome Institute and contains the genes for both subunits of succinyl-CoA synthetase. With no activity of succinyl-CoA synthetase detected in *Thermithiobacillus* sp. ParkerM it could be another example of *in vivo* biochemistry not being accurately predicted by genome sequencing. As previously mentioned with *Pseudomonas* sp. Strain T and *Achromobacter* sp. Strain B, the growth of *Thermithiobacillus* sp. ParkerM on different inorganic sulfur may influence the TCA cycle regarding amino acid synthesis; potentially in response to upregulation of certain enzymes involved in the inorganic sulfur oxidation. This, of course, will need further investigation.

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