

2020-09-28

Insights into growth kinetics and roles of enzymes of Krebs' cycle and sulfur oxidation during exochemolithoheterotrophic growth of *Achromobacter aegrifaciens* NCCB 38021 on succinate with thiosulfate as the auxiliary electron donor

Hutt, LP

<http://hdl.handle.net/10026.1/16693>

10.1007/s00203-020-02028-1

Archives of Microbiology

Springer Science and Business Media LLC

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

Insights into growth kinetics and roles of enzymes of Krebs' cycle and sulfur oxidation during exochemolithoheterotrophic growth of *Achromobacter aegrifaciens* NCCB 38021 on succinate with thiosulfate as the auxiliary electron donor

Lee P. Hutt^{1,#}, Glenn M. Harper², A. John Moody^{1,3} and Rich Boden^{1,3,4*}

1. School of Biological and Marine Sciences, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, UK.

2. Electron Microscopy Unit, University of Plymouth, Plymouth, Devon, PL4 8AA, UK.

3. Sustainable Earth Institute, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, UK.

4. Marine Institute, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, UK.

* Corresponding author: rich.boden@plymouth.ac.uk (+44 (0)1752 584452)

Present address: School of Biomedical and Healthcare Sciences, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, UK.

Keywords:

Chemolithoheterotrophy, exochemolithoheterotrophy, *Achromobacter*, thiosulfate, Krebs' cycle, sulfur cycle

ORCID IDs:

HUTT (0000-0001-5219-7579)

MOODY (0000-0002-1994-7419)

BODEN (0000-0002-4496-152X)

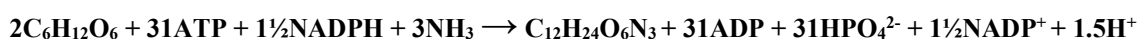
23 **Abstract**

24 *Achromobacter aegrifaciens* NCCB 38021 was grown heterotrophically on succinate *versus*
25 exochemolithoheterotrophically on succinate with thiosulfate as auxiliary electron donor. In batch culture, no
26 significant differences in specific molar growth yield or specific growth rate were found for the two growth
27 conditions, but in continuous culture in the succinate-limited chemostat, the maximum specific growth yield
28 coefficient increased by 23.3 % with thiosulfate present, consistent with previous studies of endo- and
29 exochemolithoheterotrophs and thermodynamic predictions. Thiosulfate oxidation was coupled to respiration at
30 cytochrome *c*₅₅₁, and thiosulfate-dependent ATP biosynthesis occurred. Specific activities of cytochrome *c*-
31 linked thiosulfate dehydrogenase (E.C. 1.8.2.2) and two other enzymes of sulfur metabolism were significantly
32 higher in exochemolithoheterotrophically grown cell extracts, while those of succinyl-transferring 2-
33 oxoglutarate dehydrogenase (E.C. 1.2.4.2), fumarate hydratase (E.C. 4.2.1.2) and malate dehydrogenase (NAD⁺,
34 E.C. 1.1.1.37) were significantly lower – presumably owing to less need to generate reducing equivalents during
35 Krebs' cycle, since they could be produced from thiosulfate oxidation.

36

Introduction

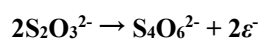
Chemolithoheterotrophy is a mixed metabolic mode in which an organocarbon compound is used as the carbon source, electron donor and energy source (*i.e.* the same as in generalist heterotrophy), with simultaneous oxidation of an inorganic species such as thiosulfate, Mn(II), molecular hydrogen *etc* as an auxiliary electron donor, generating additional proton motive force (Δp), used in turn to generate ATP (Guittoneau, 1927; Sijderius, 1947; Trudinger, 1967; Kelly and Kuenen, 1984; Boden *et al.*, 2010; Boden and Hutt 2018*a,b*). If we consider a generalist heterotroph growing on *e.g.* a hexose sugar, some carbon from the latter is dissimilated to CO₂ to produce Δp by way of NADH (from glycolytic pathways and Krebs' cycle) and quinol (Krebs' cycle), which couple to proton translocation from the cytosol to the periplasm at NADH dehydrogenase (EC 1.6.99.3) and the quinol-cytochrome *c* reductase (*bc*₁ complex, EC 1.10.2.2) or the Q-cycle, as well as further proton translocation during the terminal oxidation of the respiratory chain (one of the quinol oxidases or cytochrome-*c* oxidases). This Δp is then consumed for the formation of ATP by the H⁺-transporting ATPase ('ATP synthase', EC 7.1.2.2). ATP is additionally formed at substrate level during the synthesis of succinate in Krebs' cycle – in some organisms succinate-CoA ligase ('succinyl-CoA synthetase') yields GTP (EC 6.2.1.4), in others it yields ATP (EC 6.2.1.5) – in the case of the former, GTP is converted to ATP *via* nucleoside-diphosphate kinase (EC 2.7.4.6). Remaining hexose carbon is assimilated into biomass (C₁₂H₂₄O₆N₃, 306g/mol, Anthony (1982)) at the expense of ATP and NADPH, ultimately requiring:



This can be summarised energetically in heterotrophic organisms by the balance:

$$\Delta S_C = \Delta S_{Cb} + \Delta S_{Ce}$$

where ΔS_C is total substrate carbon consumed, ΔS_{Cb} is substrate carbon converted into biomass and ΔS_{Ce} is substrate carbon oxidised to provide energy, though it is important to note that the latter includes both energy for anabolism (biosynthesis) *sensu* cell division and new growth, as well as energy for maintenance (turnover of biomass, transport, maintaining osmotic gradients, cell motility, detoxification of metals/antimicrobials *etc*). In a chemolithoheterotrophically growing organism, an auxiliary electron donor (*B*, following the nomenclature of Boden and Hutt (2018*b*)) – an inorganic ion, molecular hydrogen *etc* – is oxidized *e.g.* thiosulfate can be oxidized by either the cytochrome *c*-linked or quinone-linked thiosulfate dehydrogenase (EC 1.8.2.2 or EC 1.8.5.2, respectively):



The electrons (ϵ^-) reduce cytochrome *c* or ubiquinone, respectively, and thus Δp is generated following translocation at the *bc*₁ complex and/or terminal oxidases. This yields additional ATP, thus the energetics of a chemolithoheterotroph can be summarised by the balance:

$$E_G = \Delta S_{ce} + \Delta Be$$

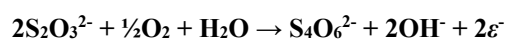
(where E_G is total energy required for growth and ΔBe is auxiliary electron donor consumed for energy).

As such, organisms growing chemolithoheterotrophically have an additional means of producing Δp and thus ATP, therefore dissimilate less substrate carbon for energy and assimilate more of it into biomass (Mason and Kelly, 1988; Boden *et al.*, 2010; Boden and Hutt, 2018a; Kelly & Kuenen, 1984; Kuenen & Beudeker, 1982; Ghosh & Dam, 2009). Such organisms are unable to fix CO₂, distinguishing them from mixotrophs, which are able to grow autotrophically and heterotrophically simultaneously – mixotrophs superficially resemble chemolithoheterotrophs but are distinct from the latter *e.g.* as outlined by Kelly and Kuenen (1984). The terms ‘chemolithoheterotrophy’ and ‘mixotrophy’ are often – incorrectly – used interchangeably as generic terms in the literature for any mixed form of metabolism, and sometimes for thiosulfate oxidation during heterotrophic growth, regardless of if there is any metabolic gain (Kelly and Kuenen, 1984; Boden *et al.*, 2010; Boden and Hutt, 2018a), often leading to confusing and inaccurate descriptions of bacterial metabolism (Kelly and Kuenen 1984). For clarity, in the current study the definitions of chemolithoheterotrophy and mixotrophy are as described in Kelly and Kuenen (1984) and updated by Boden and Hutt (2018a) to take into account more recent work, such as antioxidant properties of thiosulfate that may be easily confused with this trait and further delineating the trait into endo- and exochemolithoheterotrophy, depending upon the source of the auxiliary electron donor. Auxiliary electron donors obtained from the environment of the cell (*e.g.* culture media, river water) lead to exochemolithoheterotrophy, whereas those generated inside of the cell from *e.g.* organosulfur compounds – such as thiosulfate generated from dimethylsulfide dissimilation – give rise to endochemolithoheterotrophy. Originally these differential forms of chemolithoheterotrophy were defined by Boden *et al.* 2010 and the nomenclature adjusted in Boden *et al.* 2018a,b). In this study, we consider exochemolithoheterotrophic growth only, with exogenous thiosulfate supplied.

There are two key pathways of thiosulfate oxidation known in the non-phototrophic, unicellular *Bacteria*, the Lu-Kelly-Friedrich cycle and the Kelly-Trudinger pathway (Kelly, 1982). In the Lu-Kelly-Friedrich cycle, thiosulfate is oxidised to sulfate with no free metabolic intermediates, with the whole process acting on protein-bound sulfur intermediates. In contrast, the Kelly-Trudinger pathway produces tetrathionate and sometimes

other free polythionate intermediates from oxidation of thiosulfate and is not dominated by protein-bound-sulfur oxidation. The oxidation of thiosulfate by chemolithoautotrophic *Bacteria* is a well-studied aspect of inorganic-sulfur metabolism but such oxidation in heterotrophic *Bacteria* remains relatively poorly understood: it is usually considered to use similar or partial versions of the pathways from autotrophs. From the few studies conducted on thiosulfate oxidation in heterotrophs most appear to utilise a partial Kelly-Trudinger pathway as polythionates, particularly tetrathionate ($\text{S}_4\text{O}_6^{2-}$), are produced as the end products of sulfur oxidation (Trautwein, 1921; Starkey, 1934*a, b*; Trudinger, 1961*a*; Mason & Kelly, 1988), although a few studies have demonstrated full oxidation to sulfate (Pepper & Miller, 1978; Schook & Berk, 1978). It must be noted that the literature contains two key examples of inorganic sulfur oxidation in heterotrophic organisms – i) *bona fide* chemolithoheterotrophy, in which sulfur oxidation is coupled to ATP biosynthesis and the maximum specific growth yield (Y_{MAX}) increases (“fortuitous oxidation”) and ii) sulfur oxidation that does not appear to have any positive impact on Y_{MAX} and occurs for unknown reasons (“gratuitous oxidation”) – Kelly and Kuenen (1984), Boden *et al.* 2010; Boden and Hutt (2018*a, b*). *Sensu stricto* ‘chemolithoheterotrophy’ only applies to the former. It is worth noting that another form of apparently fortuitous oxidation that was not from chemolithoheterotrophy has been observed in *Spirillum winogradskii*, where thiosulfate is oxidised to tetrathionate by reactive oxygen species produced in aerobic metabolism (Podkopaeva *et al.*, 2005), thus Y_{MAX} increases, but ATP was not actually produced. We have previously (Boden and Hutt, 2018*b*) added to the criteria of Kelly and Kuenen (1984) that explicitly differentiate true chemolithoheterotrophy from these other examples of sulfur oxidation in heterotrophs.

Activity of the cytochrome *c*-linked thiosulfate dehydrogenase (EC 1.8.2.2) has been detected in many heterotrophic *Bacteria*:



$$\Delta G^\circ = -61.9 \text{ kJ/mol thiosulfate oxidised}$$

The standard Gibbs energy change (ΔG°) given was determined *de novo* from ΔG_f° values from Hoare (1985), Ross (1985) and Zhdanov (1985). Using the method of Boden and Hutt (2018*a*), thermodynamic ATP synthesis maxima (on the basis purely of thermodynamics, not biochemistry) can be determined from these values based on the biosynthesis of ATP from ADP and orthophosphate having ΔG° of +46.1 kJ/mol ATP formed. The thermodynamic ATP maximum for this reaction is 1.3 mol ATP/mol $\text{S}_2\text{O}_3^{2-}$. It is worth noting that multiple isoenzymes have been described in the literature and either feed electrons into the respiratory chain directly at the level of cytochrome (cyt) *c* as in *Allochromatium vinosum* DSM 180^T of the *Chromatiales* of the

Gammaproteobacteria, in which thiosulfate dehydrogenase (TsdA) is a cyt *c*₅₅₄ of c. 25 kDa (Denkman *et al.*, 2012). In other cases, electrons enter the chain *via* a second cyt *c* (TsdB) as found in *Thiomonas intermedia* in the *Burkholderiales* of the *Betaproteobacteria* and “*Sideroxydans lithotrophicus*” of the *Nitrosomonadales* in the same class, which feeds electrons into the terminal cytochrome *c* oxidase. A third isoenzyme is effectively a naturally occurring TsdA:TsdB fusion protein, in which electrons are fed from thiosulfate to TsdA then TsdB (now *sensu stricto* both domains of the same polypeptide) and onto a high potential iron-sulfur protein (HiPIP) prior to the terminal oxidase (as in *Marichromatium purpuratum* of the *Chromatiales* in the *Gammaproteobacteria*, Kurth *et al.*, 2016). The recombinant TsdA:TsdB ‘fusion’ protein forms an $\alpha_2\beta_2$ heterodimer of 108 kDa. A similarly sized thiosulfate dehydrogenase of 115 kDa was partially purified from *Thiobacillus thioparus* (Lyric and Suzuki, 1970), but the *tsdA* and *tsdB* genes are not present in the genome of the type strain, *T. thioparus* DSM 505^T (Hutt *et al.*, 2017). Larger proteins with thiosulfate dehydrogenase activity of 130-140 to >300 kDa in size have been found in other *Bacteria* (Trudinger, 1961*a, b*; Whited and Tuttle, 1983; Lu and Kelly, 1988*a*; Schook & Berk, 1979) – this may indicate the presence of more isoenzymes of thiosulfate dehydrogenase.

In this study we report central carbon metabolism and sulfur oxidation enzyme activities in a historically important strain of “*Thiobacillus trautweinii*” originally isolated by Robert L. Starkey (1934*a*) – his work on this strain (Starkey’s ‘Culture B’ = NCCB 38021) represented the first detailed physiological study of a chemolithoheterotroph. “*Thiobacillus trautweinii*” was the name used fairly informally for a number of heterotrophic strains that could oxidise thiosulfate (whether or not there was any energetic gain) but it was not included in the Approved Lists of 1980, thus has no standing – many “*T. trautweinii*” isolates are actually *Pseudomonas* spp. For many years the ability to oxidise inorganic sulfur compounds was seen as a unique taxonomic trait exclusive to the genus *Thiobacillus* (*sensu lato*), but with the advent of 16S rRNA gene phylogenetic analyses, many members of the genus *Thiobacillus* have been reclassified to *Acidiphilium*, *Acidithiobacillus*, *Annwoodia*, *Guyparkeria*, *Halothiobacillus*, *Paracoccus*, *Starkeya*, *Thermithiobacillus*, *Thiomicrospira* and *Thiomonas* (Wood & Kelly, 1995; Katayama *et al.*, 1995; Moreira & Amils, 1997; Hiraishi *et al.*, 1998; Kelly & Wood, 2000; Kelly *et al.*, 2000; Boden *et al.*, 2012; Boden, 2017; Boden *et al.*, 2017). Our recent work on Starkey’s (1934*a*) ‘Culture B’ isolate (hereafter NCCB 38021) has shown that it is an *Achromobacter* sp. (Hutt, 2016). Starkey (1934*a, b*) found that it oxidised thiosulfate to tetrathionate only when organic carbon was present, and it could not grow autotrophically. Given this, *Achromobacter* sp. NCCB 38021 was selected as a model organism to investigate the poorly understood trait of chemolithoheterotrophy *versus*

heterotrophy, already having baseline data from Starkey's (1934*a,b*) studies. It does not oxidise any inorganic sulfur oxyanions other than thiosulfate in our hands (trithionate, tetrathionate, pentathionate, hexathionate, (bi)sulfite and dithionate were tested; Hutt (2016)). We also include a more complete identification of this organism as a strain of *Achromobacter aegrifaciens*.

Materials and methods

Organism and materials

All chemicals used throughout were of analytical grade or higher from Sigma-Aldrich Corporation (Poole, UK) unless stated otherwise. Analytical grade sodium thiosulfate pentahydrate was from Fisher Scientific and sodium polythionates were synthesised according to Boden *et al.* (2010). Quartz-distilled deionised water (ddH₂O) was used for all experiments and media were made in acid-washed 'Class A' volumetric glassware. *Achromobacter* sp. NCCB 38021 (=Culture B = IAM 12564 = JCM 200642 = LMD 38.21) was supplied on nutrient agar as a kind gift of the *Westerdijk Fungal Biodiversity Institute* (formerly *Centraalbureau voor Schimmelcultures*), Netherlands. It was grown on E Basal Salts (EBS; Boden and Hutt 2018*b*) supplemented with carbon sources and/or electron donors as specified in each experiment. Succinate was selected as the carbon source for all experiments since it gave good growth without clumping of biomass, and being an intermediate of Krebs' cycle, it allows for facile energetic calculations. For maintenance, EBS supplemented with 10 mM disodium succinate was solidified with 1% (w/v) high-gel-strength granulated agar (Melford, Suffolk, UK). Unless stated otherwise all growth was at 30 °C. The genome of *Achromobacter* sp. NCCB 38021 has been sequenced (Boden, *in preparation*) and is publically available in the Integrated Microbial Genomes (IMG) database under Genome ID 2770939327.

Analytical methods

Acid-washed 'Class A' volumetric glassware was employed throughout and all analyses were conducted in an air-conditioned temperature controlled laboratory at 22°C. For spectrophotometric methods a Jenway 7315 UV-visible spectrophotometer equipped with a Peltier-heated cell was used. Determination of biomass was performed as previous described (Boden *et al.* 2010). With reference to a calibration curve of succinate-grown cell suspensions of known optical density at 440 nm (*OD*₄₄₀) dried to a constant weight, concentrations of biomass at any given *OD*₄₄₀ in mg dry biomass/L were calculated (Boden and Hutt 2018*b*). Succinate concentrations were measured with a succinic acid assay kit K-SUCC (Megazyme International, Ireland)

following the microplate protocol in a VersaMax plate reader (Molecular Devices, USA) and with reference to a calibration curve. Thiosulfate, tetrathionate and trithionate were determined using the cyanolytic methods of Kelly *et al.* (1969). Protein was quantified using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions using bovine serum albumen as the calibrant. The pH of batch culture experiments was determined using an Orion Star™ (3 Star) pH meter (ThermoFisher) equipped with a Ag/AgCl₂ combination pH micro-probe (Nijkerk, Netherlands), calibrated daily using traceable pH 4.0 ± 0.01, 7.0 ± 0.01 and 10.0 ± 0.01 buffers (Fisher Scientific), with calibrations checked periodically using a pH 5.0 ± 0.02 phthalate buffer (Fisher Scientific).

Phylogenetic analyses and identification of NCCB 38021

As NCCB 38021 is of unknown identity (we had previously tentatively identified it as an *Achromobacter* sp., but no further than that (Hutt, 2016; Boden and Hutt, 2018a)) two sets of analyses were undertaken, using the complete 16S rRNA (*rrs*) gene and the suite of 53 ribosomal protein genes (*rpsA-rpsU*, *rplA-rplF*, *rplL-rplX*, *rpmA-rpmJ*), concatenated as their translated amino acyl sequences – essentially as described in Boden and Scott (2018). The sequences from NCCB 38021 were obtained from the genome sequence, not by amplification. For other *Achromobacter* spp., analyses prioritised the type strains of species with validly published names, along with some additional, well-described strains. 16S rRNA genes were obtained from the IMG/ER and GenBank databases, prioritising those from whole-genomes rather than PCR amplicons. Concatamers of ribosomal protein genes were obtained from the ribosomal multilocus sequence typing (rMLST) server per Boden and Scott (2018). Phylogenetic analyses were undertaken in MEGA X (Kumar *et al.*, 2018) – sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and the aligned datasets model-tested on the basis of selecting the model with the lowest corrected Aikake information criterion (AICc) and maximum likelihood trees were constructed – full details of models and parameters are given in the legends for each Figure.

Growth in batch culture

Achromobacter sp. NCCB 38021 was grown in 500 mL culture volumes in 2.5-L glass Erlenmeyer flasks. Cultures were inoculated with 10% (v/v) of cell suspension washed from EBS agar slants to minimise substrate carryover. Cultures were incubated at 30 °C in a model G25 Incubator Shaker (New Brunswick Scientific, NJ, U.S.A.) at 100 orbits per minute through 5 cm radius. Three millilitre aliquots were taken at intervals for biomass, pH and substrate *etc.* determinations.

Growth in continuous culture

In chemostat experiments, EBS was supplemented to 1 mL/L with the vitamin solution of Kanagawa *et al.* (1982), comprising B-complex vitamins and α -lipoic acid, to ensure such growth factors did not become limiting. Growth in continuous culture was carried out in a customised Fermac 360 Series bioreactor system (Electrolab Biotech Ltd, Tewkesbury, UK) equipped with a 2-L nominal-volume vessel with side-arm weirs to give a culture volume (V) of 491.5 ± 0.5 mL (triplicate determination with probes *in situ*). The culture vessel was silanised to minimise wall-growth and washed thoroughly with ddH₂O prior to use, as described in Boden and Hutt (2018b). Temperature, pH and dissolved oxygen (dO₂) were monitored using the Fermac 360 Control Panel with Pt100 platinum RTM (Electrolab) temperature sensor, a FermProbe® 197 F-695 Ag/AgCl pH electrode (Broadley-James) and OxyProbe® 196 D340 polarographic oxygen electrode (Broadley-James), respectively, and stored electronically with Electrolab Fermentation Manager software and in a manual log book. EBS supplemented with an energy source was pumped into the growth vessel at multiple flow rates (F), and thus multiple dilution rates (D) using an MP-3 Micro Tube pump (Eyela, Tokyo, Japan). The pH was maintained at 7.2 by automatic titration of sterile, standardised 0.50 M sulfuric acid and 1.00 M sodium hydroxide. Temperature was maintained using a heated jacket and cooled with a cold-finger. Forced aeration was achieved with a Rena Air 400 pump (Rena, France) and outflowing air was chilled using a surgical steel Allihn condenser to prevent loss of culture volume. Stirring rates (100-350 rpm) and air flow (600-1000 mL/min) were adjusted to maintain dO₂ of 50 %. The production of foam not controlled by the Antifoam 289 in the medium was mitigated by automatic titration of 0.1 % v/v Antifoam 289 (Sigma-Aldrich) against signals from a headspace-conductivity foam-probe. Continuous cultures were inoculated with a 50 mL overnight batch culture on succinate and left in batch culture until late exponential phase. Continuous culture was initiated and steady-states established and tested exactly as described in Boden and Hutt (2018b). Samples removed for analytical work did not exceed 49 mL (following Pirt's "10 % rule" to minimise perturbation of the steady-state). A culture was determined to be in a steady-state when the specific molar growth yield (Y) was stable for the time required for five vessel volumes of medium to pass through the growth vessel (*c.* 2.5 L).

Kinetic determinations

Specific molar growth yield (Y), specific growth rate (μ) and specific rate of substrate uptake (q) were calculated according to Pirt (1965, 1975, 1982), Stouthamer (1979) and Boden and Hutt (2018b) as were the maximum specific growth yield coefficient (Y_{MAX}) by applying hyperbolic fit to yield vs dilution rate data exactly as

described in Boden and Hutt (2018b). Maximum specific growth rates (μ_{MAX}) were determined from wash-out kinetics using the method of Karagouni and Slater (1978).

Transmission electron microscopy sample preparation

A 100 mM cacodylate buffer at pH 7.2 was used as the diluent for all fixatives and for washing steps. Cells were fixed in 2.5 % w/v glutaraldehyde in cacodylate buffer for 1 h and were recovered by decanting. Cells were rinsed twice in the same buffer for 15 min, allowed to settle, the supernate discarded, and were then treated for with 0.1 % w/v osmium tetroxide in cacodylate buffer for 1 h. After rinsing twice, as before, cells were dehydrated by incubation for 15 min in each of 30, 50, 70 and 90 % v/v ethanol, then 15 min in each of 3 changes of 100 % v/v ethanol. Ethanol was replaced by medium-hardness low-viscosity resin (Agar Scientific, equivalent to Spurr resin) by 12 h incubation in each of 30, 50, 70 % v/v resin premix dissolved in ethanol, then 16 h in each 3 changes of 100 % v/v resin. Resin was polymerised in an Eppendorf tube at 60 °C for 16 h. Resin blocks were cut from tubes and sectioned with a Leica Ultracut E Ultramicrotome using a DiATOME Diamond Knife. Sections were stained in saturated aqueous uranyl acetate (*c.* 7.5 % w/v) for 15 min, then in Reynold's stain for 15 min (Reynolds, 1963). Sections were examined using a JEOL 1400 transmission electron microscope at the Electron Microscopy Unit (University of Plymouth).

Enzyme assays

Cells grown at $D = 0.10 \text{ h}^{-1}$ on 10 mM succinate as the limiting substrate, with and without 50 mM thiosulfate were prepared and used for enzyme assays. Cell pastes harvested from chemostat outflow on ice overnight were lysed by sonication in an ice-water bath within a 4 °C temperature-controlled room using a Vibra-Cell VCX 130PB fitted with a model CV188 probe sonicator set at 60 % of maximum amplitude. Six cycles of 1 min sonication followed by 2 min cooling in ice were performed (sonication protocol was optimised to give maximal specific activity of succinate dehydrogenase to ensure minimal protein damage). Unlysed cells and debris were removed from crude lysates at $14,000 \times g$ for 20 min at 4 °C - the supernates were used as cell-free extracts (CFEs), and the absence of remaining whole-cells was checked by light microscopy. Enzymes of inorganic sulfur metabolism were assayed per Kelly and Wood (1994) and Krebs' cycle enzymes per Weitzman (1969) for citrate (Si)-synthase (EC 2.3.3.1), Fanbler & Lowenstein (1969) for aconitate hydratase (EC 4.2.1.3), Cook and Sanwal (1969) for isocitrate dehydrogenase (NAD^+ , EC 1.1.1.41), Reed and Mukherjee (1969) for 2-oxoglutarate dehydrogenase (succinyl-transferring, EC 1.2.4.2), Bridger *et al.* (1969) for succinate-CoA ligase (GDP-forming, EC 6.2.1.4), Veeger *et al.* (1969) for succinate dehydrogenase (EC 1.3.5.1), Hill and Bradshaw

(1969) for fumarate hydratase (EC 4.2.1.2), and Murphey and Kitto (1969) for malate dehydrogenase (EC 1.1.1.37).

Monitoring of ATP formation

ATP formation was monitored per Kelly and Syrett (1966) and Boden *et al.* (2010). All glassware was washed in 5% v/v domestic hyperchlorite bleach solution, rinsed with ddH₂O and baked for two hours at 180 °C to destroy residual ATP. Experiments were carried out in 3 mL suspensions of cells in 100 mM potassium phosphate buffer (pH 7.2) stirred in a water-jacketted 7-mL Perspex® chamber from an oxygen electrode set-up (Rank Brothers Ltd., UK) held at 30 °C by a TC120 recirculating water bath (Grant Instruments, Cambridge, UK). After addition of thiosulfate, 250 µL of cell suspension was removed at 15 s intervals into 10 mL volumes of 1.45 M perchloric acid in glass scintillation vials held in ice. Digests were incubated for 10 min at 0 °C then neutralised with 1.9 mL 0.6 M KOH solution to pH 7.2. After 10 min further incubation for the precipitate to sediment, the clear supernates were carefully removed into fresh vials. ATP content of neutralised lysates was determined using the MAK135 ATP/ADP Ratio Kit (Sigma-Aldrich) according to manufacturer's instructions. Light emission was monitored in a Pi-102 luminometer (Hygiena International) in relative light units and ATP and ADP determined by reference to an ATP calibration curve – all calibrants were exposed to the perchloric acid/KOH steps, since potassium perchlorate somewhat quenches luminescence (Boden *et al.*, 2010).

Oxygen uptake by whole cells

Oxygen uptake rates were measured in a water-jacketed Perspex® chamber equipped with a Clark oxygen electrode (Rank Brothers, Cambridge, UK) under a 0.1 mm PTFE membrane. The chamber was held at 30 °C as described above. The electrode was regularly calibrated using fully aerated buffer ('100 %') and solid sodium dithionite ('0 %'). During oxygen uptake experiments, cells suspended in 100 mM potassium phosphate buffer (pH 7.2) were allowed to equilibrate with oxygen from the air by stirring for at least 1 h. Oxygen concentration was recorded for 3 min followed by addition of succinate or thiosulfate to give a final volume of 3 mL and oxygen concentration recorded for ≥ 5 min. It was assumed that saturated buffer at 30 °C contained 230 µM O₂ (Lu & Kelly, 1988b; Gnaiger, 2001).

Cytochrome spectra

Cytochrome spectra of CFEs were taken at 30 °C using the spectrophotometer described under *Analytical Methods*, set to 30 °C, and using optical quartz cuvettes of 1-cm path in matched-pairs for blanks and

experimental samples. Cytochromes were oxidised with 3 mM ammonium hexachloroiridate and fully reduced with 3 mM sodium dithionite. Experimental cytochrome reduction was with 10 mM sodium thiosulfate. Spectra were recorded after 1 min incubation in the Peltier-heated cell followed by brief mixing.

Statistical analysis

All statistical analyses were conducted using SigmaPlot 13.0. Pairwise comparisons were achieved with Student's *t* test or the Mann-Whitney *U* test depending on data normality. Data points represent mean \pm standard error of the mean, as indicated by error bars.

Results and Discussion

Identification of NCCB 38021

From the maximum likelihood tree based on the 16S rRNA gene given in Figure 1, it can be seen that the sequence from NCCB 38021 clusters closely with those of *Achromobacter aegrifaciens* LMG 26852^T and *Achromobacter anxifer* LMG 26858^T. The 16S rRNA gene sequence identity of these to NCCB 38021 are 99.8 % and 99.7 %, respectively, which would suggest that NCCB 38021 is a strain of *A. aegrifaciens*. From a maximum likelihood tree using 53 ribosomal protein genes concatenated as their translated amino acyl sequences (Figure 2), it can be seen that NCCB 38021 clusters with *Achromobacter insolitus* DSM 23807^T, but that there is a paucity of *Achromobacter* sequences from type strains in the rMLST database, and, as can be seen by comparison with Figure 1, the majority of strains that cluster with NCCB 38021 in the latter are absent from Figure 2 – though the topology of the tree in Figure 2 generally supports that of Figure 1. As such, we tentatively identify NCCB 38021 as a strain of *A. aegrifaciens*, the type strain of which was isolated from human sputum and is an opportunistic pathogen (VanDamme *et al.*, 2013), in contrast to NCCB 38021, which is from agricultural soil and does not appear to be pathogenic.

Growth in batch culture

Achromobacter sp. NCCB 38021 was grown in 500 mL EBS medium supplemented with 10 mM succinate with or without 20 mM thiosulfate in 2.5-L Erlenmeyer flasks at 30 °C (Fig. 3) and kinetic parameters are summarised in Table 1. No succinate was detected at or after 11 and 9 hours for media supplemented with or without thiosulfate, respectively. There were no significant differences with μ and *Y* with cells grown with or without thiosulfate. After 27 h incubation 98.5% of the thiosulfate (10.7 ± 0.31 mmol) had been oxidised to

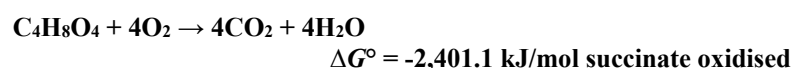
tetrathionate, with no trithionate being detected. When thiosulfate was the sole electron donor with no succinate, there was no evidence of growth (data not shown), but 22.8% of the thiosulfate (2.3 ± 0.60 mmol) had been oxidised to tetrathionate after 36 h. Starkey (1934) also noted a small amount of thiosulfate decomposition in the absence of organic carbon although thiosulfate was not directly measured. Thiosulfate dehydrogenase specific activity in cells grown on succinate alone or with auxiliary thiosulfate were 148 ± 35 and $1,357 \pm 64$ nmol min⁻¹ (mg protein)⁻¹, respectively, indicating constitutive expression at low levels that was upregulated when thiosulfate was present, which explains the ability to oxidise thiosulfate in the absence of succinate. These data support Starkey's conclusion that NCCB 38021 cannot grow autotrophically on thiosulfate and shows an apparent lack of beneficial effects from thiosulfate oxidation – and indeed it would not be typical for a strain of this species to grow autotrophically. As can be seen in Fig. 3B, most thiosulfate oxidation took place during the stationary phase when organic carbon supply had been exhausted, similar to results of batch growth obtained by Mason and Kelly (1988) using *Bacillus megaterium* from the *Bacillales* of the *Bacilli* and an *Aeromonas* sp. from the *Aeromonadales* of the *Gammaproteobacteria*. Presumably, the energy conserved from thiosulfate oxidation during this period was only used for cell maintenance as substrate carbon (or its metabolites) was no longer available for biosynthesis. A lack of beneficial effect on *Y* from thiosulfate oxidation in batch culture has been seen previously in ten different heterotrophic strains by Trudinger (1967) and Mason & Kelly (1988), where no significant differences in *Y* or μ were observed when media were supplemented with thiosulfate. Whether true chemolithoheterotrophy was taking place with these strains is unknown, but gratuitous oxidation of thiosulfate appears more likely. That said, batch culture is a poor model for natural environments due to the limited growth periods, build-up of metabolic waste, and cells being exposed to high carbon source concentrations, so this work was repeated in substrate-limited chemostat culture.

Growth in the succinate-limited chemostat

Steady-state cultures of *Achromobacter* sp. NCCB 38021 were established with 10 mM succinate as the limiting substrate from $D = 0.034$ to 0.195 h⁻¹. Steady-states were also established with 10 mM succinate as the limiting substrate, with 50 mM thiosulfate also supplied, between $D = 0.031$ and 0.163 h⁻¹. Comparison of kinetic parameters under these two growth regimes is given in Table 2. When thiosulfate was supplied as an exogenous energy source there was a small increase of 2.8% in μ_{MAX} , and an increase of 20.9% in Y_{MAX} . Similar increases in yield of approximately 20% have also been observed with “*Thiobacillus* Q” (probably a *Dechloromonas* or *Azonexus* sp. from the *Rhodocyclales* of the *Betaproteobacteria*, Boden and Hutt 2018a) and *Methylophaga*

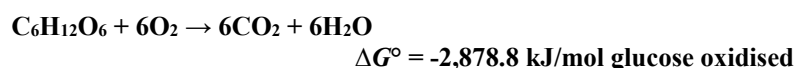
thiooxydans from the *Thiotrichales* of the *Gammaproteobacteria* when grown exochemolithoheterotrophically with thiosulfate as the auxilliary electron donor source (Gommers & Kuenen, 1988; Boden *et al.*, 2010). There was no increase in *Y* with “*Thiobacillus* Q” in batch culture on acetate with the addition of thiosulfate; however, similarly to *Achromobacter* sp. NCCB 38021, there was an increase in *Y* when it was grown in an carbon-limited chemostat and auxilliary thiosulfate was supplied. It must be noted that ATP production in “*Thiobacillus* Q” was not demonstrated and whether *bona fide* exochemolithoheterotrophy was taking place is unknown, although oxygen consumption was observed in the presence of thiosulfate, which strongly suggests that this, indeed, was the case. Comparable results were seen in *Pseudomonas aeruginosa* KF, with no apparent energetic benefit from thiosulfate oxidation in batch culture but a 30 % increase in *Y* in continuous culture was observed, albeit examined only examined at a single *D* (Mason & Kelly, 1988). Many other *Pseudomonas* spp. have been identified as thiosulfate oxidisers (Sijderius, 1946; Schook & Berk, 1979; Tuttle *et al.*, 1983; Sorokin *et al.*, 1999). Thiosulfate oxidation appears to be widespread throughout all classes of the phylum “*Proteobacteria*” and such increases in continuous culture yields, modelling the ‘real world’ scenario of carbon limitation in many environments, may demonstrate an evolutionary advantage over obligate heterotrophs as they could out-complete them when growth substrate is limited, as discussed by Boden (2010) and Boden and Hutt (2018a). Whether other species of heterotrophs capable of thiosulfate oxidation gain similar benefits would be invaluable in understanding this neglected form of bacterial metabolism.

The energetics of aerobic respiration of succinic acid are as follows, of course assuming all succinate is respired for energy metabolism:



The oxidation of thiosulfate to tetrathionate has $\Delta G^\circ = -61.9 \text{ kJ/mol thiosulfate oxidised}$. For growth on 10 mM succinate, a maximum of -24 kJ can be obtained – if we assume 50 % of succinate is dissimilated for energy and the remainder assimilated, -12 kJ energy is available in theory. This equates to formation of a maximum of 260 mmol ATP. For 50 mM thiosulfate being oxidised to tetrathionate, a maximum of -3 kJ is available, thus 67 mmol ATP. In cultivation on succinate in the presence or absence of thiosulfate, one would thus anticipate – assuming only half of the succinate provided is dissimilated for energy – a 25.8 % increase in *Y* and thus *Y*_{MAX} from the additional ATP from thiosulfate oxidation. As the *Y versus D* relation for these two cultivation conditions follows the ‘inverted uncompetitive inhibition kinetic’ we have previously described in Boden and

Hutt (2018b), in which Y with thiosulfate is always increased by the same magnitude *versus* on succinate alone, regardless of the magnitude of D , any differences in Y between the two metabolic modes would be identical to the differences in Y_{MAX} . This theoretical value is slightly higher than the 20.9 % increase observed from our laboratory data, and values in the region of 20-30 % increases in Y_{MAX} have been observed in other studies in which this kinetic also applies (*cf.* Boden and Hutt (2018b) for further discussion) – this suggests that over 50 % of succinate is dissimilated for energy (probably around 60 % in this case), thus the magnitude of the increase in Y_{MAX} is lower. In Mason and Kelly (1988), *P. aeruginosa* KF was cultivated under glucose-limitation at a single dilution rate ($D = 0.065 \text{ h}^{-1}$) on 2 mM glucose, with or without 15 mM thiosulfate, increasing Y from 21.0 g cell-carbon/mol glucose (*i.e.* 44.7 g dry biomass/mol glucose) to 27.3 g cell-carbon/mol glucose (*i.e.* 58.1 g dry biomass/mol glucose) – an increase of *c.* 30 % in terms of dry-weight. For this:



assuming that either 50 % or 60 % of glucose was dissimilated for energy and the same kinetic of exochemolithoheterotrophic Y *versus* heterotrophic Y as in *Achromobacter* sp. NCCB 38021, glucose oxidation would provide -2.88 to -3.46 kJ, thus 60 to 75 mmol ATP, and the addition of thiosulfate oxidation providing -0.92 kJ, thus 20 mmol ATP. This would result in increases in Y of between 26.7 and 33.3 % (for 60 % and 50 % dissimilation, respectively) – the experimental values of Mason and Kelly (1988) being in agreement with the latter value. It is of course well-established that the fraction of carbon source dissimilated for energy can vary considerably between organisms and carbon sources (*cf.* Boden and Murrell, 2011 and Pirt, 1975), but the general observation of Y and/or Y_{MAX} increases in the magnitude of 15-35 % in chemolithoheterotrophic *versus* heterotrophic growth is fairly consistent (Boden and Hutt, 2018a).

Examination of cells for intracellular polyphosphate or sulfur granules

Cells of *Achromobacter* sp. NCCB 38021 were harvested from steady-state chemostat cultures ($D = 0.10 \text{ h}^{-1}$) limited by 10 mM succinate with or without thiosulfate and examined for the presence of intracellular granules. It was of interest whether growth in the presence of thiosulfate caused an increase in these granules and potentially resulted in *faux* increases in yield previously observed *e.g.* in “*Thiobacillus* Q” (Gommers & Kuenen, 1988). It can be seen from transmission electron micrographs (Fig. 4) that the intracellular structures of NCCB 38021 grown with and without thiosulfate was essentially identical, with no granules present. The presence of polyphosphate (volutin) granules has been observed previously in exochemolithoheterotrophically-

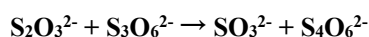
grown cells of “*Thiobacillus* Q” (Gommers & Kuenen, 1988) and elementary sulfur granules are seen in some chemolithoautrophic sulfur-oxidisers. By excluding these, we can confirm that the differences in biomass concentration seen between heterotrophically and exochemolithoheterotrophically grown *Achromobacter* sp. NCCB 38021 were not due to granule production increasing the optical density, but *bona fide* increases in biomass yield.

Enzymology of cell-free extracts obtained from heterotrophically versus chemolithoheterotrophically grown biomass.

CFEs were prepared from biomass harvested from steady-state chemostat cultures ($D = 0.10 \text{ h}^{-1}$) on succinate with or without thiosulfate. Specific activities of key enzymes of inorganic sulfur metabolism and of Krebs’ cycle were assayed (Table 3). From our current understanding of sulfur oxidation, most of the enzymes of the Kelly-Trudinger pathway were present as the activities of the cytochrome *c*-linked thiosulfate dehydrogenase (EC 1.8.2.2), sulfite dehydrogenase (EC 1.8.2.1) and trithionate hydrolase (EC 3.12.1.1) were detected in both heterotrophically and chemolithoheterotrophically grown cells. The specific activity of thiosulfate dehydrogenase was significantly higher when cells were grown with thiosulfate (Student’s *t* test, $p < 0.001$); as were sulfite dehydrogenase (Student’s *t* test, $p < 0.001$) and trithionate hydrolase (Mann-Whitney *U* test, $p < 0.001$). Up-regulation of thiosulfate dehydrogenase activity was previously shown by Boden *et al.* (2010) in *Methylophaga thiooxydans* DSM 22068^T grown in continuous culture on methanol *versus* dimethylsulfide (the sulfur moiety of the latter is oxidised to thiosulfate, which is then oxidised to tetrathionate – an example of endochemolithoheterotrophy), with a 2.5-fold increase of specific activity when grown on the latter. In *Achromobacter* sp. NCCB 38021, thiosulfate caused a 10-fold increase in specific activity, strongly indicating upregulation of gene expression from the presence of thiosulfate. A putative *tsdA* gene (IMG genome locus: 11041_ThiobacillustrauteiniiCultureB_01526) of 305 aa is present in the genome sequence of *Achromobacter* sp. NCCB 38021 (Boden, *in preparation*) directly downstream of a gene (locus: 11041_ThiobacillustrauteiniiCultureB_01525) encoding a 234 aa cytochrome *c*₅₅₃, similar to the 224 aa cytochrome *c*₅₅₃ upstream of *tsdA* in *Thiomonas intermedia* DSM 18155^T. The protein encoded by this gene in *Tma. intermedia* (TsdB) accepts electrons from TsdA during thiosulfate oxidation but does not itself oxidise thiosulfate: it is essential for feeding electrons into the respiratory chain (Kurth *et al.* 2016). The same gene cluster is also found in the genome sequences of most other *Achromobacter* spp., in which the *tsdA* and *tsdB* genes are physically distinct, thus the TsdA and TsdB proteins would be separate, per *Tma. intermedia* and “S.

lithotrophicus”, in contrast to the TsdA:TsdB fusion in *M. purpuratum*. The apparent closest relative-sequences to the TsdA from NCCB 38021 (other than *Achromobacter* spp.) were *c*-type cytochromes from *Bordetella* spp., *Burkholderia* spp. and *Pseudomonas* spp., rather than chemolithoautotrophic sulfur oxidisers such as *Thiobacillus*.

There was a 7-fold increase in the specific activity of sulfite dehydrogenase in extracts from cells grown with thiosulfate. Two copies of putative sulfite dehydrogenase genes (*sorAB*) are present in the genome sequence of NCCB 38021 (loci: 11041_ThiobacillustrauteiniiCultureB_05040, 11041_ThiobacillustrauteiniiCultureB_05041 and 11041_ThiobacillustrauteiniiCultureB_02707, 11041_ThiobacillustrauteiniiCultureB_02706) but both pairs of genes are very distant from the *tsdAB* genes, thus not part of a single operon. The presence of sulfite dehydrogenase and trithionate hydrolase activities is somewhat unexpected, since neither have a role in the thiosulfate-oxidation pathway (which terminates at tetrathionate), but a potential role of the latter could be to destroy any trithionate that becomes present in the environment of the cell (e.g. from chemical reaction of tetrathionate and sulfite – this potentially explaining the sulfite dehydrogenase activity, also), since both chemically react with thiosulfate and remove it from the environment of the cell (Roy and Trudinger, 1970):



$$\Delta G^\circ = -32.2 \text{ kJ/mol thiosulfate oxidised}$$

Thus, it is possible that the role of trithionate hydrolase in this organism is to ensure that trithionate does not interfere with the ability to utilise exogenous thiosulfate as an electron donor.

The tetrathionate hydrolase gene (*tetH*), as identified in *Acidithiobacillus ferrooxidans* in the *Acidithiobacillales* of the *Acidithiobacillia*, is not present, nor is tetrathionate hydrolase enzyme activity, although many organisms known to have tetrathionate hydrolase activity do not possess this gene, likely due to there being more than one enzyme present in the *Bacteria* with this activity, or a misidentification in the original work. Two putative tetrathionate reductase genes, *ttrAB* (loci: 11041_ThiobacillustrauteiniiCultureB_03855 and 11041_ThiobacillustrauteiniiCultureB_03853), are predicted in the genome – this is a respiratory reductase that reduces tetrathionate to thiosulfate during anaerobic respiration – however, the *ttrC* gene is missing. This encodes the membrane-bound TtrC subunit, which is likely a quinol dehydrogenase, transferring electrons to tetrathionate via TtrAB (James *et al.*, 2013). The presence of an incomplete Lu-Kelly-Friedrich cycle *sox* operon has already been shown in a number of Kelly-Trudinger pathway species (Boden *et al.*, 2016; Hutt *et al.*, 2017).

The genes of the *sox* operon are completely absent in the genome of *Achromobacter* sp. NCCB 38021, confirming a completely independent inorganic sulfur oxidation system, separate to the well-studied Lu-Kelly-Friedrich cycle.

To understand the change to central carbon metabolism during exochemolithoheterotrophy, enzymes of Krebs' cycle were examined. There was a significant decrease in the specific activities of 2-oxoglutarate dehydrogenase (EC 1.2.4.2; Student's *t* test, $p < 0.001$), fumarate hydratase (EC 4.2.1.2; Student's *t* test, $p = 0.005$) and malate dehydrogenase (E.C. 1.1.1.37; Student's *t* test, $p < 0.001$) in extracts prepared from cells grown exochemolithoheterotrophically *versus* heterotrophically. There were no significant differences in the specific activities of citrate (Si)-synthase (EC 2.3.3.1), aconitate hydratase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.41), succinate-CoA ligase (EC 6.2.1.4) and succinate dehydrogenase (EC 1.3.5.1). The decrease in 2-oxoglutarate dehydrogenase, fumarate hydratase and malate dehydrogenase specific activities may indicate a preference for the biosynthesis of the amino acid glutamate (or its downstream products) from the Krebs' cycle intermediate 2-oxoglutarate. Alternatively, as the lower specific activities of 2-oxoglutarate dehydrogenase and malate dehydrogenase result in a drop in production of NADH for feeding into the respiratory chain, this probably indicates that the provision of electrons required to generate the Δp needed for ATP biosynthesis is from another source, *viz.* thiosulfate oxidation. Decreased coupling of Krebs' cycle and the NADH pool correlates with Krebs' cycle fulfilling more of its anabolic role than its catabolic role in providing carbon skeletons rather than producing Δp (and thus ATP) and CO_2 (Quasem *et al*, 2017). During exochemolithoheterotrophic growth, diversion of substrate carbon from energy production to biosynthesis occurs, raising Y_{MAX} . This supports the findings of Tuttle (1980) who found more carbon-14 from [^{14}C]-D-(+)-glucose was incorporated into biomass when thiosulfate was being oxidised in two marine heterotrophs. A similar study with *Achromobacter* sp. NCCB 38021 and the fate and intermediates of organic carbon from succinate would be beneficial in understanding the physiological changes between heterotrophic and exochemolithoheterotrophic growth. Of course, it is possible that the decreases in specific activity of these three enzymes with decreased specific activity were owing to inhibition by thiosulfate and/or tetrathionate *in vitro*, giving a 'partial false negative' – whilst the former does not appear to inhibit any enzymes of central carbon metabolism on the basis of an inhibitor search of the BRENDA database (<http://www.brenda-enzymes.info>), the latter does inhibit some dehydrogenases *e.g.* glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12, Suzuki and Imahori (1973)) by interaction with thiol moieties present in the active site and formation of Bunte salts. Fumarate hydratase does not have conserved thiol moieties required for activity in either the active site or the 'B

site', nor does the 2-oxoglutarate complex, but malate dehydrogenase is dependent on methionine residues in the active site (Billue and Bell, 2012). The cofactors of the E2 subunit of the 2-oxoglutarate dehydrogenase complex are coenzyme A (a thiol) and lipoate (a 1,2-dithiolane), and tetrathionate and/or thiosulfate interference by chemical reaction with these cofactors could also be possible. It is well-established that thiosulfate can oxidise dihydrolipoate ('reduced lipoic acid') to lipoate with concomitant formation of bisulfide and sulfite, but there is no evidence of thiosulfate or tetrathionate reacting directly with lipoate itself – though this does not appear to have actually been examined (Villarejo and Westley, 1963; Roy and Trudinger, 1970). Coenzyme A contains a terminal thiol group, which could react chemically with thiosulfate or tetrathionate in several potential ways, including the formation of a coenzyme A sulfodisulfane by binding of either of these species to the pendent thiol moiety, as is the case with thiosulfate and/or tetrathionate with glutathione (Szczepowski, 1958). If this were the case, thiosulfate or tetrathionate present in CFEs could react with coenzyme A, effectively removing this reaction cofactor and giving a false decrease in specific activity; however, since the cells were from steady-state cultures devoid of thiosulfate and were well-washed to remove exogenous tetrathionate before lysis, no appreciable quantity of either sulfur oxyanion should be present, thus this possibility of inhibition can be disregarded.

Thiosulfate-dependent ATP biosynthesis and respiratory molecular oxygen consumption

Thiosulfate-dependent ATP production was demonstrated in resting cells of *Achromobacter* sp. NCCB 38021 obtained from a succinate-limited chemostat grown in the presence of thiosulfate ($D = 0.10 \text{ h}^{-1}$). When cells were exposed to 1 mM thiosulfate there was production of ATP at a rate of $490 \text{ pmol min}^{-1} (\text{g dry biomass})^{-1}$ and concentrations were significantly higher than in control cells that were not exposed to thiosulfate (Fig. 5). ATP production rates from auxiliary energy source oxidation in chemolithoheterotrophs have been previously reported at 280 and $570 \text{ pmol min}^{-1} (\text{g dry biomass})^{-1}$ in *Sagittula stellata* DSM 11524^T (grown exochemolithoheterotrophically on succinate with dimethylsulfide oxidised to dimethylsulfoxide for auxiliary energy) and in *Methylophaga thiooxydans* DSM 22068^T (grown endochemolithoheterotrophically on dimethylsulfide with endogenously produced thiosulfate oxidised to tetrathionate for auxiliary energy), respectively (Boden *et al.* 2011; Boden *et al.*, 2010). For comparison, in the chemolithoautotroph *Halothiobacillus neapolitanus* DSM 581, in which thiosulfate is oxidised fully to sulfate, rates of ATP production from thiosulfate oxidation are as high as $1.6 \text{ } \mu\text{mol min}^{-1} (\text{g dry biomass})^{-1}$ (Kelly and Syrett, 1966). The rate of production in our experiment started to plateau after about 60 s, presumably as cells started to

consume the ATP being produced – similar timescales were seen in *M. thiooxydans* and *H. neapolitanus* in the studies cited above. This confirms *bona fide* chemolithoheterotrophy was taking place as thiosulfate oxidation was coupled to ATP production and was not being solely used as an antioxidant, and with a very similar ATP production rate as in other thiosulfate-oxidising chemolithoheterotrophs, of *c.* 500 pmol min⁻¹ (g dry biomass)⁻¹.

In a second experiment, cells of *Achromobacter* sp. NCCB 38021 harvested from succinate-limited chemostats grown with or without thiosulfate ($D = 0.10 \text{ h}^{-1}$) were exposed to increasing concentrations of succinate (0.5 – 10.0 mM) or thiosulfate (0.5 – 10.0 mM) from which molecular oxygen consumption was recorded (Table 4). Transfer of electrons from thiosulfate to respiratory molecular oxygen *via* cytochrome *c* and cytochrome *c* oxidase was determined from the maximum rates of substrate-dependent oxygen uptake (V_{\max}) by whole cells exposed to thiosulfate (Table 4). There was a *c.*8-fold higher rate of O₂ consumption from thiosulfate and a higher affinity for thiosulfate (evident from the lower Michaelis constant, K_m) when cells were grown with thiosulfate. This coincided with a *c.*10-fold higher thiosulfate dehydrogenase specific activity previously seen in CFEs. The increase in thiosulfate-oxidation capability did not hinder succinate-dependent oxygen uptake as no significant differences in V_{\max} or K_m were observed between cells formerly grown with or without thiosulfate. The large change in K_m in cells grown with thiosulfate could potentially indicate that the apparent thiosulfate dehydrogenase activity in cells growth without it is due to a separate cytochrome *c* that shows some level of ability to oxidise thiosulfate.

Thiosulfate-dependent cytochrome reduction

Absolute cytochrome spectra were obtained from CFEs of cells harvested from a succinate limited chemostat supplemented with 50 mM thiosulfate and show peaks at 412, 514 and 551 nm when thiosulfate-reduced, corresponding to the Soret, β and α peaks of cytochrome c_{551} (Fig. 6). When dithionite-reduced, peaks at 416, 512 and 550 nm were found. Using difference spectra of thiosulfate-reduced minus hexachloroiridate-oxidised the concentration of thiosulfate-reducible cytochrome c_{551} was estimated as 1.8 nmol (mg protein)⁻¹ assuming a millimolar extinction coefficient for the reduced form at 550 nm of 28 mM⁻¹ cm⁻¹. Cytochrome *c* is the only energetically feasible point at which thiosulfate oxidation can be coupled to the respiratory chain and has been demonstrated *in vitro* in numerous studies (Trudinger 1961a, 1967; Lu *et al.*, 1983; Kelly *et al.*, 1993; Denkmann *et al.*, 2012). The standard redox potential (E°) of the thiosulfate/tetrathionate couple is +198 mV (Kurth *et al.*, 2015), meaning the only realistic entry of electrons from this reaction into the respiratory chain is cytochrome *c* (E° , oxidised/reduced +220 mV). Due to the low energy from inorganic oxidation, the reduction of

NAD⁺ to NADH in chemolithoautotrophic organisms is not feasible, thus must be achieved *via* reverse electron transport through the respiratory chain, at the expense of Δp , and thus of ATP formation (Kelly, 1971). NAD⁺/NADH levels were not quantified in our study, but it is improbable that thiosulfate oxidation in *Achromobacter* sp. NCCB 38021 would be coupled to NADH biosynthesis by reverse electron transport, as it is considerably more energetically favourable coupling it with the oxidation of succinate in Krebs' cycle.

Conclusions

Our study supports the previous work of Starkey (1934*a,b*) on this strain, but demonstrates additionally that this apparent “sulfur-oxidising heterotroph” in fact exhibits *bona fide* exochemolithoheterotrophy resulting in an increase in Y_{MAX} owing to coupling the oxidation of an inorganic electron donor to ATP biosynthesis, and the magnitude of the increase is consistent with thermodynamic predictions – but this only occurs when the organism is grown in substrate-limited continuous culture and not in batch culture. This is a property of chemolithoheterotrophs that we are now finding to be fairly common, it having demonstrated in *Methylophaga thiooxydans*, *Sagittula stellata*, various *Pseudomonas* spp. and so on (*cf.* Boden and Hutt (2018*a*) for a comprehensive list of studies). The bioenergetic basis for this observation is not immediately clear, but we suspect that intracellular ATP levels remain in relative excess in batch-culture *versus* the substrate-limited chemostat, and as such, intracellular ATP levels limit growth in the latter but not the former. This is probably not universally the case, and we would expect it to vary with the type of substrate used, based on the division of substrates into three groups by Anthony (1982) after Linton and Stephenson (1978) and Anthony (1980) – i) substrates that leave the cell biochemically limited by ATP over [H] and C – generally these are more oxidised substrates such as short-chain carboxylic acids, hexoses and those that are dissimilated without NADH-consuming steps, as is the case with succinate; ii) substrates that leave the cell biochemically limited by C over [H] and ATP – generally these are alkanes, long-chain alcohols and carboxylates, and iii) substrates that leave the cell biochemically limited by [H] – specifically NADH - over ATP and C – generally substrates that require initial oxygenase attack (consumes [H]), high [H]-consumption during assimilation (as is the case with C₁ compounds) and dissimilatory dehydrogenases that don't couple directly to NADH formation. Obviously much more work is required to study chemolithoheterotrophs growing on substrates from each of these categories to determine whether or not the “negative in batch, positive in chemostat” trait is universal or limited only to type ‘i’ substrates, and similarly whether the kinetics that relate Y and μ during heterotrophic *versus* chemolithoheterotrophic growth vary or if they always obey the ‘inverted uncompetitive inhibition’ model that

has been the case thus far in all chemolithoheterotrophs studied – the caveat being that they are predominantly cultivated on sugars or acetate, typically, both of which are type ‘i’ substrates. One exception of note is *Methylophaga thiooxydans*, which has been grown both exo- and endochemolithoheterotrophically on C₁ compounds that are type ‘iii’ substrates, and still shows this kinetic. Of course, there are considerably more factors that the nature of the *substrates*, but the nature of the core biochemistry and physiology of the heterotrophic metabolic mode is very variable – respiratory chains vary considerably, multiple glycolytic pathways can be used (often at the same time, Wood *et al.*, 1977) – and these factors also require consideration in selecting organisms for further work.

The decreases to the specific enzyme activities of Krebs’ cycle observed in chemolithoheterotrophically grown *versus* heterotrophically grown CFEs are consistent with a potential downregulation of the genes encoding [H]-generating steps in chemolithoheterotrophically grown cells, since Δp is generated from thiosulfate-oxidation, energetically ‘bypassing’ Krebs’ cycle, in a sense. One would anticipate intracellular pooling of the substrates of these enzymes, *viz.* 2-oxoglutarate and fumarate (whilst malate dehydrogenase is also downregulated, malate would of course not pool since downregulation of fumarate hydratase would result in no production of malate from fumarate in the first place). Therefrom, glutamate and pyruvate can be produced by alanine transaminase (EC 2.6.1.2), forming L-glutamate at the expense of L-alanine or by D-amino acid transaminase (EC 2.6.1.21), forming D-glutamate at the expense of D-alanine, both of which are predicted in the genome sequence of NCCB 38021. L-glutamate is the substrate for glutamine synthetase (EC 6.3.1.2), which forms L-glutamine as a key step in nitrogen assimilation from ammonia, whereas D-glutamate is involved in cell-wall biosynthesis in Gram-stain-negative *Bacteria*. Fumarate can be converted to L-arginosuccinate by arginosuccinate lyase (EC 4.3.2.1) in many organisms, however, this is not predicted in the NCCB 38021 genome. As such, it would be prudent in future work on chemolithoheterotrophs that also display changes in regulation of Krebs’ cycle enzymes to quantify free D- and L- amino acid and each corresponding amino acyl-tRNA in the cytoplasm during various stages of heterotrophic and exochemolithoheterotrophic growth, including the transition period, to establish if their pooling occurs, or if modifications in overall cellular amino acid demand from the changes in protein expression negate this.

617 **Acknowledgements**

618 The authors thank NCCB for the kind gift of strain NCCB 38021; Dr James Bray (University of Oxford) with
619 assistance in having the associated genome sequence added to the public rMLST database, and Electrolab
620 Biotech Ltd for the manufacture of the customised kinetic bioreactor for this work, funded by RG120444 (Royal
621 Society).

622 **Declaration**

623 *Funding* – the University of Plymouth provided a fully-funded Ph.D studentship to LPH and the Royal Society
624 (UK) provided Research Grant RG120444 to RB.

625 *Conflicting/Competing interests* – the authors declare that they have no conflicting or competing interests.

626 *Ethics approval* – not applicable.

627 *Consent to participate* – not applicable.

628 *Consent to publish* – not applicable.

629 *Availability of data and material* – genome sequence data for *Achromobacter aegrifaciens* NCCB 38021 are
630 publically available in the IMG database under Genome ID 2770939327. All raw data are available on request
631 from the corresponding author. The strain *A. aegrifaciens* NCCB 38021 is publically available from culture
632 collections under codes given in the Materials and Methods and Introduction.

633 *Code availability* – not applicable.

634 *Authors' contributions* – RB and LPH conceived the study with contributions from AJM; LPH carried out most
635 of the experimental work and data analyses with supervision and assistance from RB and AJM; RB conducted
636 phylogenetic and genome-related analyses and all thermodynamic determinations; GMH prepared specimens
637 and conducted electron microscopy with LPH; LPH and RB wrote the manuscript. All authors reviewed and
638 contributed comments to the manuscript and agreed it for publication.

639 References

- 640 Anthony C (1980) Methanol as a substrate: theoretical aspects. In: Harrison DEF, Higgins IJ, Watkinson R (eds)
 641 *Hydrocarbons in biotechnology*. London: Heyden. pp.220-230.
- 642
- 643 Anthony C (1982) *The biochemistry of methylotrophs*. Academic Press: London.
- 644
- 645 Akaike H (1973) Information theory and extension of the maximum likelihood principle. In Petrov, B.N., Csáki,
 646 F. (eds) *Second International Symposium on Information Theory, Tsahkadsor, Armenia, USSR*, September 2–8,
 647 1971, vol. 267–281. Akadémiai Kladó, Budapest.
- 648
- 649 Billue F, Bell E (2012) The role of methionine residues in activity and subunit communication in malate
 650 dehydrogenase. *FASEB J.* **1**, supplement: 558.9.
- 651
- 652 Boden R (2017) Reclassification of *Halothiobacillus hydrothermalis* and *Halothiobacillus halophilus* to
 653 *Guyparkeria* gen. nov. in the *Thioalkalibacteraceae* fam. nov., with emended descriptions of the genus
 654 *Halothiobacillus* and family *Halothiobacillaceae*. *Int. J. Syst. Evol. Microbiol.* **67**: 3919-3928.
- 655
- 656 Boden R, Hutt LP (2018a) Chemolithoheterotrophy: means to higher growth yields from this widespread
 657 metabolic trait. In: Rojo F (ed) *Aerobic utilization of hydrocarbons, oils and lipids (Handbook of Hydrocarbon
 658 and Lipid Microbiology)*. Springer: Cham, Switzerland.
- 659
- 660 Boden R, Hutt LP (2018b) Determination of kinetic parameters and metabolic modes using the chemostat. In:
 661 Steffan R (ed) *Consequences of microbial interactions with hydrocarbons, oils and lipids (Handbook of
 662 Hydrocarbon and Lipid Microbiology)*. Springer: Cham, Switzerland.
- 663
- 664 Boden R, Hutt LP (2018c) Bacterial metabolism of C₁ sulfur compounds. In: Rojo F (ed) *Aerobic utilization of
 665 hydrocarbons, oils and lipids (Handbook of Hydrocarbon and Lipid Microbiology)*. Springer: Cham, Switzerland.
- 666
- 667 Boden R, Murrell JC (2011) Response to mercury (II) ions in *Methylococcus capsulatus* (Bath). *FEMS Microbiol.
 668 Lett.* **324**: 106-110.
- 669
- 670 Boden R, Scott KM (2018) Evaluation of the genus *Thiothrix* Winogradsky 1888 (Approved Lists 1980) emend.
 671 Aruga *et al.* 2002: reclassification of *Thiothrix disciformis* to *Thiolinea disciformis* gen. nov., comb. nov., and of
 672 *Thiothrix flexilis* to *Thiofilum flexile* gen. nov., comb. nov., with emended description of *Thiothrix*. *Int. J. Syst.
 673 Evol. Microbiol.* **68**: 2226–2239.
- 674
- 675 Boden R, Murrell JC, Schäfer H (2011) Dimethylsulfide is an energy source for the heterotrophic marine
 676 bacterium *Sagittula stellata*. *FEMS Microbiol. Lett.* **322**: 188-193.
- 677

- Boden R, Cleland D, Green PN, Katayama Y, Uchino Y, Murrell JC, Kelly DP (2012) Phylogenetic assessment of culture collection strains of *Thiobacillus thioparus*, and definitive 16S rRNA gene sequences for *T. thioparus*, *T. denitrificans*, and *Halothiobacillus neapolitanus*. *Arch. Microbiol.* **194**: 187-195.
- Boden R, Hutt LP, Huntemann M, Clum A, Pillay M, Palaniappan K, Varghese N, Mikhailova N, Stamatis D, Reddy T, Ngan CT, Daum C, Shapiro N, Markowitz V, Ivanova N, Woyke T, Kyrpides N (2016) Permanent draft genome of *Thermithiobacillus tepidarius* DSM 3134^T, a moderately thermophilic, obligately chemolithoautotrophic member of the *Acidithiobacillia*. *Stand. Genomic. Sci.* **11**: 74.
- Boden R, Hutt LP, Rae A (2017) Reclassification of *Thiobacillus aquaesulis* (Wood & Kelly, 1995) as *Annwoodia aquaesulis* gen. nov., comb. nov., transfer of *Thiobacillus* (Beijerinck, 1904) from the *Hydrogenophilales* to the *Nitrosomonadales*, proposal of *Hydrogenophilalia* class. nov. within the 'Proteobacteria', and four new families within the orders *Nitrosomonadales* and *Rhodocyclales*. *Int. J. Syst. Evol. Microbiol.* **67**: 1191-1205.
- Boden R, Kelly DP, Murrell JC, Schäfer H (2010) Oxidation of dimethylsulfide to tetrathionate by *Methylophaga thiooxidans* sp. nov., a new link in the sulfur cycle. *Environ. Microbiol.* **12**: 2688–2699.
- Bridger WA, Ramaley RF, Boyer PD (1969) Succinyl coenzyme A synthetase from *Escherichia coli*. *Methods Enzymol.* **8**: 70-75.
- Cook RA, Sanwal BD (1969) Isocitrate dehydrogenase (NAD-specific) from *Neurospora crassa*. *Methods Enzymol.* **8**: 42-47.
- Denkmann K, Grein F, Zigann R, Siemen A, Bergmann J, van Helmont S, Nicolai A, Pereira IAC, Dahl C. (2012) Thiosulfate dehydrogenase: a widespread unusual acidophilic c-type cytochrome. *Environ. Microbiol.* **14**: 2673-2688.
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.
- Fanbler B, Lowenstein JM (1969) Aconitase from pig heart. *Methods Enzymol.* **8**: 26-30.
- Ghosh W, Dam B (2009) Biochemistry and molecular biology of lithotrophic sulfur oxidation by taxonomically and ecologically diverse bacteria and archaea. *FEMS Microbiol. Rev.* **33**: 999-1043.
- Gnaiger E (2001) Oxygen solubility in experimental media. *OROBOROS Bioenergetics Newsletter.* **6**: 1-6.
- Gommers PJF, Kuenen JG (1988) *Thiobacillus* strain Q, a chemolithoheterotrophic sulphur bacterium. *Arch. Microbiol.* **150**: 117-125.

- Guittoneau G (1927) Sur l'oxydation microbienne du soufre au cours de l'ammonisation. *Compt. Rend. Acad. Sci.* **184**: 45-46.
- Hill RL, Bradshaw RA (1969) Fumarase. *Methods Enzymol.* **8**: 91-99.
- Hiraishi A, Nagashima KVP, Matsuura K, Shimada K, Takaichi S, Wakao N, Katayama Y (1998) Phylogeny and photosynthetic features of *Thiobacillus acidophilus* and related acidophilic bacteria: its transfer to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov. *Int. J. Syst. Bacteriol.* **48**: 1389-1398.
- Hoare JP (1985) Oxygen In: Bard AJ, Jordan J, Parsons R (eds) *Standard potentials in aqueous solutions*. CRC Press: Boca Raton.
- Hurvich CM, Tsai C-L (1989) Regression and time series model selection in small samples. *Biometrika.* **76**: 297-307.
- Hutt LP (2016) Taxonomy, physiology and biochemistry of the sulfur Bacteria. *Ph.D Thesis*, University of Plymouth.
- Hutt LP, Huntemann M, Clum A, Pillay M, Palaniappan K, Varghese N, Mikhailova N, Stamatis D, Reddy T, Daum C, Shapiro N, Ivanova N, Kyrpides N, Woyke T, Boden R (2017) Permanent draft genome of *Thiobacillus thioparus* DSM 505^T, an obligately chemolithoautotrophic member of the *Betaproteobacteria*. *Stand. Genomic. Sci.* **12**: 10.
- James MJ, Coulthurst SJ, Palmer T, Sargent F (2013) Signal peptide etiquette during assembly of a complex respiratory enzyme. *Mol. Microbiol.* **90**: 400-414.
- Kanagawa T, Dazai M, Fukuoka S (1982). Degradation of *O,O*-dimethyl phosphorothioate by *Thiobacillus thioparus* Tk-1 and *Pseudomonas* AK-2. *Agric. Biol. Chem.* **46**: 2571-2575.
- Karagouini A, Slater JH (1978) Growth of the blue green alga *Anacystis nidulans* during washout from light- and carbon dioxide-limited chemostats. *FEMS Microbiol. Lett.* **4**: 295-299.
- Katayama Y, Hiraishi A, Kuraishi H (1995) *Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus. *Microbiology.* **141**: 1469-1477.
- Kelly DP (1993) Cytochromes in *Thiobacillus tepidarius* and the respiratory chain involved in the oxidation of thiosulphate and tetrathionate. *Arch. Microbiol.* **160**: 87-95.

- Kelly DP (1971) Autotrophy: concepts of lithotrophic bacteria and their organic metabolism. *Annu. Rev. Microbiol.* **25**: 177-210.
- Kelly DP (1982) Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. *Philos. Trans. R. Soc. Lond. B. Sci.* **298**: 499-528.
- Kelly DP, Syrett PJ (1966) Energy coupling during sulphur compound oxidation by *Thiobacillus* sp. c. *J. Gen. Microbiol.* **43**: 109-118.
- Kelly DP, Kuenen JG (1984) Ecology of the colourless sulphur bacteria, in *Aspects of Microbial Metabolism and Ecology*, ed. G. A. Codd (New York, NY: Academic Press), 211-240.
- Kelly DP, Wood AP (1994) Enzymes involved in the microbiological oxidation of thiosulfate and polythionates. *Methods Enzymol.* **243**: 501-509.
- Kelly DP, Wood AP (2000) Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int. J. Syst. Evol. Microbiol.* **50**: 511-516.
- Kelly DP, Chambers LA, Trudinger PA (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixtures with thiosulphate and tetrathionate. *Anal. Chem.* **41**: 898-901.
- Kelly DP, Lu W-P, Poole RK (1993) Cytochromes in *Thiobacillus tepidarius* and the respiratory chain involved in the oxidation of thiosulphate and tetrathionate. *Arch. Microbiol.* **160**: 87-95.
- Kelly DP, McDonald IR, Wood AP (2000) Proposal for the reclassification of *Thiobacillus novellus* as *Starkeya novella* gen. nov., comb. nov., in the α -subclass of the *Proteobacteria*. *Int. J. Syst. Evol. Microbiol.* **50**: 1797-1802.
- Kuenen JG, Beudeker RF (1982) Microbiology of *Thiobacillus* and other sulphur-oxidising autotrophs, mixotrophs and heterotrophs. *Philos. Trans. R. Soc. Lond. B. Sci.* **298**: 473-497.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**: 1547-1549.
- Kurth JM, Dahl C, Butt JN (2015) Catalytic protein film electrochemistry provides a direct measure of the tetrathionate/thiosulfate reduction potential. *J. Am. Chem. Soc.* **41**: 13232-13235.

- Kurth JM, Brito JA, Reuter J, Flegler A, Koch T, Franke T, Klein E, Rowe SF, Butt JN, Denkmann K, Pereira IAC, Archer M, Dahl C (2016) Electron accepting units of the diheme cytochrome *c* TsdA, a bifunctional thiosulfate dehydrogenase/tetrathionate reductase. *J. Biol. Chem.* **291**: 24804-24818.
- Le SQ, Gascuel O (2008) An improved general amino acid replacement matrix. *Mol. Biol. Evol.* **25**: 1307–1320.
- Linton JD, Stephenson RJ (1978) A preliminary study on growth yields in relation to the carbon and energy content of various organic growth substrates. *FEMS Microbiol. Lett.* **3**: 95-98.
- Lu WP, Kelly DP (1983) Thiosulfate oxidation, electron transport and phosphorylation in cell-free system from *Thiobacillus A2*. *J. Gen. Microbiol.* **129**: 1661-1671.
- Lu WP, Kelly DP (1988a) Cellular location and partial purification of the ‘thiosulphate-oxidizing enzyme’ and ‘trithionate hydrolyase’ from *Thiobacillus tepidarius*. *J. Gen. Microbiol.* **134**: 877-885.
- Lu WP, Kelly DP (1988b) Kinetic and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. *J. Gen. Microbiol.* **134**: 865-876.
- Lyric RM, Suzuki I (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidising enzyme and proposed pathway of thiosulfate oxidation. *Can. J. Biochem.* **48**: 355-363.
- Mason J, Kelly DP (1988) Thiosulfate oxidation by obligately heterotrophic Bacteria. *Microb. Ecol.* **15**: 123-134.
- Moreira D, Amils R. (1997) Phylogeny of *Thiobacillus cuprinus* and other mixotrophic thiobacilli: proposal for *Thiomonas* gen. nov. *Int. J. Syst. Bacteriol.* **47**: 522-528.
- Murphey WH, Kitto GB (1969) Malate dehydrogenase from *Escherichia coli*. *Methods Enzymol.* **8**: 145-148.
- Pepper IL, Miller RH (1978) Comparison of the oxidation of thiosulfate and elemental sulphur by two heterotrophic bacteria and *T. thiooxidans*. *Soil Sci.* **126**: 9-13.
- Pirt SJ (1965) The maintenance energy of bacteria in growing cultures. *Proc. Roy. Soc. B.* **163**: 224-231.
- Pirt SJ (1975) *Principles of microbe and cell cultivation*. Oxford: Blackwell.
- Pirt SJ (1982) Maintenance energy: a general model for energy-limited and energy sufficient growth. *Arch. Microbiol.* **133**: 300-302.
- Podkopaeva DA, Grabovich MY, Dubinina GA (2005) The functional role of reduced inorganic sulfur compounds in the metabolism of the microaerophilic bacterium *Spirillum winogradskii*. *Microbiology.* **74**: 12-19.

829 Quasem I, Achille AN, Caddick BA, Carter TA, Daniels C, Delaney JA, Delic V, Denton KA, Duran MC,
 830 Fatica MK, Ference CM, Galkiewicz JP, Garcia AM, Hendrick JD, Horton SA, Kun MS, Koch PW, Lee TM,
 831 McCabe CR, McHale S, McDaniel LD, Menning DM, Menning KJ, Mirzaei-Souderjani H, Mostajabian S,
 832 Nicholson DA, Nugent CK, Osman NP, Pappas DI, Rocha AM, Rosario K, Rubelmann H, Schwartz JA, Seeley,
 833 KW, Staley CM, Wallace EM, Wong TM, Zielinski BL, Hanson TE, Scott KM (2017). Peculiar citric acid cycle
 834 of hydrothermal vent chemolithoautotroph *Hydrogenovibrio crunogenus*, and insights into carbon metabolism
 835 by obligate autotrophs. *FEMS Microbiol. Lett.* **364**: fnx148.

836 Reed LJ, Mukherjee BB (1969) α -ketoglutarate dehydrogenase complex from *Escherichia coli*. *Methods Enzymol.*
 837 **8**: 55-62.

838 Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell.*
 839 *Biol.* **17**: 208-212.

840
 841 Ross PN (1985) Hydrogen In: Bard AJ, Jordan J, Parsons R (eds) *Standard potentials in aqueous solutions*.
 842 CRC Press: Boca Raton.

843
 844 Roy AB, Trudinger, PA (1970) *The biochemistry of inorganic compounds of sulphur*. Cambridge University
 845 Press: Cambridge.

846
 847 Schook LB, Berk RS (1978) Nutritional studies with *P. aeruginosa* grown on inorganic sulphur sources. *J.*
 848 *Bacteriol.* **133**: 1377-1382.

849
 850 Schook LB, Berk RS (1979) Partial purification and characterization of thiosulfate oxidase from *Pseudomonas*
 851 *aeruginosa*. *J. Bacteriol.* **140**: 306-308.

852
 853 Sijderius R (1946) Heterotrophe bacterien, die thiosulfaat oxydeeren. *Ph.D Thesis*, Vrije Universiteit Amsterdam.

854 Sorokin DY, Teske A, Robertson LA, Kuenen JG (1999) Anaerobic oxidation of thiosulfate to tetrathionate by
 855 obligately heterotrophic bacteria, belonging to the *Pseudomonas stutzeri* group. *FEMS Microbiol. Ecol.* **30**: 113-
 856 123.

857
 858 Starkey RL (1934a) Isolation of some bacteria which oxidise thiosulfate. *Soil Sci.* **39**: 197-219.

859
 860 Starkey RL (1934b) The production of polythionates from thiosulfate by microorganisms. *J. Bacteriol.* **18**: 387-
 861 400.

862
 863 Stouthamer AH (1979) The search for correlation between theoretical and experimental yields. In: *International*
 864 *Review of Biochemistry*, vol. 21, J. R. Quayle (eds). Baltimore, University Park Press.

- Suzuki K, Imahori K (1973) Isolation and some properties of glyceraldehyde-3-phosphate dehydrogenase from vegetative cells of *Bacillus cereus*. *J. Biochem.* **73**: 97-106.
- Szczepkowski TW (1958) Reactions of thiosulphate with cysteine. *Nature* **182**: 934-935.
- Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**: 512-526.
- Trautwein K (1921) Beitrag zur Physiologie und Morphologie der Thionsaurebakterien (Omelianski). *Zentr. Bakteriol. Parasitenk.* **53**: 513-548.
- Trudinger PA (1961a) Thiosulfate oxidation and cytochromes in *Thiobacillus* X: 1. Fractionation of bacterial extracts and properties of cytochromes. *Biochem. J.* **78**: 673-680.
- Trudinger PA (1961b) Thiosulfate oxidation and cytochromes in *Thiobacillus* X: 2. Thiosulfate-oxidising enzyme. *Biochem. J.* **78**: 680-686.
- Trudinger PA (1967) Metabolism of thiosulfate and tetrathionate by heterotrophic Bacteria from soil. *J. Bacteriol.* **93**: 550-559.
- Tuttle JH (1980) Organic carbon utilisation by resting cells of thiosulfate oxidising marine heterotrophs. *Appl. Environ. Microbiol.* **40**: 516-521.
- Tuttle JH, Holmes PE, Jannasch HW (1974) Growth rate stimulation of marine pseudomonads by thiosulfate. *Arch. Microbiol.* **99**: 1-14.
- Tuovinen OH, Kelly DP (1973) Studies on the growth of *Thiobacillus ferrooxidans*. I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison with ¹⁴CO₂-fixation and iron oxidation as measures of growth. *Arch. Microbiol.* **88**: 285-298.
- VanDamme P, Moore ER, Cnockaert M, Peeters C, Svensson-Stadler L, Houf K, Spilker T, LiPuma JJ (2013) Classification of *Achromobacter* genogroups 2, 5, 7 and 14 as *Achromobacter insuavis* sp. nov., *Achromobacter aegrifaciens* sp. nov., *Achromobacter anxifer* sp. nov., and *Achromobacter dolens* sp. nov., respectively. *Syst. Appl. Microbiol.* **36**: 474-482.
- Veeger C., DerVartanian DV, Zeylemaker WP (1969) Succinate dehydrogenase. *Methods Enzymol.* **8**: 75-81.
- Villarejo M, Westley J (1963) Rhodanese-catalysed reduction of thiosulfate by reduced lipoic acid. *J. Biol. Chem.* **238**: PC1185-PC1186.

- Weitzman PDJ. (1969) Citrate synthase from *Escherichia coli*. *Methods Enzymol.* **8**: 22-26.
- Whited GM, Tuttle JH (1983) Separation and distribution of thiosulfate-oxidising enzyme, tetrathionate reductase and thiosulfate reductase in extracts of marine heterotroph strain 16B. *J. Bacteriol.* **56**: 600-610.
- Wood AP, Kelly DP (1993) Reclassification of *Thiobacillus thyasiris* as *Thiomicrospira thyasirae* comb. nov., an organism exhibiting pleomorphism in response to environmental conditions *Arch. Microbiol.* **159**: 45-47.
- Wood AP, Kelly DP, Thurston CF (1977) Simultaneous operation of three catabolic pathways in the metabolism of glucose by *Thiobacillus* A2. *Arch. Microbiol.* **113**: 265-274.
- Zhdanov SI (1985) Sulfur, selenium, tellurium, and polonium In: Bard AJ, Jordan J, Parsons R (eds) *Standard potentials in aqueous solutions*. CRC Press: Boca Raton.

Table 1 Kinetic parameters of *Achromobacter* sp. NCCB 38021 in batch culture on 10 mM succinate, with or without thiosulfate. Parameters are specific molar growth yield (Y - g dry biomass/mol succinate), specific growth rate (μ - h⁻¹) and specific rate of substrate uptake (q – mmol succinate/h). Values shown are mean \pm standard error of mean ($n = 3$).

Kinetic Parameter	Growth condition:	
	10 mM succinate	10 mM succinate + 20 mM thiosulfate
Y (g dry biomass/mol succinate)	37 ± 0.7	35 ± 2.1
μ (h ⁻¹)	0.311 ± 0.017	0.287 ± 0.022
q (mmol succinate/h)	0.983 ± 0.070	0.905 ± 0.050

Table 2 Kinetic parameters of *Achromobacter* sp. NCCB 38021 in succinate-limited chemostats, with or without auxiliary thiosulfate. Parameters are the maximum specific growth yield coefficient (Y_{MAX} - g dry biomass/mol succinate), the maximum specific growth rate (μ_{MAX} - h⁻¹) and the Pirtian maintenance coefficient (m_s - mmol succinate (g•h)⁻¹).

Kinetic Parameter	Growth condition:	
	10 mM succinate	10 mM succinate + 50 mM thiosulfate
Y_{MAX} (g dry biomass/mol succinate)	51.98	62.89
μ_{MAX} (h ⁻¹)	0.394	0.405
m_s (mmol succinate (g•h) ⁻¹)	0.49	0.70

935

936

937

938

939

940

941

942

Table 3 Specific enzyme activities in *Achromobacter* sp. NCCB 38021 cell-free extracts obtained from succinate-limited chemostats. Cells were harvested from chemostat culture on 10 mM succinate with and without 50 mM thiosulfate under carbon limitation ($D = 0.10 \text{ h}^{-1}$). Specific activities are given in nmol/min/(mg protein). Values with asterisks represent statistically significantly higher activities when pairwise comparisons were made between heterotrophically and chemolithoheterotrophically grown cells. Values represent mean \pm standard error of mean ($n = 3$).

		Specific activities of enzymes in CFEs prepared from cells grown on: (nmol/min/(mg protein))	
Enzyme	E.C. number	10 mM succinate	10 mM succinate + 50 mM thiosulfate
<i>Inorganic sulfur metabolism:</i>			
Thiosulfate dehydrogenase (cyt <i>c</i>)	1.8.2.2	110 ± 4	1,163 ± 14*
Trithionate hydrolase	3.12.1.1	4 ± 1	50 ± 4*
Tetrathionate hydrolase	3.12.1.B1	0	0
Sulfite dehydrogenase	1.8.2.1	122 ± 14	881 ± 18*
Thiocyanate dehydrogenase	1.8.2.-	0	0
<i>Krebs' cycle:</i>			
Citrate (Si)-synthase	2.3.3.1	39 ± 1	40 ± 3
Aconitate hydratase	4.2.1.3	11 ± 1	7 ± 1
Isocitrate dehydrogenase (NAD ⁺)	1.1.1.41	19 ± 1	19 ± 2
2-oxoglutarate dehydrogenase	1.2.4.2	22 ± 1*	13 ± 1
Succinate-CoA ligase (GDP-forming)	6.2.1.4	33 ± 4	35 ± 3
Succinate dehydrogenase	1.3.5.1	48 ± 2	51 ± 2
Fumarate hydratase	4.2.1.2	178 ± 16*	83 ± 5
Malate dehydrogenase (NAD ⁺)	1.1.1.37	1,011 ± 6*	233 ± 19

943

944

945
946
947
948
949

Table 4 Oxygen uptake by whole cells of *Achromobacter* sp. NCCB 38021. Maximum rates of substrate-dependent oxygen uptake (V_{\max}) and Michaelis constant (K_m) relating to succinate or thiosulfate concentration for whole cells of *Achromobacter* sp. NCCB 38021 grown in carbon limited chemostats on 10 mM succinate with or without 50 mM 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 (μM)	V_{\max} [nmol O ₂ min ⁻¹ (mg dry biomass) ⁻¹]	K_m (μM)
Succinate	628 \pm 88	7.2	26 \pm 1	30.0
Succinate + thiosulfate	602 \pm 46	7.8	209 \pm 6	14.5

950
951

952 Figure Legends

953 **Fig 1.** Maximum-likelihood tree of the genus *Achromobacter* on the basis of the 16S rRNA (*rrs*) gene. Sequences
 954 were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA X (Kumar *et al.*, 2018). Aligned data were
 955 model tested in MEGA X on the basis of the lowest corrected Akaike information criterion (AICc, Hurvich and
 956 Tsai, 1989; Akaike, 1973). The outgroup contains equivalent gene from the type species of *Alcaligenes*. Numbers
 957 in parentheses refer to genome accession numbers in GenBank or IMG/ER databases – sequences from whole-
 958 genome sequences were preferentially used. The tree was constructed in MEGA X with partial deletion of gaps
 959 (95 % cut-off), and the final analysis involved 1,411 nt. The model of Tamura and Nei (1993) was used with a
 960 discrete gamma distribution (ten categories, gamma parameter = 0.6039) with 84.11 % of sites evolutionarily
 961 invariant. Tree shown had the highest log-likelihood (-2,888.95). Branch lengths are proportional to the number
 962 of substitutions, the bar representing 0.01 substitutions per site. Bootstrap values at nodes are on the basis of 5,000
 963 replications (values < 70 % are omitted for clarity).

964 **Fig 2.** Maximum-likelihood tree of the genus *Achromobacter* on the basis of 53 concatenated ribosomal protein
 965 gene sequences (*rpsA-rpsU*, *rplA-rplF*, *rplL-rplX*, *rpmA-rpmJ*) concatenated and translated *in silico* into amino
 966 acyl sequences. Gene concatamers were downloaded *en bloc* from the ribosomal multilocus sequence typing
 967 (rMLST) database (<http://pubmlst.org/rmlst>) and were translated then aligned using the MUSCLE algorithm
 968 (Edgar, 2004) in MEGA X (Kumar *et al.*, 2018). Aligned data were model tested in MEGA X on the basis of the
 969 lowest corrected Akaike information criterion (AICc, Hurvich and Tsai, 1989; Akaike, 1973). The outgroup
 970 contains equivalent concatamers from the type species of *Alcaligenes*. Numbers in parentheses refer to genome
 971 accession numbers in the rMLST database. The tree was constructed in MEGA X with partial deletion of gaps
 972 (95 % cut-off), and the final analysis involved 7,150 aa. The model of Le and Gascuel (2008) was used with a
 973 discrete gamma distribution (ten categories, gamma parameter = 0.6434) with 42.64 % of sites evolutionarily
 974 invariant. Tree shown had the highest log-likelihood (-27,355.45). Branch lengths are proportional to the number
 975 of substitutions, the bar representing 0.02 substitutions per site. Bootstrap values at nodes are on the basis of 5,000
 976 replications (values < 70 % are omitted for clarity).

977 **Fig 3.** Growth and sulfur oxyanion concentrations in batch cultures of *Achromobacter* sp. NCCB 38021. Growth
 978 curves represent growth on 10 mM succinate (A) or 10 mM succinate plus 20 mM thiosulfate (B), at 30 °C under
 979 air with shaking at 100 r.p.m. through 5 cm. Biomass (●), succinate (□), thiosulfate (▲), trithionate (●) and
 980 tetrathionate (◇) were determined. Values shown indicate mean ± standard error of mean (*n* = 3).

981 **Fig 4.** Transmission electron micrographs of *Achromobacter* sp. NCCB 38021 cells grown in a carbon limited
 982 chemostats (*D* = 0.10 h⁻¹) on 10 mM succinate without (A) or with (B) 50 mM thiosulfate, shown at 60,000× (left)
 983 and 40,000× (right) magnification, demonstrating the absence of any accumulated granules of elementary sulfur,
 984 polyphosphate *etc* during growth with thiosulfate.

985 **Fig 5.** ATP production from thiosulfate oxidation over time of whole cells of *Achromobacter* sp. NCCB 38021.
 986 Cells were harvested from a carbon-limited chemostat grown on 10 mM succinate with 50 mM thiosulfate (*D* =
 987 0.10 h⁻¹). Closed circles represent control cells not exposed to thiosulfate. Open circles represent cells exposed to
 988 a final concentration of 1 mM thiosulfate at time point zero. Values shown indicate mean ± standard error of mean
 989 (*n* = 3).

990 **Fig 6.** Absolute spectra of *Achromobacter* sp. NCCB 38021 cell-free extracts. Cells were grown in a carbon
 991 limited chemostats (*D* = 0.10 h⁻¹) on 10 mM succinate and 50 mM thiosulfate. Shown are 3 mM hexachloroiridate-
 992 oxidised (dashed line), 1 mM thiosulfate-reduced (dense solid line) and 3 mM dithionite-reduced (thin solid line).