Chemolithotrophic oxidation of thiosulphate and tetrathionate by novel strains of *Azospirillum* and *Pseudoxanthomonas* isolated from the rhizosphere of an Indian tropical leguminous plant

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The nutritionally fastidious bacterial strains, TTS and JT 002, were enriched and isolated on reduced sulphur compounds as the sole energy and electron source along with several other mesophilic, neutrophilic and facultatively sulphur-chemolithotrophic proteobacteria from the rhizospheric soil of *Clitoria ternatea*, a slender leguminous herb found throughout the Lower Gangetic Plains of India. 16S rRNA gene sequence-based phylogenetic analyses showed that TTS and JT 002 belonged to the α-1 and γ-2 subclasses of '*Proteobacteria*' with *Azospirillum* and *Pseudoxanthomonas* as the nearest genera respectively. The findings of the present study not only extend the phylogenetic spectrum of chemolithotrophic sulphur oxidation to these two genera hitherto unknown for sulphur-lithotrophic members but also describe distinctive lithotrophic properties. The new strain TTS exhibited more efficient utilization of, and conservation of energy from, tetrathionate than other alphaproteobacteria in terms of molar growth yield.

Keywords: *Azospirillum*, leguminous rhizosphere, *Pseudoxanthomonas*, sulphur-chemolithotrophy.

PLANT rhizospheres are mainly studied from the perspectives of biological nitrogen fixation and other nutritional facets of plant growth promotion or biocontrol, but this microhabitat is seldom viewed as a potential seat of sulphur oxidation by chemolithoautotrophic bacteria. In nature, taxonomically diverse species of aerobic chemolithotrophic[1] and anaerobic photolithotrophic[2] sulphur-oxidizing bacteria work in tandem to carry on the oxidative half of the sulphur cycle, which in its turn supplies sulphate, the utilizable form of sulphur, to all soil-dwelling organisms, including plants[3]. While a few bacteriological studies had earlier reported diverse thiosulphate-oxidizing proteobacteria from paddy field soil[4,5], more recent *in situ* experiments have shown sulphur-lithoautotrophic microorganisms to be ubiquitously active in the rhizospheres of tropical plants[6]. Rhizospheres were thus expected to be rich in phylogenetic and metabolic diversity of sulphur-chemolithotrophic bacteria. Since a systematic survey of plant rhizospheres vis-à-vis microbial sulphur oxidation was still largey wanting, the root–soil interface of a tropical leguminous herb *Clitoria ternatea* (family Papilionaceae), occurring in almost every waste land and village forest of the Lower Gangetic Plains of India, was explored for the same. This led to the isolation and characterization of several taxonomically novel and chemolithotrophically distinct, mesophilic, neutrophilic and facultatively sulphur-oxidizing proteobacteria[7,8]. Two such phylogenetically novel isolates possessing characteristic chemolithotrophic potentials for utilization of reduced sulphur compounds have been discussed here.

A number of *Clitoria ternatea* plants were uprooted and thin layers of soil adhered to the roots were dislodged by gently striking with sterile forceps. The collected pool of soil, devoid of root nodules, was enriched for 30 days with 5% $Na_2S_2O_3 \cdot 5H_2O$, 1% soluble Na_2S and 5% elemental sulphur, all in w/w ratios, by intermittent sprinkling of sterile water. The enriched soil was added to sodium thiosulphate-containing (20 mM $Na_2S_2O_3$) liquid medium having a pH of 7.5 and based on a modified basal and mineral salts (MS) solution[7] supplemented with 5 g yeast extract l^{-1} (MSTY) and neutrophilic, mesophilic and facultatively sulphur-chemolithotrophic bacteria distinguished in terms of colony morphology, and rate and extent of acid production in autotrophic MS-thiosulphate (20 mM $Na_2S_2O_3 \cdot 5H_2O$ and 50 mg yeast extract l^{-1}) (MST) and mixotrophic MSTY media were isolated as pure cultures following methods described earlier[9]. The two strains TTS and JT 002 were thus retrieved along with several other facultatively sulphur-chemolithotrophic bacterial strains. Both the strains could utilize thiosulphate as the energy and electron source, although best utilization of the same was under mixotrophic conditions, i.e. in the MSTY medium.

In order to confirm the genotypic distinctiveness of the leguminous plant rhizospheric (LPR) isolates, AFLP and/or APPCR patterns were generated for all the new strains by PCR using arbitrary primers bearing *Pseudaminobacter salicylatoxidans* KCT001-specific sequences taken randomly from discrete regions of its sulphur oxidation (*sox*) gene cluster (EMBL accession no. AJ404005). The uniqueness of the two strains, TTS and JT 002, in comparison to the other strains[7,8] retrieved in the same isolation procedure, was evident from manual as well as computer-aided comparison of the said APPCR profiles (data not shown).

Chemolithoautotrophic or chemoorganoheterotrophic growth experiments were performed at 30°C in MS solutions supplemented at a time with a single sulphur compound (12–20 mM thiosulphate, 2 mM sulphide, 3–5 mM thiocyanate, 0.5–1.0% elemental sulphur, or 10 mM tetrathionate) or a single carbon source (5 g per l) respectively. Levels of thiosulphate or tetrathionate in the media were estimated by the cyanolytic method[10]. Since the two

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strains had unknown growth factor requirements, synthetic media were supplemented with 50 mg yeast extract per litre or a vitamin mixture containing 10 mg each of nicotinic acid, pantothenic acid, pyridoxine, thiamin, para-aminobenzoic acid, riboflavin and biotin per litre. Other phenotypic tests were performed using standard techniques described elsewhere[11].

Both TTS and JT 002 were facultative chemolithotrophs and could grow on 12–20 mM sodium thiosulphate, although neither could utilize sodium sulphide, sodium thiocyanate or elemental sulphur under the experimental conditions provided. Increase in OD_{600} during growth in MST medium with concomitant lowering of pH from 7.5 to 6.0 was recorded. Additionally, TTS could oxidize tetrathionate for chemolithotrophic growth corresponding to an OD_{600} of ≥0.3 in batch cultures in mineral salts–tetrathionate (10 mM potassium tetrathionate) medium (MSTr) in four days. However, JT 002 did not utilize tetrathionate. While thiosulphate is a common substrate oxidized by most of the sulphur-chemolithotrophs, species distributed over the alpha-, beta- and gamma-proteobacteria additionally utilize many other sulphur compounds (including tetrathionate) that are however not accommodated in the so-called Sox-mediated pathway[12]. An earlier hypothesis explaining the disparate chemolithotrophic abilities of diverse species, states that all sulphur-oxidizing microorganisms had plausibly originated from some ancient stock possessing lithoautotrophic potentials governed by a primordial system that was perhaps similar to the Sox-mediated one, and additional abilities to oxidize tetrathionate or other special reduced sulphur compounds have been acquired subsequently as environment-guided characters[13].

Chemolithotrophic growth of TTS coupled with oxidation of thiosulphate or tetrathionate was also studied in continuous cultures using an initial concentration of 20 mM sodium thiosulphate and 10 mM potassium tetrathionate (both equivalent to 40 μg atoms of sulphur per ml) with subsequent addition of the same amounts of filter-sterilized sodium thiosulphate or potassium tetrathionate solutions, periodically, four times, to the respective cultures over a total incubation period of six days. Eventual utilization of 200 μg atoms of sulphur per ml supported a final OD_{600} of ≥0.35 and ≥0.75 in the two media-types, MST and MSTr, respectively. A cellular yield of 350 and 650 mg cell protein per g atoms of sulphur oxidized was observed on thiosulphate and tetrathionate respectively.

Distinctly greater tetrathionate-dependent autotrophic growth yield exhibited by TTS in comparison to another tetrathionate-oxidizing alphaproteobacterium KCT001 (Table 1) is in favour of the occurrence of different sulphur-oxidation enzymes in different bacteria[1] and reinforces the hypothesis that advocates the existence of discrete substrate oxidation pathways and modes of energy conservation from the same substrate by different organisms[1]. Notably, inadequacies of the alphaproteobacterial Sox molecular machinery[12] have been indicated earlier along with the need to make it more substrate-inclusive and enzymically elaborate, so as to account for atypical sulphur substrates like tetrathionate[13,14]. Molecular investigation of sulphur-chemolithotrophy in bacteria like the ones described here is likely to help understand the evolution, diversification and spread of this unique metabolism.

Near-complete 16S rRNA genes of the two strains were amplified, sequenced and phylogenetically analysed following methods described earlier[7,9]. Comparison with sequences available in public databases identified JT 002 (AJ864461) as a member of γ-2 subclass of *Proteobacteria*, while TTS (AJ864460) was affiliated to the α-1 subclass of *Proteobacteria*. The phylogenetically distinct LPR isolate TTS exhibited no more than 97% 16S rRNA gene sequence similarity with strains of *Azospirillum lipoferum*, while strains of *A. dobereinerae* (95.8%) and *A. brazilense* (94.4%) had further lower levels of similarity. Members of other genera like *Roseomonas* showed <94% 16S rRNA gene sequence similarity with TTS. Hitherto

Table 1. Comparison between strain TTS and another tetrathionate-oxidizing, sulphur-chemolithotrophic alphaproteobacterium *Pseudaminobacter salicylatoxidans* KCT001 in terms of utilization of tetrathionate, growth and concomitant decrease in pH of the medium after four days of growth in batch cultures in chemolithoautotrophic MSTr medium at 30°C. Initial concentration of tetrathionate in the medium was 10 mM $K_2S_4O_6$ equivalent to 40 μg atoms of sulphur per ml. To determine cellular yields (in mg of cell protein per g atoms of S oxidized) of the two bacteria on tetrathionate data from continuous cultures were also considered

Results after incubation for four days	TTS	KCT001
Final OD_{600}*	0.30–0.35	0.15–0.2
Tetrathionate consumed in μg atoms of S per ml	32–34	24–26
Percentage of amount supplied	80–85	60–65
Final pH of spent medium#	5.2–5.5	5.8–6.0
Cellular yield on tetrathionate in mg of cell protein per g atoms of S oxidized	650	350

*Increase in OD_{600} is attributed to tetrathionate utilization and values presented were calculated after eliminating background growth of the bacteria in basal salts medium without tetrathionate.

#Initial pH of autotrophic tetrathionate-containing medium was 7.5. Ranges presented for all the data were obtained from three different experiments.

reported strains of *Azospirillum* are not known to possess sulphur-lithotrophic attributes, but recent studies have revealed *sox* genes as well as chemolithotrophic thiosulphate oxidation[15] in the closely related organism, *Magnetospirillum magnetotacticum*. When grown in liquid cultures under static conditions without shaking the microaerophilic strain TTS formed biofilms and adhered to any provided substratum, including glass walls of culture flasks. The bacterium JT 002 possessed highest ≥99% 16S rRNA gene sequence similarity with strains of *Pseudoxanthomonas mexicana*[16], a member of the family *Xanthomonadaceae* within the order *Xanthomonadales* of the γ-2 subclass of *Proteobacteria*. Interestingly, JT 002 had around 98% 16S rRNA gene sequence similarity with certain uncultured betaproteobacteria, while the same with the species of *Xanthomonas* was still lower.

Under the provided experimental conditions, TTS and JT 002 could not utilize any carbon compound in synthetic heterotrophic media supplemented with vitamin mixture. Attempts were made to grow and maintain TTS and JT 002 in complex media like nutrient broth (NB) or Luria–Bertani (LB) broth or the latter with 2 g NaCl per litre. However, upon subsequent subculturing for several generations at intervals of 7–10 days, the viability of the cultures was lost and eventually the two bacteria could not be sustained. The two strains were perhaps nutritionally obligated to the rhizospheric niches as indicated by their fastidious growth requirements and apparent unculturable nature. *Azospirillum* and *Pseudoxanthomonas* spp. are known to have diverse metabolic interactions with plants. While *Xanthomonas* species are known to infect cultivated plants as facultative pathogens[17], spirilla have reported symbiotic and endophytic existence[18]. Again, some strains of *Azospirillum* exert multifaceted impact on plant pathogenicity and disease-inflicting potential of other bacteria[19]. With the present revelation of sulphur-chemolithotrophic attributes in strains belonging to these genera, future research would be attenuated towards a comprehensive understanding of the nutritional, biochemical and metabolic interactions that sulphur-oxidizing bacteria might have between themselves as well as with plants whose rhizospheres they infest.

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