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Anaerobic growth of the haloalkaliphilic denitrifying sulfur-oxidizing bacterium *Thialkalivibrio thiocyanodenitrificans* sp. nov. with thiocyanate

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Two strains of obligate chemolithoautotrophic sulfur-oxidizing bacteria were isolated from soda-lake sediments by enrichment culture with thiocyanate and nitrate at pH 9·9. The isolates were capable of growth with thiocyanate or thiosulfate as electron donor, either aerobically or anaerobically, and with nitrate or nitrite as electron acceptor. Cyanate was identified as an intermediate of thiocyanate oxidation, while sulfate, ammonia and dinitrogen gas were the final products. The anaerobic growth on thiocyanate plus nitrate was much slower ($\mu_{max}=0{\cdot}006\ h^{-1}$) than on thiosulfate plus nitrate ($\mu_{max}=0{\cdot}02\ h^{-1}$), while growth yields were similar (4·8 and 5·1 g protein mol^{-1}, respectively). On the basis of their phenotypic and genetic properties, strains ARhD 1^T and ARhD 2 are described as a novel species of the genus *Thialkalivibrio*, with the highest similarity to *Thialkalivibrio denitrificans*. The name *Thialkalivibrio thiocyanodenitrificans* sp. nov. is proposed for this novel species.

INTRODUCTION

Thiocyanate ($N{\equiv}C{-}S^-$) is a C_1 sulfur compound, and the simplest nitrile species. It can be produced as a natural metabolite in biological cyanide detoxification processes and as a waste product of coke and metal plants (Kelly & Baker, 1990; Wood, 1975). Furthermore, thiocyanate is used for manufacturing some insecticides and herbicides, and for chemical synthesis, which increases its release to the environment.

Thiocyanate can be utilized as an energy source by chemolithotrophic sulfur-oxidizing bacteria, after primary degradation by two distinct pathways. Initial cleavage of the C–S bond results in the formation of the intermediate cyanate ($N{\equiv}C{-}O^-$) which, in the presence of bicarbonate, is converted further to ammonia and CO_2 by the enzyme cyanase (Happold *et al.*, 1958; Youatt, 1954). The alternative pathway is based on the initial hydrolytic cleavage of the nitrile bond ($N{\equiv}C$), resulting in the formation of carbonyl sulfide ($S{=}C{=}O$) and ammonia (Katayama *et al.*, 1992, 1993, 1998). The carbonyl sulfide is subsequently hydrolysed further to sulfide and CO_2. In both cases, the released sulfide can serve as the energy source and electron donor for autotrophic growth.

The ability to grow with thiocyanate as an electron donor for energy generation and CO_2 fixation is restricted to a few strains of neutrophilic thiobacilli (De Kruyff *et al.*, 1957; Happold *et al.*, 1954, 1958; Katayama & Kuraishi, 1978; Smith & Kelly, 1988; Youatt, 1954). Recently, we described several new thiocyanate-oxidizing bacteria capable of chemolithoautotrophic growth with thiocyanate at high pH and salt concentration (Sorokin *et al.*, 2001a). They included two novel species within the genus *Thialkalivibrio*, which accommodates a large number of haloalkaliphilic sulfur-oxidizing chemolithotrophic bacteria from soda lakes (Sorokin *et al.*, 2001b, 2002). These bacteria degrade thiocyanate via cyanate.

Despite the substantial amount of information now available on bacterial thiocyanate degradation, almost nothing is known about the possibility of anaerobic growth with thiocyanate. An early publication of De Kruyff *et al.* (1957) reported that *Thiobacillus denitrificans* can grow with thiocyanate, aerobically or anaerobically, in the presence of nitrate as the electron acceptor, reducing the latter completely to N_2, while *Thiobacillus thioparus* only reduced

Abbreviations: NAR, nitrate reductase; NIR, nitrite reductase.

The GenBank accession number for the sequence of the 16S rRNA gene of strain ARhD 1^T reported in this paper is AY360060.

nitrate to nitrite in the presence of thiocyanate. The ability of certain strains of *Tb. thioparus* to grow with thiocyanate aerobically was independently confirmed by several research groups. On the other hand, the potential of *Tb. denitrificans* to grow with thiocyanate, either aerobically or anaerobically, has never been substantiated after the report of De Kruyff *et al.* (1957). Moreover, analysis of the quantitative data presented in De Kruyff *et al.* (1957) showed substantial deviation of the stoichiometry from the theoretical values. This makes it difficult to understand the anaerobic conversion of thiocyanate by *Tb. denitrificans*. In more recent literature, we found only a single report of thiocyanate-dependent denitrification by a mixed bacterial population in a thiocyanate waste-treatment plant (Andreoni *et al.*, 1988).

The aim of this work was to investigate the possibility of anaerobic oxidation of thiocyanate, and to obtain kinetic parameters of the process. Two pure cultures of alkaliphilic, obligate chemolithoautotrophic and facultative anaerobic sulfur-oxidizing bacteria, capable of growth with thiocyanate as energy and nitrogen source under denitrifying conditions, have been obtained from soda-lake sediments. The two strains were identified as a novel species of the genus *Thialkalivibrio*.

METHODS

Enrichment and isolation conditions. Two mixed sediment samples, composed of 10 individual samples, each from the soda lakes in Wadi Natrun (Egypt) and Kulunda steppe (Siberia, Russia), were used as inoculum to enrich for anaerobic thiocyanate-oxidizing haloalkaliphiles. The pH and total salt concentration in the lakes ranged from 9·2 to 10·5 and 20 to 380 g l^{-1}, respectively. The mineral enrichment medium contained (g l^{-1}): Na_2CO_3, 20; $NaHCO_3$, 10; NaCl, 5; K_2HPO_4, 1. After sterilization, 0·5 mM $MgCl_2.6H_2O$, 10 mM KSCN, 15 mM KNO_3 and 1 ml l^{-1} of trace elements solution (Pfennig & Lippert, 1966) were added. The final pH was 9·9. Portions (80 ml) of the complete medium were dispensed into 100 ml serum bottles, sealed with butyl rubber stoppers, made anoxic by five cycles of evacuation/argon flushing and incubated at 30 °C for 2 months. Progress of the enrichment was monitored by the measurement of thiocyanate and nitrate consumption. After complete thiocyanate degradation, several successive subcultures with a 1 % inoculum were performed to obtain a stable mixed culture. Finally, the culture was serially diluted, and the last positive dilution was plated onto SCN^- (micro-oxic conditions, 5 % O_2 in the gas phase) and SCN^-/NO_3^- (anoxic) alkaline agar. The dominant colony types were picked and inoculated into liquid media to test for the ability to grow autotrophically with thiocyanate and thiosulfate under oxic and denitrifying conditions.

Growth experiments with pure cultures. Growth kinetics and product formation of the pure cultures with different combinations of electron donor/acceptor were studied, using the same mineral medium as for the enrichment. Anaerobic growth with thiocyanate and thiosulfate as substrate, and nitrate, nitrite or N_2O as electron acceptor, was performed in 600 ml serum bottles with 500 ml cultures, using a fed-batch mode of thiocyanate and nitrate supply. During growth, the biomass, substrate consumption and product formation were measured periodically. Thiocyanate and nitrate were added on several occasions when the previous doses had been utilized. Aerobic cultures with thiocyanate and thiosulfate were incubated in 600 ml closed serum bottles, with 100 ml medium and 10 % O_2 in the gas phase, on a rotary shaker at 100 r.p.m. Higher aeration inhibited growth.

Experiments with washed cells. The respiration rates with different sulfur substrates of the washed cells grown under oxic or anoxic conditions were measured as described previously (Sorokin *et al.*, 2001a). The anaerobic activity of washed cells was tested under anoxic conditions with different electron donors/acceptors (3 ml cell suspension in 9 ml serum bottles) in sodium carbonate/bicarbonate buffer, pH 10.

Analyses. Thiocyanate was analysed colorimetrically as ferric thiocyanate (Sörbo, 1957), thiosulfate by an iodimetric titration and by cyanolysis (Kelly *et al.*, 1969), sulfate by a turbidimetric method (Cypionka & Pfennig, 1986), NH_4^+ by a phenol/hypochlorite colorimetric procedure according to Weatherburn (1967), and nitrite by a diazotation method (Gries-Romijn-van Eck, 1966). Nitrate was assayed colorimetrically with the Szechrome NAS reagent (Polysciences): to 0·25 ml sample, 2·5 ml 0·5 % Szcechrome in 1 : 1 H_2SO_4/H_3PO_4 was added, and the absorbance was measured at 600 nm after 30 min incubation. Because of a strong thiosulfate interference, accurate nitrate measurements were only possible with thiocyanate as substrate. N_2O was detected by a gas chromatograph (Fison Instruments) equipped with a Hayesep column and a ^{63}Ni-electron capture detector. Cell protein was measured by the Lowry method. Cyanate (OCN^-) was routinely assayed as NH_4^+ released after acidification of solutions to pH 2–3 with 6 M HCl and subsequent heating in boiling water for 1 min. This procedure gave 95–97 % recovery of pure cyanate added to standard sodium carbonate media at pH 10 (Sorokin *et al.*, 2001a).

Denaturing SDS-PAGE of whole-cell polypeptides was performed with a 10 % gel according to Laemmli (1970). Nitrate and nitrite reductase activities (NAR and NIR, respectively) in the cell-free extracts (obtained by sonication) were measured under anoxic conditions, with reduced methyl viologen as artificial electron donor, by analysing nitrate or nitrite consumption at pH 7–10, with boiled extract as a control. Detection of NAR and NIR polypeptides, after electrophoresis of total cell extracts, was based on the negative staining that resulted from enzyme activity in the presence of reduced methyl viologen as the electron donor and nitrate or nitrite as electron acceptors (Murillo *et al.*, 1999). Absorption spectra of the cytochromes in the cell-free extract were recorded with a UV/visible diode-array HP 8453 spectrophotometer (Hewlett Packard). Cyanase activity measurements, electron microscopy, DNA analysis and phylogenetic analysis were performed as described previously (Sorokin *et al.*, 2001b, 2002).

RESULTS

Enrichment and isolation of pure cultures

Within 30 to 40 days of incubation, development of a mixed population of alkaliphilic bacteria was observed in the anaerobic enrichment cultures with thiocyanate plus nitrate inoculated with Egyptian or Siberian soda-lake sediments. Growth resulted in the concomitant consumption of thiocyanate and nitrate at a final pH of 9·9. After several subcultures, stable mixed cultures were obtained, with motile rods as the dominant morphotype. However, plating of serial dilutions from the cultures did not result in colony formation under anoxic conditions on the same medium. In contrast, colonies developed readily under micro-oxic conditions on the medium with thiocyanate or thiosulfate as substrate. Of four different colony types, only the biggest reddish ones produced stable aerobic liquid cultures with thiocyanate as the only energy and nitrogen source. The strains originating from the Wadi Natrun and the Kulunda samples were designated ARhD 1^T and ARhD 2, respectively. The cells of both strains were motile rods, in ARhD 2 somewhat shorter, 0·4–0·6 × 1·5–5 μm, with a single polar flagellum (Fig. 1).

Growth characteristics of the isolated strains

The new isolates belonged to the obligate chemolithoautotrophic and haloalkaliphilic sulfur-oxidizing bacteria. They grew on mineral medium with thiosulfate and thiocyanate as electron donor under oxic and anoxic conditions using nitrate or nitrite as electron acceptor. Thiocyanate and ammonia, but not nitrate or nitrite (tested aerobically in the presence of thiosulfate as electron donor), could be used as nitrogen source.

The first attempts to grow the isolates anaerobically with thiocyanate and nitrate, nitrite or N_2O did not provide satisfactory results: e.g. only one culture attempt out of many resulted in stable growth. Optimization of the medium revealed that a slight pH decrease from 10 to 9·6–9·8 stabilized the culture, resulting in reproducible anaerobic growth of both strains with thiocyanate as electron donor and nitrate or nitrite (<5 mM) as electron acceptor. However, no growth was observed with N_2O as electron acceptor. Anaerobic growth with thiocyanate and nitrate was extremely slow compared to aerobic growth with thiocyanate and anoxic growth with thiosulfate plus nitrate, but the growth yield was only slightly lower (Table 1). Two parallel cultures of strain ARhD 1^T, with starting pHs of 9·6 and 9·8, demonstrated exponential growth, with concomitant thiocyanate and nitrate consumption (Fig. 2). CNO^- (up to 0·45 mM) and N_2O (0·1 mM) were detected as minor intermediates, while ammonia (65–75 % of the metabolized SCN^- nitrogen) and sulfate (90–95 % of the metabolized SCN^- sulfur) were the final products of anaerobic thiocyanate oxidation. During the exponential growth phase, the ratio of the consumed nitrate/thiocyanate was within the range 1·3–1·4. However, at the end of growth, incomplete nitrate reduction to nitrite

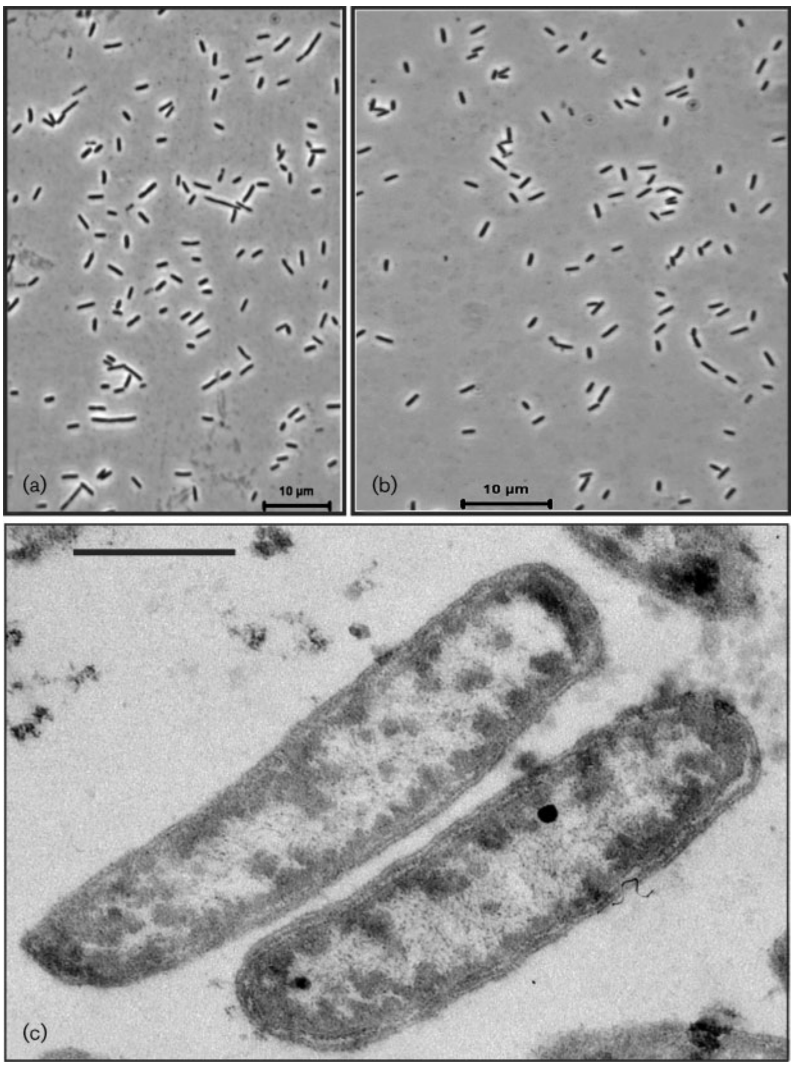

Fig. 1. Cell morphology of strains ARhD 1^T (a, c) and ARhD 2 (b), grown aerobically with thiocyanate at pH 9·8. (a, b) Phase-contrast photomicrographs; (c) electron micrograph of thin section (bar, 0·5 μm).

Table 1. Kinetic parameters of strains ARhD 1^T and ARhD 2 under oxic and anoxic growth conditions

μ, Specific growth rate; *Y*, growth yield; qO_2, respiration potential measured in washed cells suspended in sodium carbonate/bicarbonate buffer (pH 10, 0·6 M total Na^+); ND, not determined. Data represent the mean of duplicate experiments with deviations of less than 15 %. Parameters for anoxic growth are estimated for ARhD 1^T cultures.

Parameter	Oxic		Anoxic (with NO_3^-)	
	SCN^-*	$S_2O_3^{2-}$	SCN^-	$S_2O_3^{2-}$
μ (h^{-1})	0·032, 0·040	0·11, 0·14	0·006	0·02
Y (g protein mol^{-1})	5·5, 5·8	6·1, 5·9	4·2	5·8
qO_2 [nmol O_2 min^{-1} (mg protein)$^{-1}$]				
SCN^-	65, 97	0	50	0
$S_2O_3^{2-}$	45, 75	150, 135	65	70
HS^-	60, 100	160, 140	110	210
S_8	56, 90	90, 135	30	40
$S_4O_6^{2-}$	ND	55, 70	ND	ND

*Left-hand value, ARhD 1^T; right-hand value, ARhD 2.

and an increase of the consumed nitrate/thiocyanate ratio to 1·6–1·9 were observed. The maximum specific growth rate during the exponential phase was 0·006 h^{-1}. Accumulation of free ammonia as a product of thiocyanate degradation at high pH may be extremely toxic for these alkaliphilic autotrophs (Sorokin *et al.*, 2001a). Therefore, to check its effect, one of the parallel anaerobic cultures of strain ARhD 1^T was continuously flushed with argon to strip off the accumulating ammonia. Despite an almost complete removal of ammonia from the culture, no obvious growth stimulation was observed compared to the control culture grown without argon flushing. The anaerobic growth of both isolates with thiosulfate was much faster than growth on thiocyanate, but still much slower than that observed under aerobic conditions (Table 1). Similar to growth with thiocyanate, no intermediate nitrite production was observed in the anaerobic cultures grown with thiosulfate plus nitrate.

The aerobic cultures grown with thiocyanate accumulated up to 3 mM cyanate during oxidation of 19 mM thiocyanate. The bacteria did not grow below pH 8 aerobically with thiosulfate, and not below pH 9 with thiocyanate as electron donor. Aerobic growth with thiosulfate was possible between 0·3 and 2 M total Na^+ at pH 10. Thus, both isolates are moderately halophilic alkaliphiles.

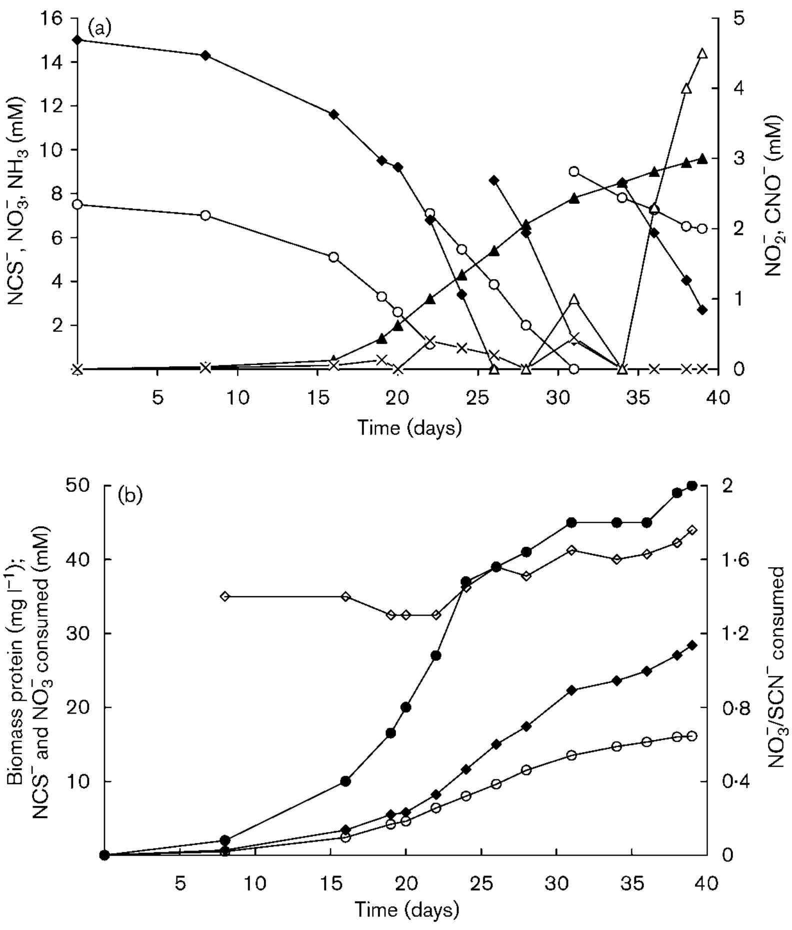

Fig. 2. Anaerobic growth of strain ARhD 1^T, with thiocyanate as electron donor and nitrate as electron acceptor, in fed-batch culture at pH 9·6. Symbols in (a): ○, SCN^-; ◆, NO_3^-; △, NO_2^-; ×, CNO^-; ▲, NH_3. Symbols in (b): ●, biomass protein; ◆, NO_3^- consumed; ○, SCN^- consumed; ◇, ratio of NO_3^-/SCN^- consumed. Graphs represent data of a single experiment.

Metabolic potential

Washed cells of ARhD 1^T and ARhD 2, grown with either thiocyanate or thiosulfate as substrate, were capable of aerobic oxidation of thiosulfate, sulfide, polysulfide, elemental sulfur and tetrathionate to sulfate. The thiocyanate-oxidizing capacity (thiocyanate-dependent oxygen uptake and thiocyanate consumption by washed cells) was induced only in the presence of thiocyanate. The aerobic activity of cells grown anaerobically, with thiocyanate or thiosulfate as substrate, was evidently repressed compared to that of cells grown in the presence of oxygen (Table 1).

Both strains expressed a high level of cyanase while growing with thiosulfate, thiocyanate or a combination of these substrates. The activity levels were within the range of 650–700, 1050–1700 and 650–870 nmol (mg protein min)$^{-1}$ in cells grown with thiosulfate plus ammonia, thiosulfate plus thiocyanate, or thiocyanate alone, respectively. The presence of active cyanase in the cells grown with thiocyanate as single energy and nitrogen source clearly differentiates the new isolates from the previously described aerobic thiocyanate-oxidizing alkaliphiles, which either completely lack cyanase activity or repress its production during growth with thiocyanate. (Sorokin *et al.*, 2001a, 2002).

The anaerobic activity (oxidation of thiocyanate and thiosulfate in the presence of nitrogen oxides) of the cells grown with thiocyanate plus nitrate was very low, ranging from 1·5 to 6 nmol SCN^- oxidized (min mg protein)$^{-1}$ with nitrate, nitrite or N_2O as electron acceptor. In contrast to the whole-cell activity with equally low nitrate- and nitrite-reduction potentials, the *in vitro* measurements of NAR–NIR activities with an artificial electron donor demonstrated the presence of a much greater NAR activity in comparison with NIR [79 and 4 nmol nitrite (min mg protein)$^{-1}$, respectively]. The pH optima for *in vitro* NAR and NIR activities were 7·0 and 9·0, respectively, suggesting that the two enzymes are located in different cellular compartments. Activity staining of native gels demonstrated the presence of two different polypeptides with NAR activity (apparent molecular masses of 150 and 230 kDa) and a 60 kDa NIR (data not shown).

Cytochrome spectra of cell extracts prepared from cells grown anaerobically with thiocyanate plus nitrate revealed several peaks typical of cytochrome cd_1-containing NIR, e.g. at 422 and 468 nm in the gamma region and 553, 613 and 667 nm in the alpha region (supplementary Fig. S1 at http://mic.sgmjournals.org). In the cells of strain ARhD 1^T grown aerobically with thiosulfate, cytochrome *c* and an unidentified cytochrome with the alpha- maximum at 585 nm were detected in the soluble fraction (supplementary Fig. S2a), and cytochromes *c*, *b* and aa_3-type in the membrane fraction (supplementary Fig. S2b).

Total protein profiles obtained from cells grown with different substrates demonstrated obvious differences from the previously described aerobic strain *Thialkalivibrio thiocyanoxidans* strain ARh 4 in the sizes of the polypeptides specifically expressed during growth with thiocyanate (Fig. 3). While strain ARh 4 overexpressed only a single polypeptide with an apparent molecular mass of 62 kDa, the new isolates had four specific bands with apparent masses of 59–61, 47–50, 28–29 and 17·5 kDa.

Identification of ARhD strains

The DNA G+C content of strains ARhD 1^T and ARhD 2 was 63·7 and 63·1 mol% (T_m), respectively. DNA–DNA hybridization between the two strains showed 65 % relatedness, suggesting membership of the same species. The phylogenetic analysis of strain ARhD 1^T on the basis of 16S rDNA sequence demonstrated that it belonged to the genus *Thialkalivibrio* (Fig. 4) in the gammaproteobacteria, with highest similarity to *Tv. denitrificans* (98·3 %),

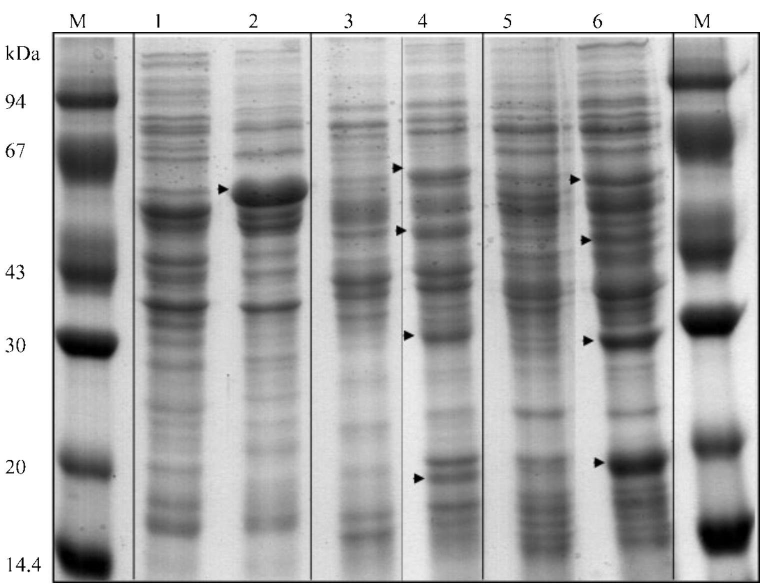

Fig. 3. Total protein profiles (10 % SDS-PAGE) of cell extracts from strain ARhD 1^T (lanes 3–4) and ARhD 2 (lanes 5–6) in comparison with the aerobic thiocyanate-oxidizing *Tv. thiocyanoxidans* strain ARh 4 (lanes 1–2). Lanes 1, 3 and 5, cells grown with thiosulphate; lanes 2, 4 and 6, cells grown with thiocyanate; M, molecular mass marker. Arrows indicate bands specific for thiocyanate-grown cells. Total amount of protein loaded was 30 μg per lane.

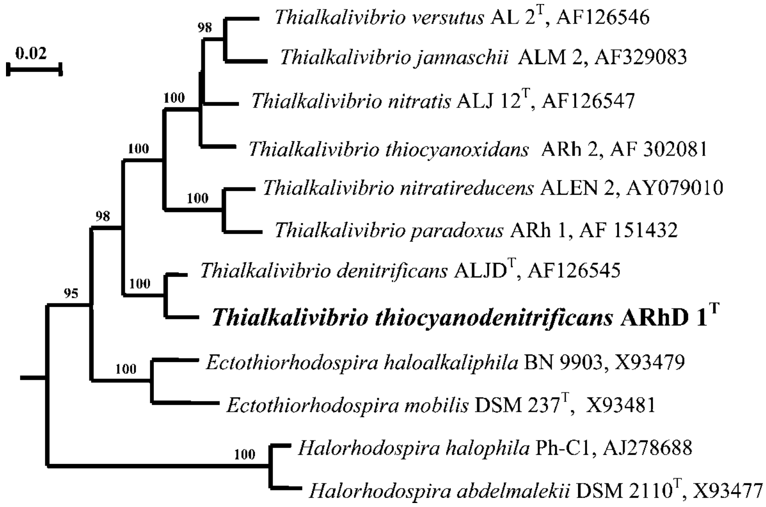

Fig. 4. Neighbour-joining phylogenetic tree demonstrating the position of strain ARhD 1^T within the genus *Thialkalivibrio* and its closest relatives in the gammaproteobacteria. Bootstrap values (expressed as percentage of 100 replications) are shown at branch points; values greater than 90 were considered as significant. The gaps were excluded from the analysis. Bar, 5 substitutions per 100 nt.

and much lower relatedness (93·9–94·2 %) to the other species of this genus. These results were in accordance with the DNA-hybridization data, which demonstrated 40 % relatedness between ARhD 1^T and the denitrifying species *Tv. denitrificans* ALJD, but only 15–19 % similarity with the aerobic thiocyanate-utilizing strain *Thialkalivibrio thiocyanoxidans* ARh 2 and the type strain of the genus *Thialkalivibrio versutus* AL 2.

DISCUSSION

The two described bacterial strains isolated from the soda-lake sediments are unique in two aspects. First, they have proved to be capable of anaerobic growth with thiocyanate under denitrifying conditions, a mode of metabolism which, to our knowledge, has never been thoroughly investigated. The anaerobic thiocyanate oxidation in the denitrifying isolates proceeded through the intermediate cyanate, similar to the aerobic thiocyanate-utilizing haloalkaliphiles of the genus *Thialkalivibrio* (Sorokin *et al.*, 2001a; 2002), with ammonia, sulfate and nitrogen gas as the final products:

$$5SCN^- + 8NO_3^- + H_2O \rightarrow 5SO_4^{2-} + 4N_2 + 5CNO^- + 2H^+$$

$$5CNO^- + 5HCO_3^- + 10H^+ \rightarrow 5NH_3 + 10CO_2$$

$$5SCN^- + NO_3^- + H_2O + 8H^+ + 5HCO_3^- \rightarrow 5SO_4^{2-} + 5NH_3 + 10CO_2 + 4N_2$$

CO_2 is not the true final product, since it is converted into HCO_3^-/CO_3^{2-} at pH 10.

According to the total reaction, 1·6 mol nitrate should be reduced to nitrogen gas per 1 mol thiocyanate oxidized to sulfate. Assuming that 10–20 % of the electrons are utilized for CO_2 reduction in anabolic reactions (Kelly, 1982; Visser *et al.*, 1997), the ratio must be 1·28–1·44 (mean 1·36). The actual stoichiometry observed in exponentially growing anaerobic ARhD 1^T cultures was very close to the theoretical values (see Fig. 2b). The extremely low specific growth rate of the anaerobic thiocyanate-utilizing cultures might indicate certain metabolic constraints in such metabolism. This is hardly surprising, since even in the presence of oxygen, instead of nitrate, as electron acceptor, the growth rate with thiocyanate in ARhD 1^T was six times lower than that with thiosulfate. However, thiosulfate and nitrate are effective substrates for sulfur-oxidizing bacteria and various denitrifiers, respectively. Therefore, organisms like strains ARhD 1^T and ARhD 2 might have a competitive advantage in using the anaerobic thiocyanate-oxidation reaction, when more effective substrates, such as suphide or thiosulfate, are not available. Of course, the question remains as to the availability of thiocyanate and nitrate in soda lakes. The relative ease of enrichment of aerobic thiocyanate-oxidizing alkaliphiles from soda-lake sediments (Sorokin *et al.*, 2001a) indirectly indicates the presence of the substrate. Nitrate production in soda lakes might be attributed to the presence of autotrophic, alkali-tolerant ammonia- and nitrite-oxidizing bacteria (Sorokin *et al.*, 1998, 2001c), although this assumption might be problematic for hypersaline lakes from which nitrifying bacteria cannot be enriched.

One of our major concerns about the possibility of anaerobic growth with thiocyanate under alkaline conditions was ammonia toxicity. However, there was no obvious growth stimulation upon removal of ammonia from the medium (see Fig. 2b). Both isolates were able to grow actively at ammonia concentrations above 5 mM, and were apparently less sensitive to free ammonia than the aerobic alkaliphilic strains (Sorokin *et al.*, 2001a). Their higher tolerance to ammonia was also reflected by the presence of high cyanase activity (producing ammonia from cyanate) in cells growing with thiocyanate. It has been shown previously for aerobic alkaliphiles that cyanase is specifically repressed when thiocyanate is utilized as energy source, to avoid toxic ammonia accumulation (Sorokin *et al.*, 2001a).

Another interesting aspect of the physiology of the new isolates is that they represent the first example of complete denitrifiers (capable of complete reduction of nitrate to nitrogen gas) among the haloalkaliphilic sulfur-oxidizing chemolithoautotrophs known so far. Our numerous attempts to find such bacteria in soda lakes using sulfide or thiosulfate as electron donor yielded partial denitrifiers, such as *Tv. denitrificans*, which started denitrification from nitrite and grew best with nitrous oxide as electron acceptor (Sorokin *et al.*, 2001d). When nitrate was used as the electron acceptor, enrichment either selected for nitrite-accumulating species or resulted in co-cultures of incomplete denitrifiers, consisting of nitrite-producing nitrate reducers and species similar to *Tv. denitrificans*, reducing nitrite to dinitrogen gas (Sorokin *et al.*, 2003). The major problem of autotrophic denitrification under haloalkaliphilic conditions seems to be the excessive nitrite accumulation, possibly due to the higher sensitivity of the (periplasmic) nitrite-reduction stage to extreme conditions (high pH/high salt) compared to nitrate reduction. The use of thiocyanate instead of thiosulfate as electron donor resulted in the selection of complete autotrophic denitrifiers from the soda lakes which, in contrast to the other haloalkaliphilic denitrifiers, did not accumulate nitrite during nitrate reduction. One of the possible explanations could be the extremely slow growth of the denitrifying strains with thiocyanate under anaerobic conditions, which might ensure a balance in the activity of NAR and NIR. While the *in vitro* measurements demonstrated a much higher (potential) NAR activity, the *in vivo* situation, measured with whole cells, showed low rates of both nitrate and nitrite reduction.

Apart from the possible ecological advantage, the potential for autotrophic growth with thiocyanate under denitrifying conditions might also be important in industrial treatment plants dealing with thiocyanate-containing wastewater, at least in the case of the simultaneous presence of thiocyanate and nitrate combined with oxygen limitation (Andreoni *et al.*, 1988). Usually, microbial degradation of thiocyanate in such plants results in the excessive production of ammonium, which is further oxidized to nitrate by nitrifying bacteria present in the activated sludge (Dictor *et al.*, 1997). In the case of low concentrations of organic electron donors and high residual thiocyanate, the additional anaerobic pathway might be useful to enhance thiocyanate degradation.

Overall, the quantitative data presented in this paper confirm the observation of De Kruyff *et al.* (1957) on the possibility of bacterial thiocyanate-dependent denitrification in neutrophilic obligate chemolithoautotrophic sulfur-oxidizing bacteria. The facultative anaerobic thiocyanate-utilizing haloalkaliphilic strains ARhD 1^T and ARhD 2, isolated from soda-lake sediments, can be regarded as a novel species of the genus *Thialkalivibrio* on the basis of their unique physiological and genetic properties. The name *Thialkalivibrio thiocyanodenitrificans* sp. nov. is proposed to accommodate these bacteria.

Description of *Thialkalivibrio thiocyanodenitrificans* sp. nov.

Thialkalivibrio thiocyanodenitrificans (thi.o.cya′n.o.de.ni.tri′fi.cans N.L. n. *thiocyanatum* thiocyanate; N.L. v. *denitrifico* denitrify; N.L. part.adj. *denitrificans* denitrifying; N.L. part.adj. *thiocyanodenitrificans* denitrifying on thiocyanate).

Cells are rod-shaped (0·5–0·7 × 1·5–5 μm), and motile by single polar flagella. Obligate alkaliphiles. Optimum pH for growth 9·6–10. Grow within a salinity range of 0·3–1·8 M total Na^+. Obligate chemolithoautotrophs. Differ from other *Thialkalivibrio* species by the ability to grow anaerobically with thiocyanate as sole energy source and nitrate or nitrite as electron acceptor. Produce cyanate as intermediate of thiocyanate oxidation. Also oxidize sulfide, thiosulfate, polysulfide, elemental sulfur and tetrathionate to sulfate. Thiocyanate and ammonia, but not nitrate, can serve as nitrogen source during growth with thiosulfate. DNA G + C content 63·1–63·7 mol% (T_m). Closest relative among the *Thialkalivibrio* species is *Tv. denitrificans*. Other properties as for the genus.

Isolated from the sediments of Egyptian and Siberian soda lakes. The type strain is ARhD 1^T (= UNIQEM 226^T).

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