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A new facultatively autotrophic hydrogen- and sulfur-oxidizing bacterium from an alkaline environment

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Abstract An alkaliphilic bacterium, strain AHO 1, was isolated from an enrichment culture with hydrogen at pH 10 inoculated with a composite sample of sediments from five highly alkaline soda lakes (Kenya). This bacterium is a gram-negative, nonmotile, rod-shaped, obligately aerobic, and facultatively autotrophic hydrogen-oxidizing organism. It was able to oxidize reduced sulfur compounds to sulfate during heterotrophic growth. It utilized a wide range of organic compounds as carbon and energy sources and grew mixotrophically with hydrogen and acetate. With sulfur compounds, mixotrophic growth was observed only in acetate-limited continuous culture. The normal pH range for autotrophic growth with hydrogen was pH 8.0–10.25, with a pH optimum at 9–9.5. Growth at pH values lower than 8.0 was extremely slow. Heterotrophic growth with acetate was optimal at pH 10.0. The hydrogen-oxidizing activity of whole cells was maximal at pH 9.0 and still substantial up to pH 11. NAD-dependent hydrogenase activity was found in the soluble fraction of the cell-free extract, but no methylene blue-dependent activity in either the soluble or membrane fractions was observed. On the basis of its pH profile, the soluble hydrogenase of strain AHO 1 was a typical pH-neutral enzyme. Phylogenetic analysis revealed that strain AHO 1 belongs to the α-3 subgroup of the *Proteobacteria* with a closest relation to a recently described alkaliphilic aerobic bacteriochlorophyll *a*-containing bacterium "*Roseinatronobacter thiooxidans*."

Key words Hydrogen · Hydrogen-oxidizing bacteria · Alkaliphilic · Sulfur-oxidizing bacteria · Soda lakes · Hydrogenase

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Introduction

Gaseous hydrogen plays an important role as an electron donor for both anaerobic and aerobic bacteria, such as acetogenic, methanogenic, sulfate-reducing, ferric iron-reducing, denitrifying, and aerobic hydrogen-oxidizing bacteria (Zeikus 1983; Aragno and Schlegel 1994). Aerobic hydrogen-utilizing bacteria were isolated mostly from neutral environments (Aragno and Schlegel 1994). Among the neutrophilic species, salt-tolerant and thermophilic species are known (Schink et al. 1983; Aragno 1992).

Natural alkaline environments with a high salt content, such as soda lakes, have been rarely investigated by microbiologists except a few studies on phototrophic and sulfate-reducing bacteria (Isachenko 1951; Abdel Malek and Rizk 1963; Imhoff et al. 1979). During the past few years, however, these environments have received more attention because of industrial needs for microbial enzymes from alkaliphilic bacteria, represented mainly by the alkaliphilic nonhalophilic *Bacillus* and halotolerant *Halomonas* species (Horikoshi 1996; Duckworth et al. 1996). Recently, investigation of bacteria important in biogeochemical cycling in soda lakes has begun (Zavarzin 1993). In particular, various groups of alkaliphilic hydrogen-utilizing secondary anaerobes have been found in soda lakes, such as sulfate-reducing, acetogenic, and methanogenic alkaliphiles (Zhilina et al. 1997; Zavarzin et al. 1996; Blotevogel et al. 1985; Worakit et al. 1986), hydrogen-dependent sulfate reduction being the most important anaerobic sink for hydrogen (Zavarzin et al. 1996). However, thus far there has been no available information concerning aerobic hydrogen-oxidizing bacteria in alkaline environments.

This article describes the isolation of a new facultatively autotrophic aerobic hydrogen-oxidizing bacterium from

a soda lake environment. The bacterium is alkaliphilic, although its NAD-dependent hydrogenase is located in the cytoplasm and has a neutral pH optimum.

Materials and methods

Samples

Two composite samples were used to enrich for alkaliphilic hydrogen-oxidizing bacteria. One was constructed from five subsamples of the surface sediments from Kenyan soda lakes (water pH and salinity are between 10 and 10.5 and 20 and 200 g/l, respectively). Another sample was composed from nine subsamples of Siberian soda lakes (Kunkur steppe in southeast Siberia) with pH and salinity ranges of 9.5–10.2 and 5–40 g/l, respectively. Samples were collected during two expeditions in 1996 and kept at 4°C before use.

Enrichment and isolation procedures

The enrichments were performed in 100-ml bottles with butyl rubber stoppers and 20 ml of mineral base medium. The mineral base included Na_2CO_3, 20 g/l; $NaHCO_3$, 10 g/l; NaCl, 5 g/l; K_2HPO_4, 0.5 g/l; KNO_3, 5 mM; $MgSO_4 \cdot 7H_2O$, 0.5 mM; trace elements (Pfennig and Lippert 1966), 2 ml/l; and a final pH of 10.1 after sterilization (20 min at 120°C). The headspace was filled with an air-H_2 mixture at 1:1 ratio with 20 kPa overpressure. The enrichment cultures were incubated at 30°C on a rotary shaker at 100 rpm. After several successful passages, a serial dilution was made and a culture from the last positive dilution was used for the isolation of a pure culture from single colonies, which appeared on mineral agar at pH 10 during prolonged incubation under air-H_2 atmosphere. Single colonies were then transferred into mineral liquid medium, and those cultures exhibiting vigorous growth with hydrogen were selected. Purity of the selected clones was checked by repeating passages onto solid medium and by light microscopy.

Culture conditions and media composition

Routine batch cultivation was conducted in closed ("anaerobic") jars mounted on a rotary shaker under an air-H_2 atmosphere (1:1) with overpressure from 20 to 50 kPa, using the mineral base medium already described. Carboxydotrophic potential was tested on mineral medium at pH 10 with 5%–50% (v/v) of CO and 10% (v/v) of O_2 in the gas phase. For heterotrophic conditions, sodium acetate (20 mM) and yeast extract (0.1 g/l) were added to the mineral base after sterilization. Yeast extract was not absolutely required, but it stimulated growth with acetate. Cultures were incubated aerobically. Media with different pH were prepared on the basis of 0.1 M HEPES-NaOH (pH 7–8) supplemented with 0.6 M NaCl or on the basis of sodium carbonate–sodium bicarbonate (pH 9–10.5) with 0.6 M total Na^+. Buffers were supplemented with KNO_3, K_2HPO_4, and trace elements before being filter sterilized. Growth of bacteria with hydrogen under pH-controlled conditions was performed in a 1.5-l laboratory fermentor with 1 l working volume and equipped with pH and pO_2 control (Applicon, Schiedam, Netherlands). pO_2 was controlled by the stirring speed at 20% air saturation. H_2 was sparged into the liquid phase at 20 ml/min.

The same equipment was used for the continuous cultivation of the heterotrophically grown bacterium. In this case, medium was supplied from the two reservoirs containing basic and acidic components, respectively. When sulfide was used as an additional substrate, it was added to the basic medium, which was kept anaerobic by means of continuous argon flushing. The maximal specific growth rate at different pH values was calculated from the dynamics of biomass washout. Ability for anaerobic growth under denitrifying conditions was tested at pH 10 using acetate or H_2 as electron donors and nitrate, nitrite, and N_2O as electron acceptors. The medium was made anaerobic by argon flushing for 20 min. Methylotrophic potential was examined at pH 10 using methanole (2 ml/l) or formate (20 mM) as substrates. Growth with various organic compounds (10–20 mM) was tested at pH 10 in the presence of 0.1 g/l of yeast extract. Culture grown only with yeast extract background served as a control. All growth experiments were performed in duplicates. Results represent averaged data.

Experiments with washed cells

The kinetic parameters for the oxidation of various substrates and the pH activity profiles were determined using cell suspensions obtained from batch cultures grown with the different substrates at pH 10. The respiratory activity was measured with a Clark type oxygen electrode in a 5-ml thermostatted cell (Yellow Spring Instruments Inc., Yellow Spring, OH, USA). In these measurements hydrogen was supplied by adding 1 ml of a H_2-saturated buffer to a 4-ml cell suspension. Elemental sulfur was supplied as a saturated acetone solution at a final concentration of 70 μM. Control experiments demonstrated that the amount of acetone added did not influence respiratory activity. For pH activity profiles, the same buffers were used as for growth experiments, namely, HEPES-NaOH + 0.6 M NaCl for the pH range 6.0–7.0 and sodium carbonate–sodium bicarbonate buffer (total Na^+, 0.6 M) for the pH range 8.0–11.0. All buffers were supplemented with 50 mM KCl.

Enzyme activity

To measure the hydrogenase activity, cells grown with hydrogen at pH 10 were harvested by centrifugation, washed with sodium carbonate buffer, pH 10, resuspended in 0.1 M Tris-HCl, pH 8.0, and disrupted by sonication. After removal of unbroken cells by centrifugation, the cell-free extract was separated into soluble and membrane fractions by ultracentrifugation at 144 000 × g for 3 h. NAD-dependent

and methylene blue-dependent hydrogenase activities were assayed as described by Aragno and Schlegel (1994) using H_2-saturated HEPES or Tris-HCl buffers with or without NaCl for the pH range 6–7 and 8–10, respectively. For the pH range 8–10, the sodium carbonate–sodium bicarbonate buffer was also used. In the case of NAD-dependent activity, the reaction was followed by H_2-dependent NADH formation measured at 340 nm at a NAD concentration of 1 mM; H_2-dependent methylene blue reduction was measured at 590 nm. Buffer (2.5 ml) supplemented with 0.2 M methylene blue was placed into a 3-ml anaerobic quartz cuvette, sparged with N_2 for 5 min, then evacuated and sparged with H_2 for another 5 min. The reaction was started by the addition of cell-free extract or its fractions. In control incubations, the H_2 sparging step was omitted.

RuBPcase activity in crude cell extract was determined with radiolabeled $NaHCO_3$ according to Romanova (1980) in Tris-HCl, pH 8.0, supplemented with 50 mM Mg^{2+} and 50 mM dithiotreitol. The final concentration of $NaHCO_3$ was 5 mM, the final ^{14}C-labeled $NaHCO_3$ was 10 μCi/ml, and RuBP, 0.25 mM. The activity was calculated from the initial linear period of increase in radioactivity of HCl-insoluble material.

Analysis

Thiosulfate and tetrathionate were determined by cyanolysis (Kelly et al. 1969); sulfide, colorimetrically according to Trüper and Schlegel (1964), and sulfate by HPLC. Biomass protein was estimated by the Lowry method after removal of interfering reduced sulfur species (soluble compounds by washing with 0.6 M NaCl solution and elemental sulfur by an acetone extraction). DNA extraction, purification, and the subsequent determination of the DNA base composition were performed according to Marmur (1961). DNA–DNA hybridization was performed according to De Ley et al. (1970).

The absorption spectra in the soluble and membrane fractions of the cell extract were recorded using a UV-visible diode-array HP 8453 spectrophotometer. To test for the possible presence of bacteriochlorophyll, wet cell biomass, grown either autotrophically with hydrogen or heterotrophically with acetate, was extracted by a 7:3 mixture of methanol/acetone. SDS-PAGE of the total proteins was performed using a Mini-Gel system (Pharmacia). Cell extracts were prepared from the cells grown under different conditions. Proteins were denatured by boiling for 5 min in sample buffer with 5% (w/v) 2-mercaptoethanol.

Electron microscopy

Intact cells were fixed with formaldehyde (2.5% final, w/v) and then were positively stained with 1% (w/v) phosphotungstic acid. Samples for ultrathin sectioning were postfixed with OsO_4 + 2% (w/v) NaCl, dehydrated, and embedded into the resin. Thin sections were stained with uranyl acetate and lead citrate.

16S *r*DNA sequence and phylogenetic analysis

The 16S *r*DNA sequence analysis was performed and the results were interpreted by the BCCM™/LMG (Bacterial Collection of Gent University) identification service. The genomic DNA was extracted and purified according to the protocol of Pitcher et al. (1989). The 16*S* rRNA gene was amplified by PCR using primers 16F38 and 16R1522 (positions 19–38 and 1541–1522; *E. coli* numbering system). Products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The complete sequence was determined using a DNA sequencer (model 377; Perkin Elmer, Applied Biosystems, Foster City, CA, USA) and the ABI PRISM dye terminator cycle sequencing ready reaction kit (with AmpliTaq DNA polymerase, Fs). For phylogenetic analysis, the sequence was aligned by using the sequence library of EMBL. The resulting tree was constructed using the neighbor-joining method.

Results

Enrichment and isolation

One of the two enrichment cultures inoculated with the mixed sample from Kenyan soda lakes started to grow with hydrogen at pH 10 after prolonged incubation (3 weeks). No growth was obtained with the sample from Siberian soda lakes. The positive culture was further enriched by several passages through 1:100 dilutions. A pure culture was isolated using serial dilution followed by plating of the final positive dilution onto mineral agar at pH 10. After 2 weeks incubation under air-H_2 atmosphere, two types of dominant colonies were found to be able to grow with hydrogen at pH 10. One isolate formed spreading and transparent colonies, and another one produced refractive and more compact colonies. Although different in macromorphology, the two isolates were identical in their cell morphology and total protein profiles. DNA–DNA hybridization experiments indicated 95% similarity. Therefore, only one of the isolates, strain AHO 1, has been studied in detail. It was deposited with LMD culture collection (Delft, Netherlands) under the number LMD 97.164.

Morphology

The cells of isolate AHO 1 are small pear- or lemon-shaped rods with tapered ends, 0.5 by 0.8–1 μm, and nonmotile. The cells are often arranged in multiple, starlike aggregates, especially in autotrophic cultures. Cells in such aggregates are bound together by a netlike matrix (Fig. 1a). Electron microscopy of ultrathin sections revealed that it has a gram-negative cell-wall structure. Cells grown under heterotrophic conditions (Fig. 1b) had a much more dense cytoplasm than cells grown autotrophically with hydrogen (Fig. 1a). The latter were also surrounded by an additional surface layer of amorphous material. Carboxysomes in

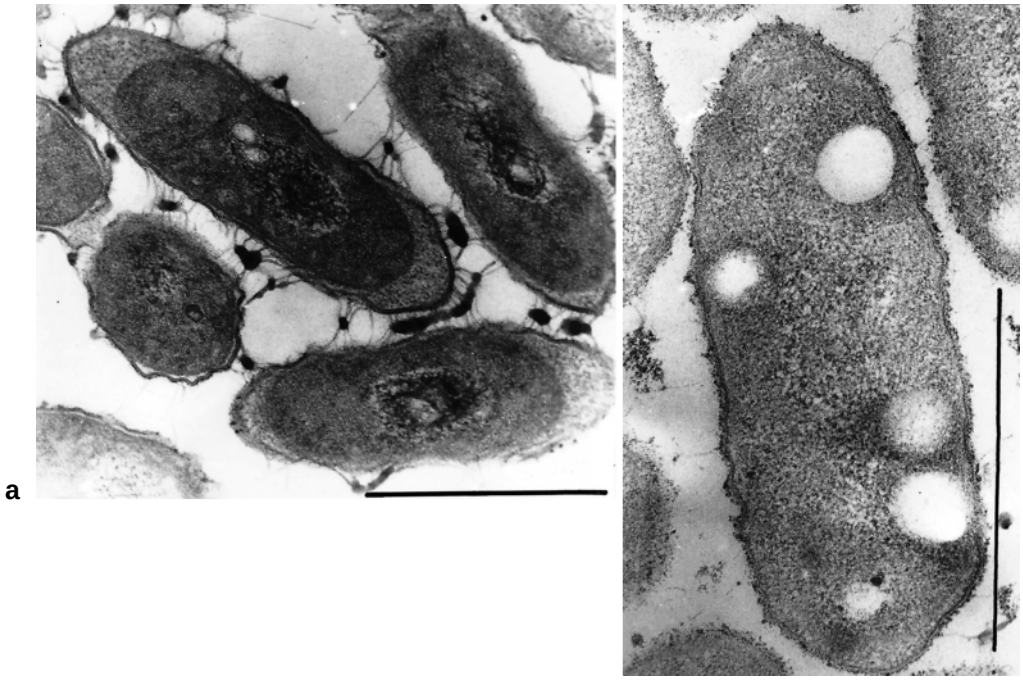

Fig. 1a,b. Morphology of strain AHO 1 grown autotrophically with hydrogen (**a**) and heterotrophically with acetate + yeast extract (**b**) at pH 10, shown by electron photomicrographs of the thin sections. *Bar*, 1 μm

autotrophically grown cells were not observed. Heterotrophically grown cells accumulated large amounts of PHB-like granules (Fig. 1b). No pigments were observed in methanol/acetone extract of the cells of AHO 1 grown in different media.

Metabolism

Strain AHO 1 is an obligately aerobic bacterium able to grow at high pH and employing three types of metabolism. It grows chemolithoautotrophically with hydrogen (but not with CO) as energy source, chemoorganoheterotrophically with organic acids and sugars, and mixotrophically, in particular with hydrogen as energy source and acetate as energy and carbon source. Moreover, this bacterium is able to oxidize thiosulfate, sulfide, polysulfide, and elemental sulfur to sulfate while growing heterotrophically. Heterotrophic growth was stimulated by the addition of yeast extract, but it was not absolutely required. A capability for carboxydotrophic and methylotrophic growth and denitrification were not observed under the experimental conditions used.

Autotrophic growth with hydrogen

In batch culture under autotrophic conditions, strain AHO 1 always started to grow after a lag phase of 1–3 days. Incubation of the flasks in closed jars and introduction of 0.1% of CO_2 into the gas phase decreased the lag phase and stimulated the growth of cultures. During growth, the pH dropped from 10 to 9.2–9.5. The addition of an extra amount of Ni^{2+} to the basic trace elements had no effect on the final growth yield, which reached maximally 600 mg of protein per liter. Autotrophic growth at pH 10 was Na^+ dependent with an optimum at 0.5 M and a tolerance up to 1 M total sodium ion. The temperature optimum for the growth was at 30°C.

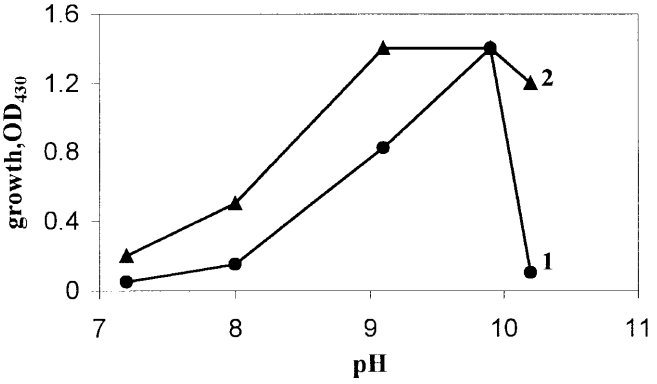

Fig. 2. pH profile for growth of strain AHO 1. *1*, Autotrophic growth with hydrogen (24 h after 48-h lag phase); *2*, heterotrophic growth with 20 mm/l of acetate + 0.1 g/l of yeast extract (24 h). The medium of pH 7–8 was buffered with HEPES and included 0.6 M NaCl; the medium of higher pH values was buffered with sodium carbonates (total Na^+, 0.6 M)

The pH profile of autotrophic growth was typical for alkaliphilic bacteria with extremely slow growth at neutral pH and an optimum at pH 9.5–9.8 (Fig. 2). pH values higher than 10 were difficult to maintain during routine cultivation. Even when highly buffered with sodium carbonate, the medium gradually acidified, presumably because of absorption of atmospheric CO_2. Therefore, to confirm the ability of strain AHO 1 to grow autotrophically at pH 10 and to find an upper pH limit, its cultivation with hydrogen was

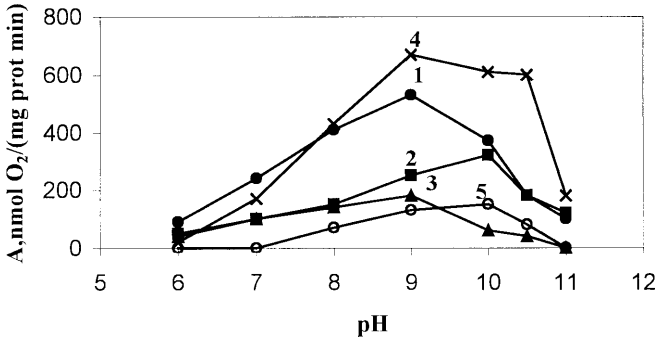

Fig. 3. pH activity profile for oxidation of various substrates by washed cells of AHO 1, grown at pH 10. *A*, Respiration activity; *1*, H_2 oxidation by cells grown autotrophically with hydrogen; *2*, acetate oxidation by cells grown heterotrophically with acetate + yeast extract; *3*, thiosulfate oxidation by cells grown with acetate + thiosulfate; *4*,*5*, sulfide and elemental sulfur oxidation by cells grown in acetate-limited continuous culture with sulfide. Buffers: pH 6.0–7.0, 0.1 M HEPES-NaOH + 0.6 M NaCl; pH 8.0, 0.6 M $NaHCO_3$, freshly prepared; pH 9.0–11.0, Na_2CO_3-$NaHCO_3$, 0.6 M Na^+

performed under pH-controlled conditions in chemostat. The results revealed that the bacterium is able to grow autotrophically with hydrogen at pH values up to 10.25. The maximum specific growth rates at pH 10.03, 10.12, and 10.25 were 0.09, 0.03, and 0.01 h^{-1}, respectively. Washed cells, grown with hydrogen at pH 10, exhibited maximal hydrogen-oxidizing capacity at pH 9.0 and were still relatively active up to pH 11.0 (Fig. 3).

Heterotrophic and mixotrophic growth

Strain AHO 1 grew rapidly (μ_{max}, 0.4 h^{-1}) with acetate as carbon and energy source at pH 10 with the addition of yeast extract (0.05–0.1 g/l). In the absence of yeast extract there was a prolonged lag phase and the growth was also slower (μ_{max}, 0.15–0.20 h^{-1}). Nevertheless, the cultures reached almost the same final biomass level as in the presence of yeast extract. The pH profile for growth (Fig. 2) and acetate oxidation by washed cells (Fig. 3) differed from the patterns obtained for autotrophic growth, in being shifted to the more alkaline region. In the presence of acetate + yeast extract, the bacterium grew optimally at a total sodium concentration of 1 M and tolerated up to 2 M.

Strain AHO 1 can grow heterotrophically with acetate, pyruvate, glycolate, fumarate, succinate, propionate, citrate, lactate, malate, glycerol, mannitol, xylitol, D-glucose, maltose, D-mannose, D-fructose, sucrose, cellobiose, trehalose, L-arabinose, D-galactose, D-melibiose, D-xylose, D-ribose, and glucosamine. When heterotrophically grown cultures were incubated in the presence of hydrogen, the biomass yield increased before acetate exhaustion, implying that mixotrophic growth took place when the bacterium utilized hydrogen as an additional energy source. After complete acetate utilization (HPLC test), cultures continued to grow autotrophically with hydrogen (Table 1).

Table 1. Mixotrophic growth of the facultatively chemolitho-autotrophic H_2-utilizing alkaliphilic strain AHO 1

Acetate, mM (+YE, 0.1 g/l)	Biomass, mg prot/l			
	Without H_2		With H_2	
	20 h	40 h	20 h	40 h
–	–	–	35	105
1	30	30	61	120
2	40	46	75	155
5	65	73	115	230

Organisms were grown in batch culture with acetate + yeast extract (YE) and with or without H_2 at pH 10; differences between duplicates did not exceed 10%

Oxidation of sulfur compounds

Strain AHO 1 oxidized thiosulfate to sulfate during heterotrophic growth in batch cultures with acetate at pH 10. Intermediate sulfur compounds (sulfur, sulfite, tetrathionate) were not produced during the thiosulfate oxidation, which started only near the end of active growth, i.e., close to the stationary phase (data not shown). A batch culture grown with 20 mmol/l of acetate, 20 mmol/l thiosulfate, and 0.1 g/l of yeast extract at pH 10 oxidized up to 12 mmol/l of thiosulfate during 30 h of the stationary phase. The final biomass yield was the same with and without thiosulfate. The bacterium was not capable of autotrophic growth with thiosulfate as the energy source. To examine the capability of strain AHO 1 to use sulfur compounds as an energy source, the bacterium was grown in acetate-limited continuous culture at pH 10. This culture induced very low thiosulfate-oxidizing potential on addition of 5 mM of thiosulfate. On the other hand, sulfide was much more efficient as cosubstrate: it increased both the specific biomass yield and the specific oxidizing potential of the cells (Table 2). The acetate + sulfide-grown cells were able to oxidize sulfide, polysulfide, elemental sulfur, and thiosulfate, but not tetrathionate or sulfite. Under these conditions, the bacterium exhibited the highest sulfur-oxidizing potential, especially for sulfide and polysulfide (Table 2). The stoichiometry of oxygen consumption during oxidation of the respective sulfur compounds by washed cells corresponded to the complete oxidation to sulfate. The reason for the low efficiency of the thiosulfate oxidation as compared to sulfide as an additional electron donor during organotrophic growth can possibly be explained by the difference in the pH optimum and the activity of their conversion (Fig. 3).

When thiosulfate was added to a batch culture grown autotrophically with hydrogen, it was oxidized parallel to growth. In this case, a thiosulfate-dependent increase of the specific growth yield was observed (Fig. 4), implying that AHO 1 can grow mixolithotrophically with two different inorganic electron donors. A likely explanation why, in this case, thiosulfate is used more efficiently than in acetate-limited continuous culture is that at the relatively high growth rate (0.1 h^{-1}) on acetate in chemostat, catabolic

Table 2. Influence of sulfur compounds on biomass yield and oxidizing potential of the alkaliphilic strain AHO 1 in acetate-limited continuous culture (Acetate, 10 mM; pH 10.05; 30°C; pO_2, 50% air saturation; D, 0.1 h^{-1})

Sulfur compound (mM)	Biomass[a] (mg prot/l)	Sulfur compound oxidized (mM)	Oxidizing potential at pH 10 nmol O_2/(mg prot·min)				
			Acetate	$S_2O_3^{2-}$	HS^-	S_6^{2-}	S_8
Thiosulfate, 0.1[b]	125	0.1	325	15	80	nd	nd
Thiosulfate, 5.0	132	2.5	320	80	100	nd	60
Sulfide, 4.5	145	4.5[c]	350	100	610	780	250

nd, not determined; S_6^{2-}, polysulfide; S_8, elemental sulfur
[a] Average data from 3 determinations at steady state (after 5 volume changes)
[b] Culture with 0.1 mM thiosulfate was used as a reference for possible growth stimulation by thiosulfate as a source of reduced sulfur
[c] No intermediate sulfur compounds (S_8, $S_2O_3^{2-}$, polythionates) were detected in the effluent

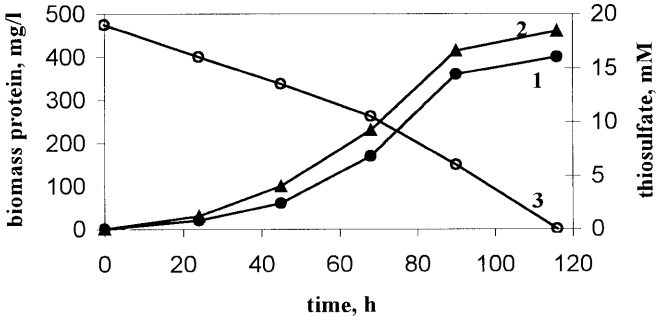

Fig. 4. Mixolithotrophic growth of strain AHO 1 with hydrogen and thiosulfate (18 mM) at pH 10. *1*, Growth without thiosulfate; *2*, growth with thiosulfate; *3*, thiosulfate concentration

repression of thiosulfate utilization may have occurred (Gottschal and Kuenen 1980a).

Nitrogen assimilation

Both hydrogen- and acetate-grown cultures utilized ammonium salts, nitrate, nitrite, urea, and organic nitrogen compounds (yeast extract, peptone) as a nitrogen source. Strain AHO 1 was not able to fix N_2 while growing with H_2 at 50% air saturation.

Biochemical properties

Hydrogenase activity

A NAD-specific hydrogenase activity was found in the soluble fraction of cell extract prepared from the autotrophically grown cells. Methylene blue-linked activity was absent in both the soluble and membrane fractions. NAD-specific activity was neutral in its pH profile, and it was inhibited by sodium carbonate (data not shown). The maximum NAD-specific activity values measured in Tris-HCl buffer at pH 8.0 were within the range of 1.0–1.2 μmol NADH mg prot^{-1} min^{-1}.

Ribulose bisphosphate carboxylase activity

Using cells grown autotrophically with hydrogen at pH 10, the ribulose bisphosphate-dependent carbon dioxide fixation rate, measured in cell-free extract at pH 8.0 in Tris-HCl buffer, was maximally 40 nmol CO_2 fixed mg prot^{-1} min^{-1}. This result means that during autotrophic growth the bacterium employed the Calvin cycle for CO_2 assimilation.

Cytochrome composition

In the membrane fraction of a cell extract prepared from autotrophically grown cells, cytochromes *b* and *c* were spectroscopically identified (data not shown). Cytochromes c_{551} and b_{557} were high potential (reduced by ascorbate), and cytochrome b_{560} was low potential (reduced only by dithionite). A membrane-bound cytochrome *b* reacted with CO. The amount of cytochromes in cells grown with hydrogen was about double that in heterotrophically grown cells, as estimated from the absorption maxima in the alpha region.

DNA base composition

The G + C content in DNA of strain AHO 1 was 63 ± 0.5 mol% as determined by a thermal denaturation analysis.

Phylogenetic analysis

The complete sequence analysis of 16*S* rDNA of strain AHO 1 allowed the determination of its phylogenetic position within the α-3 subgroup of the *Proteobacteria* with the highest similarities (about 97% sequence homology) to a recently described alkaliphilic aerobic bacteriochlorophyll *a*-containing bacterium "*Roseinatronobacter thiooxidans*" strain ALG 1 (Fig. 5). The DNA–DNA hybridization experiment demonstrated 50% homology between two alkaliphilic strains. The 16*S* rDNA sequence data of strain AHO 1 has been deposited in the EMBL and GenBank databases with the accession number AJ132383.

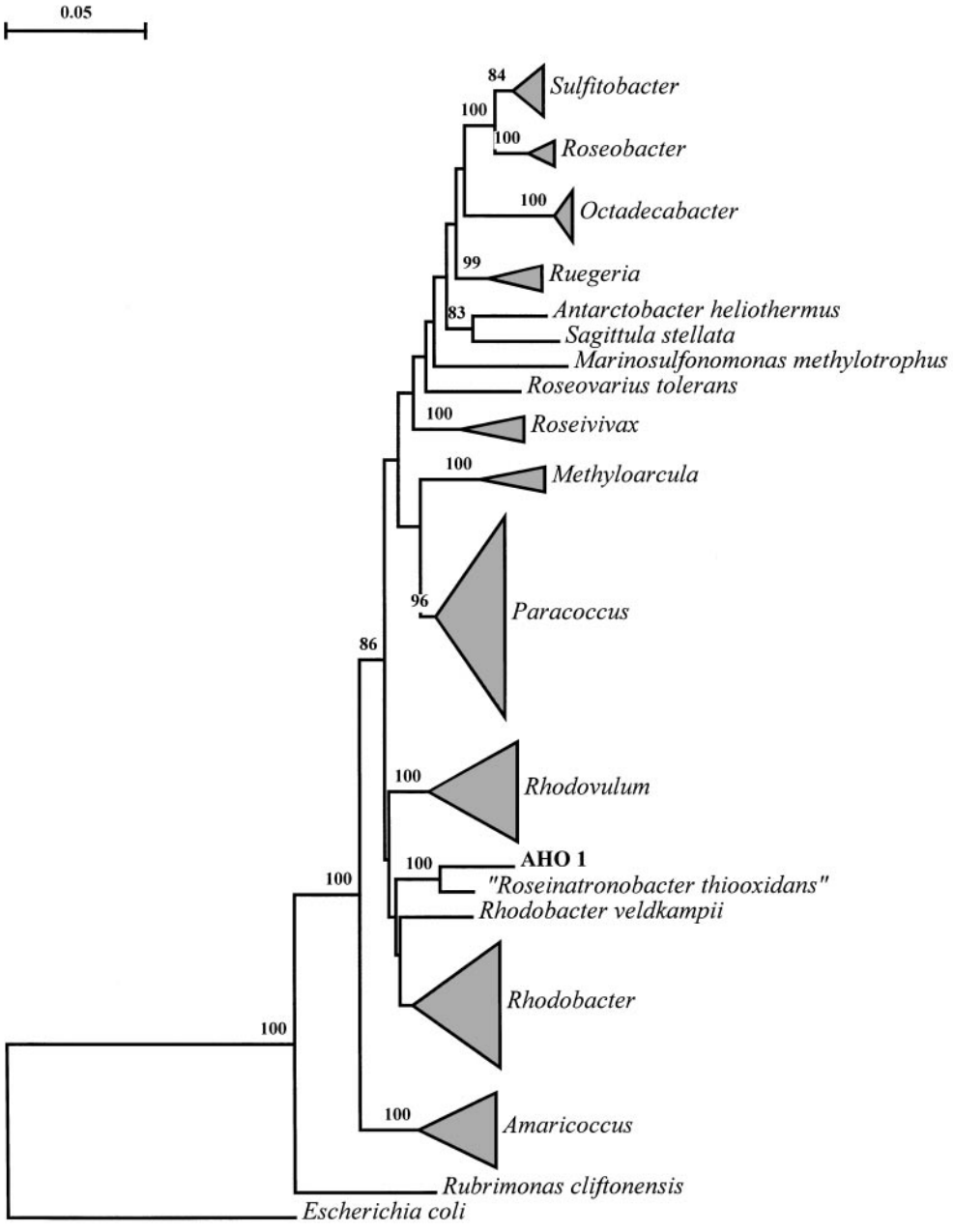

Fig. 5. A phylogenetic tree showing the position of the alkaliphilic hydrogen-utilizing strain AHO 1 inside the α-3 subgroup of the *Proteobacteria*

Discussion

The novel isolate AHO 1 represents a first example of an alkaliphilic "Knallgas" bacterium. Based on its hydrogenase type, it belongs to a relatively rare group, containing soluble NAD-dependent enzymes only. This group includes "*Alcaligenes ruhlandii*," *A. xylosooxidans*, and *Nocardia opoca* (Aragno and Schlegel 1994). It might be argued that an alkaliphilic bacterium should rather contain a membrane-bound hydrogenase exposed to the periplasm, to be active at high pH, because it is difficult to imagine how a bacterium with intracellular and neutrophilic key catabolic enzymes would manage to compete for hydrogen with other hydrogen-utilizing alkaliphiles. Because whole-cell hydrogen-dependent oxygen consumption was optimal in the alkaline region, it is evident that the respiratory chain of AHO 1 is working optimally under alkaline conditions. As a heterotroph, strain AHO 1 possesses the typical characteristics of alkaliphiles with an optimum for growth and oxidizing activity at pH values around 10. Given its soluble hydrogenase, it appears that AHO 1 represents a heterotrophic alkaliphilic bacterium that can gain some benefit from supplementary H_2 oxidation. However, there might be selective conditions (such as those created in the enrichment culture) when such bacteria could outcompete obligately heterotrophic alkaliphiles due to their ability to grow autotrophically and mixotrophically (Gottschal and

Table 3. Comparison of phenotypic properties of hydrogen-oxidizing alkaliphile strain AHO 1 and aerobic bacteriochlorophyll *a*-containing alkaliphile "*Roseinatronobacter thiooxidans*" strain ALG 1

Property	AHO 1	ALG 1
Carothenoids and bacteriochlorophyll *a*	−	+
Hydrogenase and growth with hydrogen	+	−
RBPCase and autotrophic growth	+	−
Oxidation of sulfur compounds	+	+
Lithoheterotrophy with thiosulfate	−	+
Lithoheterotrophy with sulfide	+	nd
μ_{max} in acetate-limited chemostat culture at pH 10	$0.40\,h^{-1}$	$0.11\,h^{-1}$
pH optimum for acetate-grown culture	10	10
Maximum concentration of total Na^+ during growth with acetate	2 M	2 M
Cytochrome aa_3	−	+
Content of G + C in DNA, mol%	61.5 ± 0.5	63.0 ± 0.5
Source of isolation	Soda lake in Kenya	Soda lake in Siberia

From Sorokin et al. (2000)

Kuenen 1980b) at moderately alkaline pH values. Such conditions may exist at the interface of the anaerobic hydrogen-producing sediments and the oxygen-containing waters.

During autotrophic growth of AHO 1 on hydrogen, the NADH required for CO_2 reduction can be provided directly by its NAD-dependent hydrogenase, without the need for reverse electron transport. In contrast, hydrogen-oxidizing bacteria containing a membrane-bound hydrogenase require a reverse electron transport system to reduce NAD^+. If such organisms are also able to oxidize sulfur compounds, the presence of such a reverse electron transport system might permit sulfur-dependent autotrophic growth. Thermophilic hydrogen bacteria from the genus *Hydrogenobacter* and some mesophilic hydrogen-oxidizing species represent an example of such a double chemolithotrophy (Friedrich and Mitrenga 1981; Aragno 1992). Against this background it may be speculated that the inability of AHO 1 to grow autotrophically on sulfide may result from the absence of a mechanism for reverse electron transport, which would be required when sulfur compounds serve as electron donor. The phylogenetic position of strain AHO 1 within the α-3 subgroup of alpha-*Proteobacteria* is not surprising, given the fact that many facultative and lithoheterotrophic species oxidizing sulfur compounds and hydrogen are the members of this subgroup. Such aerobic and facultatively anaerobic species, such as *Paracoccus* spp. and obligately heterotrophic sulfur-oxidizing lithoheterotrophs (Sorokin and Lysenko 1993), are phylogenetically clustered with the anaerobic phototrophic bacteria of the genus *Rhodobacter* and with aerobic bacteriochlorophyll *a*-containing erythrobacteria. One might speculate that versatile hydrogen + sulfur- oxidizing aerobic bacteria, like the alkaliphilic strain AHO 1 described here, may represent a direct aerobic descendant of the nonsulfur purple bacteria.

The most specific relative of strain AHO 1 is a recently described alkaliphilic aerobic bacteriochlorophyll *a*-containing bacterium, "*Roseinatronobacter thiooxidans.*" The latter was isolated from the Siberian soda lake as a sulfur-oxidizing lithoheterotroph (Sorokin et al. 2000). Together, these two alkaliphilic bacteria form a separate cluster located between genera *Rhodobacter* and *Rhodovulum* (see Fig. 5). High levels of 16*S* rDNA sequence similarity and of DNA–DNA homology imply that these alkaliphilic strains may belong to the same genus. However, substantial phenotypic differences (see Table 3) do not allow affiliating strain AHO 1 together with "*R. thiooxidans*." To our knowledge, there is no precedent of putting together in one genus colorless species and those containing bacteriochlorophyll. Therefore, a formal taxonomic description of strain AHO 1 at this moment seems premature. Most probably, the hydrogen-oxidizing alkaliphile AHO 1 represents a separate genus, closely related to "*Roseinatronobacter.*" Indeed, it may reflect a specific evolutionary situation within the α-3 subgroup in which mutations of the phenotypically most important genes have led to key changes in the phenotype whereas the total genetic makeup still has high similarities to the relatives. In such cases, thorough characterization of the phenotype is absolutely necessary for correct taxonomic affiliation of the organism.

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