Oxidation of Thiosulfate by a New Bacterium, *Bosea thiooxidans* (strain BI-42) gen. nov., sp. nov.: Analysis of Phylogeny Based on Chemotaxonomy and 16S Ribosomal DNA Sequencing

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A gram-negative bacterium which was capable of oxidizing reduced inorganic sulfur compounds was isolated from agricultural soil and designated BI-42. This new isolate grew on a wide range of organic substrates but was not able to grow autotrophically and lacked ribulose 1,5-bisphosphate carboxylase, a key enzyme of carbon dioxide fixation. These results suggested that strain BI-42 was a chemolithoheterotroph. Ammonia and nitrate were not used as sole nitrogen sources for growth, and strain BI-42 lacked glutamate synthase activity, which resulted in glutamate auxotrophy. The glutamate dehydrogenase activity of this organism was apparently insufficient for ammonia assimilation. On the basis of the results of additional biochemical tests, the G+C content of the DNA, the results of a respiratory ubiquinone analysis, the results of a 16S ribosomal DNA sequence analysis, the fatty acid composition, and the results of a membrane lipid analysis, strain BI-42 was identified as a phylogenetically and physiologically distinct taxon belonging to the alpha subclass of the *Proteobacteria*. *Bosea thiooxidans* gen. nov., sp. nov. is the name proposed for this taxon.

Sulfate is the end product of oxidations of reduced sulfur compounds by chemosynthetic, photosynthetic, and various heterotrophic microorganisms. Nature balances these oxidations through the biological reduction of sulfate to sulfide. Microorganisms utilize sulfur compounds for the biosynthesis of cellular material or transform these compounds as part of a respiratory energy-generating process. Most of the known sulfur-oxidizing bacteria belong to the genera *Thiobacillus*, *Thiothrix*, *Beggiatoa*, *Thiomicrospira*, *Achromatium*, *Desulfotomobium*, *Desulfomonas*, *Desulfococcus*, and *Desulfuromonas* (18, 55). Members of the genus *Thiobacillus* have been studied extensively to increase our understanding of the coupling of oxidation of reduced inorganic sulfur compounds to energy biosynthesis and assimilation of carbon dioxide (45). In addition, some phototrophic bacteria, such as members of the genus *Chromatium*, oxidize reduced inorganic sulfur compounds to provide reducing power for carbon dioxide assimilation during anaerobic photosynthesis (38).

However, oxidation of sulfur compounds is not restricted to the true sulfur bacteria; this process also occurs in heterotrophic bacteria isolated from soil (54) and the marine environment (50, 51). Most of the heterotrophic bacteria that have such activities belong to the genera *Pseudomonas* (35), *Mycobacterium*, *Arthrobacter*, *Flavobacterium* (54), and *Xanthobacter* or are *Escherichia coli* strains (42). However, few of these heterotrophs have the ability to generate biologically useful energy from the oxidation of reduced sulfur compounds (31). An exception is *Paracoccus denitrificans* (6), which also grows autotrophically in the presence of thiosulfate. Only a few heterotrophic bacteria, such as *Thiobacillus* sp. strain Q (8) and *Catenococcus thiocyclus* (39), have been shown to obtain energy from the oxidation of sulfur compounds. This type of nutrition has been designated chemolithoheterotrophy.

Because of the potential importance of chemolithoheterotrophs in soil, attempts were made to isolate chemolithoheterotrophic bacteria from soil by enrichment culture techniques. A single strain was isolated from agricultural field soil, and this strain was designated BI-42^T. Strain BI-42 oxidizes reduced inorganic sulfur compounds only in the presence of some organic substrates. In this paper we describe the isolation of this organism, its morphological and biochemical characteristics, its phylogenetic position based on the results of a 16S ribosomal DNA (rDNA) sequence analysis, its chemotaxonomic properties, and its mode of thiosulfate utilization. In addition, we studied the properties of the nitrogen-metabolizing enzymes of strain BI-42, including glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT), in order to characterize the glutamate auxotrophy of this organism.

MATERIALS AND METHODS

Chemicals. All of the chemicals used were analytical grade and were purchased from Sigma Chemical Co., St. Louis, Mo., E. Merck, Darmstadt, Germany, and SRL Pvt., Ltd., Bombay, India. Yeast extract, tryptone and agar-agar were obtained from Difco Laboratories, Detroit, Mich., and Dowex-1-chloride (50-100 dry mesh) was obtained from Aldrich Chemical, Inc. NaH¹⁴CO₃ was purchased from Bhabha Atomic Research Centre, Trombay, India.

Enrichment and isolation. Soil samples were collected from different agricultural fields around Calcutta, India. The soil samples (25 g) were moistened with 5 ml of sterile distilled water and enriched by adding sodium thiosulfate (100 mg), sodium sulfite (50 mg), or elemental sulfur (25 mg). After thorough mixing, the preparations were incubated in petri dishes at 30°C for 10 days. Further enrichment was carried out in mineral salts broth (pH 8.0) supplemented with phenol red (0.02 g/liter) and either Na₂S₂O₃ · 5H₂O (5 g/liter) or thiosulfate plus yeast extract (5 g/liter). Portions (1 g) of the enriched soil samples were transferred into 250-ml conical flasks containing 50 ml of enrichment medium, and the resulting preparations were incubated in a gyratory water bath shaker (model G76; New Brunswick) at 220 rpm and 30°C. After 2 days of incubation, one flask turned yellow, indicating that acid was formed. Serial dilutions of the contents of this flask were plated onto mineral salts-thiosulfate agar and onto mineral salts-thiosulfate-yeast extract agar. After 4 days of incubation at 30°C, a few colonies with yellow halos appeared on the mineral salts-thiosulfate-yeast extract agar plates. These colonies were purified by repeated streaking on the same medium. Thus, a purified culture was obtained, and this culture was designated strain BI-42.

Medium and growth conditions. The mineral salts medium contained (per liter) 4.0 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 0.1 g of MgCl₂ · 5H₂O, 1.0 g of

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NH_4Cl , and 2 ml of a trace metal solution (53). The pH was adjusted to 8.0 with 4 N NaOH. Glutamate-yeast extract-mineral salt (GYM) medium contained (per liter) 4.0 g of Na_2HPO_4 , 1.5 g of KH_2PO_4 , 0.1 g of $\text{MgCl}_2 \cdot 5\text{H}_2\text{O}$, 0.5 g of sodium glutamate, and 0.1 g of yeast extract; the pH of this medium was 8.0. GYM medium was used as the basic medium unless stated otherwise. Mixed-substrate medium was GYM medium supplemented with $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and an organic substrate. The media were sterilized by autoclaving them at 103 kPa for 15 min. For solid media 20.0 g of agar per liter was added.

The denitrifying activity of strain BI-42 was determined under microaerobic conditions by adding KNO_3 (1.0 g/liter) to GYM medium containing organic substrates (5 g/liter) or thiosulfate (5 g/liter) or both. Inverted Durham tubes were placed in the culture media to determine whether gas was formed. Whether anaerobic growth occurred, was determined with the BBL GasPak system (Becton Dickinson and Co., Cockeysville, Md.). Crossed-pool auxanography was performed as described by Holliday (10). All other routine biochemical tests and procedures were performed as described previously (3, 43).

Utilization of amino acids as sole nitrogen sources or sole nitrogen and carbon sources was determined in mineral salts medium lacking NH_4Cl . Utilization of organic compounds other than amino acids was determined in GYM medium containing substrates at concentrations of 1 to 5 g/liter.

Utilization of inorganic sulfur compounds was determined in GYM medium containing various concentrations (0.1 to 10 g/liter) of different inorganic sulfur sources in addition to sodium succinate (5 g/liter). All of the sulfur compounds except elemental sulfur were sterilized by membrane filtration (pore size, 0.22 μm ; Millipore, Bangalore, India). Elemental sulfur was sterilized by intermittent steaming.

For the GS, GDH, and GOGAT assays cells were grown in GYM medium supplemented with sodium acetate (10 g/liter). NH_4Cl (1 g/liter) was added at the mid-log phase for induction, and the preparations were incubated for an additional 3 h. Ribulose 1,5-bisphosphate (RuBP) carboxylase activity was assayed by using extracts of exponential-phase cells grown in GYM medium supplemented with thiosulfate or both thiosulfate and succinate.

Flagellum staining and electron microscopy. The presence of flagella was determined by using cells that were negatively stained with 2% phosphotungstic acid. Cells were placed onto a carbon-coated grid (300 mesh; diameter, 3 mm) and examined with a JEOL model JEM 200 CX electron microscope at an accelerating voltage of 80 kV (36).

Susceptibility tests. Antibiotic resistance and the MICs of different antibiotics were determined on Luria agar. The MIC of an antibiotic was determined by comparing the number of colonies that appeared on antibiotic-containing plates with the number of colonies on control plates.

Extraction of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100-mg portions of freeze-dried cultures by using the two-stage method described by Tindall (47, 48). Cells were stirred in a hexane-methanol (1:2, vol/vol) solution, the preparation was placed on ice, and the respiratory lipoquinones were recovered in the hexane layer by adding 1 volume of cold hexane. The hexane phase was removed, and the respiratory lipoquinones were extracted a second time from the methanol phase by adding 2 volumes of hexane and 2 volumes of 0.3% aqueous NaCl. The hexane phases were pooled and used for the lipoquinone analysis. Polar lipids were extracted by modifying the methanol-0.3% aqueous NaCl phase so that a chloroform-methanol-0.3% NaCl ratio of 1:2:0.8 (vol/vol/vol) was obtained. The extraction solvent was stirred, and the cell debris was removed by centrifugation. The polar lipids were recovered in the chloroform phase by modifying the preparation so that the chloroform-methanol-0.3% aqueous NaCl ratio was 1:1:0.9 (vol/vol/vol).

Analysis of respiratory lipoquinones. The respiratory lipoquinones were separated into different classes (menaquinones and ubiquinones) by thin-layer chromatography on silica gel thin layers (catalog no. 805 023; Macherey-Nagel, Düren, Germany), using hexane-tertbutylmethylether (9:1, vol/vol) as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and analyzed by high-performance liquid chromatography (HPLC). The HPLC analysis was carried out with an LDC analytical HPLC (Thermoseparations, Darmstadt, Germany) equipped with a reverse-phase column (2 by 125 mm; 3 μm ; RP₁₈; (Macherey-Nagel) by using methanol as the eluant. The respiratory lipoquinones were detected at 269 nm.

Analysis of polar lipids. Polar lipids were separated by two-dimensional silica gel thin-layer chromatography (catalog no. 818 135; Macherey-Nagel). The eluant used for the first dimension was chloroform-methanol-water (65:25:4, vol/vol/vol) and the eluant used for the second dimension was chloroform-methanol-acetic acid-water (80:12:15:4, vol/vol/vol/vol). Total lipids and specific functional groups were detected by using dodecemolybdo-phosphoric acid (total lipids) (17), zinazde reagent (phosphate) (48), ninhydrin (free amino groups) (29), periodate-Schiff stain (α -glycols) (37), dragendorff (quaternary nitrogen) (2), and anisaldehyde sulfuric acid (glycolipids) (21).

Fatty acid analysis. Fatty acids were analyzed as methyl ester derivatives prepared from 10 mg of dry cell material. Cells were saponified and then trans-methylated as described by Kuykendall et al. (20). Fatty acid methyl esters were analyzed by gas chromatography by using a nonpolar capillary column (0.2 mm by 25 m) and a flame ionization detector. The operating conditions were as follows: injection and detector temperature, 300°C; inlet pressure, 60 kPa; split

ratio, 100:1; injection volume, 2 μl ; and temperature program, 170 to 270°C, with the temperature increasing at a rate of 5°C/min.

Isolation and amplification of 16S rDNA. Genomic DNA was extracted and 16S rDNA was amplified by the method of Rainey et al. (30). The PCR to amplify the 16S rDNA was performed by using primers 5'-GAGTTTGATCCT GGCTCAG-3' (forward primer) and 5'-AGAAAGGAGGTGATCCAGCC-3' (reverse primer) as described by Stackebrandt and Chaffreitag (40). The PCR products were sequenced directly by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) and a model 373A DNA sequencer (Applied Biosystems).

16S rDNA sequence analysis. The 16S rDNA sequence which we determined was manually aligned with previously published sequences obtained from the Ribosomal Database Project for members of the actinomycete line of descent as described by Maidak et al. (24). Pairwise evolutionary distances were computed by using the corrections of Jukes and Cantor (12). A more detailed analysis was carried out with the organisms which were found to be most closely related to BI-42. Trees were reconstructed as described by De Soete (5).

Preparation of extract. The cell extract used for enzyme assays was prepared by harvesting cells at the mid-exponential phase and then washing and resuspending the resulting cell pellet in 10 mM KPO_4 buffer (pH 7.0) containing 1 mM EDTA (disodium salt) and 7 mM 2-mercaptoethanol for the GDH and GOGAT assays and in 100 mM Tris-HCl buffer (pH 8.2) containing 20 mM MgCl_2 , 5 mM NaHCO_3 , and 0.5 mM dithiothreitol for the RuBP carboxylase assay. The cells were sonicated with a Braunsonic 1510 sonicator six times (30 s each) at 150 W in an ice bath. The cell debris was removed by centrifugation at 28,000 $\times$ g for 30 min, and the supernatant was used as the enzyme source. For the GS assay, 10 ml of exponentially growing cells was treated with cetyltrimethylammonium bromide (0.1 g/liter) and then shaken thoroughly for 3 min. The treated cultures were chilled immediately and centrifuged at 4°C, and the resulting preparations were washed and resuspended in 1 ml of an ice-cold KCl solution (10 g/liter). These cell suspensions were used for the GS activity assay.

Enzyme assays. GDH activity was measured by the method of Maulick and Ghosh (26). The 1-ml reaction mixture used for the NADPH-dependent assay contained 50 mM Tris-HCl buffer (pH 8.5), 50 mM NH_4Cl , 1 mM 2-oxoglutarate, 0.1 mM NADPH, and the cell extract. The assay was performed at 30°C by measuring the rate of oxidation of NADPH at 340 nm with a Shimadzu UV-visible recording spectrophotometer (model UV 240). Similarly, the NADH-dependent GDH activity was measured by replacing NADPH with 1 mM NADH and increasing the concentration of 2-oxoglutarate to 10 mM. After incubation for 3 min at 30°C, the reaction was stopped by adding 6.5 ml of 100 mM NaOH, and the A_{340} was determined. The oxidative deamination of glutamate by GDH was assayed by measuring the formation of NADPH at 340 nm by using an assay mixture containing 100 mM Tris-HCl (pH 8.8), 25 mM glutamate, and 0.5 mM NADP in a final volume of 1 ml.

GOGAT activity was measured by using 2-ketoglutarate and determining glutamine-dependent oxidation of NADPH as described by Meers et al. (27). GS activity was measured by the γ -glutamyl transferase method described by Miller et al. (28). In every case, the γ -glutamyl transferase activities in the presence and absence of 60 mM MgCl_2 were measured to determine the active state of the enzyme. The reaction mixture was incubated for 10 min at 30°C, and the amount of γ -glutamyl hydroxamate formed was determined at 535 nm by using a reagent blank for comparison. Specific activity was expressed as the amount of enzyme which catalyzed the formation or disappearance of 1 μmol of product or substrate per min.

The RuBP carboxylase activity in the cell extract was determined as described by Kelly et al. (16). The final volume of the assay mixture, which contained cell extract (0.1 to 0.4 g of protein), was 0.3 ml. The reaction was initiated with RuBP and was terminated after 0.5, 1, 1.5, 2, 3, and 4 min by adding 0.2 ml of acetic acid. Zero-time and RuBP-free controls were included. Each reaction mixture was dried in its vial, 5 ml of scintillation fluid, which contained 10 g of 2,5-diphenyloxazole per liter, 0.25 g of 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) per liter, and 100 g of naphthalene per liter in 1,4-dioxane, was added, and ^{14}C incorporation was determined with a Beckman scintillation counter.

Analytical methods. DNA base composition was determined by the thermal denaturation method of Marmur and Doty (25). The growth of cells was monitored by measuring optical density at 660 nm. Protein contents were estimated by using the method of Lowry et al. (22) and bovine serum albumin as the standard. The concentrations of thiosulfate and tetrathionate in culture filtrates were determined as described by Kelly et al. (15). The sulfite content was determined by the method of Truper and Schlegel (49), as modified by Suzuki and Silver (45). The sulfate content was determined by the method of Berglund and Sorbo (1) and by the method of Gleen and Quastel (7); since a concentration of thiosulfate greater than 2.0 $\mu\text{mol}/5$ ml of reaction mixture interfered with the sulfate determination when the first method was used, the second method was always used to confirm the results.

Nucleotide sequence accession number. The 16S rDNA sequence of strain BI-42 has been deposited in the EMBL data library under accession number X81044.

FIG. 1. Electron micrograph of a strain BI-42 cell, showing the single polar flagellum. Bar = 200 nm.

RESULTS

Morphological and biochemical features of strain BI-42.

Colonies of the organism which we isolated grown on Luria agar or on thiosulfate-yeast extract agar were circular, 1 to 1.5 mm in diameter, smooth, mucoid, round, and creamy colored. Single colonies appeared within 4 days at 30°C. The cells stained gram negative and were motile, rod shaped, 0.85 μm wide, and 1.4 to 1.6 μm long. The cells occurred mostly singly, did not form spores, and were motile by means of a single polar flagellum (Fig. 1). Cells grown on GYM medium containing thiosulfate and yeast extract or on mixed-substrate agar containing thiosulfate and succinate did not form sulfur deposits on the colony surfaces. The isolate which we obtained was designated BI-42.

Strain BI-42 was not able to grow without small amounts of yeast extract when sugars, amino acids, and tricarboxylic acid cycle intermediates were used as the sole sources of carbon and energy. Crossed-pool auxanography revealed that nicotinic acid (0.001 g/liter) partially replaced the requirement for 0.1 g of yeast extract per liter. Strain BI-42 also was not able to grow in a chemically defined medium containing the inorganic nitrogen compounds NH_4Cl , KNO_3 , and $(\text{NH}_4)_2\text{SO}_4$ as sole nitrogen sources. Growth was observed when these nitrogen compounds were replaced by glutamate (0.5 g/liter). These observations led to the formulation of GYM medium.

Strain BI-42 did not grow anaerobically in the presence or absence of nitrate with any of the substrates tested. However, under microaerobic conditions, strain BI-42 formed gas in GYM medium containing malate, succinate, glucose, or sucrose. When thiosulfate was added to the media, gas was not

produced. The gas produced was not absorbed by NaOH. Thus, the gas was identified as N_2 or N_2O , the products of denitrification. Strain BI-42 exhibited catalase, oxidase, and nitrate reductase activities and could grow on Simmons citrate agar and MacConkey agar. Methyl red, urease, starch hydrolysis, indole production, H_2S production, Voges-Proskauer, gelatin hydrolysis, and pigment production tests were negative. The optimum temperature for growth was 30 to 32°C. The pH range for growth was 6.0 to 9.0, and the optimum pH was 7.5 to 8.0.

Utilization of organic compounds. Glutamate, glutamine, proline, cysteine, aspartic acid, serine, asparagine, alanine, and lysine were used as both nitrogen and carbon sources, while arginine and histidine were used as nitrogen sources. In contrast, leucine, glycine, isoleucine, methionine, tyrosine, tryptophan, and phenylalanine did not support growth.

Strain BI-42 grew heterotrophically in GYM medium supplemented with glucose, fructose, rhamnose, xylose, sorbose, ribose, galactose, citrate, gluconate, acetate, pyruvate, arabinose, succinate, and malate. Glycerol, raffinose, mannitol, formate, lactate, glyoxylate, propionate, salicylate, butyrate, cyclohexanol, *p*-aminobenzoate, and methanol did not support the growth of this organism.

Growth and oxidation of sulfur compounds. In GYM medium supplemented with thiosulfate the growth yield of strain BI-42 was almost the same as the growth yield in GYM medium alone. No stimulation of growth occurred, although a considerable amount of thiosulfate was consumed (data not shown). However, marked stimulation of growth was observed when GYM medium was supplemented with sodium succinate in addition to thiosulfate. The growth yield varied with the concentration of thiosulfate (Fig. 2); the greatest growth (A_{660} , 1.2 to 1.3) was observed at a concentration of 5 g/liter. Under these conditions, conversion of thiosulfate to sulfate was stoichiometric, and the pH of the medium decreased from 8.0 to 6.6 (Fig. 3). Thiosulfate added to the medium was almost

FIG. 2. Effect of thiosulfate on the growth response of strain BI-42 in GYM medium containing succinate. Symbols: ○, 0.1 g of thiosulfate per liter; +, 1.0 g of thiosulfate per liter; *, 5.0 g of thiosulfate per liter; □, 10.0 g of thiosulfate per liter; △, medium containing only sodium succinate (5.0 g/liter). O.D., optical density.

FIG. 3. Time course of utilization of thiosulfate (□), release of sulfate (Δ), and change in pH (inset) during growth of strain BI-42 in GYM medium containing thiosulfate (5.0 g/liter) and sodium succinate (5.0 g/liter).

entirely consumed within 36 h. Interestingly, the pH of the medium increased after 36 to 40 h of growth. Like thiosulfate, tetrathionate also stimulated growth in the presence of succinate, but NaSCN, Na₂SO₃, and Na₂SO₄ inhibited growth (data not shown).

CO₂ fixation. No RuBP carboxylase activity was detected in strain BI-42.

Susceptibility to antibiotics. The MICs of several antibiotics for strain BI-42 were determined. Growth of this organism was inhibited by 120 μg of streptomycin per ml, 10 μg of tetracycline per ml, 40 μg of neomycin per ml, 20 μg of chloramphenicol per ml, and 15 μg of rifampin, per ml. Even ampicillin did not inhibit growth even at a concentration of 200 μg/ml.

GS, GDH, and GOGAT activities. Strain BI-42 cells contained readily detectable levels of GS and GDH (Table 1). Addition of NH₄Cl in the exponential phase of growth reduced the GS activity. The GS activity measured in the presence of Mn²⁺ alone was significantly higher than the GS activity measured in the presence of both Mn²⁺ and Mg²⁺. NADP⁺- and NADH-dependent GDH activities were not detected. However, considerable activity in the assimilatory direction was observed when NADPH was the cofactor. Addition of ammo-

TABLE 1. Effect of NH₄⁺ on GS, GDH, and GOGAT activities of strain BI-42

Addition to medium	Sp act (nmol/min/mg of protein)			
	GS		GDH	GOGAT
	Mn ²⁺	Mn ²⁺ + Mg ²⁺		
None	1,347	422	22	<0.1
NH ₄ ⁺ ^a	827	160	18	<0.1

^a NH₄⁺ was added to a concentration of 0.1% during the exponential phase of growth. The preparation was incubated for 3 h, and then the enzyme activities were determined.

TABLE 2. Cellular fatty acid composition of strain BI-42

Fatty acid	Designation	% in strain BI-42 ^a
Nonhydroxylated acids		
Pentadecanoic acid	C _{15:0}	2.33
cis-Hexadec-9-enoic acid	C _{16:1} ω7c	3.70
Hexadecanoic acid	C _{16:0}	7.58
cis-Hepta-9-enoic acid	C _{17:1} ω8c	3.56
cis-9,10-Methylene hexadecanoic acid	C _{17:0} cyclo	2.08
Heptadecanoic acid	C _{17:0}	4.82
cis-Octadec-9-enoic acid	C _{18:1} ω7c	61.55
Octadecanoic acid	C _{18:0}	0.67
cis-Cyclo-10,11-methylene octadecanoic acid	C _{19:0} cyclo ω8c	8.35
10-Methyl octadecanoic acid	C _{19:0} 10-methyl	0.71
Hydroxylated acid		
3-Hydroxypentadecanoic acid	C _{15:0} 3-OH	0.49
3-Hydroxyhexadecanoic acid	C _{16:0} 3-OH	3.25
3-Hydroxyheptadecanoic acid	C _{17:0} 3-OH	0.90

^a Percentages of the total fatty acids.

nium chloride did not influence the GDH activity (Table 1). GOGAT activity was not observed in this organism.

DNA base composition. The G+C content of the DNA of strain BI-42 was 68.2 mol%.

Ubiquinones. Ubiquinone was the sole respiratory quinone present. The major ubiquinone contained 10 isoprenoid units (ubiquinone 10).

Fatty acid composition. The cellular fatty acid composition of strain BI-42 is shown in Table 2. The fatty acids identified were the saturated straight-chain acids C_{15:0}, C_{16:0}, C_{17:0}, C_{18:0}, and C_{19:0} 10-methyl; the unsaturated straight-chain acids C_{16:1} ω7c, C_{17:1} ω8c, and C_{18:1} ω7c; and the nonhydroxy acids C_{17:1} cyclopropane and C_{19:1} ω8c. cis-Octadec-9-enoic acid (C_{18:1} ω7c) was the predominant compound among the other non-hydroxy acids. The major hydroxylated fatty acids found were C_{15:0} 3-OH, C_{16:0} 3-OH and C_{17:0} 3-OH.

Polar lipids. The polar head groups consisted of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, and an unidentified amino lipid.

16S rDNA sequencing and phylogenetic analysis. A total of 1,377 nucleotides of the 16S rDNA were sequenced. The 16S rDNA sequence of strain BI-42 was most similar to the sequence of *Beijerinckia indica* (level of similarity, 92.8%) and to the sequences of *Rhodopseudomonas palustris*, *Nitrobacter winogradskyi*, *Blastobacter denitrificans*, and *Bradyrhizobium japonicum* (levels of similarity, 92.0 to 92.5%), which are members of the alpha subclass of the *Proteobacteria*. The distance matrix phylogenetic tree based on levels of difference between strain BI-42 and 19 reference strains that belong to the alpha subclass of the *Proteobacteria* indicated that strain BI-42 belongs to a new lineage located between the methylotrophs, the genus *Beijerinckia*, and the *Rhodopseudomonas palustris* group (Fig. 4). No close relationship was found between strain BI-42 and other sulfur-oxidizing bacteria, such as *Thiobacillus acidophilus* and *Acidiphilium* species (levels of 16S rDNA similarity, <88%).

DISCUSSION

A great deal of work has been performed to increase our understanding of the oxidation of inorganic sulfur compounds by obligately or facultatively chemolithotrophic bacteria, especially bacteria belonging to the genus *Thiobacillus* (44). These bacteria oxidize inorganic sulfur compounds under autotrophic

FIG. 4. Phylogenetic tree based on dissimilarity values, showing the relationships between strain BI-42 and related reference organisms. Bar = 5% nucleotide difference.

or mixotrophic growth conditions (19). However, it has also been reported that *Thiobacillus* sp. strain Q (8) and *C. thiocyclos* (39) oxidize sulfur compounds to yield metabolically useful energy under lithoheterotrophic growth conditions. In this context, the stimulation of strain BI-42 growth by thiosulfate observed in an organic culture medium is very similar to the chemolithoheterotrophic growth of *Thiobacillus* sp. strain Q described by Gommers and Kuenen (8).

The following major physiological characteristics of strain BI-42 distinguish it from similar gram-negative bacteria, including *Thiobacillus novellus* (13), *Thiobacillus versutus* (46), *Thiobacillus* sp. strain Q (8), *Thiosphaera pantotropha* (32), *Pseudomonas aeruginosa* (35), *Achromobacter stutzeri* (41), and *Pseudomonas* sp. strain 16B (50), which are capable of oxidizing inorganic sulfur compounds: (i) strain BI-42 cannot grow in chemically defined medium containing ammonia or nitrate as the sole nitrogen source; (ii) strain BI-42 requires trace amounts of yeast extract as a growth factor; and (iii) strain BI-42 is auxotrophic for glutamate.

A large number of organic compounds supported heterotrophic growth of strain BI-42 but this organism was not able to utilize aromatic compounds and alcohol. Unlike *Thiobacillus* sp. strain Q (8), strain BI-42 grown on mixed-substrate agar did not produce sulfur deposits on the colony surfaces. Even though both strain BI-42 and *Thiobacillus versutus* (46) denitrify under heterotrophic growth conditions, the absence of denitrification under mixed-substrate growth conditions differentiates strain BI-42 from *Thiosphaera pantotropha* and *Thiobacillus versutus* (32). This may be due to the inhibitory effect of thiosulfate for denitrifying activity under mixed-substrate growth conditions.

Despite the presence of an assimilatory GDH, strain BI-42 was not able to grow in culture medium containing ammonia as the sole nitrogen source. Thus, the assimilatory GDH may not have been sufficient for assimilation of ammonia.

The fatty acid composition of BI-42 was distinct from the fatty acid compositions of other autotrophic and facultatively

chemolithoautotrophic sulfur oxidizers (14). The uniqueness of this isolate was supported by the presence of hydroxy fatty acids along with 10-methyl and cyclic fatty acids, which are not found in any other species belonging to the alpha subclass of the class *Proteobacteria* (4, 23, 33).

The respiratory quinone present in strain BI-42 was a ubiquinone with 10 isoprenoid units (ubiquinone 10), which indicates that this organism is closely related to members of the alpha subclass of the *Proteobacteria*. The lipids of the polar head groups of strain BI-42 were not unique but were similar to those found in the obligate or restricted facultative methylo-trophs (11). The presence of ubiquinone 10 and phosphatidylcholine and the high level of the unsaturated nonhydroxy fatty acid C_{18:1} ω7C resembles characteristics of the genus *Methylobacterium* (9, 11).

Oxidation of thiosulfate in GYM medium was gratuitous since it did not increase the growth yield. Despite thiosulfate oxidation, the low cell yield clearly indicated that like other chemolithoheterotrophic sulfur oxidizers, strain BI-42 could not assimilate carbon dioxide (8, 31). The failure to detect RuBP carboxylase is consistent with this conclusion. In contrast, the increased growth yield obtained with thiosulfate and succinate implies that thiosulfate oxidation is used to enhance heterotrophic carbon assimilation. Under these growth conditions, the stoichiometry for thiosulfate oxidation to sulfate by strain BI-42 is very similar to the stoichiometry reported for *Thiobacillus thio-parus* (52) and *Thiobacillus novellus* (34).

As determined by a 16S rDNA sequence analysis, biochemical tests, and a chemical composition analysis, strain BI-42 represents a novel genus and species in the alpha subclass of the *Proteobacteria*, for which the name *Bosea thiooxidans* is proposed.

Description of *Bosea* gen. nov. *Bosea* (Bos' e.a. M. L. gen. n. *Bosea*, of J. C. Bose the founder of the Bose Institute, where the organism was isolated). Cells are gram-negative rods that occur mostly as single cells. Strictly aerobic. The optimum temperature for growth is 30 to 32°C. No growth occurs at temperatures below 20°C and above 37°C. Oxidase and catalase positive. Reduced inorganic sulfur is oxidized in the presence of organic carbon sources. No autotrophic growth occurs.

Phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, diphosphatidylglycerol, and an amino lipid are the major lipid constituents of the polar head groups. The abundant fatty acids are *cis*-octadec-9-enoic acid (C_{18:1} ω7c), *cis*-cyclo-10,11-methylene octadecanoic acid (C_{19:0} cyclo ω8c), and hexadecanoic acid (C_{16:0}). Hydroxylated fatty acids are also present. A 16S rDNA sequence analysis of strain BI-42 revealed that the genus branches intermediate to the genus *Methylobacterium*, the genus *Beijerinckia*, *R. palustris*, and related taxa. The type species is *Bosea thiooxidans*.

Description of *Bosea thiooxidans* sp. nov. *Bosea thiooxidans* (thi.o. ox'i. dans. Gr. n. *thion*, sulfur; M.L.v. *oxido*, make acid, oxidize; M.L. part. adj. *thiooxidans*, oxidizing sulfur). This bacterium was isolated from agricultural soil. Cells are straight rods that are 0.85 μm wide by 1.4 to 1.6 μm long. Cells occur singly. Motile by means of a single polar flagellum. Strictly aerobic. Spores are not formed. Colonies on agar containing thiosulfate and succinate or yeast extract are smooth, mucoid, round, and cream colored; these colonies are about 1 to 1.5 mm in diameter after 4 days of incubation. No sulfur deposition occurs on the colony surfaces. Glutamate, glutamine, and aspartate, but not NH₄⁺, NO₃⁻, and urea, can serve as nitrogen sources. This organism can denitrify and produce gas only in heterotrophic growth medium containing nitrate. It has glutamate auxotrophy and requires yeast extract (0.1 g/liter) as a

growth factor. The pH range for growth is 6.0 to 9.0, and the optimum pH is 7.5 to 8.0.

A variety of organic compounds support heterotrophic growth in GYM medium. These compounds include glucose, fructose, rhamnose, xylose, sorbose, ribose, arabinose, galactose, citrate, gluconate, succinate, malate, acetate, glutamate, glutamine, proline, cysteine, aspartic acid, serine, asparagine, alanine, lysine, common hexoses, pentoses, and organic acids, pyruvate, and some amino acids.

This species is a chemolithoheterotroph. Thiosulfate stimulates growth in the presence of succinate. Tetrathionate is oxidized slowly. Sulfite, thiocyanate, and elemental sulfur do not support growth.

The G+C content is 68.2 mol%. Ubiquinone 10 is the major ubiquinone. A culture of the type strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 9653.

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