***Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus**

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A facultatively chemolithotrophic thiocyanate-degrading bacterium, strain THI 011^T, which was previously isolated from activated sludge and tentatively named *Thiobacillus* sp., was studied taxonomically and phylogenetically. This bacterium utilizes thiocyanate as sole energy source and the specific growth rate for chemolithoautotrophic growth with thiocyanate was 0.059 h⁻¹. Molecular phylogenetic relationships of strain THI 011^T to *Thiobacillus versutus* and members of the genus *Paracoccus* were elucidated by comparing 16S rRNA gene sequences. Binary sequence comparisons showed that strain THI 011^T was most related to *Paracoccus aminophilus*, at a similarity level of 97.0%, and *T. versutus* was most similar to *Paracoccus denitrificans*, at a level of 99.1%. A neighbour-joining phylogenetic tree showed that strain THI 011^T formed a cluster together with *T. versutus* and known species of the genus *Paracoccus* within the α -3 subclass of the *Proteobacteria*. DNA–DNA hybridization assays and phenotypic studies indicated that strain THI 011^T differed from *T. versutus* and known species of the genus *Paracoccus*. On the basis of these results, we propose to classify strain THI 011^T into a new species of the genus *Paracoccus* with the name *Paracoccus thiocyanatus* sp. nov. We also propose to transfer *T. versutus* to the genus *Paracoccus* and present an emended description of the genus.

Keywords: thiocyanate degradation, *Paracoccus thiocyanatus* sp. nov., *Paracoccus versutus* comb. nov., *Thiobacillus versutus*, molecular phylogeny

INTRODUCTION

Thiocyanate, which is formed chemically from cyanide by the addition reaction of sulphur, is one of the major constituents of wastewater from coke-oven factories, and biological degradation of this sulphur compound in wastewater is a subject of major concern. Stafford & Calley (1969) isolated a *Pseudomonas stutzeri*-like chemoorganotroph that used thiocyanate as nitrogen and sulphur sources. *Pseudomonas* sp. (Putilina, 1961) and

Arthrobacter sp. (Betts *et al.*, 1979) were also reported as thiocyanate-degrading bacteria. Some obligately chemolithotrophic species of the genus *Thiobacillus*, such as *T. thioparus* (Happold *et al.*, 1958; Katayama & Kuraishi, 1978) and *T. denitrificans* (Wood, 1975), are known to use thiocyanate as an energy source for growth. Happold *et al.* (1954) isolated the obligate chemolithotroph *T. thiocyanoxidans*, which uses thiocyanate as energy and nitrogen source with production of carbon dioxide, ammonium and sulphate (Youatt, 1954). There are phenotypic similarities between *T. thioparus* and *T. thiocyanoxidans*, the latter of which has been classified as *T. thioparus* since the publication of the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Vishniac, 1974). Recently, thiocyanate hydrolase, a novel enzyme which catalyses the

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formation of carbonyl sulphide and ammonia from thiocyanate, was found in *T. thioparus* (Katayama *et al.*, 1992). However, thiocyanate degradation by the facultatively chemolithotrophic sulphur bacteria has not yet been described.

Members of the genus *Thiobacillus* are Gram-negative, aerobic, mesophilic, non-sporeforming, rod-shaped bacteria which are specialized in their ability to oxidize reduced sulphur compounds (Kelly & Harrison, 1989). Early taxonomic studies of *Thiobacillus* species showed their diversity in nutritional requirements, chemolithotrophic energy source utilized, pH ranges for growth, and DNA base composition (Hutchinson *et al.*, 1969; Jackson *et al.*, 1968), and the genus *Thiobacillus* has been considered to include heterogeneous bacterial groups above the generic level. Previously, we classified *Thiobacillus* species into three major categories, groups I, II and III, on the basis of phenotypic and chemotaxonomic criteria (Katayama-Fujimura *et al.*, 1982). Group I includes the facultative chemolithotrophs containing ubiquinone-10 (Q-10) as the principal quinone; *T. acidophilus*, *T. novellus* and *T. versutus* are members of this group. Group II encompasses the facultative chemolithotrophs with Q-8, and comprises *T. delicatus*, *T. intermedius* and *T. perometabolis*. The obligate chemolithotrophs with Q-8 are placed in group III, members of which are *T. denitrificans*, *T. ferrooxidans*, *T. neapolitanus*, *T. thiooxidans* and *T. thioparus* (the type species). In addition to the chemolithotrophy and quinone profiles, there are marked differences in sulphur requirements for growth and cellular fatty acid compositions among strains of different groups of thiobacilli (Katayama-Fujimura *et al.*, 1982, 1984a). The grouping of thiobacilli based on these phenotypic differences has been supported by sequence comparisons of small-subunit rRNAs (5S and 16S). *Thiobacillus* species of groups II and III so far examined belong to the β subclass of the *Proteobacteria* (Stackebrandt *et al.*, 1988), whereas those of group I are in the α subclass (Lane *et al.*, 1992; Huber & Stetter, 1990; Drobner *et al.*, 1992). Recently, Ludwig *et al.* (1993) verified the close relationship between *T. versutus* and *Paracoccus denitrificans*, as well as synonymy of the latter species and *Thiosphaera pantotropha*, using the 16S rRNA sequence information. In addition, phylogenetic relationships between *T. acidophilus* and *Acidiphilium* species were mentioned by Lane *et al.* (1992).

Although the ability to oxidize reduced sulphur compounds under chemolithotrophic growth conditions is an important criterion for the classification and the identification of *Thiobacillus* species, the available information strongly implies that at least the species of group I should be removed from the genus *Thiobacillus* and be reclassified into appropriate known or new genera. The main purpose of the present study was to characterize strain THI 011^T, a Gram-negative thiocyanate-degrading facultatively chemolithotrophic sulphur bacterium which in previous studies was classified into group I and tentatively called *Thiobacillus* sp. (Katayama-Fujimura *et al.*, 1983a, 1984 b, c). Since phenotypic studies are no longer thought to provide information of value, without molecular

phylogenetic data, in generic reassignment of the group I thiobacilli, we attempted to elucidate phylogenetic relationships between the new isolate and related bacteria including *T. versutus* and members of the genus *Paracoccus* by studying 16S rRNA gene sequence data. In this paper, we first describe phylogenetic relationships of strain THI 011^T and related bacteria, and then make a comparison of the physiological characteristics of these organisms. We propose to create a new species, *Paracoccus thiocyanatus*, for strain THI 011^T. We also propose to transfer *T. versutus* to the genus *Paracoccus* as *Paracoccus versutus* with emendation of the genus.

METHODS

Strains used. Strain THI 011^T (T = type strain), which was previously named *Thiobacillus* sp., two strains of *T. versutus*, and the type strains of known members of the genus *Paracoccus*, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* were studied. Detailed information on these test strains is given in Table 1. All test organisms were maintained on BY agar medium (Katayama-Fujimura & Kuraishi, 1980), except for the phototrophic bacteria, for which MYCA medium (Hiraishi *et al.*, 1991) was used, and all were subcultured monthly.

Phenotypic characterization. Morphological and physiological characteristics were examined as described previously (Katayama-Fujimura & Kuraishi, 1980; Katayama-Fujimura *et al.*, 1983b). Unless otherwise noted, all the tests were carried out at 30 °C in SM medium (Katayama-Fujimura *et al.*, 1982) supplemented with an appropriate growth substance such as thiosulphate (5 g l⁻¹) and glucose (10 g l⁻¹). For growth of strain THI 011^T in the synthetic medium, thiamin (200 µg l⁻¹) was added as the growth factor. Growth with potassium thiocyanate (3.5 g l⁻¹) was examined in TC medium (Katayama-Fujimura & Kuraishi, 1980). Concentrations of thiosulphate, sulphate and thiocyanate were determined as described previously (Katayama & Kuraishi, 1978; Katayama-Fujimura *et al.*, 1983b).

Analyses of quinones and fatty acids. Quinones were extracted with chloroform/methanol, purified by thin-layer chromatography, and then analysed by high-performance liquid chromatography (HPLC) (Ohara *et al.*, 1990). Fatty acids were extracted after saponification, methylated with diazomethane, and then analysed by gas-liquid chromatography (Katayama-Fujimura *et al.*, 1982). The results are reported as percentage composition of non-hydroxyl fatty acids, and also as the amount of hydroxyl fatty acid relative to the total amount of non-hydroxyl fatty acids. We used the combined values of [octadecenoic acid (C_{18:1}) + cyclopropane acid of C₁₉ (C_{19cyc})], because this combined value shows less variation with growth phase (Katayama-Fujimura & Kuraishi, 1980).

DNA base composition and DNA-DNA hybridization. Chromosomal DNA was extracted and purified by the method of Marmur (1961). The guanine plus cytosine (G + C) content of DNA was determined by HPLC of nuclease P1 hydrolysates of the DNA (Katayama-Fujimura *et al.*, 1984b). DNA-DNA reassociation assays were performed by the quantitative dot-blot hybridization method with photobiotin labelling and colorimetric detection (Ezaki *et al.*, 1988; Hiraishi *et al.*, 1991). The level of hybridization was determined by measuring the colour intensity of hybridized spots with a Shimadzu model CS-9000 two-dimensional computing densitometer.

Amplification and sequencing of 16S rRNA genes. Cells were grown in 5 ml NB liquid medium, harvested by centrifugation

Table 1. Bacterial strains studied and nucleotide sequence accession numbers for their 16S rDNAs

Species	Strain designation or received as:	Other designations/sources	Reference*	Sequence accession no.†
<i>Thiobacillus</i> sp.	THI 011 ^T	IAM12816 ^T , IFO 14569 ^T , H. Murooka 1-8A ^T	1	D32242
<i>Thiobacillus versutus</i>	IAM 12814 ^T	ATCC 25364 ^T , IFO 14567 ^T	2, 3	D32243
<i>Thiobacillus versutus</i>	IAM 12815	ATCC 27793, IFO 14568	3	D32244
<i>Paracoccus denitrificans</i>	IAM 12479 ^T	ATCC 17741 ^T	4, 5	
<i>Paracoccus denitrificans</i>	IFO 13301	ATCC 19367, DSM 65	4	
<i>Paracoccus aminophilus</i>	JCM 7686 ^T	ATCC 49673 ^T , T. Urakami DM-15 ^T	6	D32239
<i>Paracoccus aminovorans</i>	JCM 7685 ^T	ATCC 49632 ^T , T. Urakami DM-82 ^T	6	D32240
<i>Paracoccus alcaliphilus</i>	JCM 7364 ^T	Urakami TK 1015 ^T	7	D32238
<i>Paracoccus kocurii</i>	JCM 7684 ^T	ATCC 49631 ^T , Y. Katayama strain B ^T	8	D32241
<i>Rhodobacter capsulatus</i>	DSM 1710 ^T	ATCC 11166 ^T	9	
<i>Rhodobacter sphaeroides</i>	DSM 158 ^T	ATCC 17023 ^T , IFO 12203 ^T	9	

* 1, Katayama-Fujimura *et al.* (1983a); 2, Harrison (1983); 3, Katayama-Fujimura *et al.* (1983b); 4, Davis *et al.* (1969); 5, Ludwig *et al.* (1993); 6, Urakami *et al.* (1990); 7, Urakami *et al.* (1989); 8, Ohara *et al.* (1990); 9, Hiraishi & Ueda (1994).

† Accession numbers are indicated only for the sequences determined in this study.

in the late-exponential phase of growth, washed with sterile 1% (w/v) saline, resuspended in 100 µl sterile distilled water, and stored at -20 °C until used. Crude lysates were prepared from stock cell suspensions by protease digestion, heat treatment, and centrifugation. Fragments of 16S rDNA that corresponded to positions 8-1510 were amplified by the polymerase chain reaction (PCR) from the crude extract and sequenced directly by the combined method consisting of linear PCR sequencing and automated fluorescent detection. All numbers referring to positions of 16S rRNA were based on the *Escherichia coli* numbering system (Brosius *et al.*, 1978). Details of the procedure used here have been described previously (Hiraishi, 1992; Hiraishi *et al.*, 1994).

Phylogenetic analysis. Pairs of sequences were aligned, and percentage similarities were calculated with the GENETYX program (Software Development Co., Tokyo, Japan). Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc}) (Kimura, 1980) and construction of neighbour-joining phylogenetic trees (Saitou & Nei, 1987) were performed with the CLUSTAL V program (Higgins *et al.*, 1992). Alignment positions that included gaps and unidentified bases were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) with 1000 bootstrapped trials.

RESULTS

Phylogenetic analysis

Because strain THI 011^T has several phenotypic characteristics similar to *T. versutus* (see below), and *T. versutus* is closely related to *P. denitrificans* (Ludwig *et al.*, 1993), the phylogenetic relationship of strain THI 011^T to *T. versutus* and species of *Paracoccus* was examined. Nearly full-length fragments of the 16S rDNAs from the seven test strains were amplified by PCR and sequenced by the linear PCR sequencing method. All of the sequences which we determined covered a continuous nucleotide stretch from

positions 28 to 1492, including 1383 to 1387 residues. Signature analysis of 60 nucleotide positions important for distinguishing between the four subdivisions (Woese, 1987) of the *Proteobacteria* also suggested that these strains belonged to the α subdivision. The substantially shorter sequences of the test organisms compared to the *E. coli* sequence were due to large deletions of loop helices around positions 80, 210, 470 and 1270. Also, the sequences of all test organisms were characterized by insertion of 1 or 2 bases into the loop stem around position 1450. These features of the sequence data indicated that all test organisms belong to the α -3 subclass of the *Proteobacteria*.

The sequences determined in this study were compared with eight sequences published previously for representative members of the α -3 subclass, including *P. denitrificans* and some species of phototrophic bacteria (Hiraishi & Ueda, 1994), with the *E. coli* sequence as an outgroup. Table 2 shows the levels of binary sequence similarity and evolutionary distance (K_{nuc}) values based on the multiple alignment of the 1345 positions. Pairwise sequence comparisons showed that strain THI 011^T was most closely related to *P. aminophilus*, at a similarity level of 97.0%. The two strains of *T. versutus* had identical sequences, and exhibited the highest affinity to *P. denitrificans*, at a similarity level of 99.1%. The known species of the genus *Paracoccus* were related to each other at similarity levels between 95.5% and 97.0%, and showed lower levels of similarity to members of other genera of the α -3 subclass, including those of *Rhodobacter*, *Rhodovulum* and *Roseobacter*. These data indicate that the genus *Paracoccus* is a genetically coherent single genus and that strain THI 011^T, as well as *T. versutus*, should be reclassified as members of this genus.

On the basis of the K_{nuc} values calculated, a neighbour-

Table 2. Levels of sequence similarity and evolutionary distances for 16S rDNAs of strain THI 011^T, *Thiobacillus versutus*, and related species belonging to the α -3 subclass of the *Proteobacteria*

The values on the upper right are levels of sequence similarity, and the values on the lower left are K_{nuc} values.

Organism*	Level (%) of similarity or evolutionary distance (K_{nuc}) with:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Strain THI 011 ^T	—	96.4	96.7	97.0	96.5	96.5	96.1	93.6	92.6	92.9	91.5	91.5	91.3	92.0
2. <i>T. versutus</i>	0.0358	—	99.1	95.9	96.5	95.4	95.4	93.9	93.9	93.0	93.1	91.6	91.5	91.1
IAM 12814, IAM 12815														
3. <i>P. denitrificans</i>	0.0328	0.0082	—	96.2	95.7	95.7	95.5	93.8	93.3	93.1	92.6	92.1	91.8	91.3
4. <i>P. aminophilus</i>	0.0276	0.0395	0.0365	—	96.2	96.4	94.8	95.0	93.4	93.1	92.1	91.8	91.8	92.4
5. <i>P. aminovorans</i>	0.0328	0.0365	0.0402	0.0358	—	96.8	94.4	93.7	94.2	92.6	92.1	92.3	91.3	92.1
6. <i>P. alcaliphilus</i>	0.0328	0.0469	0.0425	0.0343	0.0313	—	95.7	92.8	92.1	92.6	91.3	93.1	93.0	91.8
7. <i>P. kocurii</i>	0.0358	0.0440	0.0417	0.0477	0.0507	0.0387	—	93.2	92.6	93.7	92.6	92.7	92.4	91.8
8. <i>Rb. veldkampii</i>	0.0596	0.0604	0.0596	0.0484	0.0589	0.0671	0.0656	—	94.8	95.0	94.6	93.9	92.9	92.5
9. <i>Rb. capsulatus</i>	0.0686	0.0611	0.0633	0.0663	0.0618	0.0790	0.0708	0.0529	—	95.4	94.9	93.2	91.7	90.2
10. <i>Rb. sphaeroides</i>	0.0648	0.0693	0.0641	0.0648	0.0686	0.0693	0.0589	0.0499	0.0462	—	94.7	92.9	93.2	91.3
11. <i>Rb. blasticus</i>	0.0797	0.0700	0.0723	0.0775	0.0753	0.0835	0.0723	0.0544	0.0507	0.0507	—	92.7	91.6	90.3
12. <i>Rv. sulfidophilum</i>	0.0835	0.0797	0.0745	0.0768	0.0775	0.0700	0.0678	0.0604	0.0648	0.0686	0.0700	—	95.5	90.0
13. <i>Rv. adriaticum</i>	0.0812	0.0835	0.0768	0.0768	0.0857	0.0745	0.0700	0.0678	0.0797	0.0648	0.0790	0.0432	—	90.0
14. <i>Ro. denitrificans</i>	0.0760	0.0842	0.0812	0.0715	0.0738	0.0768	0.0864	0.0745	0.0931	0.0872	0.0961	0.0946	0.0984	—
15. <i>E. coli</i>	0.2079	0.2101	0.2049	0.2064	0.2131	0.2109	0.2019	0.2109	0.2131	0.1997	0.2154	0.2146	0.2101	0.2206

* *Rb.*, *Rhodobacter*; *Rv.*, *Rhodovulum*; *Ro.*, *Roseobacter*.

joining phylogenetic tree was constructed (Fig. 1). As expected from the relatively high levels of interspecies sequence similarity within the genus *Paracoccus*, members of this genus formed a line of descent separate from the lineage including the genera of the phototrophic bacteria. Strain THI 011^T and *T. versutus* belonged to the cluster of *Paracoccus*, with *P. aminophilus* and *P. denitrificans*, respectively, as the closest relative. A bootstrap analysis showed that the *Paracoccus* cluster, including strain THI 011^T and *T. versutus*, is relatively stable as a monophyletic group (94.5% support).

Morphology, physiology and biochemistry

The cells of strain THI 011^T were Gram-negative, non-sporeforming, non-acid-alcohol-fast, non-motile rods measuring 0.5–0.7 μm in width and 0.8–1.3 μm in length, and occurred singly or in pairs (Fig. 2). Electron micrographs of thin sections revealed that polyhedral inclusion bodies were absent from the cells (Katayama-Fujimura *et al.*, 1984c), unlike those of *Thiobacillus* species of groups II and III. Colonies grown on BY medium were circular, entire, smooth, opaque and reddish yellow, becoming 3–4 mm in diameter after 2 d incubation. On thiocyanate medium, colonies were circular, thin, smooth, entire, opaque, yellowish white or creamy white, and 0.4–0.7 mm in diameter after 8 d incubation.

Strain THI 011^T grew as an aerobic facultative chemolithotroph with a strictly respiratory type of metabolism using oxygen as the terminal electron acceptor. Anaerobic

growth was possible when nitrate was present as a terminal electron acceptor in SM/glucose medium. Nitrate-respiring growth was accompanied by evolution of N_2 gas after 10 d incubation. The organism was neutrophilic and mesophilic: optimal growth occurred at pH 7.5–8.5 (range 6.5–9.5) in BY medium and at pH 7.0–8.0 (range 6.0–8.5) in TC medium; optimum temperature for growth was between 30 and 35 °C (range 15–40 °C). Thiamin was required as a growth factor. The organism was catalase and oxidase positive. A wide variety of organic compounds served as carbon sources: L-alanine, L-serine, L-leucine, L-isoleucine, L-glutamate, L-proline, L-histidine, L-phenylalanine, L-arabinose, D-ribose, D-glucose, D-galactose, fructose, mannitol, *n*-butanol, acetate, propionate, *n*-butyrate, lactate, pyruvate, succinate, glutarate, *m*-hydroxybenzoate and *p*-hydroxybenzoate. Carbon sources supporting weak growth were: ethanol, *n*-propanol, formate, 2-oxoglutarate and gluconate. The following were not utilized: L-aspartate, L-tryptophan, L-cysteine, maltose, lactose, sucrose, cellobiose, glycerol, methanol, cyclohexanol, citrate, L-malate, oxalate, adipate, pimelate, benzoate, *o*-hydroxybenzoate, *p*-aminobenzoate and DL-mandelate. Ammonium salts and glutamate, but not nitrate, urea or aspartate, were used as nitrogen sources. Gelatin was not hydrolysed.

Utilization of sulphur compounds

Strain THI 011^T was a facultative chemolithotroph when grown with reduced sulphur compounds such as thio-cyanate, thiosulphate or elemental sulphur as energy

Fig. 1. Distance matrix tree showing phylogenetic affiliations of strain THI 011^T, *T. versutus*, *Paracoccus* species, and related members of the α -3 subclass of the *Proteobacteria*. The bacteria examined for 16S rDNA sequences in this study are indicated by boldface type. A bootstrap confidence value (expressed as a percentage of 1000 bootstrapped trials) is given at the node placing the tested 'thiobacilli' and *Paracoccus* species as a monophyletic group. * Evolutionary distance (K_{nuc}) between *E. coli* and tested organisms in the α -3 subgroup.

Fig. 2. Electron micrograph of cells of strain THI 011^T. Cells were negatively stained with phosphotungstic acid. Bar, 1 μ m.

sulphate, but the pH of the medium changed little, presumably due to the formation of ammonium and potassium ions. Strain THI 011^T also oxidized thio-sulphate to sulphate or tetrathionate, depending upon the physiological condition of the cells: heterotrophically grown cells (grown in BY medium) converted thio-sulphate to tetrathionate, whereas autotrophically grown cells (grown in TC/thiocyanate medium) oxidized thio-sulphate to sulphate. Elemental sulphur was oxidized slowly with a decrease in pH of the medium from 7.5 to 5.5 during 1–2 weeks incubation. Tetrathionate did not support growth.

Chemotaxonomic characteristics

Strain THI 011^T contained ubiquinones as the sole respiratory quinones, with Q-10 representing 99% of the total ubiquinone content. It contained [$C_{18:1} + C_{19:0}$] as major non-hydroxyl fatty acids, representing about 80% of the total non-hydroxyl acids. Strain THI 011^T also contained 3-hydroxyl fatty acids, with $C_{10:0}$ predominating.

Genomic DNA relatedness

Experiments on genomic DNA relatedness confirmed the taxonomic positions of strain THI 011^T and *T. versutus*, as possible new species of the genus *Paracoccus* (Table 3).

source. The specific growth rate for growth with thiocyanate was 0.059 h^{-1} . During growth in a medium containing $3.5 \text{ g potassium thiocyanate l}^{-1}$, the thiocyanate was degraded with the concomitant production of

Table 3. DNA base composition of and DNA–DNA relatedness among the test organisms

Organism	G + C content of DNA (mol %)	Level (%) of hybridization with labelled DNA from:						
		Strain THI 011 ^T	IAM 12814 ^T	IAM 12479 ^T	JCM 7686 ^T	JCM 7685 ^T	JCM 7364 ^T	JCM 7684 ^T
Strain THI 011 ^T	66.5*	100	31	23	33	27	31	14
<i>Thiobacillus versutus</i> IAM 12814 ^T	66.8	24	100	49	29	29	31	21
<i>Thiobacillus versutus</i> IAM 12815	66.7	25	98	NT	NT	NT	NT	NT
<i>Paracoccus denitrificans</i> IAM 12479 ^T	66.5	29	56	100	26	26	39	16
<i>Paracoccus denitrificans</i> IFO 13301	66.5	26	51	89	NT	NT	NT	NT
<i>Paracoccus aminophilus</i> JCM 7686 ^T	63.8	38	21	32	100	22	34	17
<i>Paracoccus aminovorans</i> JCM 7685 ^T	66.8	35	26	22	36	100	37	14
<i>Paracoccus alcaliphilus</i> JCM 7364 ^T	64.6	28	22	24	34	34	100	16
<i>Paracoccus kocurii</i> JCM 7684 ^T	70.2	21	18	19	17	15	18	100
<i>Rhodobacter capsulatus</i> DSM 1710 ^T	66.0	9	11	12	11	8	9	7
<i>Rhodobacter sphaeroides</i> DSM 158 ^T	69.2	12	16	14	14	10	10	8

* Cited from Katayama-Fujimura *et al.* (1984b).

Hybridization levels of strain THI 011^T with *T. versutus* and *Paracoccus* species were between 14 and 38%. *T. versutus* had a relatively high level of hybridization to *P. denitrificans*, but this value is smaller than the lower limit for a single species defined on the basis of DNA–DNA hybridization (approximately 70% or greater; Wayne *et al.*, 1987). The G + C content of the DNA from strain THI 011^T was 66.5 mol% as determined by HPLC of the nuclease P1 hydrolysate of the DNA (Katayama-Fujimura *et al.*, 1984b). The G + C content calculated from T_m was 67.6 mol% (Katayama-Fujimura *et al.*, 1983a).

DISCUSSION

Utilization of sulphur compounds, including thiocyanate, as energy sources for growth has been found in a variety of chemotrophic bacteria, many of which have little or no taxonomic relationship to each other (Robertson & Kuenen, 1992). We have presented here the phenotypic, chemotaxonomic and genetic characteristics of a thiocyanate-utilizing facultative chemolithotroph, strain THI 011^T. To our knowledge, the present study is the first to describe a species of facultatively chemolithotrophic bacterium capable of using thiocyanate as an energy source.

Although strain THI 011^T was previously called *Thiobacillus* sp. because of its ability to grow chemolithotrophically with sulphur compounds, the phylogenetic analysis based on PCR-amplified 16S rDNA sequences (Table 2) demonstrates that this organism belongs to the lineage encompassing members of the genus *Paracoccus*, with *P. aminophilus* as the nearest neighbour. This strongly implies that the new isolate should be reclassified as a member of the genus *Paracoccus*. Genomic DNA hybridization levels between strain THI 011^T and known *Paracoccus* species are sufficiently low to

warrant classification of the isolate as a new genospecies of *Paracoccus*. Our phylogenetic data also provide evidence that *T. versutus* is a member of the genus *Paracoccus* with *P. denitrificans* as the closest relative, supporting previous findings concerning the phylogenetic affiliations of these organisms (Lane *et al.*, 1992; Ludwig *et al.*, 1993). The high level of sequence similarity (99.1%) between *T. versutus* and *P. denitrificans* indicates their close relationship. Nevertheless, the results of DNA–DNA pairing studies allow separation of *T. versutus* as a distinct genospecies from *P. denitrificans* and any other known members of the genus *Paracoccus*.

The genus *Paracoccus* now consists of the following five species: *P. denitrificans* (type species), *P. aminophilus* (Urakami *et al.*, 1990), *P. aminovorans* (Urakami *et al.*, 1990), *P. alcaliphilus* (Urakami *et al.*, 1989) and *P. kocurii* (Ohara *et al.*, 1990). The removal of *P. halodenitrificans* from the genus *Paracoccus* was recently proposed on the basis of chemotaxonomic traits (Ohara *et al.*, 1990; Urakami *et al.*, 1990) and it was suggested that it be placed in a genus of the γ subclass of the *Proteobacteria* (Miller *et al.*, 1994). There are a number of major phenotypic differences between the *Paracoccus* species and strain THI 011^T or *T. versutus* and between the latter two organisms in physiological properties and carbon source utilization (see Table 4). These phenotypic data, together with the molecular genetic information noted above, warrant official taxonomic proposals for strain THI 011^T and *T. versutus* as two new species of the genus *Paracoccus*.

On the basis of these results, we make three taxonomic proposals, as follows: the designation of strain THI 011^T as a new *Paracoccus* species with the name *Paracoccus thiocyanatus* sp. nov.; the transfer of *T. versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. (type strain, IAM 12814); and the resultant emendation of the genus *Paracoccus*. The differential characteristics of *P. thio-*

Table 4. Useful characteristics to differentiate *Paracoccus* species

Characteristic	<i>P. thio-</i> <i>cyanatus</i>	<i>P. versutus</i>	<i>P. alcali-</i> <i>philus</i> *	<i>P. amino-</i> <i>philus</i> †	<i>P. amino-</i> <i>vorans</i> †	<i>P. denitri-</i> <i>ficans</i> ‡	<i>P. kocurii</i> ‡
Motility	—	±§	—	—	—	—	—
Nitrate respiration	+	+	—	—	—	+	+
Utilization of:							
Glycerol	—	+	+	+	+	+ or —	w
Sucrose	—	+	—	—	—	+	—
D-Fructose	+	+	+	—	+	+	—
Mannitol	+	+	+	—	+	+	—
Benzoate	—	+	—	—	—	—	—
Thiosulphate	w	+	—	—	—	+	—
Thiocyanate	+	—	—	—	—	—	—
Vitamin requirement	Thiamin	—	Biotin	Thiamin	Thiamin	—	Thiamin

* Data from Urakami *et al.* (1989). † Data from Urakami *et al.* (1990). ‡ Data from Ohara *et al.* (1990).

§ There are positive and negative reports for motility (Harrison, 1983; Katayama-Fujimura *et al.*, 1983b).

cyanatus, *P. versutus* and other members of the genus *Paracoccus* are shown in Table 4.

Chemolithotrophy, with molecular hydrogen and thiosulphate as the energy sources, is found in *P. denitrificans*, the type species of the genus *Paracoccus*. Unfortunately, little information about chemolithotrophic growth of other species of the genus *Paracoccus* is available; thus we cannot compare the tested 'thiobacilli' with them in this respect. There is a need to study these *Paracoccus* species for the ability to grow chemolithotrophically with reduced sulphur compounds, including thiocyanate. In this connection, it is interesting to note that members of the phototrophic genera *Rhodobacter* and *Rhodovulum*, the phylogenetic neighbours of *Paracoccus*, can use sulphide or thiosulphate as an electron donor for anaerobic phototrophic growth (Imhoff, 1989; Hiraishi & Ueda, 1994). Species in the genus *Paracoccus* differ from the other members of the α -3 subgroup of *Proteobacteria* in their lack of phototrophic growth ability.

Emended description of the genus *Paracoccus*

Paracoccus (pa.ra.coc'cus, Gr. prep. *para*, like, alongside of; Gr. n. *coccus*, a grain, berry; ML masc. n. *Paracoccus*, like a coccus).

Cells are spherical or rod-shaped, 0.5–1.3 μ m wide and 0.9–2.0 μ m long, occurring singly, in pairs, in chains or in clusters. No resting stage is known. Gram-negative. Non-motile (one species is motile, having a tuft of polar flagella). Metabolism is respiratory with oxygen as the terminal electron acceptor. Some species are also able to use nitrate as the acceptor. Aerobic chemoorganotrophs or facultative chemolithotrophs. Facultative chemolithotrophic species utilize reduced sulphur compounds or molecular hydrogen as energy source. Mesophilic, neutrophilic and non-halophilic. Catalase and oxidase positive.

A wide variety of organic compounds are used as carbon and energy sources. Major quinone is Q-10. Major non-hydroxyl fatty acids are [C_{18:1} + C_{19:0}], which constitute 60–80% of total non-hydroxyl acids. 3-Hydroxyl fatty acids are present, with 3-OH C_{10:0} predominating. Some species also contain 3-OH C_{14:0}. The G + C content of the DNA is 64–70 mol%. Phylogenetic position is the α -3 subclass of the *Proteobacteria*.

Type species: *Paracoccus denitrificans* ([Beijerinck 1910] Davis 1969, 384) Ludwig *et al.* 1993.

Description of *Paracoccus thiocyanatus* sp. nov.

Paracoccus thiocyanatus (thi.o.cyan'atus, L. m. adj. *thiocyanatus* thiocyanate; referring to the ability to use thiocyanate).

Cells are rod-shaped, 0.5–0.7 μ m wide and 0.8–1.3 μ m long, occurring singly or in pairs. Non-sporeforming. Non-motile. Gram-negative. Not acid-alcohol-fast. Polyhedral inclusion bodies (carboxysomes) are absent in cells. Grows aerobically with oxygen as the terminal electron acceptor. Anaerobic growth is also possible with nitrate as the acceptor in heterotrophic medium, denitrification positive. Neutrophilic and mesophilic. Growth factor requirement is satisfied by thiamin. Facultatively chemolithotrophic and autotrophic. Thiocyanate serves as an energy source. Elemental sulphur is oxidized slowly. Thiosulphate is oxidized to tetrathionate by chemoorganotrophically grown cells or to sulphate by thiocyanate-grown cells. Tetrathionate does not support growth. Various organic compounds, including amino acids, carbohydrates, carboxylic acids, alcohols and aromatic compounds, are assimilated. Gelatin is not liquefied. The G + C content of the DNA is 66.5 mol% (HPLC). All other characteristics are as described in Results. Source: activated sludge. Type strain: THI 011 (= IAM 12816).

Description of *Paracoccus versutus* [*Thiobacillus versutus* Harrison 1983, 216 (*Thiobacillus rapidi* Katayama-Fujimura, Kawashima, Tsuzaki and Kuraishi, 1983, 536)] comb. nov.

Paracoccus versutus (ver.su'tus. L. adj. *versutus* versatile).

Cells are rod-shaped, 0.4–0.5 µm wide and 1.1–1.7 µm long, occurring singly or in pairs. Non-sporeforming. Motile with a tuft of polar flagella. Gram-negative. Not acid-alcohol-fast. Colonies are circular, wrinkled with raised centre, lobate, spreading, brownish yellow, opaque, becoming 3–4 mm in diameter after 2 d incubation on ordinary complex media. On thiosulphate medium, colonies are circular, convex, smooth, entire, creamy white and opaque, developing a brown centre with age, and about 2 mm in diameter after 1 week. Polyhedral inclusion body is absent in cells. Grows aerobically with oxygen as the terminal electron acceptor. Anaerobic growth is also possible with nitrate as the acceptor; denitrification positive under heterotrophic growth conditions. Optimal growth occurs at 30–37 °C (range, 17–40 °C) and at pH 7.5–8.0 (range, pH 6.5–9.5) in ordinary complex media and at pH 8.0–9.0 in thiosulphate medium (range, pH 6.5–9.5). Growth factors are not required. Facultatively chemolithotrophic and autotrophic; thiosulphate is utilized as an energy source. Elemental sulphur is oxidized slowly. Tetrathionate or thiocyanate does not support growth. Usable carbon sources are: L-alanine, L-serine, L-leucine, L-isoleucine, L-aspartate, L-glutamate, L-proline, L-histidine, L-phenylalanine, L-arabinose, D-ribose, D-glucose, D-galactose, fructose, maltose, sucrose, gluconate, glycerol, mannitol, formate, acetate, propionate, *n*-butyrate, lactate, pyruvate, L-malate, succinate, 2-oxoglutarate, glutarate, adipate, ethanol, *n*-propanol, *n*-butanol, benzoate, *m*-hydroxybenzoate and *p*-hydroxybenzoate. Methanol supports weak growth. Not utilized are: L-tryptophan, L-cysteine, lactose, cellobiose, oxalate, pimelate, cyclohexanol, *o*-hydroxybenzoate, *p*-amino-benzoate and DL-mandelate. There are positive and negative reports for growth on citrate (Wood & Kelly, 1983; Katayama-Fujimura *et al.*, 1983b). Nitrate and ammonium salts, glutamate and aspartate, but not urea, are utilized as nitrogen sources. Gelatin is not hydrolysed. The G + C content of the DNA is 67–68 mol %. All other characteristics are the same as those for the genus. Source: soil. Type strain: IAM 12814 (= ATCC 25364).

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