Arch Microbiol (1998) 169 : 148–158

ORIGINAL PAPER

Ann P. Wood · Donovan P. Kelly · Ian R. McDonald
Sarah L. Jordan · Thomas D. Morgan · Sajdah Khan
J. Colin Murrell · Elena Borodina

A novel pink-pigmented facultative methylotroph, *Methylobacterium thiocyanatum* sp. nov., capable of growth on thiocyanate or cyanate as sole nitrogen sources

Received: 17 April 1997 / Accepted: 16 September 1997

Abstract The isolation and properties of a novel species of pink-pigmented methylotroph, *Methylobacterium thiocyanatum*, are described. This organism satisfied all the morphological, biochemical, and growth-substrate criteria to be placed in the genus *Methylobacterium*. Sequencing of the gene encoding its 16S rRNA confirmed its position in this genus, with its closest phylogenetic relatives being *M. rhodesianum*, *M. zatmanii* and *M. extorquens*, from which it differed in its ability to grow on several diagnostic substrates. Methanol-grown organisms contained high activities of hydroxypyruvate reductase [3 μmol NADH oxidized min^{-1} (mg crude extract protein)$^{-1}$], showing that the serine pathway was used for methylotrophic growth. *M. thiocyanatum* was able to use thiocyanate or cyanate as the sole source of nitrogen for growth, and thiocyanate as the sole source of sulfur in the absence of other sulfur compounds. It tolerated high concentrations (at least 50 mM) of thiocyanate or cyanate when these were supplied as nitrogen sources. Growing cultures degraded thiocyanate to produce thiosulfate as a major sulfur end product, apparently with the intermediate formation of volatile sulfur compounds (probably hydrogen sulfide and carbonyl sulfide). Enzymatic hydrolysis of thiocyanate by cell-free extracts was not demonstrated. Cyanate was metabolized by means of a cyanase enzyme that was expressed at approximately sevenfold greater activity during growth on thiocyanate [V_{max} 634 ± 24 nmol NH_3 formed min^{-1} (mg protein)$^{-1}$] than on cyanate [89 ± 9 nmol NH_3 min^{-1} (mg protein)$^{-1}$]. Kinetic study of the cyanase in cell-free extracts showed the enzyme (1) to exhibit high affinity for cyanate (K_m 0.07 mM), (2) to require bicarbonate for activity, (3) to be subject to substrate inhibition by cyanate and competitive inhibition by thiocyanate (K_i 0.65 mM), (4) to be unaffected by 1 mM ammonium chloride, (5) to be strongly inhibited by selenocyanate, and (6) to be slightly inhibited by 5 mM thiosulfate, but unaffected by 0.25 mM sulfide or 1 mM thiosulfate. Polypeptides that might be a cyanase subunit (mol.wt. 17.9 kDa), a cyanate (and/or thiocyanate) permease (mol.wt. 25.1 and 27.2 kDa), and a putative thiocyanate hydrolase (mol.wt. 39.3 kDa) were identified by SDS-PAGE. Correlation of the growth rate of cultures with thiocyanate concentration (both stimulatory and inhibitory) and the kinetics of cyanase activity might indicate that growth on thiocyanate involved the intermediate formation of cyanate, hence requiring cyanase activity. The very high activity of cyanase observed during growth on thiocyanate could be in compensation for the inhibitory effect of thiocyanate on cyanase. Alternatively, thiocyanate may be a nonsubstrate inducer of cyanase, while thiocyanate degradation itself proceeds by a carbonyl sulfide pathway not involving cyanate. A formal description of the new species (DSM 11490) is given.

Key words *Methylobacterium thiocyanatum* · Thiocyanate metabolism · Cyanase · 16S rRNA sequence · SDS-PAGE · Pink-pigmented facultative methylotroph

A. P.Wood (✉) · S. L. Jordan · T. D. Morgan · S. Khan
E. Borodina
Division of Life Sciences, King's College London,
Campden Hill Road, London W8 7AH, England
Tel. + 44-171-333-4344; Fax + 44-171-333-4500
e-mail: ann.p.wood@kcl.ac.uk

D. P. Kelly
Environmental Sciences Research and Education Unit,
Institute of Education, University of Warwick,
Coventry CV4 7AL, England

I. R. McDonald · J. C. Murrell
Department of Biological Sciences,
University of Warwick, Coventry CV4 7AL, England

Introduction

Thiocyanate is produced as an end product of both animal and plant metabolism and arises from cyanide and possibly sulfide detoxification; it also arises from the breakdown of more complex plant products such as the glucosinolates and occurs in waste waters from biocides, coal and oil processing, steel manufacture, and the petrochemical industry (Beekhuis 1975; Wood 1975; Neufeld and

Valiknac 1979; Oh et al. 1987; Richards and Shieh 1989; Paruchuri et al. 1990; Brown and Morra 1993; Kelly et al. 1993). Thiocyanate is toxic to many microorganisms at relatively low concentrations (1–2 mM), but its biodegradation has been shown to be a property of a number of chemolithotrophic and heterotrophic bacteria. Complete degradation to carbon dioxide and sulfate was first demonstrated in strains of the obligate chemolithotroph *Thiobacillus thioparus* (Happold and Key 1937; Youatt 1954; De Kruyff et al. 1957; Katayama and Kuraishi 1978), which uses oxidation of the sulfur of thiocyanate to sulfate for chemolithotrophic energy generation to support autotrophic growth. At least some *T. thioparus* strains contain a hydrolase that converts thiocyanate initially to ammonia and carbonyl sulfide, the latter subsequently being hydrolyzed to sulfide, which is oxidized to sulfate (Happold et al. 1954, 1958; Smith and Kelly 1988; Katayama et al. 1992):

$$KSCN + 2\ H_2O \rightarrow COS + NH_3 + KOH \quad (1)$$

$$COS + H_2O \rightarrow H_2S + CO_2 \quad (2)$$

$$H_2S + 2\ O_2 \rightarrow H_2SO_4 \quad (3)$$

Several heterotrophic bacteria have also been shown to degrade thiocyanate during growth and to use the ammonia released as the source of nitrogen for biosynthesis (Stafford and Callely 1969; Betts et al. 1979; Paruchuri et al. 1990; Mason et al. 1994; Stratford et al. 1994; Katayama et al. 1995). Two alternative pathways for the initial cleavage of thiocyanate have been identified in heterotrophs: (1) the carbonyl sulfide pathway, producing carbonyl sulfide and ammonia as the first intermediates (Eq. 1), and (2) the cyanate pathway, producing cyanate and hydrogen sulfide as initial products of thiocyanate hydrolysis (Kelly et al. 1993; Mason et al. 1994; Stratford et al. 1994; Mason 1995), with the cyanate being subsequently hydrolyzed to release ammonia:

$$KSCN + H_2O \rightarrow KOCN + H_2S \quad (4)$$

$$KOCN + 2\ H_2O \rightarrow NH_3 + CO_2 + KOH \quad (5)$$

The use of cyanate as a nitrogen source has been separately established in *Escherichia coli* and in a *Pseudomonas* species, both of which cleave cyanate using cyanase enzymes that have been studied in some detail (Anderson and Little 1986; Kunz and Nagappan 1989; Anderson et al. 1990). Much of the sulfide released by different heterotrophs using reactions 2 and 4 is subsequently oxidized, primarily to one or more of thiosulfate, tetrathionate and sulfate (Stafford and Callely 1969; Boucabeille et al. 1994; Mason et al. 1994; Stratford et al. 1994; Mason 1995).

Prior to our studies, no facultatively methylotrophic heterotroph had been shown to use thiocyanate as sole nitrogen and sulfur sources or cyanate as a nitrogen source. We now report a novel species of *Methylobacterium* isolated by enrichment culture from the root ball of *Allium aflatunense*, a member of a family of plants that is known to produce a wide range of alkyl mono-, di-, and tri-sulfides, thiosulfinates, and sulfinyl disulfides (Kelly and Smith 1990; Kuo et al. 1990; Block et al. 1992). This novel organism, *Methylobacterium thiocyanatum*, which is described here for the first time, can use thiocyanate as its sole nitrogen source while growing heterotrophically on sugars or organic acids, or on formate as a carbon and energy source, or methylotrophically on methanol. We discuss the evidence for and against a cyanate pathway of thiocyanate degradation and the evidence for the role of a cyanase enzyme in the assimilation of nitrogen from thiocyanate and cyanate. The identity of *M. thiocyanatum* as a distinct species within the genus is demonstrated on the basis of 16S rRNA sequences and the diagnostic physiological and biochemical features that distinguish it from other *Methylobacterium* species.

Materials and methods

Medium for growth experiments and maintenance

The mineral salts medium used was that described previously for growth of *Paracoccus versutus* (Wood and Kelly 1977), with the modification that NH4Cl was replaced by 2.5 mM potassium thiocyanate and that the trace metal solution was replaced by 1 ml 2% (w/v) $FeSO_4 \cdot 7H_2O$ in 1 M HCl per litre of medium. A medium containing 25 mM glucose and 2.5 mM potassium thiocyanate was routinely used for maintenance of stocks on 1.5% (w/v) agar slants. Liquid cultures were routinely grown in 50 ml of medium in 250-ml Erlenmeyer flasks shaken at 200 rpm at 30°C in a rotary shaking incubator.

In some experiments, alternative energy sources replaced glucose, and alternative nitrogen sources replaced thiocyanate; these are detailed in Results. Where KSCN was tested as the sole sulfur source, magnesium and ferric sulfates were replaced by magnesium chloride and ferric chloride. Anaerobic growth was tested using (1) solid media in anaerobic gas jars, (2) liquid media supplemented with potassium nitrate (25 mM), and (3) fermentation tubes.

Enrichment and isolation procedures

Soil (2–3 g) removed from the root balls of *A. aflatunense* was added to basal salts medium supplemented with glucose (25 mM) as the carbon and energy source and with thiocyanate (2.5 mM) as the nitrogen source. Cultures were shaken at 30°C and were subcultured at intervals into fresh growth medium. After three subcultures, single-colony isolation on glucose-thiocyanate-agar plates produced two cultures: white-pigmented and pink-pigmented colony types. On the basis of the greater efficiency of growth of the pink organism on thiocyanate as a nitrogen source, it was selected for detailed work and is described in this paper as a new species: *M. thiocyanatum* strain ALL/SCN-P.

Morphological, biochemical and physiological tests

Standard light microscopy and staining procedures were used to detect gram reaction, capsule and spores. For scanning electron microscopy, cells from an agar slant were (1) fixed for 1 h in 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate containing 1 mM $CaCl_2$, (2) washed three times in 0.1 M phosphate buffer (pH 7.2), (3) post-fixed in 1% (w/v) OsO_4 in cacodylate/$CaCl_2$ for 1 h, (4) washed twice in phosphate buffer, and (5) dehydrated through 30, 50, 70, and 90% (v/v) acetone and three times in 100% acetone. Samples were subjected to critical point drying onto 20-μm membrane filters under pressure with liquid carbon dioxide using an EM Scope Critical Point Drier 750. Membranes were mounted

onto Cambridge-type (pinhead) stubs and transferred to an SEM coating unit E5100 (Fisons Instrument Division, VG Microtech, East Grinstead, UK). After flushing several times with argon, the unit was evacuated to 7 Pa and the cells were sputter-coated with gold (20–30 nm thickness). Catalase and oxidase activity were determined using standard tests (Barrow and Feltham 1993). Thiocyanate was assayed spectrophotometrically as ferric thiocyanate, and thiosulfate and tetrathionate were assayed using specific cyanolytic procedures (Kelly et al. 1969; Kelly and Wood 1994). Protein content of cell-free extracts was determined by the Folin-Ciocalteu method (Lowry et al. 1951).

Assay of cyanase in cell-free extracts

Cultures were grown in the basal medium on 10 mM sodium succinate with 2.5 mM KSCN, 2.5 mM KOCN or 7.5 mM NH_4Cl as a nitrogen source. Organisms were harvested by centrifuging at $23{,}000 \times g$ for 10 min at 5° C, washing in 13 mM phosphate buffer (pH 7.0), recentrifuging, and resuspending in 0.1 M Pipes (pH 7.0). Suspensions were broken by two or three passages through a chilled French pressure cell at 140 MPa. Cell debris was removed by centrifugation at $38{,}000 \times g$ for 40 min at 5° C, and the supernatant crude cell extract was used immediately or stored at –20° C prior to use.

Cyanase (cyanate amidohydrolase; EC 3.5.5.3) assays were based on the procedures of Anderson (1980) and Kunz and Nagappan (1989): for the standard assay, reaction mixtures (total volume, 4 ml in 15 ml polycarbonate or polypropylene tubes) were incubated at 30° C and contained 50 mM potassium phosphate buffer (pH 7.6), 3 mM sodium bicarbonate, and 2 mM potassium cyanate; the reaction was initiated by addition of cell-free extracts (0.1, 0.2 or 0.4 ml; 4.5–7.6 mg protein ml^{-1}). Samples (0.5 or 1 ml) were removed immediately and at intervals for up to 20 min, and were mixed with an equal volume of a 1:3 dilution of commercial Nessler's reagent. Ammonia formation from the substrate was estimated from the $OD_{420\ nm}$ using ammonium chloride as a standard. Most OD measurements were made with a Shimadzu UV-150–02 spectrophotometer (Shimadzu, Kyoto, Japan). For determination of kinetic constants and inhibitory effects, cyanate concentrations were varied between 0.2 and 50 mM in the standard assay. Effects on cyanase activity in crude extracts of thiocyanate, selenocyanate, ammonium ion, sulfide, thiosulfate, and omission of bicarbonate from the assay are detailed in the Results.

Assay methods for "thiocyanate hydrolase" and hydroxypyruvate reductase

The production of ammonia from thiocyanate was sought using a procedure similar to that for cyanase, both with and without added bicarbonate; thiocyanate concentrations between 0.4 and 18 mM and incubation times of 20 min were tested. A decrease of thiocyanate concentration by a possible thiocyanate hydrolase was sought by a method based on Katayama et al. (1992): reaction mixtures (0.8 ml) in capped tubes incubated at 30° C contained 100 mM potassium phosphate buffer (pH 7.6), 20 mM potassium thiocyanate, and a cell-free extract from bacteria grown with thiocyanate as the sole nitrogen source (1.7 mg protein). Thiocyanate concentration was measured in 0.1-ml samples at intervals for 3 h. Standard Ouchterlony gel-diffusion procedures using antibodies to the thiocyanate hydrolase of *T. thioparus* (Katayama et al. 1992) were tested to determine if this enzyme was present in strain ALL/SCN-P.

Hydroxypyruvate reductase (EC1.1.1.29) was assayed with NADH as the reductant as described previously (Kelly and Wood 1984; Wood and Kelly 1989) using either phosphate buffer (pH 7.0) or acetate buffer (pH 5.0) with 2–10 µg cell-free extract protein (ml assay)$^{-1}$. After establishing the endogenous activity of NADH oxidase, the hydroxypyruvate reductase assay was initiated by addition of 2 mM lithium hydroxypyruvate, and the increase in rate of NADH oxidation due to hydroxypyruvate reductase was determined from the rate of decrease in A340 nm.

SDS-PAGE analysis of protein in cell-free extracts

M. thiocyanatum strain ALL/SCN-P was grown in batch culture in media with (1) succinate as the carbon and energy source, supplemented with methylamine, ammonium chloride, thiocyanate or cyanate as nitrogen sources, and (2) methylamine as the sole carbon, energy and nitrogen source, or methylamine with ammonium chloride, thiocyanate or cyanate as additional nitrogen sources. Organisms were harvested by centrifuging, were resuspended in 0.1 M Pipes (pH 7.0), and were disrupted by sonication. Suspensions were chilled on ice and sonicated three times for 1 min using a 1-cm probe fitted to an MSE100-W ultrasonic disintegrator (MSE, London, UK). If necessary, the protein in extracts was concentrated by partition in aqueous methanol and chloroform, precipitation with methanol, and drying over silica gel at –20° C. Protein was then redissolved in 30 mM Tris-HCl (pH 8.0; 1 vol.) and mixed with loading buffer (4 vol.). Extracts were treated with SDS and analysed by polyacrylamide gel electrophoresis by standard procedures using 12.5% (w/v) acrylamide gels (Laemmli 1970). Samples equivalent to 15 and 20 µg protein for each preparation were loaded onto 10- and 5-well gels, respectively. Molecular masses of polypeptides were estimated by reference to a commercial standard mixture (mol.wt.: 14.4, 21.5, 31, 45, 66.2 and 97.4 kDa; BioRad, Hemel Hempstead, UK).

DNA extraction, base composition, PCR amplification, and sequencing of 16S rRNA clones

DNA was extracted using a modification of the method of Marmur (1961) from cells grown on medium supplemented with 20 mM sodium succinate and 2.5 mM potassium thiocyanate as the sole nitrogen source. Aerobic batch cultures (500 ml) were shaken in 2-l flasks at 200 rpm and 30° C for 48 h and were harvested. The mol% G+C was determined using the melting-point method (Mandel and Marmur 1968) and the procedure of Fredericq et al. (1961).

Bacterial 16S rRNA genes were amplified from all the DNA samples using the bacterial specific primers f27 and r1492 (Giovannoni 1991). Amplification reactions were performed using the reagents supplied with GIBCO-BRL Taq polymerase kits at a magnesium ion concentration of 1.5 mM, with 20 ng template DNA and 100 pmol of each primer added. The reactions were carried out in a Hybaid TR2 thermocycler with 30 cycles of 92° C for 1 min, 60° C for 1 min, and 72° C for 1 min, with a final extension at 72° C for 5 min. Reaction products were checked for size and purity on 1% (w/v) agarose gels (Sambrook et al. 1989) and then ligated into the pCR II vector supplied with a TA Cloning Kit (Invitrogen) according to the manufacturer's instructions.

Small-scale preparations of plasmids were done by the method of Saunders and Burke (1990). All sequencing reactions were carried out using the Sequenase v2.0 Sequencing Kit (USB) according to the manufacturer's instructions. Primers used for the sequencing reactions were complementary to the conserved regions of the 16S rRNA. Their sequences can be found in Lane (1991) and Giovannoni (1991).

Phylogenetic analysis

Sequences were aligned manually to representative proteobacterial sequences obtained from the Genbank database and the Ribosomal Database Project (Maidak et al. 1994). The type strains of the *Methylobacterium* species whose 16S rRNA sequences were compared in this study were *M. organophilum* (type species) ATCC 27886^T, *M. rhodesianum* ATCC 43882^T, *M. zatmanii* ATCC 43883^T, *M. extorquens* ATCC 43645^T, *M. rhodinum* ATCC 14821^T and *M. radiotolerans* ATCC 27329^T. Sequences for other Proteobacteria were those of the reference strains universally used for comparisons of sequence similarities. Dendrograms were constructed using the programs DNADIST, DNAPARS, FITCH and BOOTSTRAP from the PHYLIP v3.4 package (Felsenstein 1988). Only

regions that could be unambiguously aligned were included in the analyses. Secondary structure predictions for 16S rRNAs were constructed manually based on the published models of Neefs et al. (1991) and Gutell et al. (1994). Dendrograms were constructed with the inclusion of the bootstrap values, giving the percentage of occasions in 1,000 trees that the same branching point was obtained. Secondary structure predictions for 16S rRNAs were constructed manually based on the published models of Neefs et al. (1991) and Gutell et al. (1994).

Ubiquinone analysis

The organism was grown on succinate-thiocyanate medium, harvested, resuspended in 0.1 M Pipes (pH 7.0), and the cells were disrupted by sonication. Isolation, purification and identification of the ubiquinone fraction were as described by Jordan et al. (1995). Ubiquinone Q_8 was prepared from frozen cells of *Thiobacillus tepidarius*, and Q_{10} was obtained from Sigma-Aldrich (Gillingham, UK).

Chemicals

Potassium thiocyanate, potassium cyanate, potassium selenocyanate, lithium hydroxypyruvate, NADH, Nessler's Reagent, and piperazine-N,N'-bis[2-ethanesulfonic acid] (Pipes) were purchased from Sigma-Aldrich (Gillingham, UK). Analytical-grade reagents were used for the preparation of all other assay solutions and culture media.

Results

Morphology and growth characteristics

Strain ALL/SCN-P was isolated from soil around the root balls of *A. aflatunense* as described in Materials and methods. Colonies on glucose-thiocyanate agar are pigmented pink, slightly shiny, convex, entirely smooth, and ca. 1.5 mm in size on agar plates. Cells of strain ALL/SCN-P are gram-negative, short rods (0.8×2.0 μm) without spores or capsule (Fig. 1); motility was not observed in any preparation. The pink pigment was nondiffusible in all growth conditions tested. Catalase activity tested positive, and oxidase variably so. Biochemical tests (confirmed up to five times) for indole production and urease activity were positive, but were negative for methyl-red and for Vosges-Proskauer tests and H_2S production. Growth occurred in the temperature range of 15 to 37° C, but with no growth at 5 or 44° C. Initial pH for growth ranged from pH 7.5 to 9.4. Best growth was observed at 30–37° C and pH 7.7.

The range of energy and carbon substrates used by strain ALL/SCN-P was tested using mineral medium supplemented with 2.5 mM potassium thiocyanate as the nitrogen source. It grew on 10 mM glucose, fructose, succinate, pyruvate, fumarate, acetate, glutamate, methylamine, 5 mM citrate, 50 mM methanol, 40 mM formate, nutrient agar and Luria-Bertani agar, and it grew slowly on 10 mM arabinose. Growth was obtained on 1.25 mM formaldehyde, but not with higher concentrations (2.5 and 5 mM). Growth was not supported by 5 mM sucrose, 10 mM thiosulfate, tetrathionate, methanesulfonate, dimethylamine, trimethylamine, or 2.5–20 mM dimethylsulfone. As sole nitrogen source, thiocyanate supported growth on succinate or glucose when supplied at concentrations between 2.5 and 50 mM, but at 100 mM KSCN, growth was either completely inhibited or occurred in small aggregates. No growth was observed with 150 mM thiocyanate. Heterotrophic growth at similar growth rates (on succinate or glucose) was supported by any one of the following: thiocyanate, ammonium chloride, potassium cyanate, methylamine, or potassium nitrate. Methylamine could also serve as a sole carbon and energy substrate and acted as a sole nitrogen source in cultures lacking added nitrogen salts through at least four subcultures. Anaerobic growth was not observed on (1) glucose-thiocyanate agar plates in anaerobic jars, (2) in liquid media with potassium nitrate and glucose, succinate or pyruvate (no gas production from denitrification), or (3) on glucose or succinate in fermentation tubes (no fermentative acid production).

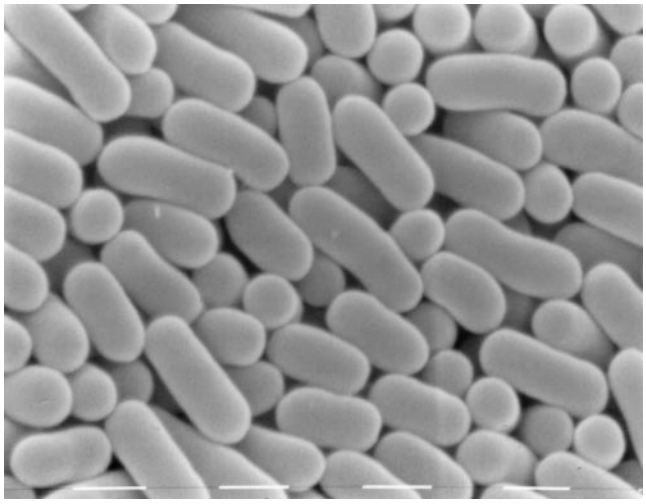

Fig. 1 Scanning electron micrograph of *Methylobacterium thiocyanatum* strain ALL/SCN-P (*white bars* 1 μm)

Growth of strain ALL/SCN-P on one-carbon substrates

With thiocyanate as the sole nitrogen source, the growth rates and biomass production observed on one-carbon substrates (Table 1) were typical of those of methylotrophs using the serine pathway to support methylotrophic growth (Harder et al. 1981). No significant difference in the growth rate or yield on methylamine was seen in the presence or absence of 2.5 mM thiocyanate. Growth was observed only on very low concentrations of formaldehyde (1.25 mM), which was a toxic substrate, giving a yield lower than that on methanol or methylamine (Table 1). Growth rates and yields on 25–50 mM formate were essentially identical, indicating no toxicity of this substrate at least up to 50 mM.

Assay of cell-free extracts of methanol-grown organisms showed the presence of very high activities of hydroxypyruvate reductase. Specific activities in assays in

Table 1 Growth rates and biomass production by *Methylobacterium thiocyanatum* strain ALL/SCN-P on methylotrophic and heterotrophic substrates with thiocyanate (2.5 mM) supplied as a nitrogen source. Biomass production was estimated from initial and final optical density values, based on the relationship that an $OD_{440\ nm}$ value of 1.0 indicated 0.26 g dry wt l^{-1}. Where mean ± standard errors are shown, data are means of 3–17 determinations (*nd* not determined)

Substrate (mM)	Doubling time (h)	Specific growth rate, μ (h^{-1})	Biomass production [g dry wt. (mol substrate)$^{-1}$]
Methanol (10)	8.5 ± 0.2	0.082	14.4 ± 1.8
Methylamine (10)	11.0 ± 1.8	0.051	16.3 ± 2.8
Formaldehyde (1.25)	nd	nd	10.4 ± 1.0
Formate (25, 40, 50)	11.0 ± 0.9	0.062	4.4 ± 0.3
Acetate (10)	15.0	0.046	23.8
Pyruvate (5)	5.5	0.126	20.7
Succinate (10)	5.0 ± 0.2	0.141	30.2 ± 4.5
Fumarate (10)	7.5	0.092	38.5 ± 4.2
Citrate (5)	11.5	0.060	34.3
Glutamate (10)	15.0	0.046	nd
Glucose (10)	11.0	0.063	nd
Fructose (10)	15.0	0.046	85.6
Arabinose (10)	53.0	0.013	nd

phosphate at pH 7.0 and acetate at pH 5.0 were 1.79 ± 0.18 μmol NADH oxidized min^{-1} (mg protein)$^{-1}$ (6 determinations) and 3.00 ± 0.11 μmol NADH oxidized min^{-1} (mg protein)$^{-1}$ (4 determinations), respectively.

Growth of strain ALL/SCN-P on succinate with combinations of thiocyanate, cyanate or ammonium ion as nitrogen sources, and thiocyanate as a sulfur source

During growth with thiocyanate as the sole nitrogen source, consumption of thiocyanate by cultures paralleled growth, with only sufficient thiocyanate being consumed to allow biosynthesis of organisms with a nitrogen content equivalent to 6–9% (w/w) of their dry weight. Growth of cultures on succinate (10 mM) ceased when limiting amounts of thiocyanate (0.31 mM) had been totally consumed as the sole nitrogen source. Inoculating succinate-thiocyanate-grown organisms into media with 10 mM ammonium chloride and 2.5 mM thiocyanate resulted in growth accompanied by disappearance of 80% of the thiocyanate. Subsequent serial subculture in the same medium, however, resulted in approximately 95% suppression of thiocyanate consumption, so that cell nitrogen was derived exclusively from added ammonium ion. This suggested that the thiocyanate-degrading activity of the initial inoculum culture was not inhibited by ammonium ion, but further synthesis of enzymes required for thiocyanate metabolism was repressed.

Because of the known difficulty of preparing media free of combined inorganic sulfur, the use of thiocyanate as both the nitrogen and the sole sulfur source was shown by passage through three subcultures on "sulfate-free" media. Comparison was made between parallel cultures supplemented with (1) 2.5 mM potassium thiocyanate alone, (2) potassium thiocyanate and 10 mM ammonium chloride, and (3) ammonium chloride only. Normal growth and biomass production were seen in sulfate-free media with thiocyanate alone and with thiocyanate and ammonium ion; but cultures without added thiocyanate ceased growth at biomass levels only 25–35% of those of the cultures with added thiocyanate-sulfur.

While 2.5 mM cyanate was readily used as a sole nitrogen source, it caused lag periods of approximately 18 h when succinate or glucose media with 10 mM potassium cyanate were inoculated with organisms previously grown with thiocyanate. When thiocyanate-grown inocula were added to media containing 2.5 mM thiocyanate, the initial doubling time of approximately 5 h was increased to approximately 55 h by the presence of 10 mM potassium cyanate, and rapid growth occurred only after a delay of approximately 50 h. Cyanate (10 mM) in the growth medium also lengthened the lag phase in medium supplemented with ammonium chloride. It was observed, however, that after maintenance on 2.5 mM thiocyanate (as the sole nitrogen source), the organism was able to grow to produce normal yields on succinate when subcultured into media with high concentrations of thiocyanate (50 mM; 46-h lag), cyanate (50 mM; 90-h lag), methylamine hydrochloride (50 mM; 90-h lag), ammonium chloride (200 mM; slow growth) or potassium nitrate (50 mM) as sole nitrogen sources.

Heterotrophic growth with thiocyanate as a nitrogen source

Specific growth rates and biomass production on multicarbon substrates in batch cultures in shaken flasks (Table 1) illustrated a broad chemoorganotrophic capacity, with growth on glucose, fructose, arabinose, citrate and glutamate being an ability not seen in some other *Methylobacterium* species (Green 1992). Both stimulation and depression of the growth rate in response to variation in the concentration of thiocyanate supplied were seen (Fig. 2a). The growth rate increased slightly with concentrations between 2.5 and 20 mM KSCN, and a double reciprocal plot of specific growth rate against thiocyanate concentration indicated a substrate affinity (K_S) of approximately 0.2 mM KSCN and a maximum specific growth rate on succinate of approximately 0.15 h^{-1} (Fig. 2a). At more than 20 mM

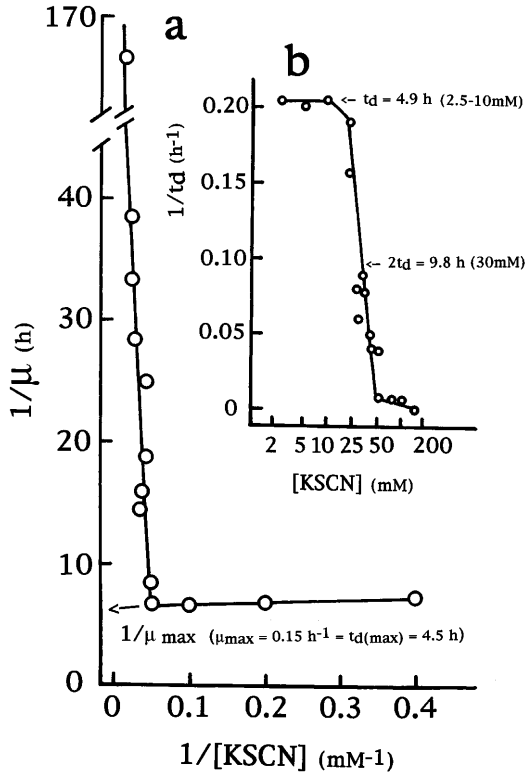

Fig. 2a, b Effect of thiocyanate concentration on the growth rate of *Methylobacterium thiocyanatum* strain ALL/SCN-P on succinate. Exponential growth rates of cultures in shaken flasks were estimated for cultures supplied with potassium thiocyanate as the sole nitrogen source at concentrations of 2.5–150 mM. **a** Plot of reciprocals of specific growth rates (μ) and thiocyanate concentrations showing increasing growth rate between 2.5 and 20 mM KSCN, and progressive inhibition at higher concentrations; **b** plot of reciprocals of doubling times (t_d) against thiocyanate concentration (mM; log scale) showing the concentration of KSCN that causes halving of the growth rate ($2t_d$) as compared to the 2.5–10 mM KSCN range

KSCN, the growth rate was progressively depressed, giving typical substrate inhibition kinetics (Fig. 2a): the maximum doubling time (t_d) was increased twofold to approximately 10 h with 30 mM KSCN, and was raised to 20 and 27 h at 40 and 50 mM thiocyanate, respectively (Fig. 2b).

Fate of the sulfur atom of thiocyanate during growth of strain ALL/SCN-P on succinate with thiocyanate as the sole nitrogen source

Cultures grown in flasks with centre wells containing lead acetate and sealed with vaccine stoppers did not show any blackening of the lead acetate papers, indicating that any free sulfide produced was not detectable by this method. Such sealed cultures did, however, have a marked sulfurous odour that was not solely hydrogen sulfide. This odour that could have been carbonyl sulfide, was not detected in thiocyanate cultures in unsealed flasks or in cultures in sealed flasks grown with ammonium ion as the sole nitrogen source.

Analysis of the medium from sealed and unsealed flasks in which the organism had been grown on succinate with 2.5–2.7 mM thiocyanate as the nitrogen source showed the only detectable soluble sulfur compounds present to be unused thiocyanate (0.1–1.4 mM) and thiosulfate (0.3–0.8 mM); tetrathionate was not detected. The total recovery of thiocyanate-sulfur was 72–86%. In unsealed flasks, 70–80% of the sulfur of the thiocyanate consumed was recovered as thiosulfate, while only approximately 50% was converted to thiosulfate in sealed (oxygen-limited) flasks.

Addition of 1 mM sodium sulfide to a culture (in an unsealed flask) that had completely consumed 2.7 mM thiocyanate (with the production of 0.94 mM thiosulfate) resulted in the production of a further 0.58 mM thiosulfate within 4 h, indicating quantitative oxidation of the added sulfide to thiosulfate.

Cyanase activity in strain ALL/SCN-P

Cyanase was present in cell-free extracts of organisms grown on succinate with thiocyanate, cyanate, or ammo-

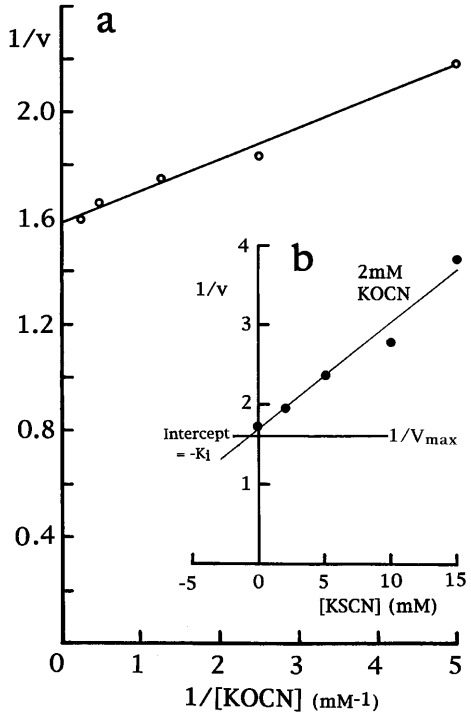

Fig. 3a, b Kinetic analysis of cyanase activity in cell-free extracts of *Methylobacterium thiocyanatum* strain ALL/SCN-P grown on succinate with thiocyanate as the sole nitrogen source. All assays received 0.55 mg protein (4 ml assay vol.)$^{-1}$. **a** Plot of 1/v [= reciprocals of rate of ammonia formation; μmol min^{-1} (mg protein)$^{-1}$] against reciprocals of potassium cyanate substrate concentration (0.2–4 mM). The intercept on the 1/v axis indicates the maximum velocity [V_{max} = 625 nmol ammonia min^{-1} (mg protein)$^{-1}$]. **b** Plot of reciprocals of rate of ammonia formation (1/v) from 2 mM potassium cyanate against concentration of inhibitory potassium thiocyanate (2–15 mM). The intercept on the $1/V_{max}$ line and the negative extrapolation of the plot of the experimental data indicates the inhibitor constant (K_i = 0.65 mM KSCN)

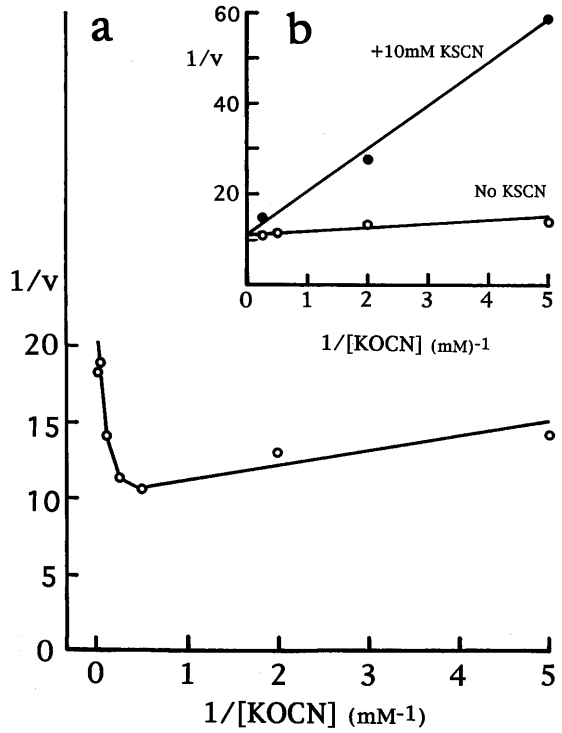

Fig. 4a, b Kinetic analysis of cyanase activity in cell-free extracts of *Methylobacterium thiocyanatum* strain ALL/SCN-P grown on succinate with cyanate as the sole nitrogen source. Assays received **a** 0.76 or **b** 1.52 mg protein (4 ml assay vol.)$^{-1}$. **a** Plot of 1/v [= reciprocals of rate of ammonia formation; μmol min^{-1} (mg protein)$^{-1}$] against reciprocals of potassium cyanate substrate concentration (0.2–50 mM). The data show substrate inhibition above approximately 4 mM KOCN; the intercept on the 1/v axis indicates the maximum velocity [V_{max} = 99 nmol ammonia min^{-1} (mg protein)$^{-1}$]. **b** Plot of reciprocals of rate of ammonia formation (1/v) against potassium cyanate concentration in the presence and absence of 10 mM potassium thiocyanate. The common 1/v intercept (= $1/V_{max}$) indicates competitive inhibition by thiocyanate and a V_{max} value of 88 nmol ammonia min^{-1} (mg protein)$^{-1}$

Table 2 Effect on the activity of cyanase (in the standard assay procedure) of ammonium ion, selenocyanate, and thiocyanate, and the absence of bicarbonate, using extracts of *Methylobacterium thiocyanatum* strain ALL/SCN-P grown with cyanate as the sole nitrogen source (0.76 mg protein per assay)

Modification to the standard assay	Specific activity [nmol ammonia min^{-1} (mg protein)$^{-1}$]	Relative activity (%)
None	90.0 (± 4)	100
0.25 mM NH_4Cl	99.4	110
0.50 mM NH_4Cl	102.1	113
1.00 mM NH_4Cl	89.6	100
Omit KOCN + 0.5 mM KSeCN	0	0
0.5 mM KSeCN	44.9	50
1.0 mM KSeCN	0	0
5.0 mM KSeCN	0	0
2.0 mM KSCN	79.2	88
5.0 mM KSCN	65.7	73
10 mM KSCN	56.7	63
Omit KOCN + 4.0 mM KSCN	0	0
Omit $NaHCO_3$	17.3	19

nium ion as the sole nitrogen source. Specific activities of the enzyme in crude cell-free extracts, using the standard assay, were maximally 613, 94 and 19 nmol ammonia produced min^{-1} (mg protein)$^{-1}$ after growth on KSCN, KOCN and NH_4Cl, respectively. The enzyme activity was very stable, with no loss being found after 12 months' storage of extracts frozen at –20° C.

Essentially identical kinetics were exhibited by the enzyme in extracts of both thiocyanate- and cyanate-grown organisms with respect to K_m for cyanate and competitive inhibition by thiocyanate (Figs. 3, 4). The apparent K_m values were not significantly different at 0.076 and 0.066 mM cyanate for extracts of thiocyanate- and cyanate-grown cultures. The V_{max} value for activity from thiocyanate-grown organisms was 634 ± 24 nmol ammonia formed min^{-1} (mg protein)$^{-1}$ compared to 89 ± 9 nmol ammonia min^{-1} (mg protein)$^{-1}$ for cyanate-grown organisms (calculated from several experiments, of which Figs. 3a and 4 are examples). This ratio of sevenfold more activity in thiocyanate-grown organisms was consistently found in the standard assay (with 2 mM cyanate) using a number of preparations of extracts of thiocyanate- and cyanate-grown organisms over a period of 5 years.

Cyanate was an inhibitory substrate, depressing its own rate of hydrolysis by cyanase above approximately 4 mM (Fig. 4a), with 10 and 50 mM cyanate causing 27 and 43% depression of the rate of ammonia production, respectively. Thiocyanate was a competitive inhibitor (Figs. 3b, 4b), with 10 mM KSCN lowering the apparent K_m (= K_p) to 0.86 mM cyanate for the enzyme in both thiocyanate- and cyanate-grown organisms (calculated from Figs. 3 and 4). The inhibitor constant (K_i) was estimated to be 0.65 mM thiocyanate from the Dixon plot of Fig. 3b (Dixon and Webb 1958).

The possible inhibitory effect of ammonium ion and the selenium analogue of thiocyanate, KSeCN, were estimated (Table 2). Ammonium ion had no inhibitory effect on cyanase activity in the standard assay at concentrations of up to 1 mM. Selenocyanate was, however, at least 20-fold more inhibitory than thiocyanate, although it was not itself a substrate for the enzyme (Table 2). The nature of this inhibition has not yet been further investigated. Omission of bicarbonate from the standard assay reduced activity by 81% (Table 2).

The possibility that cyanase inhibition by thiocyanate might be mediated by low concentrations of sulfide or thiosulfate formed from it was tested by adding sulfide or thiosulfate to the standard assay with 2 mM cyanate using extracts of thiocyanate-grown organisms (0.11 mg protein ml^{-1}). Both sulfide and thiosulfate caused some interference with the Nessler assay for ammonia, but this was corrected by means of chemical controls. Sulfide, at 0.25 mM, and 0.25 or 1 mM thiosulfate did not cause significant inhibition, but 5 mM thiosulfate reduced the rate of ammonia formation by approximately 16%.

Attempts to demonstrate thiocyanate disappearance and ammonia formation from thiocyanate using assay mixtures with extracts of thiocyanate-grown organisms

(up to 2 mg protein ml^{-1}), with KSCN concentrations between 0.4 and 20 mM have been unsuccessful. No cross-reaction was seen in Ouchterlony diffusion gels between antibodies against *T. thioparus* thiocyanate hydrolase (Katayama et al. 1992) and extracts of organisms grown on thiocyanate, cyanate or ammonium as nitrogen sources.

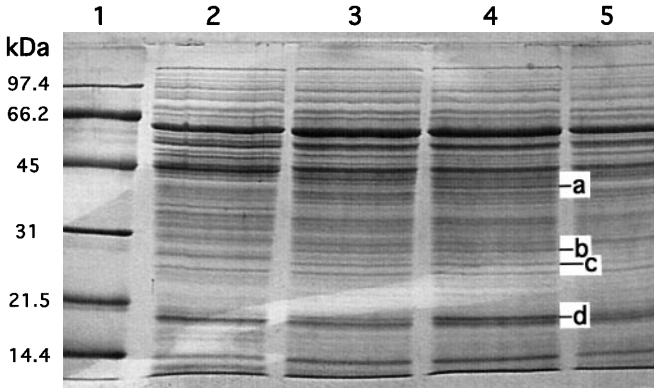

Fig. 5 SDS-polyacrylamide gel electrophoresis [12.5% (w/v) polyacrylamide separating gels] of cell-free extracts of *Methylobacterium thiocyanatum* strain ALL/SCN-P grown on succinate with four different nitrogen sources as indicated: *lane 1* protein standards (mol.wt. values shown in kDa), *lane 2* methylamine hydrochloride, *lane 3* potassium cyanate, *lane 4* potassium thiocyanate, and *lane 5* ammonium chloride. Polypeptides present most prominently in thiocyanate- or cyanate-grown organisms are indicated as *a* (mol.wt. 39.3 kDa), *b* (mol.wt. 27.2 kDa), *c* (mol.wt. 25.1 kDa), and *d* (mol.wt. 17.9 kDa)

SDS-PAGE analysis of cell-free extracts of strain ALL/SCN-P grown with different nitrogen sources

Four polypeptide bands were consistently shown to be present in extracts of organisms grown with thiocyanate or cyanate as the sole nitrogen sources, but were shown to be absent or fainter after growth on other nitrogen sources (Fig. 5). A polypeptide of mol.wt. 17.9 ± 0.2 kDa (from five separate determinations) was heavily expressed in thiocyanate-grown cells and was also quite distinct, though less intense, in cyanate-grown organisms, but it was faint in those grown on succinate with methylamine or ammonium ion as nitrogen sources (Fig. 5, band d). This band was absent from an extract of cells grown on methylamine as a sole source of carbon and nitrogen, but was present in extracts of organisms grown on methylamine with thiocyanate or cyanate (gel not shown). Two polypeptide bands, mol.wt. 25.1 ± 0.2 and 27.2 ± 0.2 kDa (four determinations), were similarly expressed in both thiocyanate- and cyanate-grown bacteria, but were fainter in ammonium-grown cells (Fig. 5, bands c and b). The fourth polypeptide, mol.wt. 39.3 ± 1.0 kDa

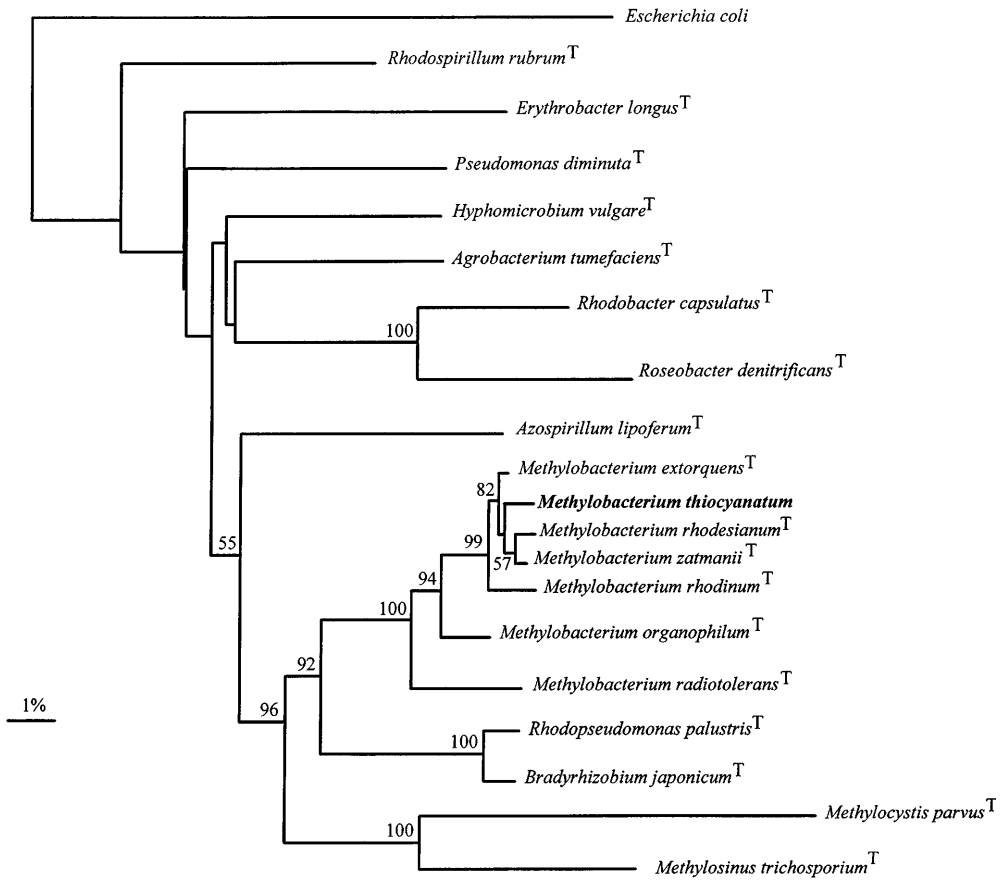

Fig. 6 Phylogenetic analysis of 16S rRNA sequences of *Methylobacterium thiocyanatum* strain ALL/SCN-P and comparison with the other species of *Methylobacterium* and other genera. The dendrogram shows the results from analyses using DNADIST, with the percentage BOOTSTRAP values also shown (Felsenstein 1988) (*bar* 1% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two species)

Table 3 16S rRNA sequence similarity values between *Methylobacterium thiocyanatum* and other species of *Methylobacterium* and *Rhodopseudomonas palustris*

Strains	1	2	3	4	5	6	7	8
1. *Methylobacterium thiocyanatum*	–							
2. *Methylobacterium rhodesianum*	98.9	–						
3. *Methylobacterium zatmanii*	98.5	99.2	–					
4. *Methylobacterium extorquens*	98.7	98.9	99.2	–				
5. *Methylobacterium rhodinum*	97.1	97.1	97.4	97.9	–			
6. *Methylobacterium organophilum*	96.2	96.5	96.1	96.9	96.3	–		
7. *Methylobacterium radiotolerans*	94.7	95.0	94.7	95.4	95.7	96.5	–	
8. *Rhodopseudomonas palustris*	89.7	90.6	90.4	90.3	89.8	90.9	90.6	–

(four determinations), was strongly expressed in succinate-grown organisms with thiocyanate as the nitrogen source, but was absent from ammonium- or methylamine-grown cells and was very faint in cyanate-grown bacteria (Fig. 5, band a).

DNA base composition, 16S rRNA sequence analysis, and ubiquinone content of strain ALL/SCN-P

Analysis of the DNA of strain ALL/SCN-P showed 69.8 and 71 mol% G+C by the melting-point method and 64.7% by spectrophotometric procedure: mean value, 68.5 ± 3.4 mol%. The complete 16S rRNA sequence of the organism was deposited with the Genbank database under accession no. U58018. Strain ALL/SCN-P was shown unequivocally to cluster exclusively with the six *Methylobacterium* species (Fig. 6), but to be quite distinct from them at the species level. Its closest relatives (with 98.5–98.9% 16S rRNA sequence similarity) were *M. rhodesianum, M. zatmanii* and *M. extorquens* (Table 3). The respiratory ubiquinone was shown to be coenzyme Q10 (ubiquinone 50).

Discussion

On the basis of cell and culture morphology, its pink pigmentation, facultatively methylotrophic growth on one-carbon compounds, biochemical tests (Green 1992), and comparative sequence analysis of 16S rRNA, the novel strain ALL/SCN-P was shown to be a new species of *Methylobacterium* that we formally describe and name *M. thiocyanatum* at the conclusion of this Discussion. It differs from its closest relatives (as identified by their 16S rRNA sequences; Table 3, Fig. 6), *M. zatmanii, M. rhodesianum* and *M. extorquens*, in its ability to grow on glucose, arabinose and citrate, while they cannot, and on fructose and glutamate, which *M. extorquens* and *M. zatmanii*, respectively, cannot (Green 1992; Green et al. 1988). In common with most *Methylobacterium* spp., it grows relatively rapidly and efficiently on formate, methanol and methylamine (but not di- or tri-methylamine), and can use methylamine as a sole source of carbon, energy and nitrogen, and as a nitrogen source when grown with succinate or glucose.

M. thiocyanatum is currently unique among described species of facultative methylotrophs in its resistance to high concentrations of the potentially toxic anions thiocyanate and cyanate, and in its ability to grow on either thiocyanate or cyanate as a sole source of nitrogen and on thiocyanate as a sole source of sulfur. Other species and strains of *Methylobacterium* have yet to be tested for any ability to tolerate and use thiocyanate and cyanate as nitrogen sources, so this character cannot at this time be claimed to be a diagnostic characteristic unique to *M. thiocyanatum*. The ability to degrade thiocyanate as a nitrogen source indicates that *M. thiocyanatum* possesses an enzyme or enzymes that release ammonia from thiocyanate. The presence of high activities of cyanase in thiocyanate- and cyanate-grown organisms suggests that cyanate may be an intermediate in thiocyanate breakdown, although we were not able to demonstrate a thiocyanate hydrolase or ammonia production from thiocyanate in cell-free extracts. The specific activity of cyanase in crude extracts of ammonium-grown organisms [up to 19 nmol NH_3 min^{-1} (mg protein)$^{-1}$] was only 20 and 3%, respectively, of the activities in cyanate- or thiocyanate-grown organisms, but it did demonstrate that there was a low level of constitutive synthesis of the enzyme. Growth in the presence of ammonium ion and thiocyanate showed that very little thiocyanate was hydrolysed in the presence of ammonium. The activity of cyanase in cyanate-grown organisms [up to 94 nmol NH_3 min^{-1} (mg protein)$^{-1}$] was similar to that seen in *Pseudomonas fluorescens* [128 at 30° C; Kunz and Nagappan (1989)], but less than that observed in *E. coli* [1,200 at 37° C; Anderson (1980)].

Protein profiles of the soluble proteins of pink-pigmented facultative methylotrophs subjected to SDS-PAGE have been used as a taxonomic tool (Hood et al. 1988), but altering the growth substrate was found to affect the quantitative comparison of such electrophoretograms. Our use of SDS-PAGE allowed us to visualize quantitative differences between polypeptide profiles of *M. thiocyanatum* grown with different nitrogen sources (Fig. 5), and hence to show polypeptides that were predominantly or uniquely expressed during growth with thiocyanate or cyanate. While none of these has been biochemically characterized, it is valid to draw comparison with data from other thiocyanate- and cyanate-degrading bacteria. By analogy with the cyanase of *E. coli* [an oligomer of eight or ten identical subunits of mol.wt. 16,350 Da; Kunz and Nagappan (1989)], the 17.9-kDa polypeptide (Fig. 5) might be the cyanase enzyme whose activity we have shown. The cyanase of *M. thiocyanatum* has not been pu-

rified, but our tests with crude extracts indicate a K_m value of approximately 0.07 mM, which is approximately tenfold better than that for *E. coli* (Taussig 1965; Anderson 1980). As with the *E. coli* enzyme, bicarbonate is required for activity and cyanate is an inhibitory substrate, but the *Methylobacterium* enzyme appears less sensitive to autoinhibition by cyanate (Anderson 1980; Anderson and Little 1986). Two other polypeptides comparably expressed in organisms grown with either thiocyanate or cyanate had molecular masses (25.1 and 27.2 kDa) that are very similar to the molecular mass of the cyanate permease of *E. coli* (26 kDa; Sung and Fuchs 1989; Anderson et al. 1990), suggesting that these polypeptides might be involved in the uptake of thiocyanate and/or cyanate. While permeases would be expected to be embedded in the cytoplasmic membrane (Kaback 1992), it is known that the cyanate permease of *E. coli* lacks the membrane-spanning segments expected of a typical permease and may be a peripheral membrane protein (Anderson et al. 1990). A permease with similar properties in *M. thiocyanatum* might thus be recovered at least in part in our SDS-PAGE analyses, especially as the crude sonicated cell preparation will have contained some membrane fragments. The fourth polypeptide (mol.wt. 39.3 kDa) appeared to be expressed only in thiocyanate-grown bacteria (Fig. 5, band a). As such, it could be a component of either a thiocyanate hydrolase or a permease protein necessary for growth on thiocyanate: these possibilities remain to be investigated.

The presence of approximately seven times as much cyanase activity in *M. thiocyanatum* grown on thiocyanate as compared to when it was grown on cyanate could be a regulatory response of the organism to a toxic substrate: thiocyanate was shown to be a competitive inhibitor of cyanase and to depress the growth rate at concentrations above 25 mM. Cyanase activity was not significantly affected by sulfide and thiosulfate at concentrations likely to arise in growing cultures, so these are not an indirect cause of inhibition of either cyanase or growth rate by thiocyanate. If thiocyanate is metabolized by a hydrolytic pathway leading to cyanate, efficient growth would depend on levels of cyanase sufficient to maintain the flow of ammonium ion for biosynthesis in the presence of inhibitory thiocyanate. Alternatively, if thiocyanate metabolism is by a carbonyl sulfide pathway (see Introduction, Eq. 1), thiocyanate may be acting as a gratuitous inducer of cyanase even though the enzyme is not involved in thiocyanate metabolism. The mechanism of thiocyanate degradation will be the subject of a future study of this organism.

Description of *M. thiocyanatum* sp. nov.

Methylobacterium thiocyanatum (sp. nov.) thio.cyan.at.'um M.L.n. using thiocyanate.

Cells are gram-negative, nonmotile, pink-pigmented rods, 0.8×2.0 μm in size, without spores or capsules. The pink pigment is nondiffusible. Catalase positive; oxidase variable; indole production and urease positive; methyl-red and Vosges-Proskauer tests and H_2S production negative. Growth occurs in the temperature range 15–37° C; no growth at 5 or 44° C. Initial pH for growth pH 7.5–9.4. Growth optimum at 30–37° C and pH 7.7. The G + C content of the DNA is 68.5 mol% (Tm and UV ratios). Respiratory ubiquinone is Q-10. Obligately aerobic, does not denitrify. Thiocyanate, cyanate, methylamine, ammonium or nitrate ion are used as a nitrogen source; does not fix dinitrogen. Methylotroph and chemoorganotroph, with the ability to derive cell-nitrogen from thiocyanate or cyanate, and cell-sulfur from thiocyanate; thiocyanate and cyanate tolerated at concentrations of at least 50 mM; thiosulfate is produced from thiocyanate during growth. Utilizes methanol, formaldehyde (1.25 mM), formate, methylamine, glucose, fructose, arabinose (slow), citrate, succinate, fumarate, pyruvate, acetate, glutamate, nutrient agar, and Luria-Bertani agar. Does not utilize sucrose, methane, methanesulfonate, dimethylamine, trimethylamine or dimethylsulfone, or grow as a chemolithotroph with thiocyanate, thiosulfate or tetrathionate as energy sources. The organism was isolated from soil from around the roots of *A. aflatunense*. The type strain is *M. thiocyanatum* strain ALL/SCN-P and has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 11490.

Acknowledgements We are grateful to N. Padden for carrying out hydroxypyruvate reductase assays, and to T. Brain (King's College London Electron Microscopy Unit, England) for assistance with the SEM. We thank Y. Katayama (Tokyo University of Agriculture and Technology, Japan) for a generous gift of antibodies raised against the thiocyanate hydrolase of *T. thioparus*.

References

Anderson PM (1980) Purification and properties of the inducible enzyme cyanase. Biochemistry 19:2882–2888

Anderson PM, Little RM (1986) Kinetic properties of cyanase. Biochemistry 25:1621–1626

Anderson PM, Sung Y, Fuchs JA (1990) The cyanase operon and cyanate metabolism. FEMS Microbiol Rev 87:247–252

Barrow GI, Feltham RKA (1993) Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge

Beekhuis HA (1975) Technology and industrial applications. In: Newman AA (ed) Chemistry and biochemistry of thiocyanic acid and its derivatives. Academic Press London New York, pp 222–249

Betts PM, Rinder DF, Fleeker JR (1979) Thiocyanate utilization by an *Arthrobacter*. Can J Microbiol 25:1277–1282

Block E, Putman D, Zhao S-H (1992) *Allium* chemistry: GC-MS analysis of thiosulfinates and related compounds from onion, leek, scallion, shallot, chive, and Chinese chive. J Agric Food Chem 40:2431–2438

Boucabeille C, Bories A, Ollivier P (1994) Degradation of thiocyanate by a bacterial coculture. Biotechnol Lett 16:425–430

Brown PD, Morra MJ (1993) Fate of ionic thiocyanate (SCN^-) in soil. J Agric Food Chem 41:978–982

De Kruyff CD, Van der Walt JP, Schwartz JM (1957) The utilization of thiocyanate and nitrate by thiobacilli. Antonie Van Leeuwenhoek 23:305–316

Dixon M, Webb EC (1958) Enzymes. Longman, London

Felsenstein J (1988) Phylogenies from the molecular sequences: inference and reliability. Annu Rev Genet 22:521–565

Fredericq E, Oth A, Fontaine F (1961) The ultraviolet spectrum of deoxyribonucleic acids and their constituents. J Mol Biol 3: 11–17

Giovannoni SJ (1991) The polymerase chain reaction. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 177–203

Green PN (1992) The genus *Methylobacterium*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd edn. Springer, Berlin Heidelberg New York, pp 2342–2349

Green PN, Bousfield IJ, Hood D (1988) Three new *Methylobacterium* species: *M. rhodesianum* sp. nov., *M. zatmanii* sp. nov., and *M. fujisawaense* sp. nov. Int J Syst Bacteriol 38:124–127

Gutell RR, Larsen N, Woese CR (1994) Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol Rev 58:10–26

Happold FC, Key A (1937) The bacterial purification of gasworks liquors. 2. The biological oxidation of ammonium thiocyanate. Biochem J 31:1323–1329

Happold FC, Johnstone KI, Rogers HG, Youatt JB (1954) The isolation and characteristics of an organism oxidizing thiocyanate. J Gen Microbiol 10:261–266

Happold FC, Jones GL, Pratt DB (1958) Utilisation of thiocyanate by *Thiobacillus thioparus* and *Thiobacillus thiocyanoxidans*. Nature 182:294–298

Harder W, Van Dijken JP, Roels JA (1981) Utilization of energy in methylotrophs. In: Dalton H (ed) Microbial growth on C_1 compounds. Heyden, London, pp 258–269

Hood DW, Dow CS, Green PN (1988) Electrophoretic comparison of total soluble proteins in the pink-pigmented facultative methylotrophs. J Gen Microbiol 134:2375–2383

Jordan SL, Kraczkiewicz-Dowjat AJ, Kelly DP, Wood AP (1995) Novel eubacteria able to grow on carbon disulfide. Arch Microbiol 163:131–137

Kaback HR (1992) The lactose permease of *Escherichia coli* – a paradigm for membrane-transport proteins. Biochim Biophys Acta 1101:210–213

Katayama Y, Kuraishi H (1978) Characteristics of *Thiobacillus thioparus* and its thiocyanate assimilation. Can J Microbiol 24: 804–810

Katayama Y, Narahara Y, Inoue Y, Amano F, Kanagawa T, Kuraishi H (1992) A thiocyanate hydrolase of *Thiobacillus thio parus*. A novel enzyme catalyzing the formation of carbonyl sulfide from thiocyanate. J Biol Chem 267:9170–9175

Katayama Y, Hiraishi A, Kuraishi H (1995) *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. Microbiology 141:1469–1477

Kelly DP, Smith NA (1990) Organic sulfur compounds in the environment. Adv Microb Ecol 11:345–385

Kelly DP, Wood AP (1984) Potential for methylotrophic autotrophy in *Thiobacillus versutus* (*Thiobacillus* sp. strain A2). In: Crawford RL, Hanson RS (eds) Microbial growth on C_1 compounds. American Society for Microbiology, Washington, DC, pp 324–329

Kelly DP, Wood AP (1994) Synthesis and determination of thiosulfate and polythionates. In: Peck HD, LeGall J (eds) Methods in enzymology. Inorganic microbial sulfur metabolism, vol 243. Academic Press, San Diego, pp 475–501

Kelly DP, Chambers LA, Trudinger PA (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulphate and tetrathionate. Anal Chem 41:898–901

Kelly DP, Malin G, Wood AP (1993) Microbial transformations and biogeochemical cycling of one-carbon substrates containing sulphur, nitrogen or halogens. In: Murrell JC, Kelly DP (eds) Microbial growth on C_1 compounds. Intercept, Andover, England, pp 47–63

Kunz DA, Nagappan O (1989) Cyanase-mediated utilization of cyanate in *Pseudomonas fluorescens* NCIB 11764. Appl Environ Microbiol 55:256–258

Kuo M-C, Chien M, Ho C-T (1990) Novel polysulfides identified in the volatile components from Welsh onions (*Allium fistulosum* L. var. maichuon) and scallions (*Allium fistulosum* L. var. caespitosum). J Agric Food Chem 38:1378–1381

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 177–203

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Maidak BL, Larsen N, McCaughey MJ, Overbeek R, Olsen GJ, Fogel K, Blandy J, Woese CR (1994) The ribosomal database project. Nucleic Acids Res 22:3485–3487

Mandel M, Marmur J (1968) Use of ultra-violet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. Methods Enzymol 12B:195–206

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218

Mason FG (1995) Bacterial degradation of thiocyanate. PhD Thesis, Queen's University of Belfast, Northern Ireland

Mason F, Harper D, Larkin M (1994) The microbial degradation of thiocyanate. Trans Biochem Soc 22:423S

Neefs J-M, Van de Peer Y, De Rijk P, Goris A, De Wachter R (1991) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 19:1987–2015

Neufeld RD, Valiknac T (1979) Inhibition of phenol degradation by thiocyanate. J Water Pollution Control Fed 51:2283–2291

Oh SY, Jalaludin S, Davis RH, Sykes AH (1987) Detoxication of cyanide in the chicken by conversion to thiocyanate, as influenced by the availability of transferable sulphur. Comp Biochem Physiol 86B:129–133

Paruchuri YL, Shivaraman N, Kumaran P (1990) Microbial transformation of thiocyanate. Environ Pollution 68:15–28

Richards DJ, Shieh WK (1989) Anoxic-oxic activated sludge treatment of cyanates and phenols. Biotechnol Bioeng 33:32–38

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Saunders SE, Burke JF (1990) Rapid isolation of miniprep DNA for double strand sequencing. Nucleic Acids Res 18:4948

Smith NA, Kelly DP (1988) Oxidation of carbon disulphide as the sole source of energy for the autotrophic growth of *Thiobacillus thioparus* strain TK-m. J Gen Microbiol 134:3041–3048

Stafford DA, Callely AG (1969) The utilization of thiocyanate by a heterotrophic bacterium. J Gen Microbiol 55:285–289

Stratford J, Dias AEXO, Knowles CJ (1994) The utilization of thiocyanate as a nitrogen source by a heterotrophic bacterium: the degradation pathway involves formation of ammonia and tetrathionate. Microbiology 140:2657–2662

Sung Y-C, Fuchs JA (1989) Identification and characterization of a cyanate permease in *Escherichia coli* K-12. J Bacteriol 171: 4674–4678

Taussig A (1965) Some properties of the induced enzyme cyanase. Can J Biochem 43:1063–1069

Wood AP, Kelly DP (1977) Heterotrophic growth of *Thiobacillus* A2 on sugars and organic acids. Arch Microbiol 113:257–264

Wood AP, Kelly DP (1989) Methylotrophic and autotrophic bacteria isolated from lucinid and thyasirid bivalves containing symbiotic bacteria in their gills. J Marine Biol Assoc UK 69:165–179

Wood JL (1975) Biochemistry. In: Newman AA (ed) Chemistry and biochemistry of thiocyanic acid and its derivatives. Academic Press, London New York, pp 156–221

Youatt JB (1954) Studies on the metabolism of *Thiobacillus thiocyanoxidans*. J Gen Microbiol 11:139–149