

Enzymes and genes of taurine and isethionate dissimilation in *Paracoccus denitrificans*

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Growth of the α -proteobacterium *Paracoccus denitrificans* NKNIS with taurine or isethionate as sole source of carbon involves sulfoacetaldehyde acetyltransferase (Xsc), which is presumably encoded by an *xsc* gene in subgroup 3, none of whose gene products has been characterized. The genome of the α -proteobacterium *Rhodobacter sphaeroides* 2.4.1 was interpreted to contain a nine-gene cluster encoding the inducible dissimilation of taurine, and this deduced pathway included a regulator, a tripartite ATP-independent transporter, taurine dehydrogenase (TDH; presumably TauXY) as well as Xsc (subgroup 3), a hypothetical protein and phosphate acetyltransferase (Pta). A similar cluster was found in *P. denitrificans* NKNIS, in contrast to an analogous cluster encoding an ATP-binding cassette transporter in *Paracoccus pantotrophus*. Inducible TDH, Xsc and Pta were found in extracts of taurine-grown cells of strain NKNIS. TDH oxidized taurine to sulfoacetaldehyde and ammonium ion with cytochrome *c* as electron acceptor. Whereas Xsc and Pta were soluble enzymes, TDH was located in the particulate fraction, where inducible proteins with the expected masses of TauXY (14 and 50 kDa, respectively) were detected by SDS-PAGE. Xsc and Pta were separated by anion-exchange chromatography. Xsc was effectively pure; the molecular mass of the subunit (64 kDa) and the N-terminal amino acid sequence confirmed the identification of the *xsc* gene. Inducible isethionate dehydrogenase (IDH), Xsc and Pta were assayed in extracts of isethionate-grown cells of strain NKNIS. IDH was located in the particulate fraction, oxidized isethionate to sulfoacetaldehyde with cytochrome *c* as electron acceptor and correlated with the expression of a 62 kDa protein. Strain NKNIS excreted sulfite and sulfate during growth with a sulfonate and no sulfite dehydrogenase was detected. There is considerable biochemical, genetic and regulatory complexity in the degradation of these simple molecules.

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INTRODUCTION

Taurine (2-aminoethanesulfonate) (Fig. 1a) and isethionate (2-hydroxyethanesulfonate) are widespread natural products whose utilization as sulfur or as carbon and energy

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Abbreviations: ABC transporter, ATP-binding cassette transporter; DCPIP, dichlorophenol indophenol; IDH, isethionate dehydrogenase; MALDI-TOF-MS, matrix-assisted, laser-desorption ionization time-of-flight mass spectrometry; Pta, phosphate acetyltransferase; TDH, taurine dehydrogenase; ThDP, thiamin diphosphate; Tpa, taurine:pyruvate aminotransferase; TRAP transporter, tripartite ATP-independent transporter; Xsc, sulfoacetaldehyde acetyltransferase.

The GenBank accession numbers for the sequences reported in this paper are AY498613 (gene cluster in *Paracoccus denitrificans* NKNIS), AY498615 (gene cluster in *Paracoccus pantotrophus* NKNCYSA), AY498614 (gene cluster in *P. pantotrophus* DSM 65), AY498616 (partial *tauY* in *Achromobacter xylosoxidans* NCIMB 10751), AY498617 (partial *tauY* in *Comamonas* sp. strain SFCD1) and AY498618 (partial *tauY* in *Ralstonia* sp. strain EDS1).

sources for growth has been recognized for many decades, though these phenomena have only recently been explored at the molecular level (Huxtable, 1992; Lie *et al.*, 1998; Kertesz, 2000; Cook & Denger, 2002). Transport of sulfonates into the cell is axiomatic, but little information is available on transport in dissimilatory pathways (Kertesz, 2001; Cook & Denger, 2002), though an ABC (ATP-binding cassette) transporter (e.g. Ehrmann *et al.*, 1998) has been postulated for taurine in *Sinorhizobium meliloti* (Ruff *et al.*, 2003). The dissimilatory pathways, which are usually inducible, converge at sulfoacetaldehyde (Cook & Denger, 2002) (Fig. 1a). A taurine:pyruvate transaminase (Tpa) (EC 2.6.1.77) (Laue & Cook, 2000) is widespread, whereas a taurine dehydrogenase (TDH) (EC 1.4.99.2) (Fellman *et al.*, 1980; Kondo & Ishimoto, 1987) was thought to be rare (Cook & Denger, 2002) until a gene(s) for TDH was hypothesized in the genome sequence of *Sin. meliloti* (Ruff *et al.*, 2003) (Fig. 1b). The enzymic conversion of isethionate to sulfoacetaldehyde has been detected (Kondo *et al.*, 1977) as has the metabolism of isethionate via

The established and putative *xsc* genes can be attributed to three subgroups, 1–3 (Denger *et al.*, 2001; Ruff *et al.*, 2003). Representative gene products of subgroups 1 and 2 have been characterized and confirmed to be encoded by the appropriate gene. All genes in subgroup 3 are found in α -*Proteobacteria* (Ruff *et al.*, 2003) and they are known *in silico* only, so the function of the gene product has not been confirmed.

We postulated that one genomic locus (SMb21525–SMb21532) encodes enzymes involved in the inducible degradation of taurine, the only sulfonate known to be degraded by *Sin. meliloti* Rm1021 (Fig. 1b) (Ruff *et al.*, 2003), a member of the *Rhizobiaceae* in the α -*Proteobacteria*. A similar pathway involving a TRAP transporter (tripartite ATP-independent periplasmic transporters were reviewed recently by Kelly & Thomas, 2001) can be derived from genomic data from *Rhodobacter sphaeroides* 2.4.1, a member of the *Rhodobacteraceae*, in the α -*Proteobacteria* (see below). Three other *Rhodobacteraceae* are known to dissimilate at least one sulfonate, *Paracoccus denitrificans* NKNIS (isethionate and taurine) and *Paracoccus pantotrophus* strains NKNCYSA (isethionate, sulfoacetate and taurine are relevant here) and DSM 65 (taurine) (Denger *et al.*, 1997; Mikosch *et al.*, 1999; Ruff *et al.*, 2003). The initial work with the latter organisms was done under conditions of nitrate respiration, but it has since been observed that each organism dissimilates its sulfonate substrate(s) with oxygen as the terminal electron acceptor (K. Denger, unpublished). When several pathways of desulfonation of C₂-sulfonates are present in one organism, Xsc seems to be induced in each case, but the convergent pathways are induced individually (Denger & Cook, 2001). Furthermore, the reactive sulfite ion, which is released by Xsc (or other enzymes), is sometimes actively detoxified by sulfite dehydrogenase, as has been detected in *Burkholderia* sp. strain ICD (King *et al.*, 1997; see also Reichenbecher *et al.*, 1999) and in *P. pantotrophus* NKNCYSA (Rein, 1999), but sulfite is sometimes found transiently in large amounts outwith the cell (e.g. Thurnheer *et al.*, 1986). Little is known about bacterial sulfite dehydrogenases (EC 1.8.2.1) [or sulfite oxidases (EC 1.8.3.1)] (Kappler & Dahl, 2001).

This report shows the development of a hypothesis on the nature of the locus encoding an inducible pathway of taurine dissimilation in *Rho. sphaeroides* whose genes show high

levels of similarity with a gene cluster found in *P. denitrificans* NKNIS. An analogous cluster in *P. pantotrophus* NKNCYSA (and DSM 65) presumably encodes an ABC transporter in place of the TRAP transporter. Inducible TDH, Xsc and Pta were detected in strain NKNIS and the reaction products were identified. The enzymes were subject to partial or complete purification and characterization; as predicted, Xsc was encoded by the *xsc* gene. A separately induced isethionate dehydrogenase (IDH) was detected in *P. denitrificans*.

METHODS

Materials. The preparation of sulfoacetaldehyde, as the bisulfite addition complex, has been described previously (Denger *et al.*, 2001). Commercial chemicals were of the highest purity available and were purchased from Fluka, Merck, Roth, Serva or Sigma. Native proteins were separated on a Mono Q column (10 × 10 mm) (Pharmacia).

Organisms, growth, harvesting of cells and preparation of cell-free extracts. *P. denitrificans* NKNIS (DSM 15418), *P. pantotrophus* NKNCYSA (DSM 12449), *Ralstonia* sp. strain EDS1 (DSM 13640) and *Alcaligenes defragrans* NKNTAU (DSM 11046) were isolated in this laboratory (Denger *et al.*, 1997; Mikosch *et al.*, 1999; Denger & Cook, 2001; Ruff *et al.*, 2003). *P. pantotrophus* DSM 65 and *Ralstonia eutropha* JMP134 (DSM 4058) were obtained from the DSM Culture Collection (DSMZ, Braunschweig, Germany). *Achromobacter xylosoxidans* NCIMB 10751 (Kondo *et al.*, 1971) is available from NCIMB, Aberdeen. *Comamonas* sp. strain SFCD1 (King & Quinn, 1997) (DSM 15091) was provided by J. Quinn, University of Belfast. *Sin. meliloti* Rm1021 was provided by Stefan Weidner, University of Bielefeld, Germany. *Burkholderia* sp. strain LB400 was obtained from J. Tiedje, Michigan State University, USA. Cells were grown aerobically at 30 °C in mineral-salts medium (Thurnheer *et al.*, 1986) with 10–20 mM taurine, isethionate or acetate as the sole added source of carbon and energy for growth, unless otherwise stated. On occasion, the ammonium ion was omitted and taurine served as sole source of carbon and nitrogen for growth. Precultures (3 ml) were grown in 30 ml screw-cap tubes in a roller. Growth experiments were done on the 100 ml scale in 500 ml Erlenmeyer flasks shaken in a water bath. Samples were taken at intervals to measure OD₅₈₀, to assay protein and to determine the concentrations of sulfate, sulfite, carbon source and, on occasion, ammonium ion. Similar cultures were used to generate small amounts of cells for enzyme assays. When large amounts of cells were needed, strain NKNIS was grown in a 4 l fermenter with 3.5 l working volume in 20 mM taurine (or isethionate or acetate)-salts medium, harvested at OD₅₈₀ = 0.8–0.9 (300 mg protein l⁻¹) by centrifugation (15 000 g, 20 min, 4 °C), washed in 50 mM potassium phosphate buffer, pH 7.5 (containing 2.5 mM MgCl₂), and stored frozen. The same

Fig. 1. The degradative pathways for taurine and isethionate in *P. denitrificans* NKNIS (a) and the genetic organization of corresponding genes in selected α - and β -*Proteobacteria* (b). The reannotated cluster (SMb21525–SMb21532; line 5) presumably encoding degradation of taurine in *Sin. meliloti* Rm1021 comprises genes encoding a potential regulator (TauR), an ABC transporter, putative TDH (TauXY), Xsc, unknown function (TauZ) and Pta (Ruff *et al.*, 2003). The gene cluster in the *Rho. sphaeroides* 2.4.1 genome (Rsph2616–2608; line 2) presumably encodes TauR, a TRAP transporter (TauKLM), TDH, Xsc, TauZ and Pta (see Table 1). The cluster in *P. denitrificans* NKNIS (line 1) resembles that in *Rho. sphaeroides* (line 2) (Table 1), whereas the cluster in *P. pantotrophus* (lines 3, 4) resembles that in *Sin. meliloti* (line 5). The incomplete genome sequence of the α -proteobacterium *Sil. pomeroyi* DSS-3 (line 6) contains adjacent *tauR*-, *xsc*- and *pta*-like genes. The gene cluster around *xsc* in *Burkholderia* sp. strain LB400 (line 9), data from the *L. major* genome project (clone P1023) (line 7), and, to a lesser extent, data from *Ral. eutropha* JMP134 (line 8), indicate the relatively close proximity of *tauXY* and *xsc*, together with regulation, transport and the *cobST* and *orfX* (relevance unknown) genes. Key: arrows, direction of transcription; ~, incomplete sequence; *, data from genome sequencing project.

buffer served as extraction buffer. Cell-free extracts free of nucleic acids were generated after disruption by three passages through a chilled French press set at 138 MPa (Junker *et al.*, 1994) and they could be stored for several weeks at -20°C without significant loss of activity. The membrane/particulate fraction was obtained by ultracentrifugation (170 000 g, 30 min, 4°C). If the extract was required in a different buffer, 2.5 ml portions were rebuffered with equilibrated PD 10 (Pharmacia) columns according to the manufacturer's instructions. Cells, which were to provide template DNA, were washed twice in water and frozen and thawed prior to use to make genomic DNA more available.

Enzyme assays. TDH was routinely assayed spectrophotometrically as the taurine-dependent reduction of dichlorophenol indophenol (DCPIP) at 600 nm (molar extinction coefficient $16\,100\text{ M}^{-1}\text{ cm}^{-1}$; Jones, 1979). The reaction mixture contained (in a final volume of 1.0 ml): 40 μmol potassium phosphate buffer, pH 7.2, 25 μmol taurine, 100 nmol DCPIP and 0.1–1 mg protein with which the reaction was started. The reaction was proportional to the protein content up to 2 mg ml^{-1} and was linear for at least 2 min. The routine assay was occasionally augmented by following the disappearance of taurine and the formation of sulfoacetaldehyde. DCPIP and taurine tended to interfere with the routine determination of the other product of oxidative deamination, ammonium ion, so lower amounts were used in the assay (10 μmol taurine, 50 nmol DCPIP). DCPIP could be replaced with beef-heart cytochrome *c* (50 nmol), and the optimized photometric enzyme assay also contained 100 μmol Tris/HCl buffer, pH 9.0, 25 μmol taurine and 0.01–0.05 mg protein with which the reaction was started: the wavelength used was 550 nm and the molar extinction coefficient was $21\,000\text{ M}^{-1}\text{ cm}^{-1}$ (Ensley *et al.*, 1982).

IDH was routinely assayed spectrophotometrically as the isethionate-dependent reduction of DCPIP at 600 nm. The reaction mixture contained (in a final volume of 1.0 ml): 40 μmol potassium phosphate buffer, pH 7.2, 25 μmol isethionate, 100 nmol DCPIP and 0.1–1 mg protein with which the reaction was started. The reaction was proportional to the protein content up to 2 mg ml^{-1} and was linear for at least 2 min. DCPIP could be replaced with beef-heart cytochrome *c* (50 nmol).

Xsc was assayed by GC as the ThDP- and phosphate-dependent release of acetate after acidification to hydrolyse the acetyl phosphate formed (Ruff *et al.*, 2003). The routine assay was occasionally augmented by colorimetric determination of acetyl phosphate, by the disappearance of substrate or by the formation of sulfite (Ruff *et al.*, 2003).

Pta was assayed photometrically as the HS-CoA-dependent formation of acetyl-CoA (Bergmeyer *et al.*, 1983).

Tpa was assayed discontinuously at 30°C as the pyruvate-dependent disappearance of taurine concomitant with the formation of alanine. The reaction mixture contained (in a final volume of 1.0 ml): 40 μmol Tris/HCl, pH 9.0, 5 μmol taurine, 10 μmol pyruvate, 100 nmol pyridoxal-5-phosphate and 0.1–1 mg protein with which the reaction was started. Samples were taken at intervals, derivatized with Sanger's reagent, and subject to separation by HPLC (Laue *et al.*, 1997).

Sulfite dehydrogenase was assayed spectrophotometrically with $\text{K}_3\text{Fe}(\text{CN})_6$ or cytochrome *c* as electron acceptor (Reichenbecher *et al.*, 1999).

Cytochromes were assayed spectrophotometrically. Cell extracts of taurine- or acetate-grown cells were examined as redox difference spectra of dithionite-reduced samples versus air-oxidized samples. Cytochrome *c* was considered to have an M_r value of 12 000 and a molar absorption coefficient for the α -band of $19\,000\text{ mM cm}^{-1}$ (Chance & Williams, 1955).

Purification of Xsc and separation of Pta. Particulate matter was removed from crude extract by ultracentrifugation (see above) and the soluble fraction (the supernatant fluid) was diluted 1:2:5 with distilled water to generate the correct buffer concentration for chromatography. This generated a precipitate, which was removed by centrifugation (10 000 g, 3 min, room temperature). The clear supernatant fluid was loaded on to a Mono Q anion exchange column and protein was eluted as described elsewhere (Ruff *et al.*, 2003). Representative fractions were assayed for Xsc, which was found to be essentially homogeneous, and for Pta.

Fractionation of TDH. The membrane fraction (e.g. 2.0 ml) was washed in 0.1 M Tris/HCl buffer, pH 9.0, and subjected to ultracentrifugation (see above). The pellet was resuspended in the same volume of fresh buffer.

Analytical methods. Absorbance was measured in a Uvikon 922 spectrophotometer (Kontron). Sulfate was quantified as turbidity in the presence of BaCl_2 (Sörbo, 1987). Sulfite was quantified as the fuchsin derivative as described elsewhere (Kondo *et al.*, 1982; Thurnheer *et al.*, 1986). Ammonium ion was routinely assayed colorimetrically by the Berthelot reaction (Gesellschaft Deutscher Chemiker, 1996) and occasionally confirmed enzymically in the specific reaction with glutamate dehydrogenase (Bergmeyer, 1983). Acetyl phosphate was determined chemically as iron(III) acetyl hydroxamate (Stadtman, 1957; Racker, 1962). Reverse-phase HPLC was used to quantify taurine (Laue *et al.*, 1997) or sulfoacetaldehyde (Cunningham *et al.*, 1998) after derivatization. Acetate was quantified by GC (Laue *et al.*, 1997). SDS-PAGE and staining were done by standard methods (Laemmli, 1970) to monitor protein purification and to estimate molecular masses under protein-denaturing conditions. Values for the molecular mass of native proteins were assayed by gel filtration chromatography on a Superose 12 column as described previously (Ruff *et al.*, 2003). The sequencing of the N-terminal amino acids in Xsc was done by Edman degradation under contract by the sequencing facility of the Fachhochschule Bingen, Germany (<http://zpa.fh-bingen.de>). Values of apparent K_m (K_m^{app}) were derived by hyperbolic curve-fitting as cited elsewhere (Ruff *et al.*, 2003). The identity of sulfoacetaldehyde as the product of TDH and of IDH was confirmed by matrix-assisted, laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) which was done under contract at the University of Saarbrücken, Germany: the negative ion mode was used for the underivatized aldehyde and, after reaction with hydroxylamine, for the corresponding oxime (Tholey *et al.*, 2002).

Amplification, nucleotide sequencing and sequence analysis of genes. PCR was done in 20 μl reaction mixtures with whole cells of *P. denitrificans* NKNIS (or of strains of *P. pantotrophus* or other organisms) as template, with *Taq* polymerase (Genaxxon Bioscience) in Long Template Expand PCR buffer P2 or P3 (Roche Diagnostics). Primers were purchased from Hermann GbR Synthetische Biomoleküle or biomers.net GmbH. The key primer pair, used in the initial amplification of the *xsc* gene (subgroup 3), was *xsc3-f* (5'-TGGGCTACAACG-GYTCGAA-3') with *xsc3-r* (5'-GCSCCCACTGGTAGTTGCG-3'). Consensus primers to amplify a 1 kb fragment of the *tauY* gene were *tauYcons-f* (5'-TAYGCGCCGACCTACTGGRYCG-3') and *tauYcons-r* (5'-CGGGCATCATGTCGTGGCTSAC-3').

Nucleotide sequences were determined by cycle sequencing and primer walking using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit. The ABI DNA sequencers were operated by GATC GmbH. Sequence analysis was done using the DNASTAR LASERGENE program package version 5.5. The NCBI BLAST programs were used to search for similarities to the obtained sequences (Altschul *et al.*, 1997). Multiple sequence alignments were carried out using the CLUSTALW method with a pairwise progress of MEGALIGN from the DNASTAR program package. Searches for motifs were done using the algorithms of the

Profile Scan Server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) or the Neural Network Promoter Prediction tool (NNPP; <http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html>) (Reese *et al.*, 1996).

Sequence data on *Burkholderia* sp. strain LB400, *Ral. eutropha* JMP134, *Rho. sphaeroides* 2.4.1 and *Rhodopseudomonas palustris* CGA009 were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/index.html). Preliminary sequence data on *Silicibacter pomeroyi* DSS-3, and information on funding the project, were obtained from The Institute for Genomic Research (<http://www.tigr.org>). Sequence data on *Rhodococcus* sp. strain RHA1 were generated by the Microbial Envirogenomics Team at the University of British Columbia (<http://www.bcgsc.bc.ca/cgi-bin/rhodococcus/blast-rha1.pl>). The accession number for clone P1023 in the *Leishmania major* genome project is AC091510. Sequence data for *Rhodobacter capsulatus* SB1003 are available from PEDANT (<http://pedant.gsf.de>).

RESULTS

The putative taurine locus on the chromosomes of *Rho. sphaeroides* and *P. denitrificans*

We have partially reannotated the putative taurine locus of *Sin. meliloti* (SMb21525–SMb21532) for our own convenience (Fig. 1b) and expanded the annotation to cover the analogous putative taurine locus on the chromosome of *Rho. sphaeroides* (Rsph2616–Rsph2608) (Fig. 1b): the major difference between the two loci is that the *tauABC* genes in *Sin. meliloti* are replaced by *tauKLM* (encoding a putative TRAP transporter) in *Rho. sphaeroides* (Fig. 1b) (Table 1). We thus postulated for *Rho. sphaeroides* an inducible, taurine-degradative pathway involving a regulator protein TauR, TRAP transporter TauKLM, TDH (hypothetically TauXY), Xsc, a hypothetical protein (TauZ) and Pta, and we wondered whether this was widespread in *Rhodobacteraceae*.

PCR primers from each gene common to the SMb and Rsph loci were derived and tested in pairs with, as template DNA, whole cells of *P. denitrificans* NKNIS or of strains of *P. pantotrophus*. The amplified fragments from each organism supported the presence of an *xsc* gene (primer pair *xsc3-f* and *xsc3-r*), but only in *P. denitrificans* were we immediately able to generate contiguous sequence (about 10 kbp; Fig. 1b) which represented the whole gene cluster. The cluster *tauRKLMXY-xsc-tauZ-ptA* corresponded to that found in *Rho. sphaeroides* and the two DNA sequences shared 80 % identity. An inducible degradative pathway involving a TRAP transporter, TDH, Xsc and Pta was thus inferred and *P. denitrificans* NKNIS was chosen for biochemical work.

Expansion of the initial sequence (*xsc* gene) in the taurine locus in both strains of *P. pantotrophus* initially indicated the same nine-gene cluster as in *Rho. sphaeroides* (Fig. 1b, Table 1). However, when the transport genes were sequenced, *tauABC*-like genes were found (Fig. 1b). *P. pantotrophus* thus appears to encode an ABC transporter for taurine, as postulated for *Sin. meliloti* (Fig. 1b). The high levels of identity between the corresponding genes in *Rho. sphaeroides* and *P. denitrificans* (Table 1) and the widespread recurrence of the putative regulator in *P. pantotrophus* strains NKNCYSA and DSM 65 (Fig. 1b) and in other organisms (see below) allowed use of the TauR from *Rho. sphaeroides* to predict the class of regulator involved: it is a subgroup of the GntR family (see Rigali *et al.*, 2002).

Growth of *P. denitrificans* with taurine, isethionate or acetate

P. denitrificans NKNIS grew with taurine as sole source of carbon and energy under aerobic conditions with a specific

Table 1. Analysis of the gene cluster inferred to encode taurine dissimilation in *Rho. sphaeroides* 2.4.1 compared with that in *P. denitrificans* NKNIS

Gene number	Length in Rsph (NKNIS) [bp]	Identity with gene in strain NKNIS (%)	Identity with nearest homologue in database (%)	Function of product in domain search	Function in Rsph and strain NKNIS
Rsph2616 (<i>tauR</i>)	1470 (incomplete)	76*	51, SMb21525	Transcriptional regulator (ARO8)	Transcriptional regulator (putative)
Rsph2615 (<i>tauK</i>)	1056 (1011)	80	33, Bh2673	TRAP periplasmic binding protein	TRAP periplasmic binding protein (putative)
Rsph2614 (<i>tauL</i>)	561 (561)	80	27, Ob0242	TRAP small permease component	TRAP small permease component (putative)
Rsph2613 (<i>tauM</i>)	1341 (1341)	83	45, Bh2671	TRAP large permease component	TRAP large permease component (putative)
Rsph2612 (<i>tauX</i>)	396 (420)	81*	61, SMb21670	None	TDH subunit (putative)
Rsph2611 (<i>tauY</i>)	1389 (1392)	81*	66, SMb21529	D-Amino acid oxidase	TDH subunit (putative)
Rsph2610 (<i>xsc</i>)	1782 (1782)	92*	78, SMb21530	ThDP-requiring enzymes	Xsc
Rsph2609 (<i>tauZ</i>)	1020 (1017)	81*	64, Sm21531	Hypothetical membrane protein	Hypothetical membrane protein
Rsph2608 (<i>pta</i>)	999 (597)	78*	76, <i>P. denitrificans</i>	Pta	Pta

*The level of identity to sequence in strains NKNCYSA and strain DSM 65 was usually >90 %.

rate of 0.19 h^{-1} . The molar growth yield was $7 \text{ g protein (mol C)}^{-1}$, and 0.7 mol NH_4^+ (mol taurine) $^{-1}$ and $0.93 \text{ mol sulfate (mol taurine)}^{-1}$ were excreted into the growth medium. Sulfite ion (up to 1.6 mM) was detected in the growth medium. These data correspond to quantitative dissimilation of taurine at a specific degradation rate of $3.8 \text{ mkat (kg protein)}^{-1}$, with assimilation of the anticipated amounts of ammonium and sulfate ions. The release of about 15% of the sulfonate sulfur as sulfite implied low levels of any sulfite dehydrogenase.

Corresponding experiments with isethionate gave a specific growth rate of 0.18 h^{-1} , a molar growth yield of $7 \text{ g protein (mol C)}^{-1}$ and excretion of $0.92 \text{ mol sulfate (mol isethionate)}^{-1}$; some 10% of the sulfonate group was recovered transiently as sulfite. Here again we conclude quantitative substrate utilization at $3.8 \text{ mkat (kg protein)}^{-1}$.

Extracts of strain NKNIS grown with acetate contained no detectable TDH, IDH, Xsc or sulfite dehydrogenase, but they did contain traces of Pta (Table 2). Extracts of taurine-grown cells contained TDH, Xsc and high levels of Pta, but no IDH was detected (Table 2). The isethionate-grown cells, in contrast, contained IDH, Xsc and high levels of Pta, but no TDH (or sulfite dehydrogenase) was detected. The specific activities of Xsc [$2\text{--}4 \text{ mkat (kg protein)}^{-1}$; Table 2] were in the range calculated to be needed for growth of strain NKNIS [$3.8 \text{ mkat (kg protein)}^{-1}$; see above], as was Pta, so we presume that we have assayed enzymes involved in the degradation of taurine and isethionate. The specific activity of TDH [$7.3 \text{ mkat (kg protein)}^{-1}$; Table 2] was also sufficient to explain the growth rate, whereas the level of IDH was apparently somewhat too low (Table 2). Cytochrome *c*, identified from a redox difference spectrum with maxima at 425, 522 and 551 nm (Voet *et al.*, 1999), was detected at $5\text{--}20 \text{ g (kg protein)}^{-1}$ in extracts of taurine- and acetate-grown cells: this later led us to test an analogous protein as electron acceptor in assays of TDH and IDH. As predicted from literature data in other organisms (Cook & Denger,

2002), Xsc and Pta were induced during growth with each sulfonate substrate, whereas TDH (or IDH) was present solely during growth with taurine (or isethionate). Constitutive Tpa was detected, but with negligible activity [$<0.01 \text{ mkat (kg protein)}^{-1}$] which we attribute to the side reaction of another enzyme.

The reactions catalysed by TDH, Xsc, Pta and IDH

The routine assay of TDH involved the reduction of DCPIP which was not in itself specific. However, the reduction of DCPIP depended absolutely on the presence of taurine, and boiled extract was inactive. The apparent K_m value (K_m^{app}) for DCPIP was about $50 \mu\text{M}$. The K_m^{app} value for taurine was about 7 mM . The reaction mixture initially contained no sulfoacetaldehyde, but after exhaustion of the DCPIP, sulfoacetaldehyde was tentatively identified by co-chromatography (HPLC) of the azine derivative with the azine derivative of authentic material. The identification was confirmed by negative-ion MALDI-TOF-MS where the same base peak, $m/z = 123 (M-1)^-$, was observed, as with authentic material. The release of ammonium ion during the reaction was also detected (Berthelot reaction) (cf. Fig. 1); no ammonium ion was detected in the absence of taurine. The preliminary identification of ammonium ion was confirmed by the specific reaction with glutamate dehydrogenase. We presume the stoichiometry of TDH to be 1:1:1 (taurine/sulfoacetaldehyde/ammonium ion). About 3 mM sulfoacetaldehyde was indeed formed during the disappearance of about 3 mM taurine, but ammonium ion could not be measured in the same reaction because of interference from DCPIP and taurine. When we compared the amount of sulfoacetaldehyde formed (e.g. 3 mM) with the amount of DCPIP apparently reduced (0.5 mM) in a reaction mixture, we presumed that spontaneous reoxidation of DCPIP occurred during the reaction.

The following compounds were tested as the potential electron acceptor for the oxidation of taurine: FAD, FMN, riboflavin, PQQ, 1,4-naphthoquinone, ubiquinone, menadione, $\text{K}_3\text{Fe}(\text{CN})_6$ and cytochrome *c*. Only cytochrome *c* functioned as an acceptor, but although the reaction rate was increased by a factor of about 15, the value for K_m^{app} was scarcely altered (6.2 mM). TDH was detected as an inducible enzyme in *Sin. meliloti* Rm1021 [$0.6 \text{ mkat (kg protein)}^{-1}$], the organism in which the *tauXY* genes were first suspected to encode TDH, in *P. pantotrophus* NKNCYSA [$0.7 \text{ mkat (kg protein)}^{-1}$] where the *tauXY* genes are found (Fig. 1) and in *Burkholderia* sp. strain LB400 [$0.2 \text{ mkat (kg protein)}^{-1}$], where the *tauXY*-like genes (Bcep5174–5173) are in close proximity to the *xsc* gene (Bcep5167) (Fig. 1b). *A. defragrans* NKNTAU, in which Tpa is active (Ruff *et al.*, 2003), contained neither measurable TDH nor a *tauY*-like fragment detectable by PCR with the appropriate consensus primers.

TDH was discovered in *Achromobacter xylosoxidans* NCIMB 10751 (Kondo *et al.*, 1971, 1973), but the difficulty we experience in growing the organism with taurine (Ruff *et al.*, 2003)

Table 2. Specific activities of TDH, Xsc, Pta, IDH and sulfite dehydrogenase in extracts of cells of *P. denitrificans* NKNIS grown with different substrates

Enzyme	Specific activity of enzymes in extracts of cells grown with the following carbon source [mkat (kg protein) $^{-1}$]*		
	Acetate	Taurine	Isethionate
TDH	ND	7.3	ND
Xsc	ND	2.1	4.2
Pta	0.4	10	13.6
IDH	ND	ND	0.6
Sulfite dehydrogenase	ND	ND	ND

*ND, Not detectable [$<0.005 \text{ mkat (kg protein)}^{-1}$].

meant that we could not measure the activity directly. We could, however, amplify a DNA fragment (1 kb) from strain NCIMB 10751 with the consensus primer pair designed to detect *tauY* genes. The fragment showed 67 % identity to the corresponding portion of the *tauY* gene in *Burkholderia* sp. strain LB400 and 70 % identity at the level of the derived amino acid sequence. *Achromobacter* sp. has been assigned, with scanty evidence, to a risk group which makes its legal transfer to other laboratories impracticable. Given the availability of more easily grown organisms that utilize taurine and isethionate (e.g. *Burkholderia* sp. strain LB400) and may be transported easily, and the pending complete genome sequence for strain LB400, it seems rational to make this bacterium the basis for comparisons.

Genes, like those attributed to taurine degradation in *Burkholderia* sp. strain LB400 (Fig. 1b), were found in *Ral. eutropha* JMP134 (Fig. 1b) and we confirmed our postulate that the latter organism could grow with taurine as sole source of carbon and energy for growth. *Ralstonia* sp. strain EDS1 dissimilates taurine (Denger & Cook, 2001), so we postulated that this organism would also contain the *tauY* gene. The DNA fragment (1 kb) obtained with the consensus primer pair shared 67 % sequence identity with the corresponding portion of the *tauY*-like gene in strain LB400. *Comamonas* sp. strain SFCD1, which dissimilates taurine, also contains a 1 kb DNA sequence with 67 % identity to the *tauY* gene of strain LB400. There is a strong correlation between the *tau(X)Y* genes and the dissimilation of taurine.

The Xsc reaction in strain NKNIS depended absolutely on the presence of inorganic phosphate, and yielded acetyl phosphate and sulfite, as anticipated (Cook & Denger, 2002). Simple rebuffing experiments showed the loss of 80 % of activity in the absence of ThDP and of 20 % in the absence of Mg^{2+} : we presume that both factors are required for activity, but that the rebuffing was not rigorous enough. The following values for K_m^{app} were obtained: sulfoacetaldehyde, 2.8 mM; phosphate, 2.4 mM; ThDP, 3.1 μ M.

The assay of Pta, based on the formation of acetyl CoA, was

positive, so the degradation of taurine yields the substrate for the Krebs and glyoxylate cycles, as foreseen in Fig. 1.

The assay of IDH was initially followed as the reduction of DCPIP. This reduction depended absolutely on the presence of isethionate, and boiled extract was inactive. The K_m^{app} value for isethionate was about 1 mM. The reaction mixture initially contained no sulfoacetaldehyde, but after exhaustion of the DCPIP, sulfoacetaldehyde was tentatively identified by co-chromatography (HPLC) of the azine derivative with the azine derivative of authentic material. The identification was confirmed by MALDI-TOF-MS, both as the underivatized compound and as the oxime. We did not determine isethionate, so no data on stoichiometry are available. An inducible protein with a molecular mass of 62 kDa under denaturing conditions was observed in crude extracts (Fig. 2, lane 3) and in the membrane fraction (Fig. 2, lane 9) of isethionate-grown cells. We then detected that cytochrome *c* served as electron acceptor for IDH.

Separation of Xsc and Pta

A diluted soluble fraction of taurine-grown *P. denitrificans* NKNIS was loaded onto an anion exchange column. Two enzymes were analysed in the eluate, Xsc, which eluted at 140 mM sodium sulfate, and Pta, which eluted at 110 mM sodium sulfate (Fig. 3). The separation of Xsc was effectively a one-step purification (Fig. 2, lane 5; Table 3). The Pta fraction contained several proteins, the occurrence of only one of which was representative of the Pta activity in the fractions 43–47 and also corresponded in molecular mass (33 kDa) to that derived from the *pta* gene in strain NKNIS. This protein was present in small amounts and Pta was not examined further.

Xsc from strain NKNIS was estimated to have a molecular mass of about 64 kDa (Fig. 2, lane 5). Gel filtration chromatography (not shown) of the native protein on a calibrated column indicated a molecular mass of about 217 kDa, so, given a unique N-terminal sequence (see below), a homomultimeric, possibly tetrameric, Xsc was assumed. The

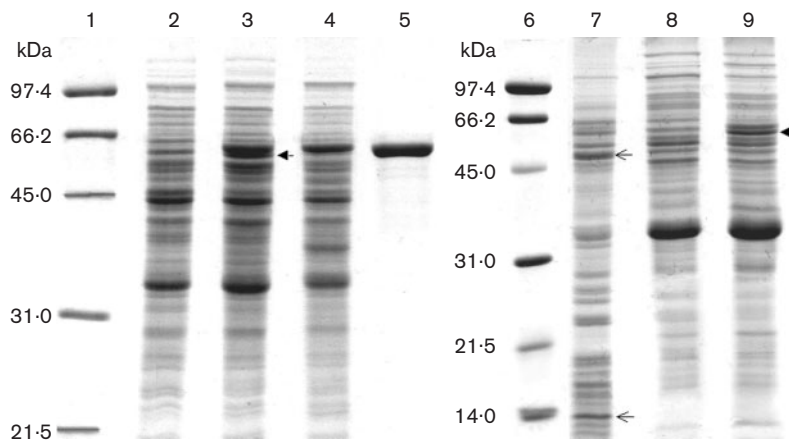


Fig. 2. Electropherograms (SDS-PAGE) of denatured proteins from crude extracts or membrane fractions of *P. denitrificans* NKNIS grown with different substrates, and of a fraction from a chromatographic separation of Xsc. Lanes: 1, 6, molecular mass markers; 2, crude extract of acetate-grown cells; 3, crude extract of isethionate-grown cells; 4, crude extract of taurine-grown cells; 5, separated Xsc; 7, membrane fraction from taurine-grown cells; 8, membrane fraction from acetate-grown cells; 9, membrane fraction from isethionate-grown cells. Arrows mark the 62 kDa protein tentatively attributed to IDH (lanes 3, 9) and the 50 and 14 kDa bands tentatively attributed to TDH (lane 7).

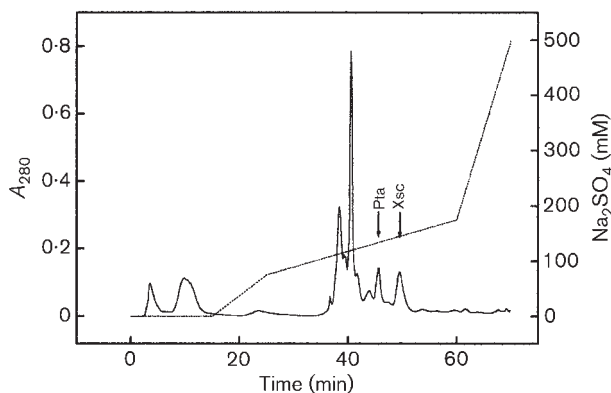


Fig. 3. The separation of soluble proteins in an extract from taurine-grown *P. denitrificans* NKNIS on an anion-exchange column and the elution of Xsc and Pta (arrows). About 50 mg protein was loaded onto the column and a non-linear gradient of Na_2SO_4 (dotted line) was used to elute protein (solid line), which was detected at A_{280} .

N-terminal amino acid sequence was determined to be MRMTTEES; there was no ambiguity, so all subunits were identical. This peptide was present in the deduced sequence of the putative *xsc* gene and allowed the *xsc* gene to be defined, because there were several possible start codons in the DNA sequence. The data thus confirm the identity of the *xsc* gene and allowed the molecular mass of the protein to be derived as 64.4 kDa, in agreement with the data from SDS-PAGE (Fig. 2, lane 5).

TDH from strain NKNIS was found exclusively in the particulate fraction [at 25 mkat (kg protein^{-1})]. The membrane proteins of differently grown cells were examined, and inducible formation of two proteins (14 and 50 kDa) in membranes of taurine-grown cells was detected by SDS-PAGE (Fig. 2, lane 7). We thus maintain the hypothesis that the gene products TauXY represent TDH.

DISCUSSION

Genomic sequence data from *Sin. meliloti* (Finan *et al.*, 2001), together with biochemical data on Xsc in *A. defragrans*, allowed us to postulate a regulated degradative pathway for taurine in *Sin. meliloti* (Ruff *et al.*, 2003). That, in turn, allowed a similar pathway to be proposed for *Rho.*

sphaeroides (Cook & Denger, 2002) (Fig. 1, Table 1). This paper shows that one cluster of nine genes is present in both *Sin. meliloti* and *P. pantotrophus* DSM 65 (Fig. 1) and that a similar cluster is found in both *Rho. sphaeroides* and *P. denitrificans* (Table 1). There was, thus, a biological system with which to test the hypotheses derived from data *in silico*. The confirmation that the hypothetical *xsc* gene in *P. denitrificans* NKNIS does indeed encode Xsc infers that all *xsc* genes in subgroup 3 encode this product, and this indicates that the gene clusters in both *Sin. meliloti* [SMb21525–SMb21532, where inducible TDH (see above), Xsc and Pta are known (Ruff *et al.*, 2003)] and in *Rho. sphaeroides* [Rsph2616–2608, where catabolism of taurine has been observed (K. Denger, unpublished)] do encode the enzymes of taurine dissimilation.

The putative regulator, TauR, was suggested because the corresponding gene was adjacent to the then tentative taurine gene cluster in *Sin. meliloti* (Fig. 1b) (Ruff *et al.*, 2003). A similar, putative regulator gene is found in all sequenced taurine utilizers (Fig. 4a), which represent α - and β -Proteobacteria and high-G+C Gram-positive bacteria. It is possible that TauR represents a new subgroup related to the ARO8 regulators (the result in all BLAST domain searches) of the MocR-group of GntR-like regulators, analogous to a recent finding in *Anabaena* (Lee *et al.*, 2003). Analyses of mutants are now required to explore the role and function of TauR.

Little is known about the transport of sulfonates in catabolic pathways, whereas ABC transporters are widespread in scavenging systems associated with sulfate starvation (Kertesz, 2001; Cook & Denger, 2002). We suspect an ABC transporter for taurine catabolism in the strains of *P. pantotrophus* (Fig. 1b), in concurrence with that inferred for taurine transport in *Sin. meliloti* (Fig. 1b) (Ruff *et al.*, 2003) and with the ABC transporter for methanesulfonate (de Marco *et al.*, 1999; Kertesz, 2001). The sequences of the genes encoding putative TauABC transporters in *Sin. meliloti*, *Burkholderia* sp. strain LB400, *L. major* and *Ral. eutropha* were examined in dendrograms (not shown) and they group very close to the corresponding genes encoding TauA (or TauB or TauC) in *Escherichia coli* and at a distance from the corresponding component of the TRAP transporters. In contrast to ABC transporters in e.g. *Sin. meliloti*, we suggest that TRAP transporters are involved in the transport of taurine in *P. denitrificans* and *Rho. sphaeroides* (Table 1). Here again, the generation of mutants is needed, as are assays

Table 3. Purification of Xsc

Fraction	Volume (ml)	Total protein (mg)	Total activity (μkat)	Specific activity [$\text{mkat} (\text{kg protein}^{-1})^{-1}$]	Purification factor (-fold)	Yield (%)
Crude extract	3.0	149	0.24	1.6	1	100
Soluble fraction	2.4	110	0.24	2.1	1.3	97
Dilution	6.0	108	0.23	2.2	1.3	95
Mono Q eluate	4.8	2.4	0.04	16.5	10	16

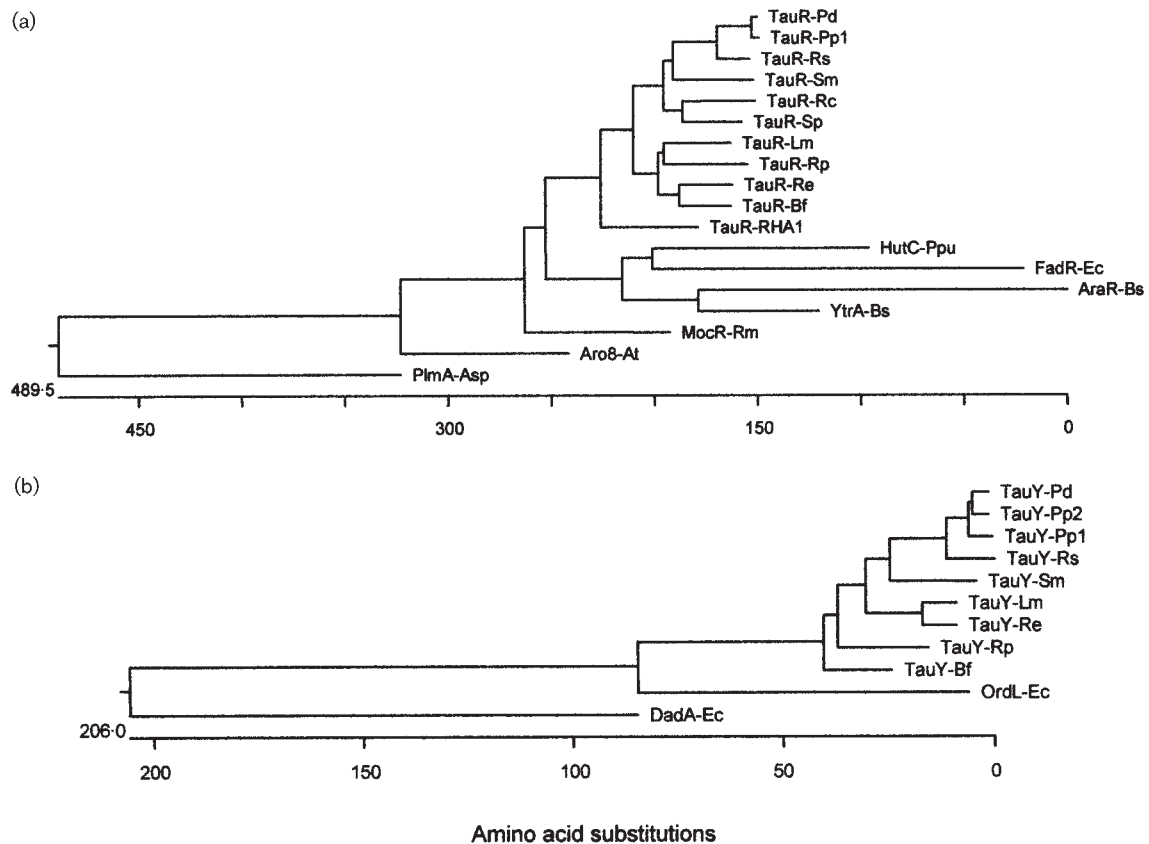


Fig. 4. Dendrograms with TauR in the GntR family of regulators, and TauY sequences with the best hit (OrdL) in BLAST searches and in domain searches (DadA). The deduced amino acid sequences of the gene products and the relevant comparisons were processed in CLUSTALW software to generate the dendrograms. The abbreviation for each protein is followed by an abbreviation for its source: At, *Agrobacterium tumefaciens*; Bf, *Burkholderia* sp. strain LB400; Bs, *Bacillus subtilis*; Ec, *E. coli* K-12; Lm, contaminant DNA (clone P1023) in the *L. major* genome sequence project; Pd, *P. denitrificans* NKNIS; Pp1, *P. pantotrophus* NKNCSA; Pp2, *P. pantotrophus* DSM 65; Ppu, *Pseudomonas putida*; Rc, *Rho. capsulatus* SB1003; Re, *Ral. eutropha* JMP134; RHA1, *Rhodococcus* sp. strain RHA1; Rp, *Rps. palustris* CGA009; Rs, *Rho. sphaeroides* 2.4.1; Sm, *Sin. meliloti* Rm1021; Sp, *Sil. pomeroyi* DSS-3. The accession numbers of sequences used as outgroups or comparisons are: AraR, P96711; Aro8, AAL42844; DadA, AAC74273; FadR, P09371; HutC, P22773; MocR, P49309; OrdL, AAC74383; PlmA, AAO92602; YtrA, CAB15024.

of transport, to test these hypotheses. The TRAP transporter presumably represents a new member of the TC 2.A.56.1.-family (Saier, 1999), whereas the ABC transporter is a tentative member of TC 3.A.1.17.1 (see Kertesz, 2001).

There are reasonable data to support a mass balance for the oxidative deamination of taurine to sulfoacetaldehyde by TDH, but although difficulties with DCPIP have left an incomplete picture of the overall stoichiometry of the reaction, there is no reason to doubt the representation in Fig. 1. Our biochemical approaches to purify TDH, where proteins of 14 and 50 kDa were observed (Fig. 2), all support the hypothesis that the enzyme is encoded by *tauXY*, which potentially encode proteins of 15 and 51 kDa, respectively, but other possibilities have not been excluded. All tested organisms with *tauXY*-like genes express TDH, whereas organisms with no apparent homologue of *tauXY* [*A. defragrans* (see

above) and *Rhodococcus* sp. strain RHA1] and an active Tpa, do not (K. Denger, unpublished). We infer that the gene products of *tauXY* contribute to the function of TDH and we anticipate that heterologous expression of the genes will allow the question to be answered conclusively. The TDH described or mentioned previously (Fellman *et al.*, 1980; Kondo & Ishimoto, 1987) is a particulate system for which no molecular description is available, presumably because the respiratory chain was used for the enzyme assay in an oxygen electrode. This poor definition is reflected in enzyme nomenclature, where the accession number is EC 1.4.99.2. The recognition that cytochrome *c* is the natural electron acceptor potentially allows the enzyme to be reclassified as taurine-ferricytochrome *c* reductase EC 1.4.2.-, a group which currently has only one entry (Sanders *et al.*, 1972).

Analyses in Prosite indicated significant similarity of TauY

binding motifs to the FAD-linked amino acid oxidases only, as represented by DadA (see Fig. 4b). The addition of FAD had no effect on activity. The sequence motifs of TauXY give no reason to suspect that these are membrane proteins, but TDH is found in the membrane fraction. BLASTP searches with the sequence of TauY yield, after a set of TauXY homologues, a large number of TauY-like hypothetical proteins. Those *tauY*-like genes, which have an adjacent *tauX* gene, fall into one group (Fig. 4b) which is independent of the phylogeny of the organisms concerned. The other, larger group of TauY-like putative proteins has no known function, but one has been named in *E. coli*, *ordL* (Jovanovic & Model, 1997). *OrdL* falls into a separate group from TauY and both are distinctly separate from DadA (Fig. 4b).

The Xsc purified here is encoded by a subgroup-3 *xsc* gene. The enzyme has many similarities to those in subgroup 1, namely the relatively high K_m^{app} value for sulfoacetaldehyde (mM value) and the requirement for ThDP in the reaction mixture, in contrast to the subgroup-2 enzyme, with its higher affinity for sulfoacetaldehyde and tightly bound ThDP (Denger *et al.*, 2001; Ruff *et al.*, 2003). The newly characterized Xsc from the subgroup-3 genes is thus not biochemically different from the Xscs of subgroup 1. As observed in many organisms (Ruff *et al.*, 2003), Xsc is strongly induced in taurine- (and isethionate-) grown cells of strain NKNIS (Fig. 2), which is in agreement with the 10-fold purification to yield an essentially homogeneous protein (Fig. 3, Fig. 2). This is the first Xsc to be isolated with the initiatory M retained in the protein, but this observation does fit the consensus for the *E. coli* aminopeptidase, where the M followed by R or K is not cleaved (Ben-Bassat *et al.*, 1987). Indeed, *xsc* subgroup 3 is the only known subgroup to have a conserved N-terminal sequence, so presumably the initiatory M is never cleaved. This conserved sequence results in the thiamin-phosphate-activating glutamate in the biochemical reaction (Bar-Ilan *et al.*, 2001) always being E49. The expansion of *xsc* subgroup 3 from three to eight members has not altered the general structure of the dendrogram generated earlier (see Ruff *et al.*, 2003) to illustrate the three subgroups, or required any further alteration of the consensus pattern for the ThDP-binding site (see Ruff *et al.*, 2003). Little work has been put into the Pta enzymes in the taurine pathway, except to confirm their presence, but there is no evidence that they fall into different subgroups (dendrogram not shown).

This discussion of the genes and gene products in the taurine pathway tends to imply a common regulation of expression of the eight genes presumed to be under the control of TauR, and this is a fair assumption during growth with taurine, with the need for transport, as well as the expression of TDH (presumably TauXY), Xsc and Pta (Table 2). When the cells utilize isethionate, however, the same Xsc seems to be present (Fig. 2), with Pta, though there is no TDH (presumably TauXY), but there is expression of IDH (Table 2). The intergenic space between *tauY* and *xsc* is only 12 bp, so there must be an unusual regulatory system to allow

expression of a downstream region, when the upstream genes are not expressed.

The present work with IDH in strain NKNIS would appear to be the first direct assay of the enzyme since its discovery in the particulate fraction of strain NCIMB 10751 in 1977 (Kondo *et al.*, 1977). IDH in strain NKNIS is also in the particulate fraction, and we suspect that a 62 kDa protein may catalyse the reaction (Fig. 2). The rules of enzyme nomenclature enable a cytochrome *c*-dependent alcohol dehydrogenase to be attributed to EC 1.1.2.-, which has relatively few entries, as yet. Given an inducible IDH (Table 3), we infer the presence of an isethionate regulator, a transport system and IDH, presumably encoded in a cluster.

P. denitrificans NKNIS is one of the organisms which excretes some sulfite during growth with a sulfonate substrate (Fig. 1a). We could not detect sulfite dehydrogenase (oxidase), so we presume that sulfite is subject to spontaneous oxidation to sulfate. It is unclear where this oxidation occurs, possibly in part inside the cell, but a portion is certainly oxidized extracellularly. It is thus unclear whether sulfite or sulfate, or both, is subject to export, and what mechanisms are used for this export and to sense the need for it. *P. pantotrophus* NKNCYSA expresses a sulfite dehydrogenase and excretes only sulfate (Rein, 1999). *Sin. meliloti* potentially encodes at least one sulfite dehydrogenase (SMA2103) and one sulfite oxidase (SMc04049), whereas *Rho. sphaeroides* appears to encode none, so we anticipate release of sulfite into the medium when *Rho. sphaeroides* utilizes taurine, but not when *Sin. meliloti* utilizes it. When the presumed requirement for the export of sulfate/sulfite to maintain a constant ionic strength within the cell is valid, another regulatory system and an export system must be required for the ammonium ion (or for the neutral species) (Fig. 1). Ammonium/methylammonium transport (Amt) proteins (e.g. Meier-Wagner *et al.*, 2001) may fill the latter role.

Degradation of taurine, in particular, is a very complex system with many unresolved questions which could well yield to analyses by gene arrays in a sequenced organism such as *Sin. meliloti* or *Rho. sphaeroides*.

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