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The DUF81 protein TauE in *Cupriavidus necator* H16, a sulfite exporter in the metabolism of C_2 sulfonates

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The degradation of taurine, isethionate and sulfoacetate in *Cupriavidus necator* (*Ralstonia eutropha*) H16 was shown by enzyme assays to be inducible, and each pathway involved sulfoacetaldehyde, which was subject to phosphatolysis by a common sulfoacetaldehyde acetyltransferase (Xsc, H16_B1870) to yield acetyl phosphate and sulfite. The neighbouring genes encoded phosphate acetyltransferase (Pta, H16_B1871) and a hypothetical protein [domain of unknown function (DUF)81, H16_B1872], with eight derived transmembrane helices. RT-PCR showed inducible transcription of these three genes, and led to the hypothesis that H16_B1872 and orthologous proteins represent a sulfite exporter, which was named TauE.

INTRODUCTION

Much information is available on the fate of natural sulfoaliphatics (Cook & Denger, 2002, 2006; Cook *et al.*, 2006), and one key step in the dissimilation of these compounds is the desulfonation reaction, which in all known cases generates sulfite (Cook *et al.*, 2007). In the dissimilation of the C_2 sulfonates [taurine (2-aminoethanesulfonate), isethionate (2-hydroxyethanesulfonate) and sulfoacetate], the point of convergence of different pathways is sulfoacetaldehyde, and the desulfonative enzyme is the phosphatolytic sulfoacetaldehyde acetyltransferase (Xsc), which converts the substrate to acetyl phosphate and sulfite (Ruff *et al.*, 2003) (Fig. 1a). This sulfite is finally recovered as sulfate in the supernatant fluid of the culture, though in some cases, transient extracellular sulfite is detected (Cook *et al.*, 2007). The sulfite is oxidized to sulfate by a sulfite dehydrogenase (Cook *et al.*, 2007), but little is known about the location of sulfite dehydrogenase(s), the nature of the exported oxyanion, or the transporters involved.

A gene (*tauZ*) which encodes a potential sulfate exporter, TauZ (TC 9.B.63.1.1), and is located in a cluster of genes that encode taurine catabolic enzymes, is inducibly transcribed when *Paracoccus pantotrophus* NKNCYSA dissimilates taurine (Brüggemann *et al.*, 2004; Rein *et al.*, 2005). Orthologues of this protein are encoded in the 'taurine gene cluster' of several taurine-degrading bacteria (Brüggemann *et al.*, 2004), associated with the metabolism of L-cysteate by *P. pantotrophus* NKNCYSA (SuyZ; Rein *et al.*, 2005) and found for example in the sulfate-excreting *Chlorobium tepidum* (CT0845) in a 'sulfur island'; in *Ruegeria* [formerly *Silicibacter* (Yi *et al.*, 2007)] *pomeroyi* DSS-3, the orthologue CuyZ is a sulfite exporter (Denger *et al.*, 2006a). The *cuyZ* gene is not induced in *R. pomeroyi* DSS-3 when the organism grows with taurine, and the nature of the sulfate exporter under these conditions is still unknown (Gorzynska *et al.*, 2006). Many other bacteria, in which no orthologue of TauZ is found, need to export sulfite or sulfate.

The complete genome sequence of *Cupriavidus necator* (*Ralstonia eutropha*) H16 was published recently (Pohlmann *et al.*, 2006), and it contains no potential orthologue of *tauZ*. The genome contains genes predicted to encode a complete taurine degradative pathway (Fig. 1a) in two gene clusters (Fig. 1b) located on chromosome 2: one cluster encodes the putative transcriptional regulator (*tauR*, locus tag H16_B1891) and taurine dehydrogenase (*tauXY*), the other cluster encodes Xsc (locus tag H16_B1870), phosphate acetyltransferase (Pta, locus tag H16_B1871), and what we now designate *tauE* (taurine, excretion; locus tag H16_B1872), which encodes a protein of the domain of unknown function (DUF)81 family. The DUF81 family seems to contain about 1500 orthologues, but only one relatively close orthologue has been assigned a function, TsaS, in 4-toluenesulfonate transport (Mampel *et al.*, 2004). CysZ, a distant orthologue, is a sulfate transporter (Rückert *et al.*, 2005). The TauE protein is a presumptive membrane protein, which is predicted to contain eight membrane-spanning helices. Orthologues of this protein, previously called OrfX, have been found in *Burkholderia xenovorans* LB400 and *C. necator* JMP134 (Brüggemann *et al.*, 2004), and they are now predicted to

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Abbreviations: DUF, domain of unknown function; SDH, sulfite dehydrogenase.

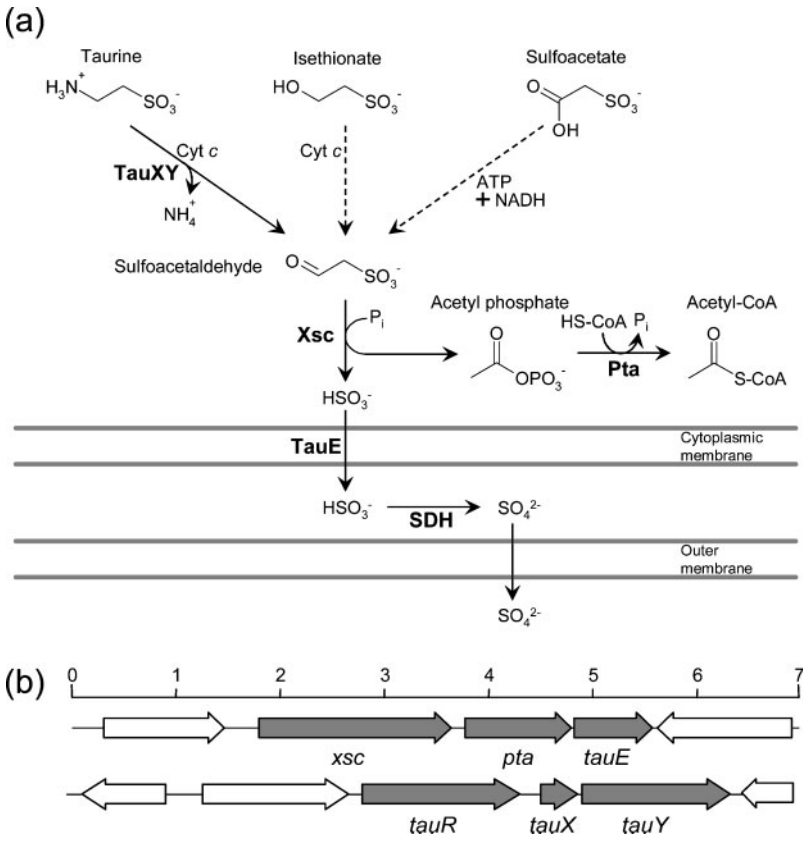

Fig. 1. Pathways for the dissimilation of taurine, isethionate and sulfoacetate in *C. necator* H16 (a) and gene clusters for taurine metabolism in *C. necator* H16 (b). Conversions for which the genes are currently unknown are indicated by dashed lines. The gene loci of *tauR–tauY* and *xsc–tauE* are H16_B1891 to H16_B1893 and H16_B1870 to H16_B1872, respectively. Cyt *c*, cytochrome *c*.

be sulfite exporters (Cook *et al.*, 2007). TauE does not show significant sequence homology to TauZ [TC 9.B.63.1.1] or CuyZ.

We now confirm the role of sulfoacetaldehyde as a point of convergence in the degradative pathways of three C_2 sulfonates (Fig. 1a). Further metabolism involves a common downstream pathway, consisting of Xsc and Pta to generate acetyl-CoA and sulfite, as well as the sulfite exporter TauE. We also present the first experimental evidence that *tauE* is transcribed inducibly when *C. necator* H16 is grown with the C_2 sulfonates taurine, isethionate and sulfoacetate.

METHODS

Organisms, growth, harvesting of cells and preparation of cell-free extracts. *B. xenovorans* LB400 and *C. necator* H16 (DSM 428) were grown aerobically under carbon-limited conditions at 30 °C in a phosphate-buffered mineral-salts medium (Thurnheer *et al.*, 1986) with 10–20 mM taurine, sulfoacetate, isethionate or acetate. Precultures (3 ml) were grown in 30 ml screw-capped tubes in a roller. Growth experiments were done on the 50 ml scale in 300 ml Erlenmeyer flasks on a shaker. Samples were taken at intervals to measure growth and to determine the concentrations of substrates and products. Similar cultures were used to generate large amounts of cells. Cells were harvested in the mid-exponential growth phase by centrifugation (30 000 ***g***, 15 min, 4 °C), washed in 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$, and stored frozen. The same buffer served as extraction buffer. Cell-free extracts were generated by four passages through a chilled French pressure cell at 138 MPa (Junker *et al.*, 1994).

Cells for the preparation of total RNA were harvested in the mid-exponential phase of growth (OD_{580} 0.3–0.6) by centrifugation at 5000 ***g***; RNA was extracted immediately. Storage of intact cells at –70 °C before RNA extraction resulted in complete loss of mRNA.

Enzyme assays. Taurine dehydrogenase (TauXY) was measured photometrically with dichlorophenol indophenol as the electron acceptor (Brüggemann *et al.*, 2004). Sulfoacetaldehyde acetyltransferase (Xsc) was assayed as the formation of acetyl phosphate (Ruff *et al.*, 2003). Phosphate acetyltransferase (Pta) was assayed photometrically as the HS-CoA-dependent formation of acetyl-CoA (Bergmeyer *et al.*, 1983); *B. xenovorans* LB400 served as the positive control for the enzyme assay. Sulfite dehydrogenase (SDH) was assayed with potassium ferricyanide (Reichenbecher *et al.*, 1999) as the electron acceptor.

Analytical methods. Growth was followed as turbidity (OD_{580}) and quantified as protein in a Lowry-type reaction (Cook & Hütter, 1981). Taurine was derivatized with dinitrofluorobenzene and quantified after separation by HPLC (Denger *et al.*, 1997). Isethionate and sulfoacetate were determined by ion chromatography (Denger *et al.*, 2004; Styp von Rekowski *et al.*, 2005). Sulfite was quantified as the fuchsin derivative (Denger & Cook, 2001). Sulfate was determined turbidimetrically as a suspension of $BaSO_4$ (Sörbo, 1987).

Molecular methods. Oligonucleotides were synthesized by Microsynth. *Taq* DNA polymerase and M-MuLV reverse transcriptase were from MBI Fermentas and used as specified by the supplier.

Table 1. Primers used in this study

Target	Primer	Sequence (5′→3′)	Reference
16S rRNA	16S-27F	CAGAGTTTGATCCTGGCTCAG	Weisburg *et al.* (1991)
	16S-533R	TTACCGCGGCTGCTGGCAC	Weisburg *et al.* (1991)
xsc	H16xscF	ACCGACATCGGCAACATCAACTC	This study
	H16xscR	GGTTGTAGAAGTCCACCTGGTTCT	This study
pta	H16ptaF	TGGTGTCGAGCTTCTTCCTGAT	This study
	H16ptaR	GCCGGCTTCCAGGCTGGGAAAC	This study
tauE	H16tauEF	GGCACCTATTTCCAGACGGTGAC	This study
	H16tauER	CAGGGTGGCTGCGCTGGAACTC	This study

Chromosomal DNA was isolated from bacteria, as described by Desomer *et al.* (1991). Total RNA was isolated using the E.Z.N.A. Bacterial RNA kit (Omega Bio-Tek) and contaminant DNA was removed with RNase-free DNase (MBI Fermentas). The RNA was tested for residual DNA before reverse transcription by PCR using the primer set H16xscF/H16xscR. The PCR primers listed in Table 1 were used for RT-PCR reactions, which were done as described elsewhere (Innis *et al.*, 1990). PCR products were visualized on 1.5 % agarose gels according to standard methods (Sambrook *et al.*, 1989). The GeneRuler 50 bp DNA ladder (MBI Fermentas) was used as a molecular marker.

Software for DNA sequence analyses. Sequence analyses of the *C. necator* H16 genome [accession nos AM260479 (chromosome 1); AM260480 (chromosome 2) and AY305378 (megaplasmid pHG1) (Pohlmann *et al.*, 2006)] were done using the BLAST algorithm (Altschul *et al.*, 1990) at NCBI. The Lasergene Package (DNASTAR) was used for routine sequence analyses *in silico*. Primers for reverse transcription and PCR were designed using the program Amplify (version 1.2). Transmembrane helices were predicted using the program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Neighbour-joining trees were generated from CLUSTAL_X alignments using the program NJ-Plot.

RESULTS

Growth of *C. necator* H16 with sulfonates and enzyme activities involved in sulfonate metabolism

C. necator H16 grew exponentially with taurine (μ=0.25 h^{-1}), isethionate (μ=0.26 h^{-1}), sulfoacetate (μ=0.14 h^{-1}) and acetate (μ=0.27 h^{-1}). Each sulfonate was utilized concomitantly with growth, and sulfate was excreted quantitatively and concomitantly with growth (data not shown). No sulfite was detected at any time point sampled during growth of any culture.

Taurine dehydrogenase (TauXY) activity could be measured in cell extracts of taurine-grown cells only (Table 2). This corresponded to the inducibility of this enzyme noted elsewhere (Brüggemann *et al.*, 2004; Denger *et al.*, 2006b). Sulfoacetaldehyde acetyltransferase (Xsc) was active in extracts from all sulfonate-grown cells, and absent in extracts from acetate-grown cells (Table 2). This confirmed the previous observations that both isethionate and sulfoacetate are degraded via sulfoacetaldehyde (Fig. 1a; Brüggemann *et al.*, 2004; Denger & Cook, 2001; S. Weinitschke, unpublished data). Enzyme activity of phosphate acetyltransferase (Pta) was not detected (Table 2), even though the assay worked well for *B. xenovorans* LB400. We presumed that this was due either to an inappropriate assay for this particular Pta, or to an unstable Pta (Lawrence *et al.*, 2006; Weinitschke *et al.*, 2006; see below). The specific activity of SDH was high in all extracts from sulfonate-grown cells, and low in extracts from acetate-grown cells (Table 2).

Transcription of genes involved in sulfoacetaldehyde metabolism

RT-PCR experiments were done to confirm the role of the candidate genes in the degradation of sulfoacetaldehyde during metabolism of taurine, isethionate and sulfoacetate. All tested genes (*xsc*, *pta* and *tauE*) were transcribed when cells grew with sulfonate, whereas transcripts were absent in acetate-grown cells (Table 2). The presence of a *pta* transcript indicates that the enzyme does indeed play a role in the metabolism of the three sulfonates, as shown in Fig. 1(a), despite the lack of a measurable enzyme (Table 2). No amplicon was obtained using H16tauER-cDNA and the primer set for *pta*. This implies (i) that no transcriptional link between *pta* and *tauE* exists, and (ii) that each gene is transcribed monocistronically.

DISCUSSION

The first metabolic step(s) in the degradation of each C_2 sulfonate studied was induced specifically and yielded sulfoacetaldehyde: for taurine, the enzyme involved is taurine dehydrogenase (TauXY; Table 2). The current hypotheses (Fig. 1a) of cytochrome *c*-dependent isethionate dehydrogenase (Brüggemann *et al.*, 2004) and sulfoacetate reduction to sulfoacetaldehyde involving CoA, ATP and NADH (Denger & Cook, 2001) have new experimental support (S. Weinitschke, unpublished data). The enzymes (Xsc, Pta) necessary to transform sulfoacetaldehyde to an amphibolic intermediate (acetyl-CoA), and those for sulfite excretion and oxidation (TauE and sulfite dehydrogenase; see below) were induced in all relevant cultures (Table 2). This confirms the prediction (Cook &

Table 2. Specific activities of enzymes and transcription of genes under different growth conditions of *C. necator* H16

Enzyme or gene transcription	Specific activity or transcription in cells grown with:			
	Taurine	Isethionate	Sulfoacetate	Acetate
Taurine dehydrogenase (TauXY)	0.3*	ND†	ND	ND
Sulfoacetaldehyde acetyltransferase (Xsc)	1.2	2.8	2.5	ND
Transcription of *xsc*	+‡	+	+	−
Phosphate acetyltransferase (Pta)	ND	ND	ND	ND
Transcription of *pta*	+	+	+	−
Transcription of *tauE*	+	+	+	−
SDH	30	16	37	1

*Enzyme specific activity in crude cell extracts [mkat (kg protein)$^{-1}$].
†ND, Not detected.
‡RNA transcript; the intensity of amplicons is scored as: −, absent; +, strong band.

Denger, 2002) that the converging pathways in sulfonate metabolism are regulated independently, whereas a single set of genes is expressed to convert sulfoacetaldehyde from different sources to products.

The product (of sulfonate dissimilation) whose metabolism is least understood is sulfite. The only characterized SDH (SorAB from *Starkeya novella*) is periplasmic (Kappler *et al.*, 2000); we argue that this location is common (Cook *et al.*, 2007), and preliminary data indicate that the SDH in *C. necator* H16 is periplasmic (K. Denger, unpublished results). Consequently, *C. necator* H16 needs a sulfite exporter to bring the inorganic product of the Xsc reaction into contact with its periplasmic SDH (Fig. 1a), and our candidate for this function is TauE, whose gene is inducibly transcribed when *xsc* is inducibly transcribed (Table 2).

The sequences of orthologues of TauE (all DUF81 proteins) from the NCBI database were compared and depicted in a dendrogram (Fig. 2). Each protein in the

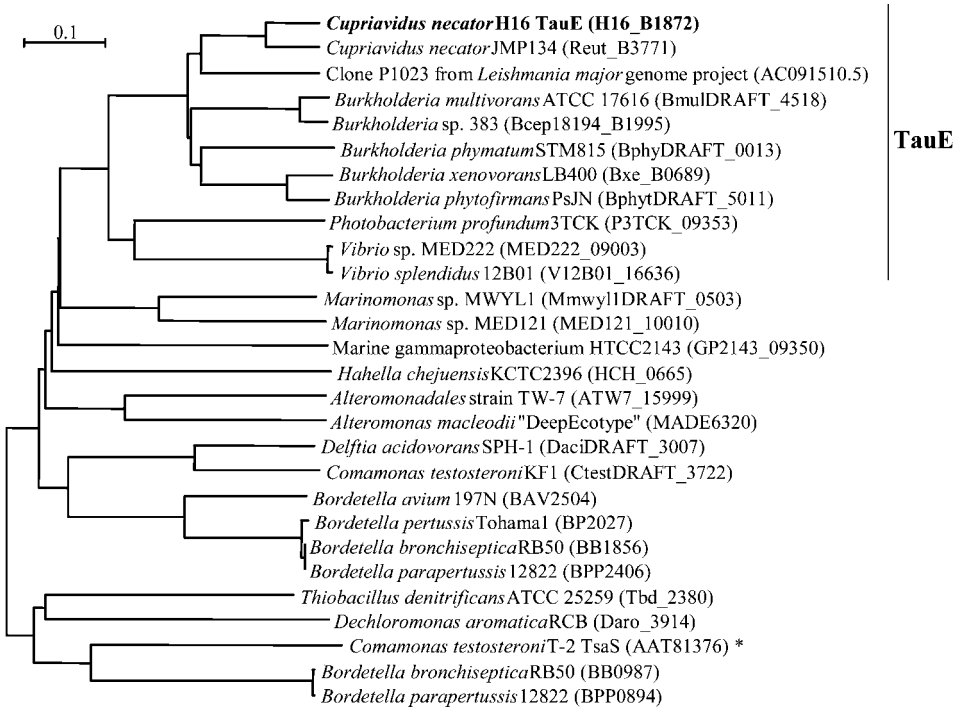

Fig. 2. Phylogenetic relationships of the *C. necator* H16 TauE (bold type) and orthologues. The neighbour-joining (NJ) plot is from a CLUSTAL_X alignment of amino acid sequences most closely related to TauE. The locus tag from the genome sequencing project or the GenBank accession no. is given in parentheses. TsaS is indicated by an asterisk. Scale bar, 10% sequence divergence.

TauE cluster (Fig. 2) is encoded in a locus of 'taurine genes'. These TauE orthologues share >47 % sequence identity. The closest orthologues in other (presumably non-desulfonative) organisms have <43 % sequence identity to all proposed TauE sequences. The most closely related DUF81 protein of known function, TsaS (19–25 % identity to all TauE orthologues), is involved in the uptake of 4-toluenesulfonate in *Comamonas testosteroni* T-2 (Mampel *et al.*, 2004), and is found in a different clade in the dendrogram (Fig. 2). Other DUF81 proteins, including the sulfate-uptake protein CysZ (Rückert *et al.*, 2005), have lower sequence identities to the TauE cluster. We hypothesize that DUF81 proteins are involved in the transport of anions across the cytoplasmic membrane.

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