The DUF81 protein TauE in Cupriavidus necator H16, a sulfite exporter in the metabolism of C₂ sulfoacetates

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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 C₂ sulfoacetates [taurine (2-aminoethanesulfonate), isethionate (2-hydroxyethanesulfonate) and sulfoacetate], the point of convergence of different pathways is sulfoacetaldehyde, and the desulfonating 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 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 sulfite 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, of which eight contain eight membrane-spanning helices. Orthologues of this protein, previously called OrfX, have been found in Burkholderia xenovorans LB400 and C. necator IMP134 (Brüggemann et al., 2004), and they are now predicted to
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 C₂ 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 \( \text{tauE} \) is transcribed inducibly when \( C. \text{necator} \) H16 is grown with the C₂ sulfonates taurine, isethionate and sulfoacetate.

**METHODS**

**Organisms, growth, harvesting of cells and preparation of cell-free extracts.** \( B. \text{xenovorans} \) LB400 and \( C. \text{necator} \) 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 MgCl₂, 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 (OD₅₈₀ 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); \( B. \text{xenovorans} \) 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 (OD₅₈₀) 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). Sulfate was determined turbidimetrically as a suspension of BaSO₄ (Sorbo, 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.
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 H16.xscF/H16.xscR. 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 ($\mu = 0.25 \text{ h}^{-1}$), isethionate ($\mu = 0.26 \text{ h}^{-1}$), sulfoacetate ($\mu = 0.14 \text{ h}^{-1}$) and acetate ($\mu = 0.27 \text{ h}^{-1}$). 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 & Cook, 2006b). Sulfoacetalddehyde 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 sulfoacetalddehyde (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 sulfoacetalddehyde metabolism**

RT-PCR experiments were done to confirm the role of the candidate genes in the degradation of sulfoacetalddehyde 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 C2 sulfonate studied was induced specifically and yielded sulfoacetalddehyde: 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 sulfoacetalddehyde involving CoA, ATP and NADH (Denger & Cook, 2001) have new experimental support (S. Weinitschke, unpublished data). The enzymes (Xsc, Pta) necessary to transform sulfoacetalddehyde to an amphibolic intermediate (acytlen-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 &

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Table 1. Primers used in this study

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<td></td>
<td>16S-533R</td>
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<td>Weisburg et al. (1991)</td>
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<tr>
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Table 2. Specific activities of enzymes and transcription of genes under different growth conditions of C. necator H16

<table>
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<th>Enzyme or gene transcription</th>
<th>Specific activity or transcription in cells grown with:</th>
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<tr>
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<td>Taurine dehydrogenase (TauXY)</td>
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<tr>
<td>Sulfoacetaldehyde acetyltransferase (Xsc)</td>
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<tr>
<td>Phosphate acetyltransferase (Pta)</td>
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<tr>
<td>Transcription of pta</td>
<td>+</td>
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<tr>
<td>Transcription of tauE</td>
<td>+</td>
</tr>
<tr>
<td>SDH</td>
<td>30</td>
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</tbody>
</table>

*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-desulfonating) organisms have <43% sequence identity to all proposed TauE sequences. The most closely related DUF81 protein of known function, Tsα5 (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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