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Isethionate formation from taurine in *Chromohalobacter salexigens*: purification of sulfoacetaldehyde reductase

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Bacterial generation of isethionate (2-hydroxyethanesulfonate) from taurine (2-aminoethanesulfonate) by anaerobic gut bacteria was established in 1980. That phenomenon in pure culture was recognized as a pathway of assimilation of taurine-nitrogen. Based on the latter work, we predicted from genome-sequence data that the marine gammaproteobacterium *Chromohalobacter salexigens* DSM 3043 would exhibit this trait. Quantitative conversion of taurine to isethionate, identified by mass spectrometry, was confirmed, and the taurine-nitrogen was recovered as cell material. An eight-gene cluster was predicted to encode the inducible vectorial, scalar and regulatory enzymes involved, some of which were known from other taurine pathways. The genes (Csal_0153–Csal_0156) encoding a putative ATP-binding-cassette (ABC) transporter for taurine ($TauAB_1B_2C$) were shown to be inducibly transcribed by reverse transcription (RT-) PCR. An inducible taurine : 2-oxoglutarate aminotransferase [EC 2.6.1.55] was found (Csal_0158); the reaction yielded glutamate and sulfoacetaldehyde. The sulfoacetaldehyde was reduced to isethionate by NADPH-dependent sulfoacetaldehyde reductase (IsfD), a member of the short-chain alcohol dehydrogenase superfamily. The 27 kDa protein (SDS-PAGE) was identified by peptide-mass fingerprinting as the gene product of Csal_0161. The putative exporter of isethionate (IsfE) is encoded by Csal_0160; *isfE* was inducibly transcribed (RT-PCR). The presumed transcriptional regulator, TauR (Csal_0157), may autoregulate its own expression, typical of GntR-type regulators. Similar gene clusters were found in several marine and terrestrial gammaproteobacteria, which, in the gut canal, could be the source of not only mammalian, but also arachnid and cephalopod isethionate.

INTRODUCTION

Isethionate (2-hydroxyethanesulfonate) is found at high concentrations in several compartments of terrestrial and marine environments (e.g. about 200 mM in red algae and the squid giant axon and 2 M in the orb spider's secretion on to its web) (Barrow *et al.*, 1993; Hellio *et al.*, 2004; Holst *et al.*, 1994; Koechlin, 1954; Townley *et al.*, 2006), at lower concentrations in mammalian tissue (1.5–4.8 mM) and urine (about 0.05 mM) (Jacobsen *et al.*, 1967; Kumpulainen *et al.*, 1982), and as exudate from red algae (e.g. Hellio *et al.*, 2004; Williamson *et al.*, 2000). These large supplies of carbon and energy presumably explain the occurrence of widespread aerobic and anaerobic respirations and fermentations of isethionate in bacteria (Cook &

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Abbreviations: ABC, ATP-binding cassette; Gldh, glutamate dehydrogenase; IsfD, sulfoacetaldehyde reductase; IsfE, isethionate exporter; RT, reverse transcription; $TauAB_1B_2C$, ABC transporter for taurine; TauD, taurine dioxygenase; TauR, transcriptional regulator of taurine genes; TauXY, taurine dehydrogenase; Toa, taurine : 2-oxoglutarate aminotransferase; Tpa, taurine : pyruvate aminotransferase; Xsc, sulfoacetaldehyde acetyltransferase.

Supplementary material, with details of (RT-)PCR primers, two other organisms used, routine enzyme assays, analytical methods, the bioinformatic software and accession numbers used, dendrograms and gene maps, and sequencing of the *tau* region of *K. oxytoca* TauN1, is available with the online version of this paper.

Denger, 2002), for which some pathways are now known (Weinitschke *et al.*, 2010).

Whereas these reserves of isethionate are an obvious nutrient for bacteria, the sources of these reserves are often unclear. Mammalian isethionate (Jacobsen *et al.*, 1967; Kumpulainen *et al.*, 1982), however, is generated from taurine (2-aminoethanesulfonate; see below) by bacteria in the gut canal and taken up by the mammal, which does not generate significant amounts of its own (Fellman *et al.*, 1980). Fellman *et al.* (1980) had to cope with considerable analytical problems (e.g. with doubly radioactively labelled compounds) to generate reliable data in faeces and tissue samples. The development of a simple, ion-chromatographic determination of isethionate (Styp von Rekowski *et al.*, 2005), and the availability of a pure culture (*Klebsiella oxytoca* TauN1), helped us to detect a pathway of inducible enzymes to reduce taurine to isethionate (Styp von Rekowski *et al.*, 2005). It involved a taurine transaminase and a partially separated, soluble, 28 kDa, NADPH-coupled sulfoacetaldehyde reductase, which we now term IsfD (isethionate formation, reductase). The N-terminal amino-acid sequence of IsfD from strain TauN1 was determined to be ATSKVVFITG (Styp von Rekowski *et al.*, 2005). A complete pathway (Fig. 1a) required, in addition, a system for the uptake of taurine and another for the excretion of isethionate, which we term IsfE (isethionate formation, exporter).

The peptide sequence of IsfD from *K. oxytoca* TauN1 and the partial genome sequence of *K. oxytoca* M5al allowed us to suggest candidate genes to encode some proteins in the pathway (Fig. 1a) (see Results). However, we needed an organism with a complete pathway encoded in the gene cluster, and searches with the BLAST algorithm yielded appropriate gene clusters in other gammaproteobacteria (see Supplementary Fig. S1, available with the online version of this paper). *Chromohalobacter salexigens* DSM 3043 (Fig. 1a) was chosen for further work (see Results).

Taurine is a major solute in vertebrates, marine invertebrates and red algae, but rare in plants (Allen & Garrett, 1971; Huxtable, 1992; Ito *et al.*, 1977; Shibuya *et al.*, 1963; Yancey *et al.*, 2002). There is wide biodiversity of taurine degradation as a sole source of carbon and energy, and of taurine as a sole source of nitrogen for growth (Baldock *et al.*, 2007; Cook & Denger, 2006). Of relevance here are a GntR-type transcriptional regulator, TauR (Wiethaus *et al.*, 2008), and an ATP binding-cassette (ABC) taurine transporter [TC 3.A.1.17.1] (Eichhorn *et al.*, 2000). One of three initial scalar reactions might be expected. Firstly, taurine dehydrogenase (Kondo & Ishimoto, 1987) is now established as cytochrome-*c*-coupled TauXY [EC 1.1.2.–]

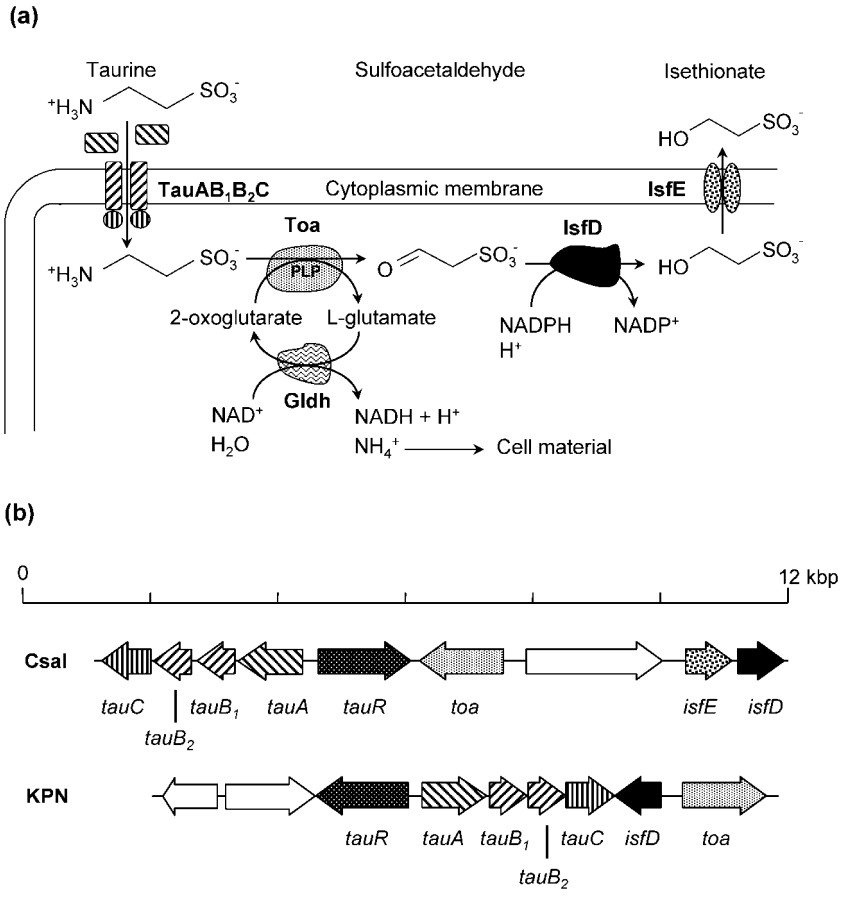

Fig. 1. (a) The published pathway of taurine-nitrogen utilization and isethionate formation, adapted to the candidate genes of *C. salexigens* DSM 3043. (b) Map of the cluster of candidate genes (Csal_0153–Csal_0161, *tauC–isfD*) in *C. salexigens* DSM 3043 (Csal) and the similar cluster of genes in *K. pneumoniae* MGH 78578 (KPN_00571–KPN_00577, *tauR–toa*) (KPN). Csal_0159 is annotated as a catalase/peroxidase.

(Brüggemann *et al.*, 2004; Weinitschke *et al.*, 2007). Secondly, taurine : pyruvate aminotransferase (Tpa) [EC 2.6.1.77] was discovered in 1979 (Shimamoto & Berk, 1979) and has been characterized in many bacteria (e.g. Denger *et al.*, 2004; Laue & Cook, 2000). Thirdly, taurine : 2-oxoglutarate aminotransferase (Toa) [EC 2.6.1.55] (Yonaha *et al.*, 1985) is an option. The second scalar reaction is presumably IsfD. The second vectorial reaction, here IsfE, is often a 'DUF81' protein (membrane protein, domain of unknown function). The DUF81 group [TC 9.A.29.–.–] is large (>2800 orthologues); the few orthologues studied are known in assimilatory sulfate uptake (Rückert *et al.*, 2005), and believed to export sulfite or sulfonates (Krejčík *et al.*, 2008; Mayer & Cook, 2009; Weinitschke *et al.*, 2007).

We now report inducible, stoichiometric formation of isethionate from taurine by *C. salexigens* DSM 3043. The genes encoding the putative vectorial proteins were transcribed inducibly. Toa was identified, and IsfD was isolated, as was constitutive, isofunctional IsfD2.

METHODS

Some additional methods are provided in the supplementary material.

Materials. Taurine (≥99.0 %) and sodium isethionate (>98 %) were purchased from Fluka, and the tetrasodium salt of NADPH was obtained from Biomol. Sulfoacetaldehyde, as the bisulfite addition complex, was characterized previously (Denger *et al.*, 2001). Succinic semialdehyde solution (purum, ~15 % in water) was from Fluka. Phosphonoacetaldehyde was provided by the late H. B. F. Dixon (Cambridge University, UK). Other chemicals were of the highest purity available and were purchased from Biomol, Fluka, Merck, Roth, Serva or Sigma. Glutamate dehydrogenase [EC 1.4.1.3] was from Sigma.

Organisms, growth, harvesting of cells and preparation of cell-free extracts. *Chromohalobacter salexigens* DSM 3043 (Arahal *et al.*, 2001) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Strain DSM 3043 showed optimal growth at 7.5–10 % (w/v) salt content (Arahal *et al.*, 2001), but this high concentration was incompatible with many routine analytical methods, so we used the modified, Tris-buffered, marine-salts medium containing 2.6 % (w/v) salt (Krejčík *et al.*, 2008), which was designed for work with sulfonates under nitrogen-limited conditions. Cultures were grown at 30 °C in the dark; the carbon source was routinely 10 mM sodium succinate and the nitrogen source was routinely 2 mM taurine or 2 mM ammonium chloride.

Precultures (5 ml) of *C. salexigens* DSM 3043 were grown in 30 ml screw-cap tubes in a roller. Growth experiments were done on the 50 ml scale in shaken 300 ml Erlenmeyer flasks. Samples were taken at intervals to measure optical density (at 580 nm; OD_{580}), to assay protein, and to determine the concentrations of taurine, isethionate, sulfoacetaldehyde, sulfoacetate, and the ammonium and sulfate ions. Similar cultures were used to generate small amounts of cells for enzyme assays or for molecular analyses.

Cells for the preparation of total RNA were harvested in the early exponential phase of growth (OD_{580} 0.14–0.16) (for *C. salexigens* an OD_{580} of 1 is equivalent to 218 mg protein l^{-1}) by centrifugation at 5000 ***g***. Suspended cells were stored in RNA*later* stabilizing solution (Ambion/Applied Biosystems) as recommended by the manufacturer prior to extraction of RNA.

Cultures (1 l) for protein purification were grown in 5 l Erlenmeyer flasks on a shaker. Cells were harvested at OD_{580} 0.40–0.45 by centrifugation (15 000 ***g***, 15 min, 4 °C), washed with 20 mM Tris/H_2SO_4 buffer, pH 9.0 (IsfD and IsfD2) or with 25 mM potassium phosphate buffer, pH 7.2 (Toa) and stored frozen. These buffers, augmented with 0.05 mg DNase I ml^{-1}, served as extraction buffers. Cell-free extracts were generated by disruption in a chilled French pressure cell (three passages) set at 140 MPa (Junker *et al.*, 1994). The membrane/particulate fraction was pelleted by ultracentrifugation (340 000 ***g***, 40 min, 4 °C) and the supernatant fluid was called the soluble fraction.

Analytical methods. Isethionate was determined by ion chromatography with suppression (Styp von Rekowski *et al.*, 2005): chloride ion (retention time 1.67 min) in the growth medium interfered with the determination of isethionate (retention time 1.40 min), so chloride ion had to be removed prior to analysis by addition of excess silver nitrate solution (0.4 mmol silver nitrate per ml growth medium) and centrifugation of the precipitated silver chloride. The standard curve was prepared with the same pre-treatment, and it was linear to at least 3.0 mM isethionate in growth medium (R^2=0.99, n=7).

Enzyme assays. Sulfoacetaldehyde reductase was routinely assayed spectrophotometrically (340 nm) as the sulfoacetaldehyde-dependent oxidation of NADPH at room temperature (about 23 °C). The reaction mixture (1.0 ml) contained 20 μmol Tris/H_2SO_4 buffer pH 9.0, 200 nmol NADPH, 5–50 μg protein and 800 nmol sulfoacetaldehyde (as the bisulfite adduct), with which the reaction was started. Variants of this method were used to determine the stoichiometry of the reaction or to generate data which allowed kinetic constants to be derived by curve-fitting. Toa (Tpa) was assayed discontinuously as the formation of glutamate (alanine) from 2-oxoglutarate (pyruvate) and taurine (Weinitschke *et al.*, 2005).

Purification of the sulfoacetaldehyde reductases. IsfD(s) was stable in Tris/H_2SO_4 buffer, pH 9.0, which was also the condition of optimal enzyme activity. Purification (separation) protocols were developed for both isoenzymes, IsfD and IsfD2, from 1–4 l of culture; the first two separative steps were identical.

We could purify IsfD2. The soluble fraction from cells grown with ammonium as nitrogen source was made up to 10 ml with 20 mM Tris/H_2SO_4 buffer, pH 9.0, and loaded (2 × 5 ml) on to an anion-exchange column (Mono Q 10/10, Pharmacia) equilibrated with the same buffer; the flow rate was 1 ml min^{-1}, and the gradient described elsewhere (Ruff *et al.*, 2003) was used. IsfD2 eluted as a single peak at 0.25 M sodium sulfate. The fraction with the highest activity was rebuffered in 20 mM Tris/H_2SO_4 buffer, pH 9.0, containing 1.7 M ammonium sulfate, and subjected to hydrophobic interaction chromatography on Phenyl Superose HR (5/5 column; Pharmacia) at a flow rate of 1.0 ml min^{-1}. A linear decreasing gradient of ammonium sulfate (1.7–0.0 M) in 20 mM Tris/H_2SO_4 buffer, pH 9.0, was applied over 60 min, and the IsfD2 eluted as a single peak at 0.25 M ammonium sulfate.

We could separate, but not purify, IsfD. The *isfD* gene, modified to encode an N-terminal His-tag, was cloned, and His-IsfD was expressed and purified under contract (Apronex, Prague, Czech Republic).

Molecular methods. Reverse transcription- (RT-) and the PCR primers (Supplementary Tables S1 and S2) were synthesized by Microsynth (Balgach, Switzerland). Total RNA was isolated using the

E.Z.N.A. bacterial RNA kit (Omega Bio-Tek) or the peqGOLD TriFast DNA/RNA/protein purification system (PEQLAB), following the manufacturers' instructions. Isolated RNA was tested for residual DNA by PCR using the primer pair Cs_isfD_F and Cs_isfD_R (Supplementary Table S1); contaminant DNA was removed with RNase-free DNase I (50 units μl^{-1}) (Fermentas), following the manufacturer's instructions. M-MuLV reverse transcriptase (20 units μl^{-1}) was from Fermentas and the RT reactions were set up as recommended by the supplier using the RT-PCR primers listed in Supplementary Table S1. cDNA integrity after RT was tested by PCR using the 16S rRNA-specific primers 16S-27f and 16S-533r (Weisburg *et al.*, 1991). Chromosomal DNA of *C. salexigens* DSM 3043 was used as a positive control for PCRs. *Taq* DNA polymerase (5 units μl^{-1}) was from Fermentas and the PCR mixture (routinely 25 µl) contained 1× Taq buffer with $(NH_4)_2SO_4$, 10 % DMSO, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 0–0.5 µg template DNA, 2.5 units *Taq* DNA polymerase and water (up to 25 µl total volume). Cycling conditions were as follows: initial denaturation step for 3 min at 95 °C, denaturation step 0.5 min at 95 °C, primer annealing step for 0.5 min at 49 °C (for 16S-27f and 16S-533r primers) or at 58 °C (for all the other primers) and extending step for either 0.5 min or longer (calculated as 1 min per 1 kb of PCR fragment) at 72 °C. Number of cycles was routinely 35: the final extending step was performed for 10 min at 72 °C. PCR products were visualized on 1.5 % or 2 % agarose gels stained with ethidium bromide (Sambrook *et al.*, 1989); as a marker, a 50 bp DNA ladder (Fermentas) was used.

RESULTS

Derivation of the deaminative pathway of taurine in *C. salexigens* DSM 3043

The N-terminal amino-acid sequence determined for IsfD in *K. oxytoca* TauN1 (see Introduction) was used to search the partial genome sequence of *K. oxytoca* M5al. One perfect match was observed in the derived product from a gene in a seven-gene cluster, whose identically structured orthologues in the finished genome sequence of *Klebsiella pneumoniae* MGH 78578 are illustrated for easy numbering (Fig. 1b).

The gene cluster for an incomplete deaminative pathway in *K. oxytoca* allowed us to search more widely in genomic data, especially for clustered orthologues of *tauR* and *isfD*. This led us to four strains of *K. pneumoniae* (Supplementary Fig. S1), to the non-pathogenic bacteria *Psychromonas ingrahamii* 37 (Supplementary Fig. S1), *Marinomonas* sp. MED121 (Supplementary Fig. S1) and *C. salexigens* DSM 3043 (Fig. 1 and Supplementary Fig. S1), and to the plant pathogen *Dickeya dadantii* Ech703 (Supplementary Fig. S1). We chose to work with *C. salexigens* DSM 3043, because of the low calculated probability of membrane association with IsfD. Further, the gene upstream of IsfD is a candidate isethionate exporter, IsfE (Fig. 1a). In addition, corresponding to this hypothetical pathway (Fig. 1a), no gene to encode an orthologue of Xsc (desulfonative sulfoacetaldehyde acetyltransferase (Cook & Denger, 2006) or TauD (desulfonative taurine dioxygenase under sulfur limitation; Eichhorn *et al.*, 1997) or TauXY (deaminative taurine dehydrogenase; Brüggemann *et al.*, 2004) was detected on the genome of *C. salexigens* DSM 3043.

Growth physiology of *C. salexigens* DSM 3043

Aerobic, Tris-buffered, ammonium-ion-free marine-salts medium with succinate as carbon source did not support growth of *C. salexigens*: addition of the ammonium ion as a source of nitrogen enabled growth to occur [specific growth rate, $\mu = 0.22\ h^{-1}$; molar growth yield, $Y = 53 \pm 3$ g protein (mol N)$^{-1}$], so neither dinitrogen nor Tris served as a nitrogen source for growth. Taurine did not serve as a source of carbon in ammonium-containing medium, consistent with the absence of an *xsc* gene (see above). Taurine was not tested as a source of sulfur because the medium contained 1 mM sulfate, which represents a 30-fold excess of the sulfur nominally required for this level of growth, and no *tauD* gene was detected (see above).

Taurine served as a sole source of nitrogen for growth in Tris-buffered succinate-marine-salts medium (Fig. 2a), and no sulfate was released (Fig. 2b). A compound, which had the ion-chromatographic properties of isethionate, and

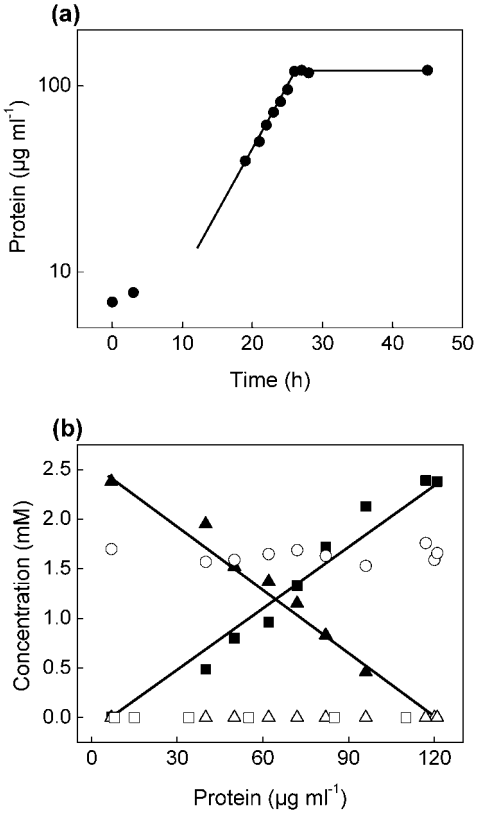

Fig. 2. (a) Growth of *C. salexigens* DSM 3043 with taurine as nitrogen source. (b) Concentrations of substrate and product as a function of protein concentration: ▲, taurine; ■, isethionate; ○, sulfate ion; △, ammonium ion, □, sulfoacetaldehyde.

which was absent in the uninoculated medium, was detected during growth (Fig. 2b). Analyses of samples of growth medium by MALDI-TOF-MS in the negative-ion mode showed that the peak at m/z=124 $[M-H^+]^-$ representing taurine (M=125) disappeared during growth: correspondingly, a peak at m/z=125 $[M-H^+]^-$, identical with that of authentic isethionate (M=126), confirmed that isethionate was generated during growth.

C. salexigens DSM 3043 grew in buffered taurine(nitrogen)-succinate-marine salts medium with a specific growth rate (μ) of 0.16 h^{-1} (Fig. 2a). The disappearance of taurine was concomitant with growth and quantitative (Fig. 2b), and the molar growth yield was 50 ± 3 g protein (mol taurine-nitrogen)$^{-1}$. Correspondingly, the formation of isethionate was concomitant with growth and the recovery of the sulfonate moiety in isethionate was stoichiometric (Fig. 2b). The mass balance for the sulfonate group was confirmed by the observation that no sulfate was formed during growth (Fig. 2b). Similarly, no detectable ammonium ion was released to the medium during growth (Fig. 2b). No sulfoacetaldehyde, an intermediate in the pathway (Fig. 1a), was detected in the growth medium (Fig. 2b). The specific degradation rate of taurine, derived from the specific growth rate and the growth yield, was calculated to be 0.9 mkat (kg protein)$^{-1}$.

Enzyme activities in *C. salexigens* DSM 3043

The specific activities of relevant enzymes (Fig. 1a) were measured in extracts of cells grown with either the ammonium ion or taurine as the sole added source of nitrogen. Each enzyme was found in the soluble fraction and not in the particulate fraction of the extracts. Taurine transaminase was anticipated to exhibit taurine : pyruvate aminotransferase (Tpa) activity, forming sulfoacetaldehyde and alanine, but neither compound was detected. Instead, taurine : 2-oxoglutarate aminotransferase (Toa) activity was observed, which yielded sulfoacetaldehyde and glutamate [0.5 mkat (kg protein)$^{-1}$]; low Toa activity [0.07 mkat (kg protein)$^{-1}$] was detected in extracts of ammonium-grown cells. The release of the ammonium ion from glutamate was postulated to involve glutamate dehydrogenase (Gldh). A significant level of Gldh was observed in extracts of both taurine- and ammonium-grown cells [1.8 and 1.2 mkat (kg protein)$^{-1}$, respectively]. We were unable to purify Toa, so we will present data on the heterologously expressed protein elsewhere.

Activity of a crucial enzyme in the pathway, IsfD, was detected at high levels in extracts of taurine-grown cells [12.4 mkat (kg protein)$^{-1}$]; low levels were found in extracts of ammonium-grown cells [0.8 mkat (kg protein)$^{-1}$]. No activity of Xsc or TauXY was detected, in agreement with the absence of *xsc* and *tauXY* genes (see above). The soluble enzymes required for the pathway (Fig. 1) were thus expressed.

Separation of IsfD, of paralogue IsfD2, and of heteromultimers thereof

Proteins from the soluble fraction of extracts from taurine-grown cells were loaded on to an anion-exchange column and IsfD activity eluted from the column as three peaks (fractions 34, 38 and 40; not shown). Proteins from the soluble fraction of extracts from ammonium-grown cells were separated on the anion-exchange column under the same conditions, and IsfD-like activity eluted as a single peak (fraction 41). It was shown by peptide mass fingerprinting analysis that (i) IsfD-like activity in extracts of ammonium-grown cells was catalysed by IsfD2 (91 % coverage of deduced sequence), (ii) the activity from fraction 34 was attributed to IsfD (Csal_0161) (91 % coverage of deduced sequence), and (iii) the enzyme(s) in fractions 38 and 40 contained both IsfD and IsfD2 (which were separable on SDS-PAGE), and were presumed to represent heteromultimers.

We concluded that *C. salexigens* DSM 3043 expresses IsfD2 (Csal_2684) constitutively, and that this enzyme is responsible for the activity of 0.8 mkat (kg protein)$^{-1}$ observed in extracts of ammonium-grown cells as described in the previous section. IsfD (Csal_0161), in contrast, is inducible, and represents the major portion of the IsfD activity described for taurine-grown cells in the previous section. The products of Csal_0161 and Csal_2684 (IsfD and IsfD2, respectively) share 76 % identity.

Purified His-IsfD

As the complete purification of IsfD was not attained (see below), we decided to clone the *isfD* gene (Csal_0161) into an expression vector and purify the heterologously expressed protein. His-IsfD was active [130 mkat (kg protein)$^{-1}$]. The predicted molecular mass (28.0 kDa) of the denatured protein was confirmed experimentally (Fig. 3a). The native protein eluted from a calibrated gel filtration column as two

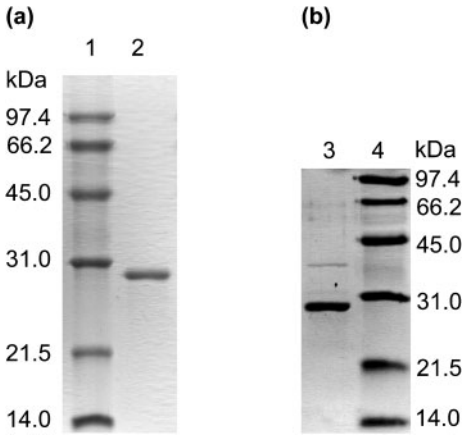

Fig. 3. Electropherograms (SDS-PAGE) of purified His-IsfD (a) and IsfD2 (b) from *C. salexigens* DSM 3043. Lanes: 1 and 4, protein markers; 2, purified His-IsfD; 3, purified IsfD2.

peaks with retention times which corresponded to molecular masses of 74 and 25 kDa, so, apart from monomers, a homomultimeric enzyme was present: the errors in this method can be ±50 % (le Maire *et al.*, 1996), so we presume that a homodimeric or a homotetrameric quaternary structure will be established, in common with other enzymes in the short-chain dehydrogenase/reductase superfamily (Kavanagh *et al.*, 2008).

Some properties of IsfD and IsfD2

IsfD2 (Csal_2684) could be extensively purified from fraction 41 of proteins from non-induced cells (Fig. 3b): a purification factor of about 3500 was achieved with a recovery of 14 %. The predicted molecular mass (27.3 kDa) of the denatured protein was confirmed experimentally (Fig. 3b). We observed that sulfoacetaldehyde was converted to isethionate, which was detected by ion chromatography, concomitant with loss of sulfoacetaldehyde, and that the enzyme was specific for NADPH; NADH was not a substrate. No reverse reaction was detected.

IsfD could be extensively separated from fraction 34 (see above), but a complete purification was not attained (not shown). A purification factor of 600 was observed with 0.1 % recovery of activity. IsfD was identified as the only common protein band in sets of analyses by SDS-PAGE of different variants of the separation protocols (not shown). This common protein was identified by peptide mass fingerprinting as Csal_0161 (not shown). The predicted molecular mass (27.1 kDa) of the denatured protein was confirmed experimentally (not shown).

Enzyme activity of IsfD showed a broad pH optimum from about pH 6.5 to pH 9.5 (not shown). There was a steep increase in activity from pH 4.5 (negligible activity) and an equally steep decrease above pH 9.5. Enzyme activity was routinely assayed as oxidation of NADPH at pH 9.0 in Tris buffer. However, when we examined the stoichiometry of the reaction, we found that some isethionate was formed in the absence of enzyme. We then changed to CAPS buffer at pH 9.3, where no spontaneous reaction was detected in the time-frame of the experiments.

IsfD was found to convert e.g. 0.20 mM sulfoacetaldehyde (as the bisulfite addition complex) to 0.19 mM isethionate in the presence of NADPH only. The conversion of 0.20 mM sulfoacetaldehyde to product oxidized 0.19 mM NADPH; unit stoichiometry was thus established. Free sulfoacetaldehyde, generated by *Acinetobacter calcoaceticus* SW1 (Weinitschke *et al.*, 2005), was also converted to isethionate by IsfD in the presence of NADPH. Values of K_m [V_{max}] of 0.061±0.009 mM [26±1.4 mkat (kg protein)$^{-1}$] for NADPH and of 0.13±0.01 mM [22±0.4 mkat (kg protein)$^{-1}$] for sulfoacetaldehyde (as the bisulfite adduct) were determined in the presence of saturating concentrations of the other substrate.

We anticipated that IsfD would catalyse the reverse reaction, to generate sulfoacetaldehyde from isethionate in the presence of $NADP^+$. No reaction was observed with isethionate concentrations in the range from 0.4 to 7 mM and 1 mM $NADP^+$, tested at pH 9 and pH 6.

The substrate range of IsfD was explored with 10 substrate analogues at 1 mM and at 5 mM: formaldehyde, acetaldehyde, betaine aldehyde, propionaldehyde, DL-glyceraldehyde, phosphonoacetaldehyde, glyoxylate, 2-oxobutyrate, 4-oxobutyrate and 3-sulfopropanaldehyde. No compound inhibited the reaction with sulfoacetaldehyde. Only one analogue, 4-oxobutyrate, was converted to product; the K_m value was very high (millimolar range) and substrate inhibition reached 90 % at 20 mM 4-oxobutyrate.

K. oxytoca TauN1

The pathway in Fig. 1(a) was formulated during work with *K. oxytoca* TauN1 (Styp von Rekowski *et al.*, 2005), where enzyme data were interpreted to represent a low level of inducible Tpa (taurine : pyruvate aminotransferase). We now tested 2-oxoglutarate in place of pyruvate and found the former to be the main amino-group acceptor. Strain TauN1 obviously expresses an inducible Toa, but, in contrast to *C. salexigens*, this Toa does accept pyruvate as an amino-group acceptor at low activity.

Given the sequence data from *K. oxytoca* M5al, we amplified a 3 kbp DNA fragment from strain TauN1 by PCR and sequenced it. The amplified genes [*tauC* (partial), *isfD* and *toa*] (GenBank accession number FJ711704) shared >99 % sequence identity (DNA) and >99 % identity (proteins) with the genome data. The N-terminal amino-acid sequence of IsfD was confirmed and the derived molecular mass of the mature protein (27.2 kDa) corresponded to the experimental data (28 kDa) (Styp von Rekowski *et al.*, 2005).

Marinomonas sp. strain MED121

The marine gammaproteobacterium *Marinomonas* sp. strain MED121 was found to utilize taurine quantitatively as sole source of nitrogen, with stoichiometric release of isethionate, which was identified by mass spectrometry (not shown). No sulfate, ammonium ion or sulfoacetaldehyde was released during growth (not shown). The activity of IsfD could not be detected in crude extracts of induced cells or in the soluble fraction, although in washed membrane fractions traces could be detected. We detected Tpa (and not Toa) activity in these extracts.

Transcription of *tau* and *isf* genes in *C. salexigens* DSM 3043

Published data indicate inducible transcription of taurine genes, whether they encode proteins in pathways of degradation, nitrogen assimilation or sulfur assimilation, and in the latter case TauR has been shown to be a typical GntR-type regulator (Denger *et al.*, 2006; Gorzynska *et al.*,

2006; Krejčík *et al.*, 2008; Wiethaus *et al.*, 2008). In *C. salexigens*, transcript of *tauR* was obviously stronger in the presence of taurine (Fig. 4), which supports its function as a GntR-type transcriptional activator (Wiethaus *et al.*, 2008). Genes for taurine uptake, $tauAB_1B_2C$, were transcribed inducibly (Table 1). Similarly, inducible transcription of *isfE*, the candidate isethionate exporter, was detected (Fig. 4; Table 1). Transcripts of *toa* and *gldh* were present in induced cells and detectable in non-induced cells (Fig. 4; Table 1), which is in agreement with biochemical data (see above). Inducible IsfD has been characterized (see above): correspondingly, inducible transcription of *isfD* was detected (Fig. 4). Constitutive expression of IsfD2 has been shown experimentally; similarly, constitutive transcription of *isfD2* was observed (Fig. 4; Table 1).

Table 1. Annotation and transcription of the taurine-related genes in the genome of *C. salexigens* DSM 3043

Scoring: −, no transcription detected; +, transcription detected; *, significantly higher level of transcription than in ammonium-grown cells.

Gene number	Annotation	Transcription in cells grown with	
		Taurine	Ammonium
Csal_0157	*tauR*	+*	+
Csal_0156	*tauA*	+	−
Csal_0155	$tauB_1$	+	−
Csal_0154	$tauB_2$	+	−
Csal_0153	*tauC*	+	−
Csal_0158	*toa*	+*	+
Csal_1340	*gldh*	+	+
Csal_0161	*isfD*	+	−
Csal_2684	*isfD2*	+	+
Csal_0160	*isfE* (DUF81)	+	−

DISCUSSION

The prediction that *C. salexigens* DSM 3043 would utilize taurine and excrete isethionate (see Introduction) (Fig. 1a) was confirmed and the transformation was shown to be quantitative and stoichiometric (Fig. 2b). The organisms in which the pathway is present (*C. salexigens*, *K. oxytoca*, *Marinomonas* sp.) (Supplementary Fig. S1), or presumed to be present (*P. ingrahamii, D. dadantii* and four strains of *K. pneumoniae*) (Supplementary Fig. S1), are all marine or terrestrial gammaproteobacteria. *K. oxytoca* TauN1 is one of many terrestrial strains of the organism isolated with this property; *Pseudomonas* sp. (another gammaproteobacterium) was also isolated (Weinitschke *et al.*, 2005). We presume that the trait in *Klebsiella* spp. is normal in the gut, because the genus belongs to the normal flora (e.g. Madigan & Martinko, 2006): this fits the observed generation of isethionate in faeces, and uptake of the compound by mammals (Fellman *et al.*, 1980). The trait will thus be widespread in soil, waters and sewage works, to which faecal matter is distributed by wild animals or sewerage systems.

One essential component of the inducible pathway (Fig. 1a) is the uptake of taurine. The candidate genes, which are inducibly transcribed (Table 1), represent an ABC transporter ($TauAB_1B_2C$; Fig. 1b), orthologues of which have been identified by mutational analyses (Eichhorn *et al.*, 2000). The direct key to the nitrogen supply for growth is transamination by a taurine aminotransferase, which turned out to be Toa, coupled to the activity of Gldh to release the amino group as the ammonium ion (Fig. 1a). The key to the formation of isethionate is IsfD, which was separated from the cell material (not shown) and identified by its peptide-mass fingerprint; additionally, His-IsfD was expressed heterologously and purified (Fig. 3a). The pathway is completed by the release of isethionate (Fig. 1a). The candidate for the exporter, IsfE (a DUF81 protein), is transcribed inducibly (Fig. 4), and thus presumably

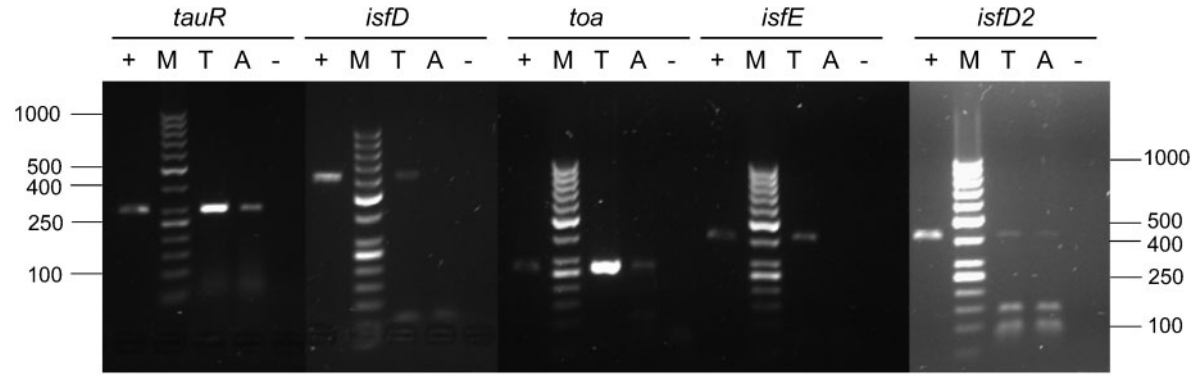

Fig. 4. Transcription of *tauR* (292 bp fragment), *isfD* (590 bp fragment), *toa* (264 bp fragment), *isfE* (401 bp fragment) and *isfD2* (414 bp fragment) from *C. salexigens* DSM 3043 during the exponential phase of growth. Lanes: M, marker, 50 bp DNA ladder; T, cDNA from taurine-grown cells; A, cDNA from ammonium-grown cells; +, positive control (genomic DNA); −, negative control (H_2O as template).

translated. This is another example of a DUF81 protein being associated with the export (or import) of highly charged anions (e.g. Krejčík *et al.*, 2008; Mayer & Cook, 2009; Rückert *et al.*, 2005; Weinitschke *et al.*, 2007). TauR, the presumed transcriptional regulator of isethionate generation (Fig. 1b), seems to be autoregulatory (Fig. 4), typical of GntR-type regulation, as observed elsewhere (Wiethaus *et al.*, 2008).

Sulfoacetaldehyde reductase (IsfD, Csal_0161) (Fig. 1) reduces the aldehyde moiety of sulfoacetaldehyde to the alcohol (isethionate) dependent on the presence of NADPH, although no reverse reaction was detected. The enzyme thus belongs to the IUBMB Nomenclature Committee's group EC 1.1.1.–. We propose the systematic name 'isethionate: $NADP^+$ oxidoreductase'. This enzyme in the superfamily of short-chain alcohol dehydrogenases is very different from the 62 kDa isethionate dehydrogenase [EC 1.1.2.–] which is involved in utilization of isethionate as a carbon source (Brüggemann *et al.*, 2004; Weinitschke *et al.*, 2010).

A dendrogram comparing proteins similar to IsfD shows a centrally placed set of 19 proteins, which include all known and predicted IsfDs (eleven) and eight other proteins (Supplementary Fig. S2). The latter proteins include one IsfD2 (Csal_2684), and none of them is found in a cluster resembling that in Fig. 1(b). So proteins with an IsfD-like sequence need not be involved in the generation of isethionate; the rest of the pathway is needed, too. The four paralogues of IsfD (Supplementary Fig. S2), one of which (Csal_2684), at least, catalyses the sulfoacetaldehyde reductase reaction, presumably play no practical role in the pathway of isethionate formation, because they are not found in all organisms with the pathway, and, indeed, there was no constitutive activity of IsfD in the original isolate, *K. oxytoca* TauN1 (Styp von Rekowski *et al.*, 2005).

Fellman *et al.* (1980) discovered that bacteria provide mammals with isethionate generated by the gut flora from taurine. Styp von Rekowski *et al.* (2005) observed the generation of isethionate (as in Fig. 2) in pure culture by a typical gut organism, *K. oxytoca*. The present paper illustrates the generation of isethionate at the molecular level (Fig. 1), apparently not only in the marine organism *C. salexigens* but also in *Klebsiella* spp. (Fig. 1b; Supplementary Fig. S1). Some biodiversity in the pathway is apparent, in that *Marinomonas* sp. involves a Tpa, and not Toa, and we infer from Supplementary Fig. S1 that *P. ingrahamii* and *D. dadantii* also do so. We speculate that two other eukaryotes which require isethionate, the orb spider (Townley *et al.*, 2006) and the squid (Koechlin, 1954), absorb isethionate generated in the gut canal: squid tissue, at least, cannot generate isethionate from taurine (e.g. Hoskin & Noonan, 1980).

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