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Different bacterial strategies to degrade taurocholate

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Abstract Aerobic enrichment cultures with taurocholate or alkanesulfonates as sole sources of carbon and energy for growth were successful and yielded nine bacterial isolates, all of which utilized taurocholate. Growth was complex and involved not only many, usually transient, excretion products but also sorption of taurocholate and cholate to cells. Three metabolic strategies to dissimilate taurocholate were elucidated, all of which involved bile salt hydrolase cleaving taurocholate to cholate and taurine. *Comamonas testosteroni* KF-1 utilized both the taurine and the cholate moieties for growth. *Pseudomonas* spp., e.g. strain TAC-K3 and *Rhodococcus equi* TAC-A1 grew with the cholate moiety and released taurine quantitatively. *Delftia acidovorans* SPH-1 utilized the taurine moiety and released cholate.

Keywords Bile salt hydrolase · *Comamonas testosteroni* · *Delftia acidovorans* · Desulfonation · Excretion of cholate · Excretion of taurine · *Pseudomonas* spp. · Taurine dissimilation

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Introduction

Taurocholate (Fig. 1) is a bile salt, which is probably best known as an emulsifier involved in the uptake of fats from the gut (e.g. Berg et al. 2007). A portion of this taurocholate is excreted in the faeces (e.g. Metzler 2003) in significant amounts (Hylemon and Harder 1998), so the compound can be considered as a significant source of available carbon in terrestrial and aquatic environments. The role of taurocholate as a selective inhibitor in microbial growth media (e.g. MacConkey Agar) has been known for about a century (MacConkey 1900); the compound is also used in other selective media, e.g. to improve the recovery of clostridial spores (Buggy et al. 1985). Further, considerable research has been invested in bile salt hydrolase (Bsh: also termed choloylglycine hydrolase) [EC 3.5.1.24] and its inferred role in (1) detoxifying the anti-microbial activity of, e.g. taurocholate to cholate and taurine in the gut, (2) pathogenicity, or (3) probiotics (e.g. Moser and Savage 2001; Sue et al. 2003; McAuliffe et al. 2005; Kumar et al. 2006; Delpino et al. 2007). The enzyme is generated from a precursor protein, that is not autocatalytically cleaved to maturity (Kumar et al. 2006), but that often contains a putative leader peptide, which indicates to the prediction software (SignalP, THMM) that the enzyme is periplasmic (e.g. CtesDRAFT_1890 in *Comamonas testosteroni* KF-1). The N-terminal cysteinyl residue of the mature protein is involved in catalysis (e.g. Tanaka et al. 2000; Kumar et al. 2006).

In contrast to the extensive work on Bsh, little effort seems to have been expended on the microbial dissimilation of taurocholate or on the pathways concerned. Taurocholate was found to be a source of taurine for *Bilophila wadsworthia* RZATAU (Schumacher et al. 1996; Laue et al. 1997), but neither taurocholate nor cholate was determined, and

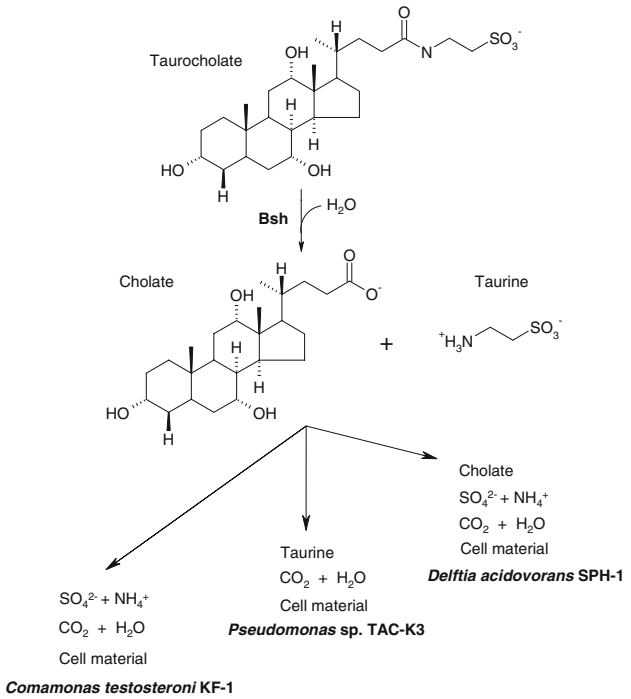

Fig. 1 Possible bacterial strategies to degrade taurocholate

the pathway of taurine degradation in *B. wadsworthia* is still incompletely understood. However, an understanding of taurine degradation in other organisms has proceeded further (Cook and Denger 2002, 2006; Denger et al. 2006; Gorzynska et al. 2006; Cook et al. 2007): relevant pathway variants have been found in *Delftia acidovorans* SPH-1 and in *C. testosteroni* KF-1 (see below). An understanding of cholate degradation is also being developed (Philipp et al. 2006).

We now report on successful enrichment cultures, which indicate widespread utilization of taurocholate by aerobes, the relevance of periplasmic Bsh, and three general bacterial strategies to dissimilate the compound.

Materials and methods

Organisms, enrichment cultures, isolations, growth media, growth conditions and cell disruption.

Comamonas testosteroni KF-1 (DSM 14576) and *D. acidovorans* SPH-1 (DSM 14801) were isolated in this laboratory (Schleheck et al. 2004). Enrichment cultures were done in phosphate-buffered salts medium (Thurnheer et al. 1986) and isolates were obtained as described previously (Mayer et al. 2006). Growth experiments and the growth of larger cultures to allow cells to be harvested and disrupted for analysis of enzyme activities were described elsewhere (Mayer et al. 2006). Harvested cells were disrupted by sonication.

Enzyme assay

Bile salt hydrolase was assayed discontinuously at 37°C in 50 mM potassium phosphate, pH 7.2, as the taurocholate-dependent formation of taurine and cholate, which were determined by HPLC. Taurocholate was determined after samples were stopped by addition of acetonitrile to 25% (v/v). Taurine was determined after samples were stopped by addition to 10 volumes 0.2 M $NaHCO_3$.

Analytical methods and units

Growth was followed as turbidity at 580 nm or quantified as protein in a Lowry-type reaction (Cook and Hütter 1981). Sulfate was determined turbidimetrically as a suspension of $BaSO_4$ (Sörbo 1987); samples containing taurocholate had to be diluted, to prevent taurocholic acid (>0.25 mM) forming a precipitate. Reversed phase HPLC was used to quantify taurine after derivatisation with 2,4-dinitrofluorobenzene (DNFB) (Laue et al. 1997), or to quantify cholate and taurocholate. The method of Philipp et al. (2006) to determine cholate, isocratic elution from a reversed-phase column with a neutral mobile phase and UV detection at 195 nm, was modified to gradient elution with acetonitrile (20–60% over 9 min) as the organic modifier in an acidic mobile phase (10 mM potassium phosphate buffer, pH 2.0) (Fig. 3). Standard methods were used to establish Gram reaction, catalase and cytochrome *c* oxidase activities (Gerhardt et al. 1994). A fragment (about 1,500 bp) of the 16S rRNA gene of the different isolates was amplified by PCR, sequenced and analyzed as described elsewhere (Brüggemann et al. 2004). The partial 16S-rRNA sequences of *Pseudomonas* sp. strain DDS-W1 (AM937256), *Pseudomonas* sp. strain HPS-W1 (AM937257), *Pseudomonas* sp. strain TAC-W1 (AM937258), *Rhodococcus equi* TAC-A1 (AM937259), *C. testosteroni* TAC-K2 (AM937260) and *Pseudomonas* sp. strain TAC-K3 (AM937261) were deposited at EMBL under the given accession numbers. Amplified ribosomal DNA restriction analysis (ARDRA) was performed as initial screening method to group isolates based on their genotype. The amplified 16S rRNA fragment of each strain was used for digestion with either of the restriction enzymes HaeIII and MspI (enzymes used as prescribed by the manufacturer) and the resulting fragments were loaded on 2% agarose gels. Unique genotypes from taurocholate enrichments were sequenced, as were both isolates from DDS and HPS.

The sequence data for *C. testosteroni* KF-1 and *D. acidovorans* SPH-1 were generated by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/. Sequence analyses were done using the BLAST algorithm on the National Center for Biotechnology Information

website (http://www.ncbi.nlm.nih.gov/). Sequence data were manipulated with different subroutines from the LASERGENE programme package (DNASTAR, Madison, USA). Transmembrane regions were predicted using the programme TMHMM, while leader peptides were predicted by SignalP (Bendtsen et al. 2004), both at the Center for Biological Sequence Analysis (CBS; http://www.cbs.dtu.dk/services/).

SI units are used throughout the text. The unit of catalytic activity, the katal (kat), is 1 mol s^{-1} (1 μmol min^{-1} = 16.7 nkat). Specific activity is given in kat (kg protein)$^{-1}$.

Materials

Commercial chemicals were of the highest purity available, and they were purchased from Fluka, Merck, Roth, Serva or Sigma.

Results and discussion

Enrichment cultures, isolates and their identification, substrate spectra, and excretion of products

Naturally occurring (taurocholate) and xenobiotic (*n*-heptanesulfonate, *n*-dodecanesulfonate, *s*-tetradecanesulfonate) sulfonated surfactants were supplied as sole sources of carbon (about 20 mM carbon) and energy in aerobic enrichment cultures, which were inoculated with activated sludge (K, "Kläranlage"), forest soil (W, "Wald") or arable soil (A, "Acker"). Negative (no added source of carbon) and positive controls (3 mM glucose) in the "K" series showed (1) that the activated sludge contained negligible available carbon, and (2) overnight growth where carbon was available. The cultures inoculated with soil contained too much humic material to allow optical evaluation of growth. Cultures were transferred to fresh medium after 3 days, thereafter, overnight growth with taurocholate (all relevant cultures), heptanesulfonate or dodecanesulfonate (one culture each, forest soil) was observed. There was no growth with *s*-tetradecanesulfonate, though other bacteria utilize it (e.g. Schleheck and Cook 2005).

The five enrichment cultures yielded nine isolates, all bacteria (light microscopy; see also below), seven from taurocholate-containing enrichments and one each from the alkanesulfonate-containing cultures (Table 1). All nine isolates utilized taurocholate, but no taurocholate-isolate could utilize an alkanesulfonate. Utilization of taurocholate seems to be widespread.

Some isolates seemed to be duplicates. ARDRA (not shown) indicated that strains TAC-A1 and TAC-A2 were similar, as were strains TAC-K1 and TAC-K2, and strains TAC-W1 and TAC-W2. The Gram-positive isolates TAC-A1 and TAC-A2 were unusual, because they were hydrophilic (rather than hydrophobic) coryneforms. However, the 16S-rRNA gene sequence of strain TAC-A1, with physiological data (Table 1), indicated that the potential equine pathogen *R. equi* (100% sequence identity with the type strain) had been isolated. These strains, from arable soil, seemingly lacked the major marker for pathogenicity (*vapA*, assayed by PCR), which is typical of soil isolates (Lührmann et al. 2004; Muscatello et al. 2006), but they were still unsuitable for research in a non-veterinary laboratory. Isolate TAC-K3 (Table 1), from activated sludge, potentially represented the same metabolic trait (see below), and was identified as *Pseudomonas* sp. strain TAC-K3, with 99.9% sequence identity to "*Burkholderia*

Table 1 The isolates obtained, their degradative and desulfonative abilities, as well as the phylogenetic identification of the organisms

Isolate	Utilization of					SO_4^{2-} formed	Identification by 16S-rRNA gene	Morphology	Gram type	Cyt *c* oxidase	Catalase
	TAC	HPS	DDS	Cholate	Taurine						
TAC-A1	+	–	–	+	–	–	*Rhodococcus equi*	Coryneform rod	+	–	+
TAC-A2	+	–	–	+	–	–	ND	Coryneform rod	+	–	+
TAC-K1	+	–	–	+	+	+	ND	Motile rod	–	+	+
TAC-K2	+	–	–	+	+	+	*C. testosteroni*	Motile rod	–	+	+
TAC-K3	+	–	–	+	–	–	*Pseudomonas* sp.	Motile rod	–	+	+
TAC-W1	+	–	–	+	+	+	*Pseudomonas* sp.	Motile rod	–	+	+
TAC-W2	+	–	–	+	+	+	ND	Motile rod	–	+	+
HPS-W1	+	+	+	+	–	+[a]	*Pseudomonas* sp.	Motile rod	–	+	+
DDS-W1	+	+	+	+	–	+[b]	*Pseudomonas* sp.	Motile rod	–	+	+

ND not determined

[a] From HPS

[c] From DDS

caryophyllii" WAB194 (acc. nr. AM184283) and 99.1% identity to *P. putida* BCNU106 (acc. nr. DQ229315). Isolate TAC-K2 was identified as a strain of *C. testosteroni* (Table 1), having 99.8% sequence identity with *C. testosteroni* KF-1. Isolate TAC-W1 was identified as *Pseudomonas* sp.; its 16S rRNA gene sequence is identical with that of *Pseudomonas* sp. strain WDL 5 (Dejonghe et al. 2003). Isolate HPS-W1 was also identified as *Pseudomonas* sp., probably a new species with a maximum of 98.8% sequence identity to *Pseudomonas* sp. NZ047 (acc. nr. AY014820), while isolate DDS-W1 was another *Pseudomonas* sp., this time similar to *Pseudomonas plecoglossidica* FPC951 (Nishimori et al. 2000).

Only six of the nine isolates released sulfate (a marker of cleavage of the carbon–sulfonate bond) from the sulfonate used in the enrichment cultures (Table 1). The three which did not release sulfate were all taurocholate-utilizers, strains TAC-A1, TAC-A2 and TAC-K3. There were, thus, differences in the degradative pathways for taurocholate in different organisms. Taurocholate dissimilation in *Pseudomonas* spp. HPS-W1 and DDS-W1 also did not involve desulfonation (Table 1). All organisms could utilize cholate, and those which released sulfate from taurocholate could also dissimilate taurine (Table 1). We hypothesized that all isolates in Table 1 contained Bsh (bile salt hydrolase), but that not all could dissimilate the taurine which was released. This was tested with *Pseudomonas* sp. strain TAC-K3, which did, indeed, release taurine into the growth medium (see below): no taurine was formed during growth with cholate (not shown). Both strains of *R. equi* were found to release taurine during growth with taurocholate.

This release of taurine by *Pseudomonas* sp. strain TAC-K3 was considered to be one strategy to degrade taurocholate (Fig. 1, centre), whereas the release of sulfate by *C. testosteroni* TAC-K1 (Fig. 1, left) was considered to be another. *C. testosteroni* KF-1, for which a draft genome sequence is available (acc. nr. AAUJ00000000), had been isolated in this laboratory and was found to show the same growth physiology as *C. testosteroni* TAC-K1 (not shown), so strain KF-1 was used for further work. Logically, it must be possible to utilize the taurine of taurocholate but not the cholate moiety (e.g. the anaerobic *B. wadsworthia*; see "Introduction"), and data in the genome sequence of *D. acidovorans* SPH-1 (acc. nr. AAVD00000000), also isolated in this laboratory, led us to predict this behaviour as depicted in Fig. 1 (right-hand arrow).

Growth with taurocholate as sole source of carbon and energy

Comamonas testosteroni KF-1 grew rapidly and exponentially ($\mu = 0.39\ h^{-1}$) with 0.9 mM taurocholate (Fig. 2a).

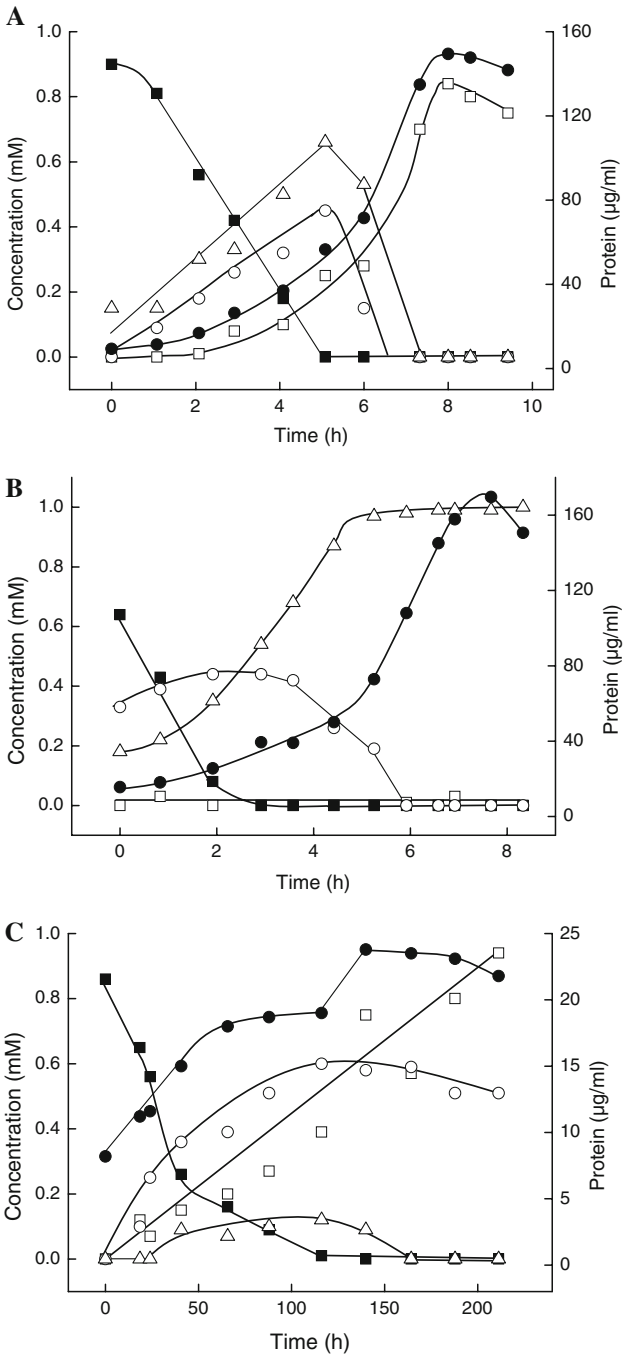

Fig. 2 Growth of (**a**) *C. testosteroni* KF-1 or of (**b**) *Pseudomonas* sp. strain TAC-K3 or of (**c**) *D. acidovorans* SPH-1 in taurocholate-salts medium. *Filled circle* protein, *filled square* taurocholate, *open circle* cholate, *open triangle* taurine, *open square* sulfate. In the case of *D. acidovorans*, there was little growth, so protein could not be assayed directly, and the values for "protein" were derived from the measured turbidity using a turbidity:protein correlation curve. This measurement was, thus, subject to large errors during the phase when cells clumped

The disappearance of taurocholate was quantitative, but was complete within 5 h, some 3 h before the end of growth (Fig. 2a). Both taurine and cholate were observed transiently

in the growth medium, reaching their maximum concentrations at 5 h (corresponding to the disappearance of taurocholate), and both compounds were utilized quantitatively (Fig. 2a). Corresponding to the complete utilization of taurine, stoichiometric recovery of the sulfonate moiety as sulfate was also detected. The extracellular cholate disappeared at least 1 h before the end of growth (Fig. 2a), so some 30% of the biomass formed was presumably generated largely from at least seven transient intermediates derived from cholate and visualized by HPLC (Fig. 3, chromatogram *t*5). These intermediates, presumably representing compounds reported elsewhere (Philipp et al. 2006), were absent at the beginning of the experiment (Fig. 3, chromatogram *t*0) and had disappeared by the end of growth (Fig. 3, chromatogram *t*9). The molar growth yield was 6 g protein (mol C)$^{-1}$, which represents complete dissimilation of the carbon source (Cook 1987). This value, with the specific growth rate, allows the minimum specific activity of taurocholate utilization during growth to be calculated as 0.7 mkat (kg protein)$^{-1}$.

Pseudomonas sp. strain TAC-K3 utilized 1 mM taurocholate with a maximum specific growth rate of 0.52 h^{-1}, though linear growth was observed over the final generation (Fig. 2b). The disappearance of taurocholate was complete by about 3 h, some 4 h before growth stopped (Fig. 2b). Both taurine and cholate were observed in the medium: indeed, the initial turnover of taurocholate was so fast, that 35% had disappeared before the first sample had been processed (Fig. 2b). The maximum concentration of cholate was at about 3 h, corresponding to the disappearance of taurocholate, but taurine was released linearly for another 2 h, when it reached 1 mM, corresponding to the initial concentration of taurocholate (Fig. 2b). The absence of degradation of taurine was confirmed by the absence of additional sulfate in the medium (Fig. 2b; Table 1). We presume that taurocholate sorbed to the cell surface, as observed earlier with linear alkylbenzenesulfonate surfactant (Kertesz et al. 1994; Schleheck and Cook 2005), and that bound taurocholate steadily desorbed to suffer attack by Bsh. The steady release of cholate under these conditions presumably explains the slow disappearance of cholate from the medium. Cholate disappeared at least 1 h before growth stopped. As observed with *C. testosteroni* KF-1, however, a large number of other transient intermediates was formed, and their disappearance corresponded with the end of growth (not shown). The molar growth yield, calculated for the cholate moiety, was 6.3 g protein (mol C)$^{-1}$ and the minimum value for the specific degradation rate of taurocholate in the culture was calculated to be 1.0 mkat (kg protein)$^{-1}$.

Delftia acidovorans SPH-1 utilized taurine with a specific growth rate of 0.2 h^{-1} (not shown), but taurocholate supported an initial specific growth rate of only 0.014 h^{-1} for 2 days, during which no taurine was detectable, sulfate

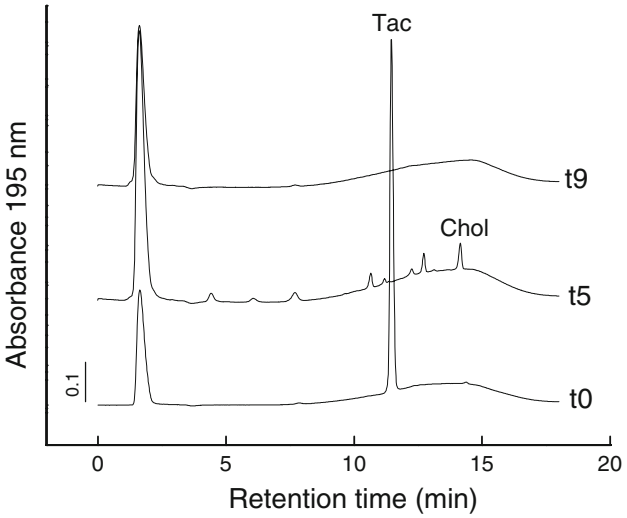

Fig. 3 Separation by HPLC of taurocholate (*Tac*), cholate (*Chol*) and presumed degradative intermediates from samples taken at different times during the growth of *C. testosteroni* KF-1 in taurocholate-salts medium (Fig. 2a). The data represent three samples taken during growth, at zero time (*t0*), at 5 h (*t5*) and at 9 h (*t9*)

was formed and about 25% of the initial taurocholate was detected in the medium as cholate, though about 80% of the taurocholate had disappeared from solution (Fig. 2c). The cells then formed clumps, taurine was detected in the growth medium and the rate of disappearance of taurocholate slowed markedly: the almost uniform formation of sulfate was maintained (Fig. 2c). The taurocholate disappeared at about day 5, when the concentrations of both cholate and taurine reached maxima, and the clumps of cells disintegrated, shown in a jump in protein (measured as turbidity; see figure legend) (Fig. 2c). Taurine disappeared over the next day, but the steady release of sulfate was apparently maintained till day 8. The concentration of cholate dropped from its maximum of about 50% to about 40% of the added taurocholate at the end of growth; there was no sign of intermediates from cholate metabolism in the HPLC analyses (not shown). We interpret that the steady release of sulfate indicated that bound taurocholate was desorbing and being cleaved by Bsh: the missing cholate was assumed to be bound to the cell surface, whose sorptive capacity was increased by declumping. The specific degradation rate of taurocholate was about 0.17 mkat (kg protein)$^{-1}$.

We have confirmed the hypothesis that aerobic bacteria have at least three strategies to dissimilate taurocholate, the catabolism of one or other or both of the products released by Bsh (see below). We presume that the rapid appearance of both taurine and cholate in the medium represents the periplasmic location of Bsh, and that metabolism of each compound follows only after transport into the cell. If the behaviour in our growth media, excretion of many compounds (Fig. 2), is representative of, e.g. behaviour in soil,

not individual species but communities will be involved in the dissimilation of taurocholate. Little work has been done on the catabolism of taurocholate: it is only recently that the analytical chemical tools (Philipp et al. 2006 and our modification to separate cholate and taurocholate) have become available to approach the problem quantitatively.

Activity of bile salt hydrolase

Bsh activity was observed in the crude extracts of taurocholate-grown cells of *C. testosteroni* KF-1 (Fig. 4; Table 2) and *Pseudomonas* sp. strain TAC-K3 (Table 2), whereas negligible activity was detected in extracts of acetate-grown cells (Table 2). Bsh in these two organisms is presumed to be inducible. In contrast, the Bsh in *D. acidovorans* SPH-1 seems to be largely constitutive (Table 2). It is difficult to compare these data with published reports, because most organisms were grown in complex medium or the *bsh* gene was expressed heterologously.

The data in Fig. 4 confirm the nature and the stoichiometry of the Bsh reaction. Effectively, one mol each of taurine and cholate was formed from one mol of taurocholate. The experiment was done with negligible particulate matter from the cells, which presumably explains the absence of significant sorptive effects (cf. Fig. 2b, c). The identity of the substrate [$M = 514.5$] was confirmed by MALDI-TOF-MS in the negative ion mode ($m/z = 513.5 = [M - 1]$) and the identity of each product was confirmed by co-chromatography with authentic material (cholate) or derivatized authentic material (taurine).

Table 2 Specific activities [mkat (kg protein)$^{-1}$] of bile salt hydrolase in extracts of taurocholate-grown or acetate-grown cells of *C. testosteroni* KF-1, *Pseudomonas* sp. strain TAC-K3 or *D. acidovorans* SPH-1

	Taurocholate-grown	Acetate-grown
C. testosteroni KF-1	0.7	≤0.1
Pseudomonas sp. strain TAC-K3	8.3	≤0.1
D. acidovorans SPH-1	11.7	4.8

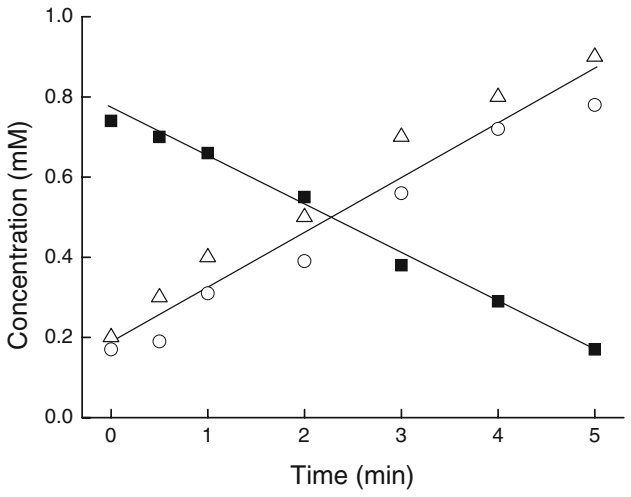

Fig. 4 Bsh activity in crude extract from taurocholate-grown cells of *C. testosteroni* KF-1 visualized as substrate disappearance and product formation. *Filled square* taurocholate, *open triangle* taurine, *open circle* cholate

The specific activity of Bsh observed in extracts of *C. testosteroni* KF-1 [0.7 mkat (kg protein)$^{-1}$] corresponded to the minimum value calculated for growth [0.7 mkat (kg protein)$^{-1}$; see above] so the observed and the calculated values were in the same order of magnitude. Similar conclusions can be drawn for *Pseudomonas* sp. strain TAC-K3. In contrast, Bsh in *D. acidovorans* SPH-1 operated at a much lower specific activity in vivo (about 1%) than measured in vitro (Table 2). We speculate that the sorbed taurocholate might function as a steric hindrance to the active site.

The most complex degradative pathway of taurocholate is in *C. testosteroni* KF-1, where both reaction products of periplasmic Bsh are transported into the cell and degraded (Fig. 1). The cholate pathway, with its many transient excretion products (Fig. 3), will be described elsewhere. A set of genes is now proposed to encode the degradation of taurine in strain KF-1 (Fig. 5a). The presumptive regulator (TauR), an orthologue of which has defined regulatory properties (Wiethaus et al. 2008), enables induction of an ABC transporter for taurine uptake (TauABC) (see Eichhorn et al. 2000) and degradation proceedes via taurine dehydrogenase (TauXY), as well as sulfoacetaldehyde acetyltransferase (Xsc) and phosphotransacetylase (Pta). The sulfite exporter is unknown, while the sulfite dehydrogenase is presumably one of four candidates for periplasmic SorA indicated elsewhere (Denger et al. 2008). We do not expect that the ammonium ion (released by TauXY) is exported, because taurocholate, with its 26 carbon atoms, requires about 2.5 mol nitrogen to allow balanced growth. The taurocholate pathway in *Pseudomonas* sp. strain TAC-K3 involves the complexity of cholate metabolism (cf. Fig. 3), but the taurine is released into the growth medium (Fig. 2b). The simplest pathway is in *D. acidovorans* SPH-1, where the candidate Bsh (Daci_3467) releases cholate and taurine in the periplasm, and taurine is taken up and then degraded via TauXY (Daci_20019/2020), Xsc (Daci_1992), Pta (Daci_1991), a sulfite exporter (TauZ, Daci_1990) (Denger et al. 2006) and sulfite dehydrogenase (SorAB, Daci_0054/0055) (Denger et al. 2008).

Hypothetical bile salt hydrolases in genomes of taurine-degrading bacteria

Earlier data indicate that some 34 bacteria with sequenced genomes can, or can be predicted to, dissimilate taurine as a sole source of carbon and energy for growth, and that at

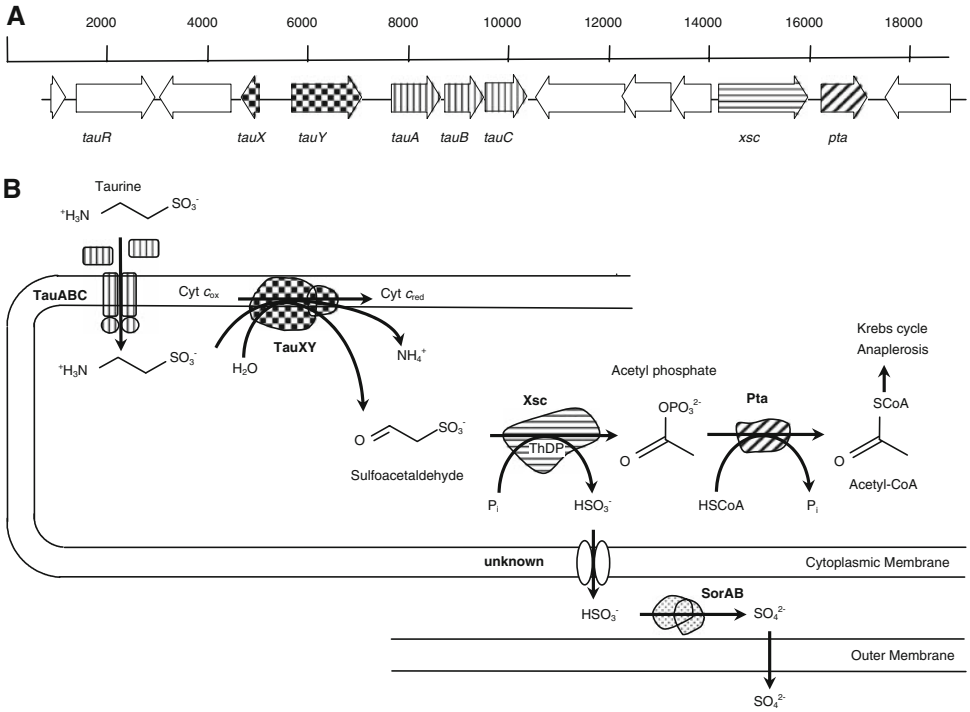

Fig. 5 Degradation of the taurine moiety of taurocholate in *C. testosteroni* KF-1. The genes concerned (**a**) and the vectorial and scalar reactions involved (**b**). The provisional numbering of the taurine cluster comprises CtesDRAFT_2571 (*tauR*) to CtesDRAFT_2762 (*pta*). Abbreviations: see text, *ThDP* thiamine diphosphate

least one organism can utilize taurine as a sole source of nitrogen (Denger et al. 2004, 2008). Application of the BLAST algorithm with these genomes indicated that, apart from *C. testosteroni* KF-1 and *D. acidovorans* SPH-1 (see above), both *P. denitrificans* PD1222 and *Burkholderia phytofirmans* PsJN might encode Bsh and utilize the taurine-carbon of taurocholate.

We conclude that taurocholate is widely used as a source of carbon and energy by bacteria. We presume that communities are involved in the complete degradation of the compound, when excretion of taurine or cholate by individuals occurs.

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