

New intermediates, pathways, enzymes and genes in the microbial metabolism of organosulfonates

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„In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hier und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.“

Sir Isaac Newton

Meiner Familie gewidmet

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SUMMARY

Taurine (2-aminoethanesulfonate), isethionate (2-hydroxyethanesulfonate) and sulfoacetate are natural C₂ sulfonates which exist in the environment and are – to our current knowledge – solely degraded by microorganisms which are able to cleave the chemically stable C-sulfonate bond.

Anaerobic and aerobic degradation of taurine is well investigated in diverse (marine and terrestrial) bacteria. The aerobic dissimilation of taurine proceeds via the central intermediate sulfoacetaldehyde (SAA) and subsequent desulfonation by sulfoacetaldehyde acetyltransferase (Xsc) to acetylphosphate and sulfite. Acetylphosphate is then metabolized (by two different possible pathways, e.g. by Pta, phosphate acetyltransferase) to acetyl-CoA and thereby channeled into central metabolism. Sulfite is, for the purpose of detoxification, oxidized to sulfate by sulfite dehydrogenase.

It is a generally accepted hypothesis that the degradative pathways of isethionate and sulfoacetate converge at SAA with other (C₂) sulfonates.

1. In this study, this hypothesis was confirmed in the bacterium *Cupriavidus necator* H16: the organism was able to utilize taurine, isethionate and sulfoacetate as a sole source of carbon and energy for growth, and it excreted stoichiometric amounts of sulfate into the growth medium. Inducible enzyme activities of Xsc and sulfite dehydrogenase (*here*: SorAB) were measured during growth with each of the three sulfonates.

Additionally, transcription experiments (RT-PCR, reverse transcription PCR) with the appropriate genes (*xsc*, *pta*, *sorAB*) showed inducible transcription of each of the genes in mRNA extracted from sulfoacetate-grown cells. Furthermore, it was shown that a gene (now termed *tauE*) adjacent to *pta*, encoding a putative membrane protein of unknown function, was also inducibly transcribed during growth with each of the three sulfonates. The similarity of TauE to known transport proteins as well as the inducible transcription of *tauE* during growth with taurine, isethionate and sulfoacetate suggests that TauE is somehow involved in the degradative pathway of these compounds. We presume TauE to represent the sulfite exporter which is responsible for the translocation of sulfite into the periplasm, its site of oxidation by SorAB.

In addition, the previously uncharacterized initial steps of isethionate and sulfoacetate, leading to SAA, were investigated:

2. The microbial dissimilation of isethionate was not elucidated in detail until now, except for the involvement of a membrane-associated protein, isethionate dehydrogenase (IseJ), in the oxidation of isethionate to SAA.

C. necator H16 as well as *Ruegeria pomeroyi* DSS-3 were able to utilize isethionate as a sole source of carbon and energy. In both organisms, inducible activity of IseJ could be measured during growth with isethionate.

Adjacent to genes of the 'central pathway' (*xsc*, *pta*, see 1.), a gene cluster was found which presumably encodes the isethionate degradative genes. The gene products include a putative transcriptional regulator (IseR), isethionate dehydrogenase (IseJ) as well as a transport system (IseU in strain H16, IseKLM in strain DSS-3). The inducible transcription of those genes (*iseJU* in strain H16, *iseJKLM* in strain DSS-3) during growth with isethionate was confirmed in both bacteria using RT-PCR.

Thus, different transport systems for isethionate exist in marine and terrestrial bacteria: whereas in terrestrial organisms, mainly MFS (major facilitator superfamily) transporters (IseU) were found, in marine bacteria, TRAP (tripartite ATP-independent periplasmic) transport systems (IseKLM) predominated. In some bacteria (e.g. *Rhodobacterales* sp. HTCC2150), a third system, TerC (tellurium ion resistance) family transporter, was postulated instead of IseU or IseKLM.

3. The microbial degradation of sulfoacetate was investigated in several bacteria, but the main subject of this study was *C. necator* H16. When convergence of the sulfoacetate degradative pathway with those of other (C₂) sulfonates at SAA was confirmed, the initial reactions responsible for reduction of the carboxylic acid to the aldehyde (SAA) were further investigated. The first step was identified as the ATP-dependent activation of sulfoacetate to the novel CoA-ester sulfoacetyl-CoA. This intermediate was identified by MALDI-TOF mass spectrometry. The enzyme catalyzing this reaction was sulfoacetate-CoA ligase (SauT) which could be measured in a discontinuous enzyme assay at the HPLC. In the next step, sulfoacetyl-CoA was converted to SAA by sulfoacetaldehyde dehydrogenase (SauS). Both SauT and SauS were inducibly active during growth with sulfoacetate. SauS was purified to homogeneity, characterized and assigned to the coding gene (H16_A2747) by PMF (peptide mass fingerprinting).

The gene *sauS* was part of a cluster consisting of four genes encoding regulation (SauR), activation (SauT), reduction (SauS) and transport (SauU). The catabolic genes *sauSTU* were inducibly transcribed during growth with sulfoacetate, as confirmed by RT-PCR. In addition, each of the three genes was also deleted by site-directed in-frame deletion mutagenesis: each of the resulting single mutants was defective in growth with sulfoacetate whereas they were still able to utilize other sulfonates including the intermediate SAA. Additionally, the mutants H16 Δ *sauS* and H16 Δ *sauT* lacked the enzyme activity of the corresponding gene which was deleted. The deletion mutagenesis results underlined the above mentioned findings and also confirmed the involvement of the MFS transporter SauU in sulfoacetate degradation.

By comparative genomics, 25 microorganisms, both marine and terrestrial, were found to contain sulfoacetate gene clusters. Thereby, a different putative activation enzyme (heteromeric sulfoacetate-CoA ligase SauPQ), different types of regulators (SauI, SauV) and an alternative transport system (TTT, tripartite tricarboxylate transporter, SauFGH) for sulfoacetate were discovered. Some of these variants of the newly discovered sulfoacetate degradation pathway were investigated in e.g. *Roseovarius nubinhibens* ISM and *Oligotropha carboxidovorans* OM5 by means of growth experiments, enzyme activity tests and RT-PCR.

ZUSAMMENFASSUNG

Taurin, Isethionat und Sulfoacetat sind natürlich vorkommende C₂-Sulfonate, die in der Umwelt vorliegen und dort nach heutigem Wissensstand ausschließlich von Mikroorganismen abgebaut werden können, da nur diese in der Lage sind, die chemisch stabile Kohlenstoff-Sulfonat-Bindung zu spalten.

Der aerobe sowie anaerobe Abbau von Taurin in verschiedenen Bakterien wurde bereits weitgehend geklärt. Die aerobe Dissimilation von Taurin verläuft immer über das zentrale Intermediat Sulfoacetaldehyd (SAA) und dessen anschließende Desulfonierung zu Acetylphosphat und Sulfit, katalysiert durch die Sulfoacetaldehyd-Acetyltransferase (Xsc). Das dabei entstehende Acetylphosphat wird (auf verschiedenen möglichen Reaktionswegen) zu Acetyl-CoA umgesetzt (beispielsweise durch Pta, Phosphat-Acetyltransferase) und kann somit in den zentralen Stoffwechsel eingeschleust werden. Das ebenfalls entstandene Sulfit wird durch eine Sulfit-Dehydrogenase zu Sulfat oxidiert.

Von Isethionat sowie Sulfoacetat wird seit einigen Jahren angenommen, dass sie wie Taurin ebenfalls über SAA abgebaut werden.

1. In der vorliegenden Arbeit wurde im Bakterium *Cupriavidus necator* H16 diese Vermutung bestätigt: Das Bakterium war in der Lage, Taurin, Isethionat und Sulfoacetat als jeweils alleinige Kohlenstoffquelle zu verwenden und schied in stöchiometrischem Verhältnis Sulfat aus. Weiterhin konnten Enzymaktivitäten gemessen werden (Xsc und Sulfit-Dehydrogenase, *hier*: SorAB), welche induzierbar waren, das heißt nur beim Wachstum mit den drei oben genannten Sulfonaten zu beobachten.

Zusätzliche Transkriptions-Experimente (Reverse transcription PCR, RT-PCR) mit den jeweiligen Genen (*xsc*, *pta*, *sorAB*) zeigten, dass die getesteten Gene induzierbar transkribiert wurden.

Es wurde weiterhin gezeigt, dass ein von *pta* benachbartes Gen (*tauE*), welches ein potentiell Membranprotein unbekannter Funktion kodiert, ebenfalls induzierbar transkribiert wurde. Die Verwandtschaft des Proteins TauE mit bekannten Transportproteinen sowie die Transkription des Gens während Wachstum mit Taurin, Sulfoacetat und Isethionat legen nahe, dass es sich bei diesem Protein um einen Sulfit-Exporter handelt. Dieser transportiert

vermutlich Sulfit aus dem Cytoplasma in das Periplasma, wo es durch die Sulfit-Dehydrogenase zu Sulfat oxidiert wird.

Als weitere Themen der vorliegenden Doktorarbeit wurden die initialen, bislang unbekanntenen Schritte des Abbaus von Sulfoacetat sowie Isethionat auf dem Weg zum gemeinsamen Zwischenprodukt (SAA) untersucht.

2. Der mikrobielle Abbau von Isethionat wurde bisher nicht gänzlich geklärt. Ein nicht näher charakterisiertes membrangebundenes Enzym, die Isethionat-Dehydrogenase (IseJ), ist vermutlich in vielen Mikroorganismen in die Oxidation von Isethionat zu SAA involviert. Die Bakterienstämme *C. necator* H16 sowie *Ruegeria pomeroyi* DSS-3 sind beide in der Lage, Isethionat als alleinige Kohlenstoff- und Energiequelle zu nutzen. Es konnte in beiden Stämmen induzierbare IseJ-Enzymaktivität gemessen werden während des Wachstums mit Isethionat.

Des Weiteren wurden Gene gefunden, die in mehreren Bakterienstämmen direkt benachbart sind zu den Genen des weiteren Abbauweges *xsc* und *pta*, welche vermutlich die für den Isethionat-Abbau benötigten Enzyme kodieren und in allen Isethionat-verwertenden Bakterienstämmen ein definiertes Cluster bilden. Diese Genprodukte kodieren vermutlich einen Regulator (IseR), die Isethionat-Dehydrogenase (IseJ) sowie verschiedene Transportsysteme (IseU in H16, IseKLM in DSS-3). Die induzierbare Transkription der Gene *iseJKLM* bzw. *iseJU* wurde in beiden verwendeten Bakterienstämmen mittels RT-PCR belegt. Hierbei wurde festgestellt, dass unterschiedliche Transportsysteme für Isethionat in marinen sowie terrestrischen Bakterienstämmen existieren. Während terrestrische Bakterien zumeist MFS (major facilitator superfamily)-Transporter für den Isethionat-Transport kodieren, besitzen marine Bakterien überwiegend TRAP (tripartite ATP-independent periplasmic)-Transporter, die aufgrund ihrer – meist hochaffinen – extracytoplasmatischen Bindeproteine sehr gut geeignet sind für Umgebungen mit geringeren Substratkonzentrationen.

Ein weiteres marines Bakterium (*Rhodobacterales* sp. HTCC2150), welches ebenfalls in der Lage ist, Isethionat abzubauen, weist eine dritte Art von Transportsystem auf (TerC aus der ‚Tellurium ion resistance family‘), welches für den Isethionat-Transport zuständig sein könnte.

3. Der mikrobielle Abbau von Sulfoacetat wurde in mehreren Bakterienstämmen untersucht. Hauptobjekt der Studie war *C. necator* H16, der – wie oben beschrieben – mit Sulfoacetat als alleiniger Kohlenstoff- und Energiequelle wuchs. Nachdem klar war, dass Sulfoacetat über

SAA abgebaut wird, wurden verstärkt die Reaktionen untersucht, die für diese Reduktion von der Säure zum Aldehyd benötigt werden. Es wurde herausgefunden, dass der erste Schritt eine ATP-abhängige Aktivierung von Sulfoacetat zu Sulfoacetyl-CoA, einem neuartigen CoA-Ester, darstellte. Dieser CoA-Ester wurde mittels HPLC gemessen und mit Hilfe von MALDI-TOF-Massenspektrometrie als Sulfoacetyl-CoA identifiziert. Das katalysierende Enzym, Sulfoacetat-CoA-Ligase (SauT), wurde in einem diskontinuierlichen Enzymtest mittels HPLC nachgewiesen. Im nächsten Schritt wurde Sulfoacetyl-CoA von der Sulfoacetaldehyd-Dehydrogenase (SauS) zu Sulfoacetaldehyd umgesetzt. Beide Enzyme waren nur bei Wachstum mit Sulfoacetat aktiv. SauS wurde gereinigt und charakterisiert, und mittels PMF (Peptide Mass Fingerprint) konnte das Enzym seinem Genlocus (H16_A2747) zugeordnet werden.

Das Gen *sauS* ist Teil eines Genclusters bestehend aus 4 Genen. Deren Genprodukte umfassen einen transkriptionellen Regulator (SauR), die Sulfoacetaldehyd-Dehydrogenase (SauS), ein als Acetat-CoA-Ligase annotiertes Protein, welches wir für die Sulfoacetat-CoA-Ligase (SauT) halten, sowie ein MFS-Transportprotein (SauU), das den Sulfoacetat-Transporter darstellt. Die katabolischen Gene *sauSTU* waren induzierbar transkribiert, wie mittels RT-PCR bestätigt. Weiterhin wurden diese drei Gene einzeln durch gerichtete Mutagenese deletiert. Die daraus resultierenden Mutanten konnten nicht mehr mit Sulfoacetat wachsen (wohl aber mit anderen Sulfonaten inklusive des Zwischenproduktes SAA). Diese Ergebnisse stützen und ergänzen einerseits die oben beschriebenen Resultate, und zusätzlich bestätigen sie insbesondere die tatsächliche Beteiligung des Transportproteins SauU am Sulfoacetat-Abbau.

Durch Vergleich von Sulfoacetat-Genclustern in 25 verschiedenen Genom-sequenzierten Bakterien wurden alternative Transportsysteme (TTT, tripartite tricarboxylate transporter, SauFGH), Regulatoren (SauI, SauV) sowie eine heteromere Variante der Sulfoacetat-CoA-Ligase (SauPQ) gefunden. Einige dieser Variationen (SauFGH, SauPQ) wurden in *Roseovarius nubinhibens* ISM sowie *Oligotropha carboxidovorans* OM5 mittels Wachstumsversuchen, Enzymaktivitätstests sowie RT-PCR untersucht und bestätigt.

GENERAL INTRODUCTION

Sulfonates – an overview

Sulfonates are organic sulfur compounds containing the $R-SO_3^-$ moiety which can be linked to oxygen (O-sulfonates, sulfate esters), nitrogen (N-sulfonates) or carbon atoms (C-sulfonates). The C-sulfonates are compounds containing a $C-SO_3^-$ group which is chemically very stable: even treatment with strong acids does not result in cleavage of the C-S-bond (Busby 1966; Freney *et al.* 1970; Johnson and Nishita 1952). To our present-day knowledge, only microorganisms can cleave the C-sulfonate bond, while eukaryotes are incapable of desulfonation (Huxtable 1992). Sulfonates occur naturally or as xenobiotics and can be either aliphatic or aromatic. Sulfonate anions are the conjugate bases of sulfonic acids which are predominantly water soluble and strong acids because the sulfone group has a high electron withdrawing effect on the hydroxyl group. Sulfonic acids and their salts are used in a range of products, e.g. as detergents, dyes or strong cationic exchangers in water treatment.

In fact we are surrounded by – mostly aliphatic – sulfonates (Fig. 1): our atmosphere contains the gases methanesulfonate and ethanesulfonate which arise from oxidation of atmospheric DMS (dimethylsulfide), and which precipitate to the earth by rain, snow and dry deposition (Kelly and Murrell 1999). The microbiological formation of methane, another constituent of the atmosphere, requires coenzyme M (2-mercaptoethanesulfonate) as a methyl carrier (Taylor *et al.* 1974; White 1988). Humic substances in our soils contain sulfonate groups (van Loon *et al.* 1993), and plants as well as photosynthetic algae and bacteria in our environment contain the sulfolipid sulfoquinovosyldiacylglycerol (SQDG) in their thylakoid membranes (Benson *et al.* 1959; Kates 1959; Wintermans 1960). The concentration of the sulfolipid in photosynthetic plant tissue ranges from 1 to 6 mM and approximates the concentration of phosphatidylcholine (Benson 1963). Some other sulfolipids, called capnoids (capnine, 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid), are major constituents of the cell envelope of gliding bacteria, e.g. *Capnocytophaga* (Godchaux III and Leadbetter 1980, 1983). The sulfolipid 1-deoxyceramide-1-sulfonate was found to be one of the four major sulfolipids of the non-photosynthetic diatom *Nitzschia alba* (Anderson *et al.* 1978). Thus, sulfolipids account for a significant part of the biogeochemical cycling of sulfur (Harwood and Nicholls 1979).

The plant sulfolipid can be degraded by plant enzymes to sulfoquinovose (6-deoxy-6-sulfo-D-glucose) which can be subsequently metabolized by bacteria to sulfoacetate (Martelli and Benson 1964), sulfopropanediol (2,3-dihydroxy-propanesulfonate) or sulfolactate (Roy *et al.* 2003; see below). The latter is also found as a major constituent of bacterial endospores (Bonsen *et al.* 1969). In the animal kingdom, sulfonates can be found as well: taurine occurs mainly in animals, e.g. as one of the major organic solutes in mammals or as an osmolyte in invertebrates (Huxtable 1992; Yancey 2005; see below). The taurine derivative taurocholate plays a role in digestive processes (Bergström and Gloor 1954; Elliott 1956), and another taurine derivative, bulgecin A (a sulfonated glycopeptide), is a bacterial metabolite which is used as β -lactamase-inhibitor (Imada *et al.* 1982; Simm *et al.* 2005). *N*-acetyltaurine was found in molar concentrations in the adhesive droplets of orb spiders webs (Higgins *et al.* 2001; Vollrath *et al.* 1990), and *N*-methyltaurine functions as an osmolyte of deep-sea worms (Yin *et al.* 2000). Sulfoacetaldehyde (SAA) is a well known intermediate of C₂ sulfonate degradation (Kondo *et al.* 1971; Shimamoto and Berk 1980; Toyama and Soda 1972). Isethionate (2-hydroxyethanesulfonate) was found in nervous tissue (Koechlin 1954) but also in algae (Barrow *et al.* 1993; Hellio *et al.* 2004; Holst *et al.* 1994) and spiders webs (Vollrath *et al.* 1990). The amino acid cysteate (2-amino-3-sulfopropionate) can be found in hydrolysates of wool, deriving from oxidation of cystine (Consdén *et al.* 1946), and in human hair (Zahn and Gattner 1997). The compound was also found as a precursor of the above mentioned taurine, sulfolactate and capnine (Abbanat *et al.* 1985; Pasantes-Morales *et al.* 1980; Weinstein and Griffith 1988).

Only few naturally occurring aromatic organosulfonates are known: aeruginosin B (Herbert and Holliman 1964) was found as a pigment in *Pseudomonas aeruginosa*, petrobactine sulfonate was found as a siderophore in a marine bacterium (Hickford *et al.* 2004), and echinosulfonic acids were found as antibacterial agents in marine sponges (Ovenden and Capon 1999). And only recently, a new class of terpenes, the diterpenyl-taurines (phorbasins D-F), were isolated from *Phorbas* sp., an Australian marine sponge (Lee *et al.* 2008; Zhang and Capon 2008).

Due to their ubiquitous occurrence, sulfonates can serve as growth substrates in many bacterial habitats, e.g. in sediments (Vairavamurthy *et al.* 1994), microbial mats (Visscher *et al.* 1999), soils (Autry and Fitzgerald 1990; Mirleau *et al.* 2005; Schmalenberger *et al.* 2008) and probably also in freshwater and oceans.

In addition to naturally occurring sulfonates, a wide range of xenobiotic sulfonates (Fig. 2) is produced in industry and released into the environment, e.g. in the form of detergents (e.g.

linear alkylbenzenesulfonates, LAS; Cook 1998), hydrotropic agents (e.g. *p*-toluenesulfonate; González *et al.* 2000), dyestuffs or whitening agents (Poiger *et al.* 1998).

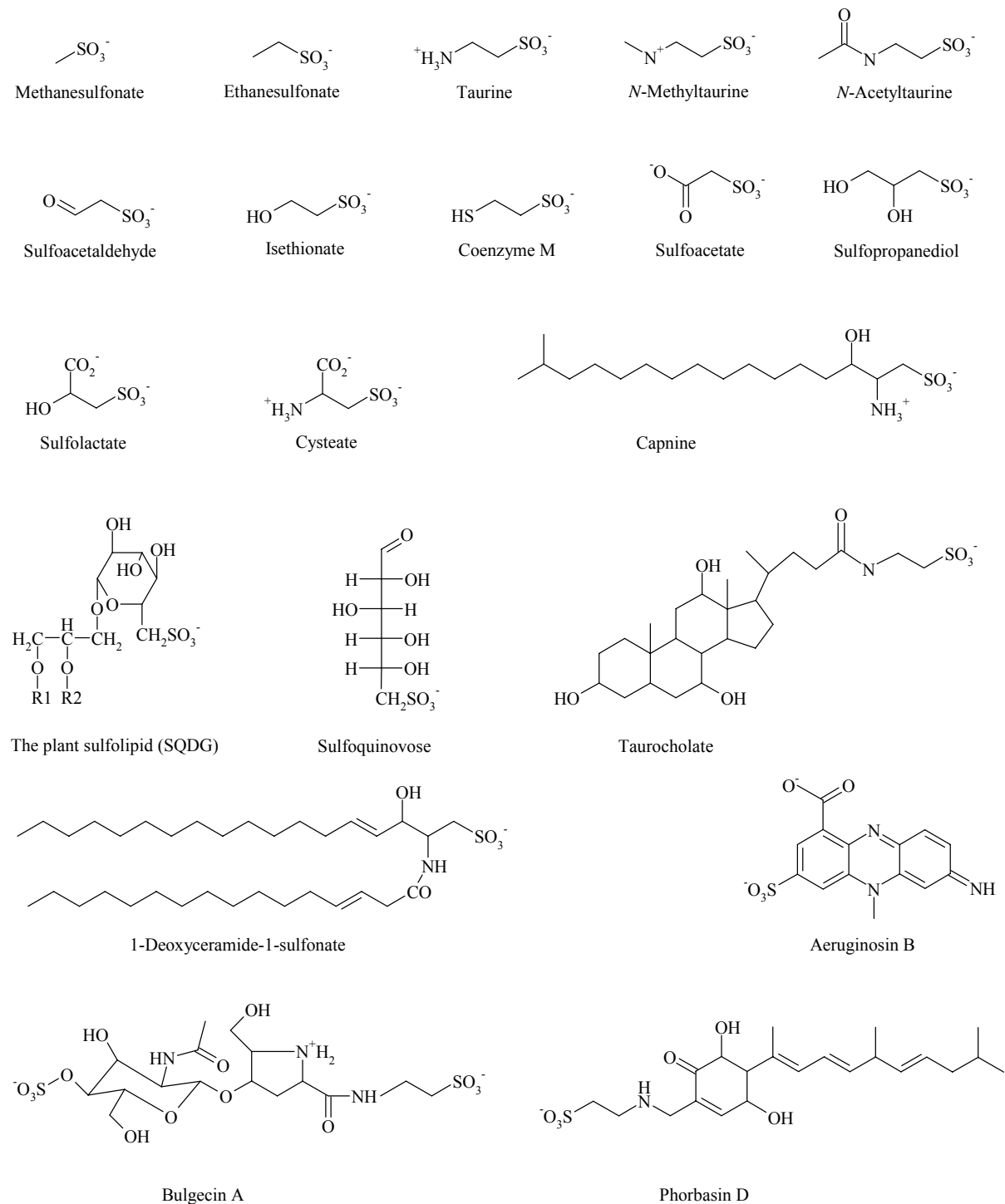


Fig. 1. Examples of naturally occurring aliphatic and aromatic organosulfonates. For systematic names see text above.

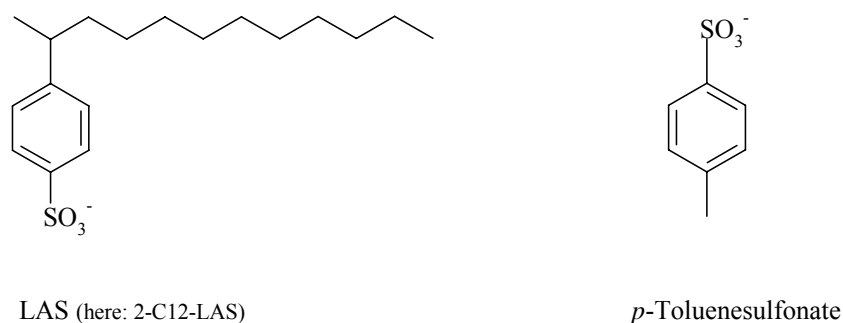


Fig. 2. Examples of xenobiotic organosulfonates. LAS, linear alkylbenzenesulfonates.

C₂ sulfonates – discovery, occurrence and functions

Taurine (2-aminoethanesulfonate), isethionate (2-hydroxyethanesulfonate) and sulfoacetate, which were the main subjects of this thesis, are C₂ sulfonates occurring naturally in the environment (see above). Their discovery, distribution and (already known pathways of) degradation are described in the following.

Taurine

Discovery, natural occurrence and functions of taurine

Taurine was discovered in the bile of the ox, *Bos taurus*, from which its current name derived (Demarcay 1838). It was first isolated in 1827 by the German scientists Friedrich Tiedemann and Leopold Gmelin and described as ‘bile asparagine’ because of its apparent similarity to asparagine. Taurine is often referred to be a (non-proteinogenic) amino acid (Brosnan and Brosnan 2006). In the strict sense, it is not an amino acid because of the lack of a carboxylic group, but it is a water-soluble β-aminosulfonic acid which is zwitterionic at pH 7 and thus very polar and lipophobic. It has a high melting temperature which mirrors the stability of the compound (see above).

Taurine is found in high amounts in the animal kingdom (see above) and in some algae (Ericson and Carlson 1954; Schweiger 1967), whereas it is present in only low amounts in higher land plants (Kataoka and Ohnishi 1986). Taurine is known to be an osmolyte in marine invertebrates and fish (Kurtz and Luck 1935; Potts 1958), and in mammals, it is one of the most abundant low-molecular-weight organic constituents, occurring at 1 g taurine per kg body weight (Huxtable 1992). Taurine is found predominantly in muscle, brain and blood and is involved in bile salt synthesis (Bergström and Gloor 1954; Spaeth *et al.* 1974). It is postulated to be involved in several more physiological functions, e.g. the stabilization of

retinal photoreceptors (Pasantes-Morales and Cruz 1984), development of the brain (Sturman and Gaull 1975) and stabilization of membranes in muscle (Huxtable and Bressler 1973).

Natural synthesis of taurine

Taurine can be synthesized in all mammals, but in varying amounts. Some mammals (e.g. cats) lack a sufficient taurine synthesizing capacity and need to supply themselves with dietary taurine, others (e.g. guinea pigs) manage to synthesize enough taurine to fulfill the diverse functions which the compound holds in metabolism (Huxtable and Lippincott 1982). Mammals can synthesize taurine from the sulfur-containing compounds L-cysteine (Ida *et al.* 1985) or cysteamine (Aruoma *et al.* 1988) by two different thiol dioxygenases (Fig. 3).

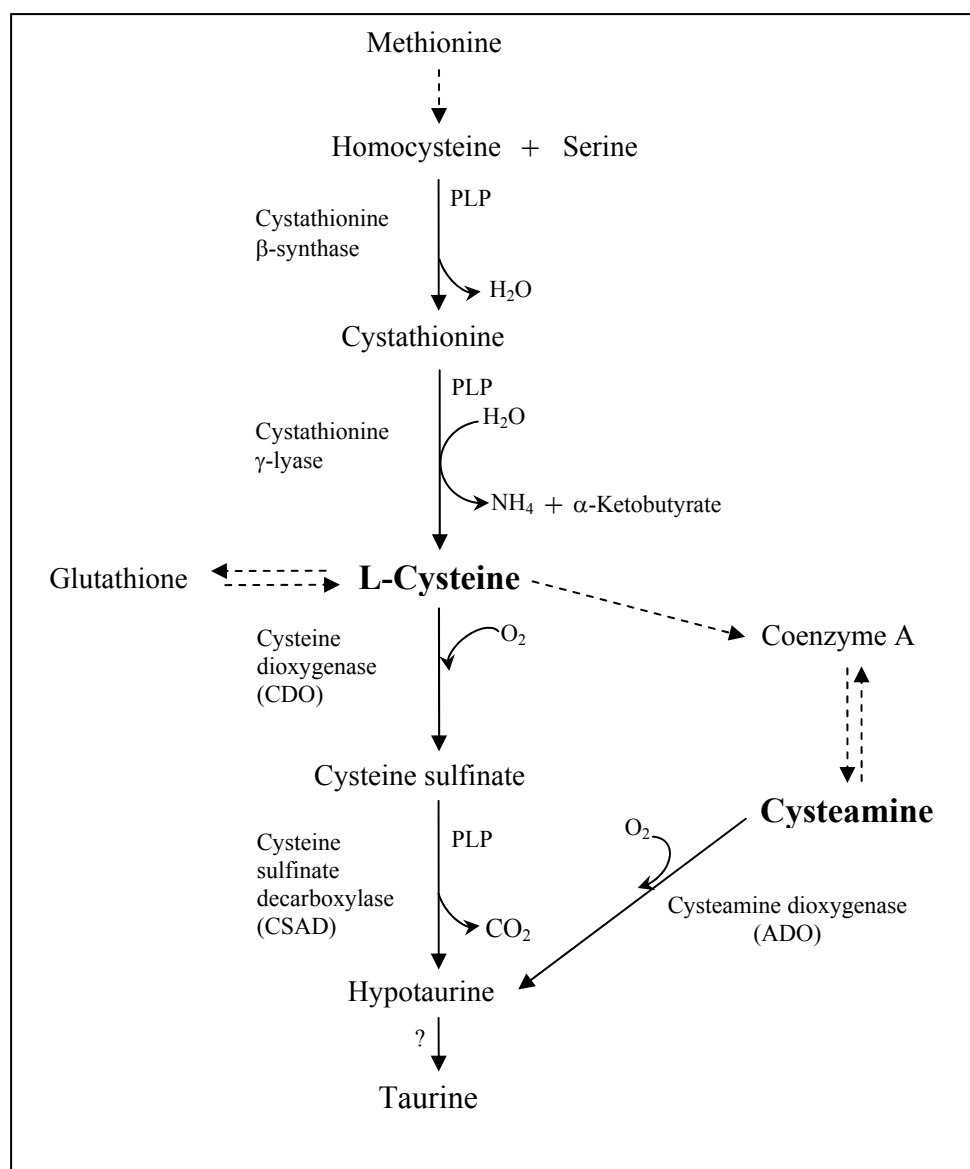


Fig. 3. Taurine biosynthesis in mammals. The two precursors of taurine biosynthesis mentioned in the text above are depicted in bold face type.

The synthesis of taurine from the amino acid L-cysteine proceeds via dioxygenation, catalyzed by cysteine dioxygenase (CDO, [EC 1.13.11.20]), yielding cysteine sulfinate which is decarboxylated by cysteine sulfinate decarboxylase (CSAD, [EC 4.1.1.29]) to hypotaurine (Daniels and Stipanuk 1982; Dominy *et al.* 2007; Simmons *et al.* 2006). Hypotaurine can then be oxidized to taurine by an unknown enzyme (Stipanuk 2004a). The rate of taurine synthesis seems to be controlled mainly by cysteine supply to the liver and by upregulation of CDO in the presence of high cysteine concentrations (Stipanuk 2004b). In the presence of excess concentrations of cysteine which must be kept under the toxicity threshold, glutathione can serve as a cysteine reservoir (Stipanuk *et al.* 2006).

Cysteamine is the second possible precursor for taurine synthesis, it is a constituent of coenzyme A and thus derives from coenzyme A turnover in the cell (Leonardi *et al.* 2005). Cysteamine can be converted to hypotaurine which was already observed in horse kidney in the 1960s (Cavallini *et al.* 1966), but the responsible enzyme was characterized and given the name cysteamine dioxygenase (ADO) 40 years later (Coloso *et al.* 2006; Dominy *et al.* 2007). It is not yet clear which proportions the two possible pathways contribute to the total taurine biosynthesis (Stipanuk *et al.* 2006).

However, as taurine is produced in the human body every day, but the stable C-S-bond can not be cleaved by eukaryotes, excess of the compound is excreted unaltered in urine (Daniels and Stipanuk 1982). Therefore, taurine is released into the environment by diverse animals in quite high amounts, ranging from 150 to 800 μmol per day in human urine (Irving *et al.* 1986) depending on taurine synthesis and dietary taurine uptake.

Antropogenic sources of taurine

Another source of taurine is industry where the compound is produced as a food additive for cats (which are not able to synthesize sufficient amounts of taurine themselves), as an ingredient for energy drinks (van den Eynde *et al.* 2008) and as a dietary supplement for athletes, the latter of which is controversially discussed (Galloway *et al.* 2008; Imagawa *et al.* 2009).

Isethionate

Discovery and occurrence of isethionate

The chemist Heinrich Gustav Magnus was the first to describe isethionate in 1833 (Magnus 1833). The compound was chemically synthesized (Goldberg 1942), but physiological isethionate was discovered more than one century after its first mention: the compound was found to be the major anion in the axoplasm of the squid's (*Loligo pealii*) giant nerve fibres

with concentrations up to 220 mM (Koechlin 1954, 1955). Isethionate was also found in marine sponges and in red algae, where the concentration accounts for about 250 mM (Barrow *et al.* 1993; Hellio *et al.* 2004; Holst *et al.* 1994). In the sticky droplets of spiders webs, where it was discovered only recently, the concentration is about 2 M (Townley *et al.* 2006).

Isethionate was also found in dog heart (Welty *et al.* 1962) and was established to be a common compound in mammalian tissue (Kumpulainen *et al.* 1982), but the biological functions of isethionate remained unclear.

Natural synthesis of isethionate

Isethionate was first thought to be synthesized from taurine by mammalian tissue (Read and Welty 1962) which was later refuted by the fact that germ-free mice were not able to form isethionate from taurine (Fellman *et al.* 1980). But as gut anaerobes were found to metabolize taurine to isethionate, the gut flora is suspected to be the source of isethionate in mammalian tissue (Fellman *et al.* 1980). Several possible ways of microbial generation of isethionate from taurine have been elucidated over the last years in our laboratory: the terrestrial bacterium *Klebsiella oxytoca* TauN1 assimilates taurine-nitrogen and excretes isethionate after deamination (Styp von Rekowski *et al.* 2005). The same phenomenon was found and thoroughly investigated in the marine bacterium *Chromohalobacter salexigens* DSM 3043 (Krejčík *et al.* 2009; see below).

Antropogenic sources of isethionate

Isethionate is also funneled into the environment by households, as industry uses the compound as a counterion for active pharmaceutical ingredients, e.g. for pentamidine which is used in the treatment of leishmaniasis, sleeping sickness and *Pneumocystis* pneumonia (Delobel and Pradinaud 2003; Fortuny *et al.* 1970; Gelfand and Alves 1954; Nguewa *et al.* 2005), or for propamidine whis is involved in the treatment of *Acanthamoeba* keratitis (Illingworth and Cook 1998). In derivatized form, sodium cocoyl isethionate serves as a mild foaming surfactant in skin care products like skin creams, shampoos and other cosmetic products (Ghosh and Blankschtein 2007; Korting *et al.* 1992).

Sulfoacetate

Discovery and occurrence of sulfoacetate

Sulfoacetate was first discovered in derivatized form as an alkaloid ester (C₁₉H₂₁NO₇S) called erysothiopine (Folkers *et al.* 1944) and later as a product of the plant sulfolipid breakdown

(Martelli and Benson 1964). Thus, the compound mainly derives from plant material and is – in contrast to taurine and isethionate – rarely found in animal tissue.

The plant sulfolipid as a source of sulfoacetate

The plant sulfolipid (SQDG) is an anionic lipid, containing a sulfonated sugar moiety (sulfoquinovose) as a polar headgroup, and is a regular component of the thylakoid membrane in many organisms carrying out photosynthesis (Harwood 1980; Pugh *et al.* 1995). There are several exceptions of phototroph organisms which lack SQDG, e.g. the cyanobacterium *Gloeobacter violaceus* sp. PCC 7421 (Selstam and Campbell 1996) or the purple nonsulfur bacterium *Rhodospseudomonas viridis* (Linscheid *et al.* 1997). Despite of this ubiquitous occurrence in photosynthetic membranes, no specific function could be attributed to the plant sulfolipid until now (Weissenmayer *et al.* 2000). Against early speculations (Barber and Gounaris 1986), a direct role in photosynthesis itself was later excluded (Benning *et al.* 1993; Güler *et al.* 1996). A further hint that the role of SQDG is not connected with or at least not limited to the photosynthetic reactions is the fact that the sulfolipid is also present in non-photosynthetic organisms. It occurs e.g. in the nodule-forming *Sinorhizobium* (formerly *Rhizobium*) *meliloti* (Cedergreen and Hollingsworth 1994; Weissenmayer *et al.* 2000), the diatom *Navicula alba* (Harwood and Jones 1989), the gram-positive thermoacidophilic *Alicyclobacillus acidocaldarius* (Langworthy *et al.* 1976) and in sperm and egg of the sea urchin *Pseudocentrotus depressus* (Isono *et al.* 1967). The only functions which could be assigned to SQDG until now are: (1) stabilization of chloroplast structure and function, (2) SQDG as a substitute for phosphatidylglycerol especially during severe phosphate limitation in *Arabidopsis* (Yu and Benning 2003) and (3) SQDG as an internal sulfur source for protein synthesis during sulfur starvation in *Chlamydomonas reinhardtii* (Sugimoto *et al.* 2007). Recently, it was also discussed to be involved in salt tolerance strategies of the halophytic rock samphire *Crithmum maritimum* (Ben Hamed *et al.* 2005).

On a quantity basis, the estimated annual global production of the plant sulfolipid is about 3.6×10^{13} kg (Harwood and Nicholls 1979) as it can represent up to half of the total lipid content in some marine brown algae (Dembitsky *et al.* 1990) and accounts for 10 % of the thylakoid membrane lipid in leaves of higher plants (Middlebrook *et al.* 1959). The total sulfur content of leaves is composed mainly by sulfolipids and proteins, thus, significant amounts of the sulfolipid or its degradation products enter the soil during senescence of deciduous plants. Plants are in general capable of deacylation of the sulfolipid by acyl hydrolases yielding sulfoquinovosylglycerol (Benson 1963; Burns *et al.* 1980) which can be further metabolized by plant β -galactosidases to sulfoquinovose (Fig. 4; Harwood 1980;

Shibuya and Benson 1961). Some plants are able metabolize sulfoquinovose further: in lucern (*Medicago sativa*), sulfolactate accumulated, and in the coral tree (*Erythrina crista-galli*) sulfoquinovose degradation resulted in accumulation of sulfoacetate (Lee and Benson 1972) which was considered to be a decarboxylation product of sulfolactate as was observed earlier in *Chlorella ellipsoidea* (Lee 1970). During degradation of labeled sulfolipid in *Scenedesmus* and *Chlorella*, sulfonated intermediates were found as well: sulfoquinovose, sulfolactate, sulfolactaldehyde and also sulfoacetate (Shibuya *et al.* 1963). To our present knowledge, desulfonation is mainly left to bacteria: *Flavobacterium* sp. was the first organism in pure culture which degraded labeled methyl-sulfoquinovose and produced intracellular sulfoacetate and cysteate which was obviously further metabolized and desulfonated since sulfate was found in the growth medium (Martelli and Benson 1964). Sulfolactate and additionally sulfopropanediol (2,3-dihydroxypropanesulfonate), resembling the glycolysis intermediate 1-phosphoglycerol, were later found as intermediates during studies of bacterial sulfoquinovose degradation with several strains of *Agrobacterium* and *Klebsiella* (Roy *et al.* 2000; Roy *et al.* 2003). As sulfoquinovose is a sulfonated analogue of glucose-6-phosphate and the intermediates which were found represent sulfonic acid analogs of phosphoglycerate, glyceraldehyde phosphate and phosphoglycolic acid, respectively, it was assumed that the degradation reactions of sulfoquinovose are probably related to glycolysis reactions (Embden-Meyerhoff-Parnas-pathway) in carbohydrate metabolism (Fig. 4; Lee and Benson 1972). In addition to identification of the intermediates, enzyme activities of phosphofructokinase were found in crude cell extract of both strains which showed the same activity levels no matter if the substrate was fructose-6-phosphate, glucose-6-phosphate or sulfoquinovose (Roy *et al.* 2003). This strongly supports the idea of a sulfoglycolytic breakdown of sulfoquinovose. Considering these broad capacities of sulfolipid degradation in both plants and bacteria, the sulfolipid and therefore also sulfoquinovose obviously represent an important potential source of sulfate, and some of the intermediates of sulfolipid degradation (e.g. sulfoacetate), can also serve as carbon sources for bacteria (see below). A confirmation of the assumed sulfoquinovose-degrading capacity of soil-communities was given by Strickland and Fitzgerald who observed the mineralization of sulfoquinovose to inorganic sulfate and sulfate esters in surface soil from watersheds in California (Strickland and Fitzgerald 1983). Since organisms containing the sulfolipid occupy both terrestrial and aquatic ecological niches (see above), this precursor of sulfoacetate is presumed to be ubiquitous in nature.

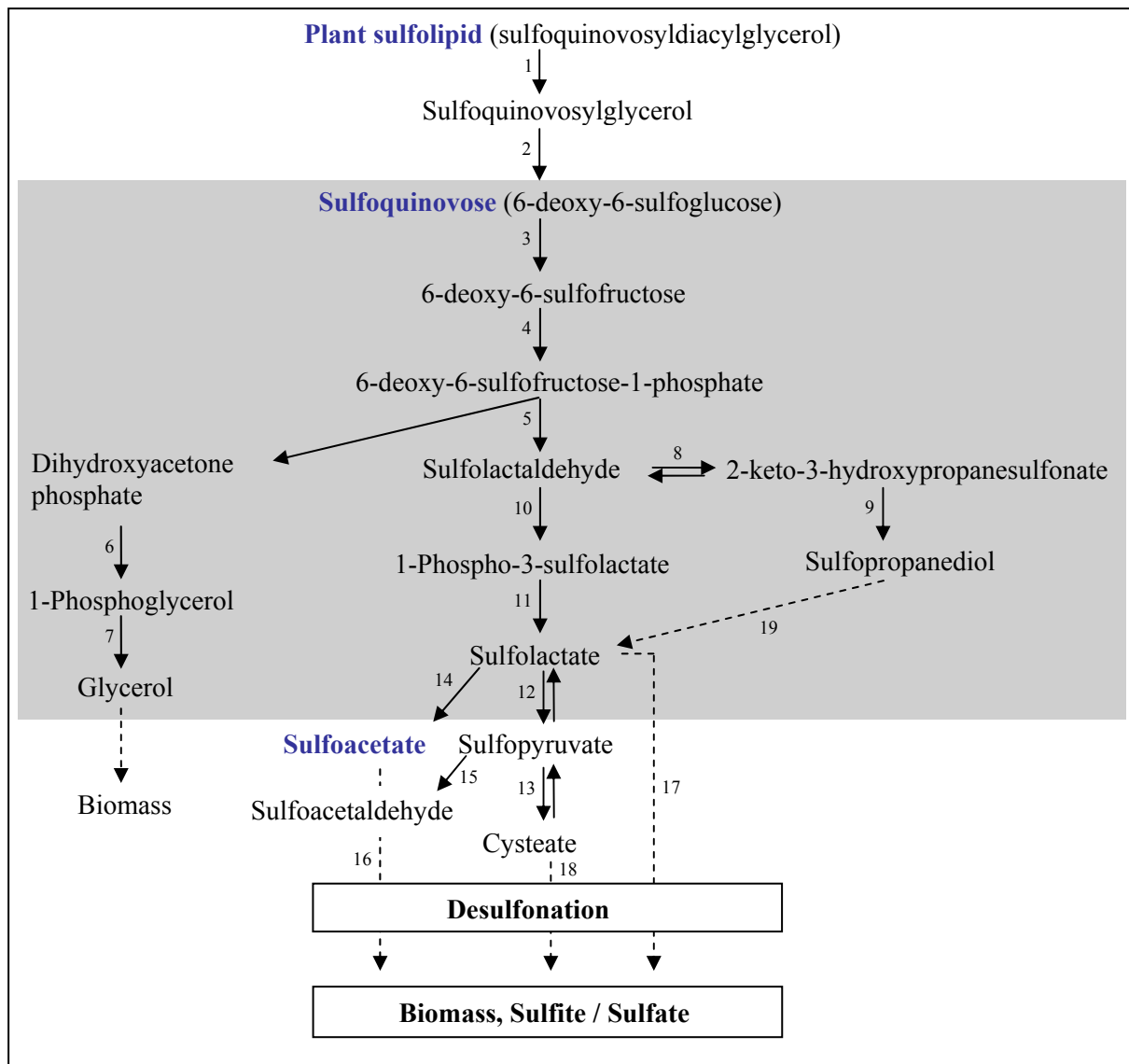


Fig. 4. Simplified scheme of the plant sulfolipid degradation. The sulfoglycolytic part (shaded in grey) was adapted from (Roy *et al.* 2003) and expanded to our current knowledge. 1, plant acylhydrolases; 2, β -glycosidases; 3, phosphoglucose isomerase; 4, phosphofruktokinase; 5, aldolase; 6, glycerolphosphate dehydrogenase; 7, glycerophosphatase; 8, triosephosphate isomerase; 9, glycerophosphate dehydrogenase; 10, glyceraldehyde-3-phosphate dehydrogenase; 11, phosphoglycerate kinase; 12, sulfolactate dehydrogenase; 13, cysteate:2-oxoglutarate aminotransferase; 14, putative decarboxylase; 15, 3-sulfoacetaldehyde decarboxylase; 16, sulfoacetaldehyde acetyltransferase; 17, L-sulfolactate sulfo-lyase; 18, L-cysteate sulfo-lyase; 19, dihydroxypropanesulfonate dehydrogenases (HspNOP; Mayer, unpublished data).

Taurine as a source of sulfoacetate

Previously, sulfoacetate was believed to originate solely from decay of the plant sulfolipid, but recently, it was established that sulfoacetate can also be a product of bacterial taurine nitrogen assimilation in marine and terrestrial bacteria: the terrestrial bacterium *Rhodopseudomonas palustris* CGA009 (Denger *et al.* 2004b) utilizes taurine as a source of nitrogen and excretes sulfoacetate. The same phenomenon was found in the marine bacterium

Neptuniibacter caesariensis MED92: taurine is deaminated yielding SAA which is subsequently oxidized to sulfoacetate (see below for details; Krejčík *et al.* 2008). As this precursor, taurine, is also very widespread in the environment (see above), the microbial conversion of taurine to sulfoacetate apparently also represents an important source of environmental sulfoacetate.

Anthropogenic sources of sulfoacetate

Sulfoacetate is not only of natural origin, but it is also used in households and is thus introduced into the environment via this route as well. Derivatized sulfoacetate (sodium lauryl sulfoacetate) serves as a foaming agent for body care products, e.g. toothpaste, cream soaps, shampoos and powdered bubble baths. Sulfoacetate also serves as a counterion for the synthetic antileukemic agent coralyne (Cho *et al.* 1975). Recently, sulfoacetate has also been discussed as a potential inhibitor of eukaryotic PEPCK (phosphoenolpyruvate carboxykinase), the key enzyme of glyconeogenesis. Hence, sulfoacetate could become an interesting target for the treatment of (diabetes-induced) hyperglycemia (Stiffin *et al.* 2008). Sulfoacetate and its derivatives are thus introduced in significant amounts into the environments by industry.

Microbial degradation of C₂ sulfonates

Degradation of taurine

Taurine is a multifunctional substrate as it contains three of the macroelements (C, N, S) to support bacterial growth. Thus, the compound can serve as a source of carbon, nitrogen or sulfur for aerobic bacteria (Chien *et al.* 1999; Denger *et al.* 2004a). Furthermore, it can be utilized by anaerobic bacteria as electron donor for nitrate reducing bacteria yielding sulfate and CO₂ (Denger *et al.* 1997b). It can also serve as an electron donor for photoautotrophs yielding sulfate and acetate (Novak *et al.* 2004) or as electron acceptor during anaerobic respiration yielding sulfide and acetate (Laue *et al.* 1997). During fermentation of taurine, sulfide or thiosulfate can be formed from the sulfonate moiety (Chien *et al.* 1997; Denger *et al.* 1997a; Denger *et al.* 1999).

Taurine as a source of carbon and energy

The pathway of aerobic taurine dissimilation is well established (Fig. 5) and proceeds via initial deamination, which can be catalyzed by two different types of enzymes: the first one is taurine dehydrogenase (TDH, [EC 1.4.99.2]), where the amino group is cleaved off and free ammonia is released (Kondo and Ishimoto 1987). Genes putatively encoding taurine dehydrogenase (*tauXY*) were found in several organisms, marine as well as terrestrial bacteria,

e.g. *Paracoccus*, *Burkholderia* or *Vibrio* spp. The second possibility is a combination of taurine:pyruvate aminotransferase (Tpa, [EC 2.6.1.77]) (Laue and Cook 2000a; Shimamoto and Berk 1979), where the amino group is transferred to pyruvate yielding alanine, and alanine dehydrogenase (Ald, [EC 1.4.1.1]) (Laue and Cook 2000b) which releases ammonia. Genes encoding this type of enzyme are also widespread and found in many bacteria, e.g. *Rhodococcus*, *Roseovarius* or *Roseobacter* spp. In both cases, during deamination catalyzed by TDH or Tpa, respectively, sulfoacetaldehyde (SAA) is formed. The enzyme for the subsequent desulfonation of SAA was first thought to be a sulfoacetaldehyde sulfo-lyase [EC 4.4.1.12] yielding acetate and sulfite (Kondo and Ishimoto 1972), but was more recently characterized as sulfoacetaldehyde acetyltransferase (Xsc, [EC 2.3.3.15]) yielding acetyl phosphate and sulfite (Ruff *et al.* 2003). Acetyl phosphate is converted to acetyl CoA by phosphate acetyltransferase (Pta, [EC 2.3.1.8]) (Pta, [EC 2.3.1.8]; Stadtman *et al.* 1951) which can then be funneled into central metabolism. The second product of desulfonation, sulfite, can be partly excreted unaltered (Weinitschke *et al.* 2006), but is completely oxidized to sulfate in most bacteria which is catalyzed by sulfite dehydrogenase (SDH), e.g. SorAB (Denger *et al.* 2008; Kappler *et al.* 2000). The above mentioned degradation pathway for taurine (sometimes differing in transport systems and sulfite dehydrogenases) was observed in several microorganisms, e.g. in terrestrial bacteria like *Rhodococcus opacus* ISO-5 (Denger *et al.* 2004a), *Paracoccus denitrificans* NKNIS (Brüggemann *et al.* 2004) or *Rhodobacter sphaeroides* 2.4.1 (Denger *et al.* 2006b), and also in marine organisms like *Ruegeria* (formerly *Silicibacter*) *pomeroyi* DSS-3 (Gorzynska *et al.* 2006).

Recently, a variant of this pathway was discovered in the marine bacterium *Roseovarius* sp. strain 217 (Baldock *et al.* 2007). The organism lacks Pta but rather harbors acetate kinase and acetate-CoA ligase which convert acetyl phosphate to acetate and subsequently activate acetate to acetyl-CoA by a ligation reaction (Fig. 5). Comparison of taurine degradative gene clusters amongst a variety of genome-sequenced organisms indicates that this new variant for acetyl-CoA formation would be less abundant in taurine-degrading bacteria.

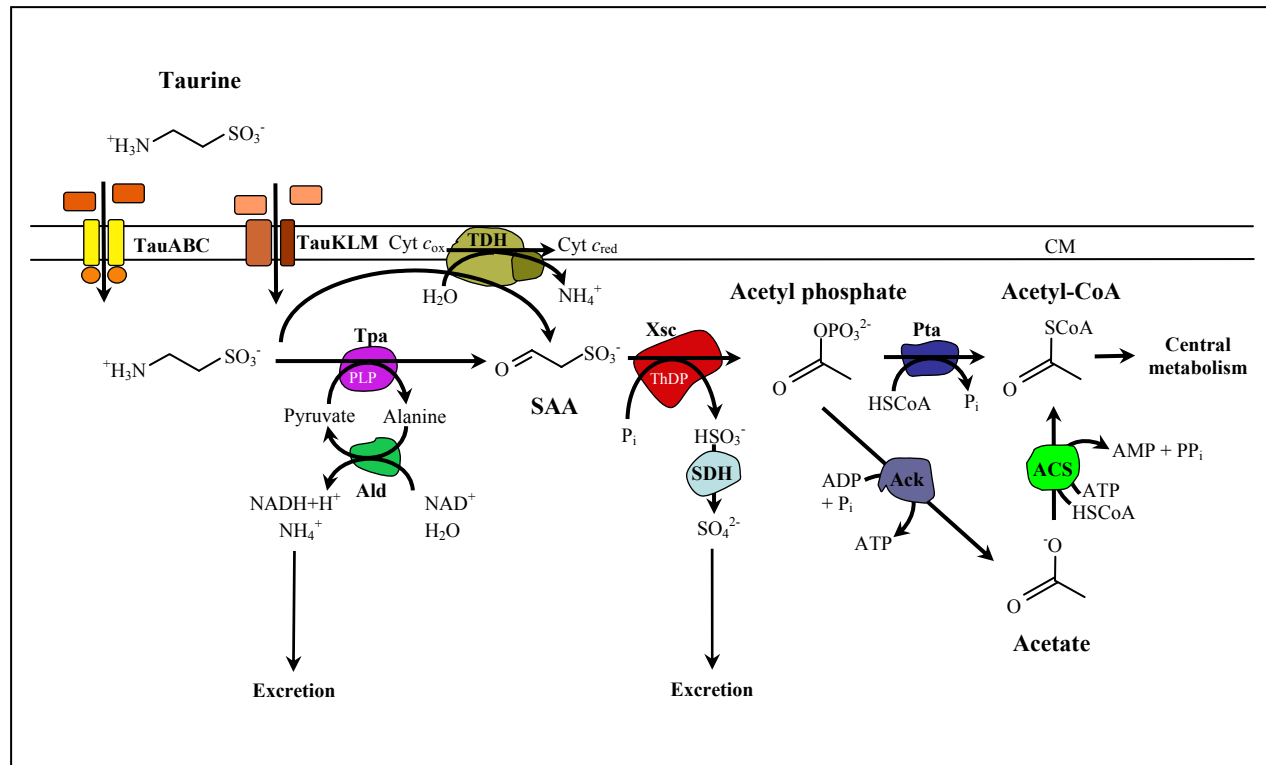


Fig. 5. Degradation pathway for taurine as a carbon source including all variations mentioned in the text above. CM, cytoplasmic membrane; TauABC, ABC (ATP-binding cassette) transporter for taurine; TauKLM, TRAP (tripartite ATP-independent periplasmic) transporter for taurine; TDH, taurine dehydrogenase; Tpa, taurine:pyruvate aminotransferase; PLP, pyridoxal 5'-phosphate; Ald, alanine dehydrogenase; SAA, sulfoacetaldehyde; Xsc, sulfoacetaldehyde acetyltransferase; ThDP, thiamin diphosphate; SDH, sulfite dehydrogenase; Pta, phosphate acetyltransferase; Ack, acetate kinase; ACS, acetyl-CoA synthetase (or acetyl-CoA synthetase).

Taurine as a nitrogen source

Den Dooren de Jong was probably the first microbiologist who tested taurine as a nitrogen source for bacteria but his cultures did not support growth (den Dooren de Jong 1926; Stapley and Starkey 1970). Now, about eight decades later, the assimilation of taurine-nitrogen is still under investigation, but great progress has been made. The first tested organisms to assimilate taurine-nitrogen, e.g. *Rhodococcus opacus* ISO-5 and *Rhodococcus jostii* RHA-1, used the enzymes of the dissimilatory pathway (see Fig. 5) and degraded taurine completely to use taurine-nitrogen. In contrast, other bacteria do not degrade taurine completely, but just cleave the C-N-bond and excrete a sulfonated compound into the growth medium (Fig. 6). For example, the terrestrial bacterium *R. palustris* CGA009 and the marine bacterium *N. caesariensis* MED92 both deaminate taurine to SAA which is subsequently oxidized to sulfoacetate by sulfoacetaldehyde dehydrogenase (SafD, [EC 1.2.1.73]) and excreted into the growth medium (see above). The ammonium ion is incorporated into cell material.

Other organisms, e.g. the terrestrial bacterium *K. oxytoca* TauN1 and the marine bacterium *C. salexigens* DSM 3043 reduce the intermediate SAA to isethionate and excrete the latter compound. The pathway of taurine to isethionate in *C. salexigens* DSM 3043 includes two reactions: (1) the deamination of taurine to sulfoacetaldehyde (SAA) by a novel taurine:2-oxoglutarate aminotransferase (Toa, [EC 2.6.1.55]), and (2) the reduction of SAA to isethionate by NADPH-dependent sulfoacetaldehyde reductase (IsfD, [EC 1.1.1.-]), both of which were separated, identified and characterized (Krejčík *et al.* 2009).

As a third possibility, even the intermediate SAA itself can be excreted unaltered after deamination which was observed in *Acinetobacter calcoaceticus* SW1 (Weinitschke *et al.* 2005). The same phenomenon is hypothesized (on the basis of comparative genomics) for *Loktanella vestfoldensis* SKA53, but this is awaiting further experimental support.

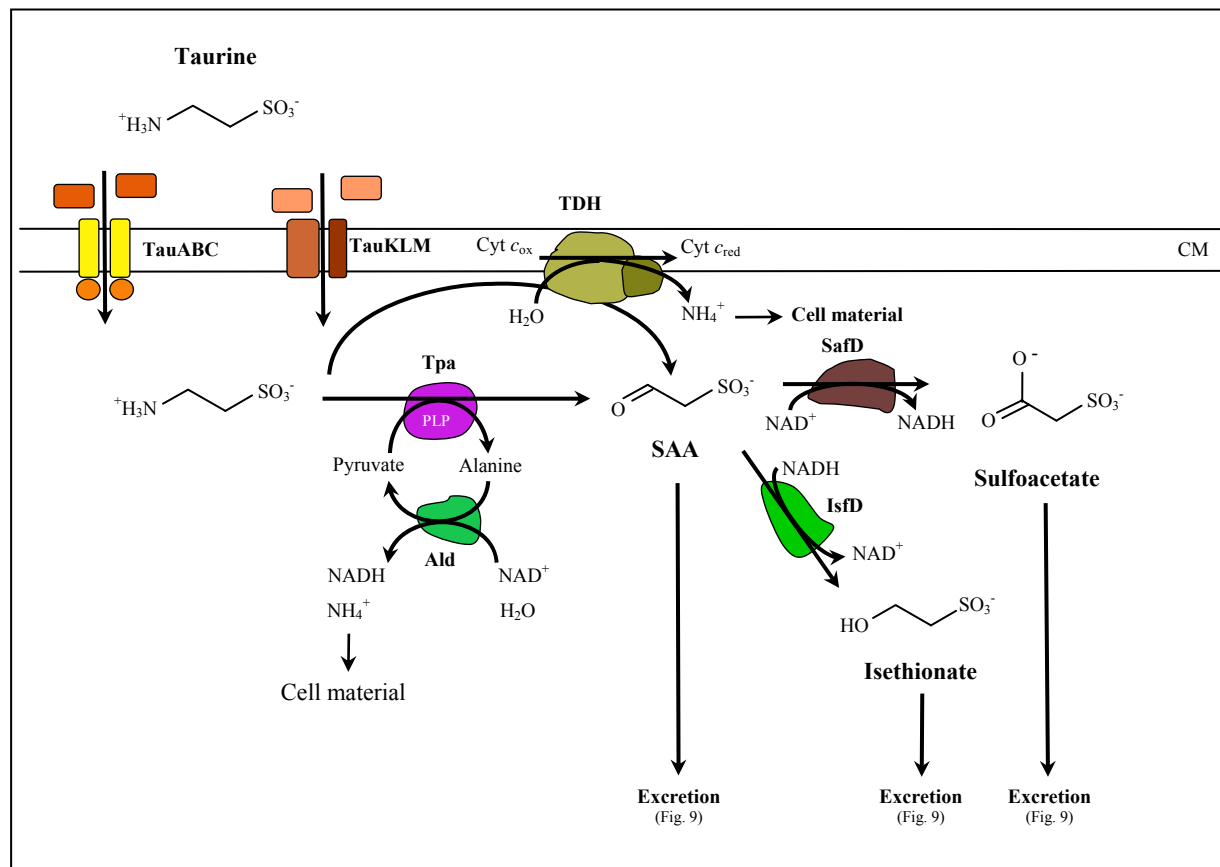


Fig. 6. Possible routes for bacterial assimilation of taurine-nitrogen. CM, cytoplasmic membrane; TauABC, ABC (ATP-binding cassette) transporter for taurine; TauKLM, TRAP (tripartite ATP-independent periplasmic) transporter for taurine; TDH, taurine dehydrogenase; Tpa, taurine:pyruvate aminotransferase (can be replaced by Toa, taurine:2-oxoglutarate aminotransferase); PLP, pyridoxal 5'-phosphate; Ald, alanine dehydrogenase; SAA, sulfoacetaldehyde; SafD, sulfoacetaldehyde dehydrogenase (sulfoacetate forming dehydrogenase); IsfD, sulfoacetaldehyde reductase (isethionate forming dehydrogenase).

Taurine as a sulfur source

While the pathways for utilization of taurine as a carbon source and as a nitrogen source share some of the enzymes (Tpa, TDH, partly Xsc, Pta), the assimilation of taurine-sulfur needs a completely different enzyme: an α -ketoglutarate-dependent taurine dioxygenase (TauD, [EC 1.14.11.17]) is responsible for oxygenolytic desulfonation of taurine (Fig. 7). The enzyme in *Escherichia coli* is only induced during sulfur starvation (Eichhorn *et al.* 1997; van der Ploeg *et al.* 1996) and was also found to desulfonate taurine to yield aminoacetaldehyde in *R. opacus* ISO-5 in sulfate-deprived medium (Denger *et al.* 2004a). During utilization of taurine as a sulfur source, the ABC (ATP-binding cassette) transporter TauABC was found to be responsible for transport of the compound (van der Ploeg *et al.* 1996).

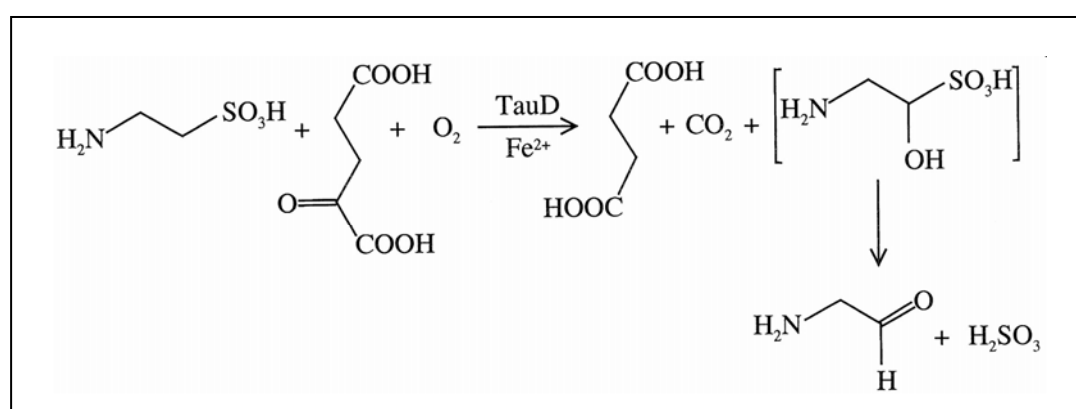


Fig. 7. Reaction of taurine dioxygenase (TauD). Taurine is oxygenolytically desulfonated to a labile intermediate (1-hydroxy-2-aminoethanesulfonate) which is decomposed spontaneously to aminoacetaldehyde and sulfite. Scheme from (Eichhorn *et al.* 1997).

Regulation of taurine degradation

The expression of TauD during utilization of taurine as a sulfur source under aerobic conditions in *E. coli* is dependent on CysB, the transcriptional activator of the cysteine regulon responsible for cysteine biosynthesis (Tei *et al.* 1990). TauD is only expressed in case of sulfur-starvation.

During utilization of taurine as a source of carbon or nitrogen, other regulatory proteins must be present. When Xsc was characterized (Ruff *et al.* 2003), the flanking regions of the *xsc* loci in different bacteria were investigated, and a gene which was adjacent to taurine degradative genes in all organisms was identified. The nearest homologues of the encoded protein were GntR family transcriptional regulators, thus, the gene product was presumed to represent a potential regulator of taurine metabolism under non-sulfur starvation conditions. This regulatory protein was later named ‘TauR’ and tentatively assigned to the MocR subfamily (Rigali *et al.* 2002) of GntR family transcriptional regulators (Brüggemann *et al.* 2004). Since

then, *tauR* was recognized in almost all taurine dissimilating and taurine-nitrogen assimilating bacteria, which supported the hypothesis.

Only recently, the involvement of TauR in taurine metabolism has been proven in *Rhodobacter capsulatus*: it was shown that TauR is necessary for transcription of *tpa*, and that TauR binds to direct repeats of the *tpa* promoter using a helix-turn-helix (HTH) motif (Wiethaus *et al.* 2008), as shown for the MocR-like protein GabR of *Bacillus subtilis*. This negative autoregulator acts as an activator of genes involved in γ -aminobutyrate (GABA) nitrogen utilization (Belitsky 2004).

Degradation of isethionate

Degradation of isethionate under anoxic conditions

Under anoxic conditions, isethionate can serve as an electron donor during nitrate reduction in *P. pantotrophus* NKNCYSA and *P. denitrificans* NKNIS (Denger *et al.* 1997b; Mikosch *et al.* 1999) yielding CO₂ and sulfate. The compound can also be utilized as an electron acceptor, e.g. in *Desulfovibrio desulfuricans* IC1, *Desulfovibrio* sp. strain RZACYSA, *Bilophila wadsworthia* RZATAU and *Desulfitobacterium* spp. yielding acetate and sulfide (Laue *et al.* 1997; Lie *et al.* 1996; Lie *et al.* 1999), or can be fermented by yielding acetate and a mixture of sulfate and sulfide (Denger *et al.* 1999).

Isethionate as a source of carbon and energy

Several aerobic bacteria can utilize isethionate as a sole source of carbon and energy, e.g. *Achromobacter xylosoxidans* NCIMB 10751, *Burkholderia* sp. ICD (formerly *Acinetobacter* sp. ICD), *Ralstonia* sp. strain EDS1, and *P. denitrificans* NKNIS (Brüggemann *et al.* 2004; Denger and Cook 2001; King *et al.* 1997; Kondo *et al.* 1977). Isethionate degradation under those conditions is assumed to proceed via SAA and subsequent desulfonation by Xsc (Cook and Denger 2002; King *et al.* 1997; Kondo *et al.* 1977). The enzyme responsible for oxidation of isethionate to SAA was postulated to be a membrane-bound, FAD-dependent dehydrogenase in *A. xylosoxidans* (Kondo *et al.* 1977). Three decades later, isethionate utilization in another bacterium, *P. denitrificans* NKNIS, was investigated, and a particulate, presumably 62 kDa protein was found to catalyze the reaction (Brüggemann *et al.* 2004). The enzyme used cytochrome *c* as an electron acceptor. Genes coding for isethionate dehydrogenase, now tentatively attributed to the class of cytochrome *c*-dependent alcohol dehydrogenases [EC 1.1.2.-], were unknown. Therefore, there were also no candidate genes available for regulatory or transport phenomena.

Isethionate as a source of sulfur

Isethionate can also serve as a sulfur source for aerobic bacteria, e.g. *Pseudomonas aeruginosa* PAO1, *Comamonas acidovorans* 14 and 105, *Acidovorax facilis* 332 and some enteric bacteria including several *E. coli* spp. (Seitz *et al.* 1993; Uria-Nickelsen *et al.* 1993). King and Quinn tested 100 enrichment cultures of which more than 90 % were able to use isethionate (and other C₂ sulfonates) as a sulfur source showing the widespread potential of soil and freshwater bacteria to assimilate sulfonate-sulfur (King and Quinn 1997b). Desulfurization of isethionate seems to require an intact sulfite reductase enzyme system and thus makes sulfite a likely intermediate of sulfonate-sulfur assimilation (Kertesz *et al.* 1999; Uria-Nickelsen *et al.* 1994).

Whereas taurine is desulfonated by the dioxygenase TauD when used as a source of sulfur (see above), there is a second known oxygenolytic desulfonation system which desulfonates a range of aliphatic sulfonates other than taurine under sulfur-limited conditions: SsuDE is a two-component alkanesulfonate monooxygenase consisting of (1) SsuD, an FMNH₂-dependent monooxygenase, and (2) SsuE, an NAD(P)H-dependent FMN reductase which provides the FMNH₂ for SsuD (van der Ploeg *et al.* 1998; van der Ploeg *et al.* 2001). SsuD is assumed to be responsible for utilization of isethionate as a sulfur source. Under these conditions, isethionate was shown to be transported into the cell via two different ABC transport systems, TauABC and SsuABC (Eichhorn *et al.* 2000). Several yeasts such as *Saccharomyces cerevisiae* are also able to utilize sulfonates as alternative sulfur sources in the absence of sulfate (Hogan *et al.* 1999). A sulfonate/ α -ketoglutarate dioxygenase seems to catalyze desulfonation of a variety of sulfonates including isethionate.

Degradation of sulfoacetate

The fact that sulfoacetate is susceptible to bacterial degradation has been known for decades (Martelli and Souza 1970). Sulfoacetate can be degraded under anaerobic conditions by diverse organisms: it serves as electron acceptor for growth of *B. wadsworthia* and *Desulfovibrio* sp. strain RZACYSA, as an electron donor for *P. pantotrophus* NKNCYSA during nitrate reduction where the sulfonate moiety was recovered as sulfate (Mikosch *et al.* 1999; Rein and Cook unpublished data).

Under aerobic conditions, the degradation of sulfoacetate in a *Pseudomonas* strain has been observed concomitant with transient glycolate excretion (Martelli and Souza 1970). Analogous to the degradation of e. g. fluoroacetate (Goldman 1965; Kurihara and Esaki 2008) or chloroacetate (Liu *et al.* 1998) which is initiated by haloacetate dehalogenases

[EC 3.8.1.3], hydrolytic cleavage of sulfoacetate was assumed. Unfortunately, the proposed pathway could not be resolved in detail because the organism was lost.

In contrast to this hypothesis, it was later discussed that sulfoacetate degradation could also proceed via sulfoacetaldehyde (King and Quinn 1997a) in *Aureobacterium* sp. strain SFCD2, *Comamonas acidovorans* SFCD1 and *Ralstonia* sp. strain EDS1 (Denger and Cook 2001).

However, until now it remained unclear which enzymes lead to the formation of this common intermediate of C₂ sulfonate metabolism. The first steps of such a degradation pathway of sulfoacetate would include the reduction of a carboxylic acid to an aldehyde. This type of reaction is known from anabolism and could proceed via phosphorylated intermediates (Denger and Cook 2001) as described for the biosynthesis of proline (Adams and Frank 1980; Krishna *et al.* 1979) and lysine (Viola 2001).

Sulfoacetate can also serve as a sulfur source for bacteria as reported for *Aureobacterium* sp. strain SFCD2. Under these conditions, sulfoacetaldehyde acetyltransferase seems to be not involved (King and Quinn 1997a), which is analogous to taurine-sulfur assimilation where also a different set of enzymes is necessary opposed to its utilization as a source of carbon (see above).

Transport phenomena involved in sulfonate metabolism

Sulfonates are highly polar and are charged molecules at a physiological pH, thus, transport of the compounds to enter the cell is regarded as axiomatic (Graham *et al.* 2002). But not much is known about transport phenomena related with sulfonates. To our knowledge from physiological data, there is a wide range of sulfonates which have to be imported into the cell, and there are also sulfonates (e.g. isethonate, sulfoacetate, sulfoacetaldehyde) or degradation products of sulfonates (e.g. sulfite, sulfate, ammonia) which have to be exported.

Import of sulfonates

Taurine import in mammals

Mammals possess a taurine transporter called TauT which belongs to the group of taurine:Na⁺ symporter [TC 2.A.22.3.3] in the Neurotransmitter:Sodium Symporter (NSS) family (Tappaz 2004). Mammalian TauT proteins have 12 transmembrane helices and share approximately 90 % sequence identity amongst each other. They are high-affinity transporters for taurine and require at least two Na⁺ and one Cl⁻ ions to transport one molecule of taurine. In addition to TauT, another taurine transporter in mammals involved in intestinal taurine transport was found recently: the electrogenic, proton-dependent amino acid:H⁺ symporter PAT1 [TC 2.A.18.8.1], a Cl⁻-independent low-affinity transporter, belonging to the family of amino

acid/auxin permeases (AAAP). PAT1 seems to predominate at higher taurine concentrations in contrast to TauT which is predominant at low taurine concentrations. Thus, it was hypothesized that PAT1 could be responsible for the bulk taurine uptake, whereas TauT could be important for taurine capture between meals (Anderson *et al.* 2009).

Taurine import in bacteria

Currently, two different transport systems are presumed to be responsible for taurine import (Fig. 5), and one additional novel transport protein is under investigation (see General Discussion).

The best characterized transport system is the ATP-binding cassette (ABC) transporter TauABC [TC 3.A.1.17.1] of *E. coli* whose involvement in taurine transport was confirmed by mutational analysis (Eichhorn *et al.* 2000). TauABC consists of three components: TauA, an extracytoplasmic taurine binding protein (Javaux *et al.* 2007), TauC, a permease, and TauB, an ATPase. TauABC is involved in assimilation of taurine-sulfur in *E. coli*, and *tauABC*-like genes were also found in many other genomes of taurine-sulfur assimilating bacteria (Cook and Denger 2002; Kertesz 2001; Masepohl *et al.* 2001).

We presume TauABC systems to be also involved in taurine import in taurine dissimilating or taurine-nitrogen assimilating bacteria: *tauABC* gene candidates are often located adjacent to taurine degradative gene clusters (containing *xsc*, *pta* etc.), e.g. in *P. pantotrophus* NKNCYSA (Brüggemann *et al.* 2004), *S. meliloti* (Ruff *et al.* 2003), *R. pomeroyi* DSS-3 (Gorzynska *et al.* 2006), and *N. caesariensis* MED92 (Krejčík *et al.* 2008). Transcriptional analysis in *R. pomeroyi* DSS-3 (Gorzynska *et al.* 2006) and in *N. caesariensis* MED92 (Krejčík *et al.* 2008) showed substrate-dependent inducible transcription of *tauABC* which supports the involvement of TauABC in taurine dissimilation and taurine-nitrogen assimilation, respectively. In some bacteria, the *tauABC* genes are not directly adjacent to the taurine degradative gene clusters, but are located elsewhere on the chromosome, e.g. in *Cupriavidus necator* H16.

The second transport system for taurine is the tripartite ATP-independent periplasmic transporter TauKLM [TC 2.A.56.4.1], where TauK is a periplasmic substrate binding receptor, TauL is a small integral cytoplasmic membrane protein with 4 transmembrane helices (TMHs), and TauM is a large integral cytoplasmic membrane protein with 12 TMHs. TauKLM was first described as a potential alternative for TauABC in *R. sphaeroides* 2.4.1 and *P. denitrificans* NKNIS (Brüggemann *et al.* 2004) where the *tauKLM* genes are present in the taurine gene clusters instead of *tauABC*. Reverse transcription PCR in *R. sphaeroides* 2.4.1 recently showed inducible transcription of *tauKLM* and thus gave the

first experimental evidence for involvement of the transport system in taurine import (Denger *et al.* 2006b).

Import of other sulfonates

A member of the DUF81 (domain of unknown function) family of proteins, TsaS, was proven to be involved in 4-toluenesulfonate import in *Comamonas testosteroni* (Mampel *et al.* 2004). TsaST are two membrane spanning proteins, and reverse transcription data showed inducible transcription of both components during growth with toluenesulfonate. TsaT is an outer membrane protein, whereas TsaS has the characteristics of an inner membrane protein and is a member of the DUF81 family. Heterologous expression of TsaT in porin-deficient *E. coli* showed that TsaT alone was not able to transport TSA but TsaS was needed as well. This finding is interesting for us because members of the same protein family (DUF81) are currently postulated to be involved in sulfite export and in export of sulfonates. This hypothesis is supported by inducible transcription of *tauE*, which encodes a DUF81 family protein, during growth with an organosulfonates (see Chapter 1; Mayer and Cook 2009; Weinitschke *et al.* 2007).

Export of sulfonates or their metabolites

Export of sulfonates

Recently, DUF81 family transporters were also considered to be responsible for export of sulfonates (Fig. 8). For example, during growth of *N. caesariensis* MED92 with taurine as a nitrogen source, sulfoacetate is formed (see above) and recovered in the growth medium, thus requiring a transport mechanism. Adjacent to the genes encoding enzymes for taurine-nitrogen assimilation in this organism, two genes (*safE1* and *safE2*) are present encoding putative transport proteins of the DUF81 family. Thus, it was assumed that SafE1 and SafE2 could be responsible for sulfoacetate export in *N. caesariensis* MED92. The fact that *safE1* and *safE2* were inducibly transcribed during growth with taurine (*safE1* throughout growth, *safE2* in the early growth phase only), supported the above mentioned hypothesis (Krejčík *et al.* 2008).

C. salexigens DSM 3043, which excretes isethionate when assimilating taurine-nitrogen (see above), as well encodes a DUF81 family protein (termed IsfE) adjacent to other genes whose products are involved in taurine utilization. Inducible transcription of *isfE* is observed making it likely that isethionate is exported by IsfE (Krejčík *et al.* 2009). Considering these data, DUF81 family transporter may play an important role in bacterial sulfonate utilization, being involved in both import and export of diverse sulfonates and their metabolites.

There are also organisms excreting the intermediate sulfoacetaldehyde itself (*A. calcoaceticus* SW1, see above) during taurine-nitrogen assimilation, thus, an exporter for the compound has to be present, but it is still unidentified (Weinitschke *et al.* 2005).

Export of ammonium

In organisms which utilize taurine as a carbon source, the excess ammonium ion is found in stoichiometric amounts in the culture medium, thus implying transport of the ion across the cytoplasmic membrane. Ammonia-methylammonia transporter of the ammonia transporter channel family (Amt, [TC 1.A.11]) are presumed to be responsible for transport of ammonia over biological membranes (Thomas *et al.* 2000). When the genome of the taurine degrading organism *R. pomeroyi* DSS-3 (Moran *et al.* 2004) showed to encode several AmtB homologues, it was presumed that one of those could be responsible for excretion of excess ammonia during growth with taurine. Evidence for this hypothesis was provided when the gene *amtB* was found to be transcribed during growth of *R. pomeroyi* DSS-3 with taurine (Gorzynska *et al.* 2006).

Export of sulfite and sulfate

In organisms which utilize taurine or other sulfonates as carbon sources, sulfite is formed from the sulfonate moiety after desulfonation. Under nitrogen-limited conditions, the same situation is found in some organisms (e.g. *R. opacus* ISO-5, see above). Therefore, during growth of these bacteria, sulfite – or more often sulfate – is found in the culture supernatant, implying export of the respective ion across the cytoplasmic membrane. Sulfite can be toxic and can also cause osmotic stress in the cell, a problem which is presumably solved by either (1) oxidation by cytoplasmic sulfite dehydrogenase and export of the generated sulfate, or (2) transport of sulfite into the periplasm where periplasmic sulfite dehydrogenases like SorAB can oxidize the compound to sulfate (Denger *et al.* 2008; Kappler *et al.* 2000).

The protein CuyZ [TC 9.B.7.1.1], was assumed to be responsible for sulfite excretion during cysteate degradation: in reverse transcription experiments with *R. pomeroyi* DSS-3, the gene *cuyZ* was inducibly transcribed and co-transcribed with the gene encoding the desulfonative enzyme for cysteate (Denger *et al.* 2006a). Inducible transcription of *cuyZ* was also found in *Roseovarius nubinhibens* ISM during growth with sulfolactate which proceeds via cysteate (Denger *et al.* 2009).

The second possibility, i.e. oxidation of sulfite directly in the cytoplasm by soluble sulfite dehydrogenases, would imply the need for a sulfate exporter instead of a sulfite exporter. TauZ [TC 9.B.63.1.1] was assumed to be such a sulfate exporter (Brüggemann *et al.* 2004)

which was later supported by reverse transcription experiments in two different organisms where *tauZ* was inducibly transcribed during growth with taurine (Denger *et al.* 2006b; Rein *et al.* 2005). In addition, *SuyZ*, which is a homologue of *TauZ*, was presumed to be the exporter responsible for sulfate translocation during growth with cysteate (proceeding via sulfolactate): the gene *suyZ* was found to be inducibly transcribed and co-transcribed with the genes encoding the desulfonative reactions (Rein *et al.* 2005).

As the nature and location of the sulfite oxidizing enzymes in different bacteria is not yet fully elucidated, it remains unclear in most of the organisms whether sulfite is oxidized in the cytoplasm or in the periplasm, and therefore the question of sulfite or sulfate transport is largely unanswered. But considering recent results concerning sulfite dehydrogenase (Denger *et al.* 2008; Denger & Cook, unpublished data), it seems to be likely that most sulfite dehydrogenases in bacteria are periplasmic, and thus primarily sulfite needs to be transported across the cytoplasmic membrane. Therefore, we now presume that the transport proteins previously described as sulfate transporters (*TauZ*, *SuyZ*) are rather sulfite exporters.

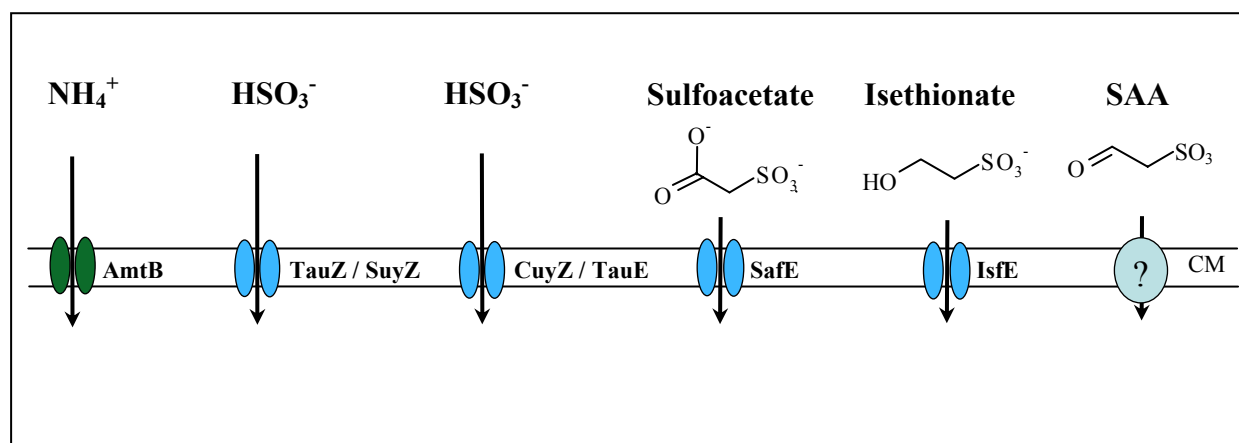


Fig. 8. Different export systems for metabolites and end products of taurine degradation. CM, cytoplasmic membrane; AmtB, ammonia-methylammonia transporter of the ammonia transporter channel family; TauZ / SuyZ, sulfite transporter of the PSE (putative sulfate exporter) family; CuyZ / TauE, DUF81-sulfite exporter of the TSUP (putative 4-toluene sulfonate uptake permease) family; SafE (sulfoacetate formation / excretion), putative DUF81-sulfoacetate exporter of the TSUP family; IsfE (isethionate formation / excretion), putative DUF81-isethionate exporter of the TSUP family.

Objectives of this study

The central aim of this PhD thesis was to establish or to verify, respectively, degradative pathways for (1) taurine, (2) isethionate and (3) sulfoacetate, combining physiological, biochemical and molecular experimental setups. The study focuses on *C. necator* H16, but to assess biodiversity, enzyme activities as well as gene transcription patterns associated with sulfonate degradation were compared in various other organisms belonging to diverse bacterial clades and living in different environments.

There was also a strong interest – besides identifying the catabolic enzymes of the respective pathways – to investigate transport systems involved in C₂-sulfonate degradation.

Different stages of previous knowledge were at hand (see above) for the three central topics of this study:

For taurine degradation (1), the degradative enzymes (taurine dehydrogenase / taurine:pyruvate aminotransferase, sulfoacetaldehyde acetyltransferase, and phosphate acetyltransferase) were already characterized in other organisms, just awaiting confirmation in *C. necator* H16 because the encoding genes were found in the genome. But of particular interest was the export of sulfite: due to our recently gained knowledge of a periplasmic sulfite dehydrogenase in H16 (Denger *et al.* 2008), sulfite export into the periplasm was obviously necessary for detoxification and oxidation of sulfite to sulfate.

For isethionate degradation (2), convergence of isethionate degradative pathway with those of other C₂ sulfonates at SAA was presumed but not confirmed in *C. necator* H16. Although some facts about isethionate dehydrogenase were known from previous studies (Brüggemann *et al.* 2004), no purification has been achieved, and the coding genes remained unknown. One of the major aims of this study was to identify the corresponding genes encoding the enzymes responsible for isethionate transport and degradation.

For sulfoacetate degradation (3), least was known at the beginning of this study. We intended to answer the question if sulfoacetate degradation in *C. necator* H16 would proceed via SAA – as indicated by King and Quinn (King and Quinn 1997a) – or via glycolate, as suggested by Martelli and Souza (Martelli and Souza 1970). However, we wanted to define the initial steps of sulfoacetate leading either from sulfoacetate to glycolate or from sulfoacetate to SAA. The biochemical reactions should be investigated, the possible intermediate(s) should be identified and the corresponding genes ought to be assigned to the enzyme activities.

CHAPTER 1

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

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SUMMARY

The degradation of taurine, isethionate or 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 and 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 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 (Fig. 1a; Ruff *et al.* 2003). 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*] *pomeroyi* DSS-3 (Yi *et al.* 2007), the orthologue, CuyZ, is a sulfite exporter (Cook *et al.* 2006). 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, were found in *Burkholderia xenovorans* LB400 and *C. necator* JMP134 (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 *tauE* is transcribed inducibly when *C. necator* H16 is grown with the C₂ sulfonates taurine, isethionate and sulfoacetate.

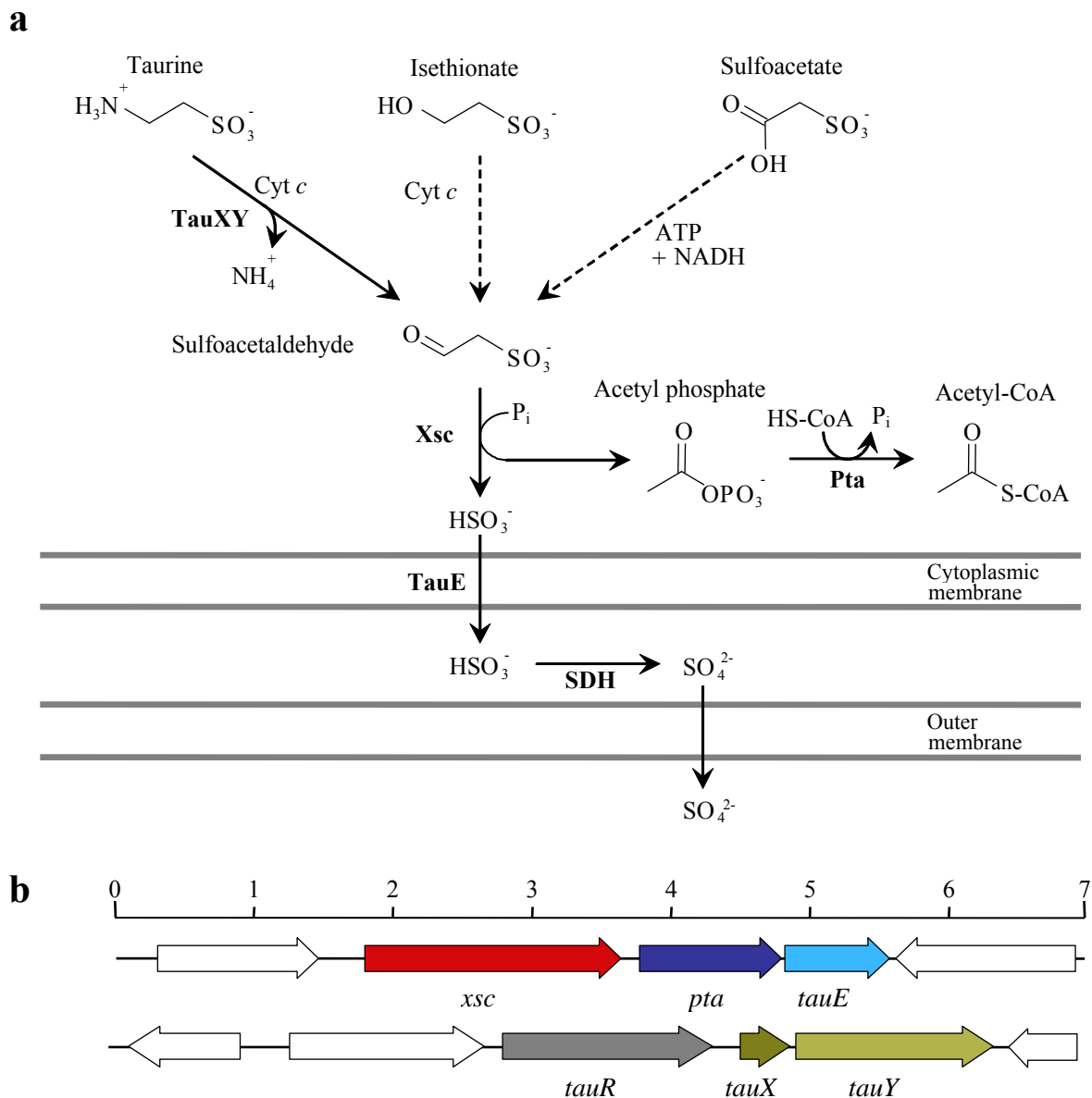


Fig. 1. Pathways for the dissimilation of taurine, isethionate and sulfoacetate in *C. necator* H16 (a) and gene clusters for taurine metabolism in *C. necator* H16 (b). Conversions for which genes and enzymes are currently unknown are indicated by dashed lines. The gene loci of *tauR-tauY* and *xsc-tauE*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

MATERIAL AND 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-cap 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 5 000 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 (OD₅₈₀) and quantified as protein in a Lowry-type reaction (Cook and Hütter 1981). Taurine was derivatized with dinitrofluorobenzene and quantified after separation by HPLC (Denger *et al.* 1997b). Isethionate and sulfoacetate were determined by ion chromatography (Denger *et al.* 2004b; Styp von Rekowski *et al.* 2005). Sulfite was quantified as the fuchsin derivative (Denger and Cook 2001). Sulfate was determined turbidimetrically as a suspension of BaSO₄ (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. Chromosomal DNA was isolated from bacteria, as described by Desomer *et al.* (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 molecular marker.

Table 1. Primers used in this study

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

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 *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 were degraded via sulfoacetaldehyde (Fig. 1a; Brüggemann *et al.* 2004; Denger and 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 sulfite dehydrogenase (SDH) was high in all extracts from sulfonate-grown cells, and low in extracts from acetate-grown cells (Table 2).

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 <i>xsc</i>	+ [‡]	+	+	-
Phosphate acetyltransferase (Pta)	ND	ND	ND	ND
Transcription of <i>pta</i>	+	+	+	-
Transcription of <i>tauE</i>	+	+	+	-
SDH	30	16	37	1

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

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. 1a, 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 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 and 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 and 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 (Denger *et al.* 2008). 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 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 it 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.

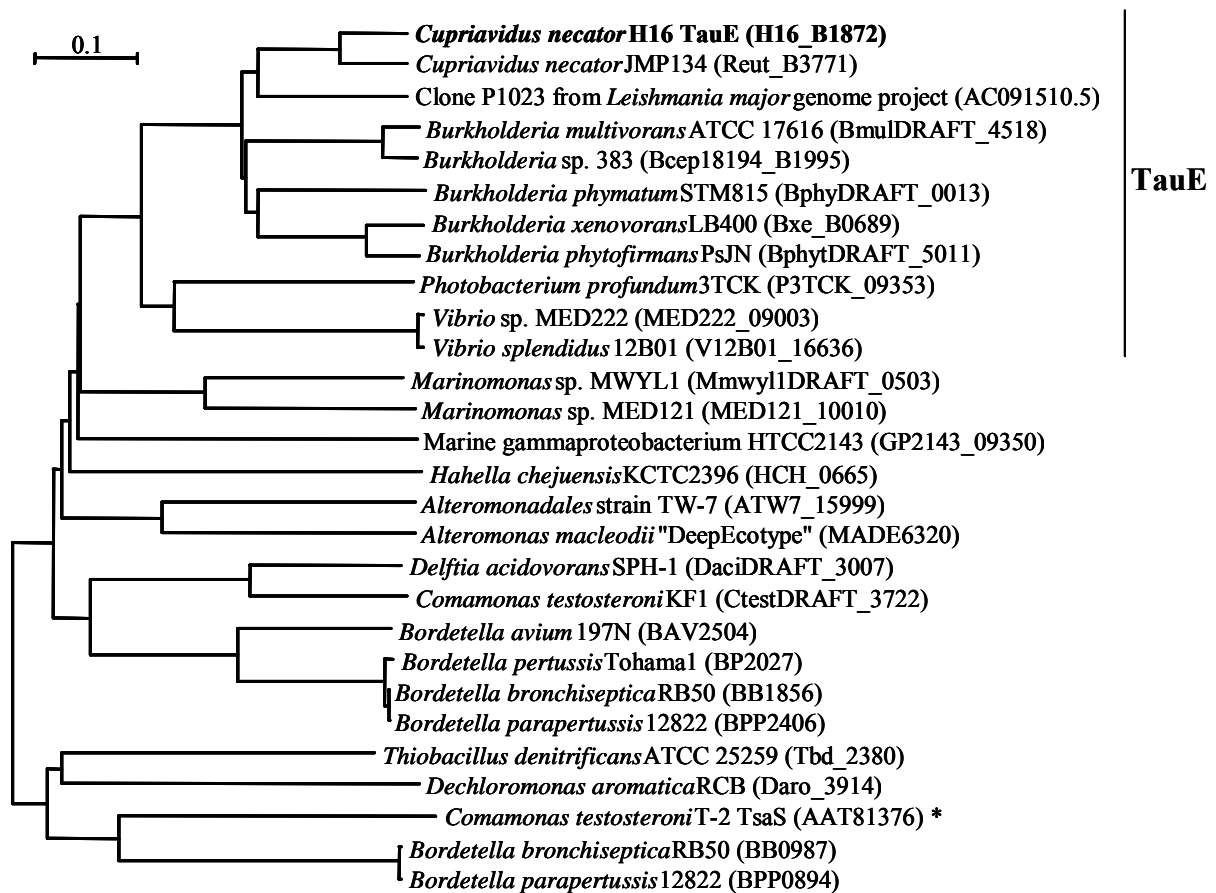


Fig. 2. Phylogenetic relationships of the *C. necator* H16 TauE (bold type) and orthologues. The neighbour-joining (NJ) plot is from a CLUSTAL_X alignment of amino acid sequences most closely related to TauE. The locus tag from the genome sequencing project or the GenBank accession no. is given in parentheses.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

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CHAPTER 2

Gene clusters involved in isethionate degradation in terrestrial and marine bacteria

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SUMMARY

Isethionate (2-hydroxyethanesulfonate) is found at high concentrations in compartments of marine and terrestrial environments, and its bacterial dissimilation, recognized for three decades, apparently involves an undefined, 62-kDa, membrane-bound isethionate dehydrogenase [EC 1.1.2.-] as well as sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15] in *Paracoccus denitrificans* NKNIS. We deduced, from a flanking region (termed *iseJKLMR*) of the *xsc* pseudogene in *Ruegeria pomeroyi* DSS-3, that (i) isethionate dehydrogenase, IseJ, could be a member of the glucose-methanol-choline oxidoreductase-family, (ii) isethionate is transported into the cell by a TRAP transporter, IseKLM, and (iii) the cluster is controlled by the IclR-type transcriptional regulator, IseR. Orthologues of this cluster were found in several marine isolates, and the available representatives utilized isethionate: further, inducible transcription of *iseJKLM* was observed in *R. pomeroyi* DSS-3. Orthologues of IseR and IseJ were found in terrestrial isolates, together with a candidate major facilitator superfamily transporter (IseU). Growth kinetics of *Cupriavidus necator* H16 with isethionate could be followed in detail and complete mass balance was observed. Inducible, membrane-bound isethionate dehydrogenase was observed as was inducible transcription of *iseJU*. Orthologues of *iseRJ* were also found in *Rhodobacterales* sp. HTCC2150, which was shown to utilize isethionate, and this implicated a third transport system for isethionate. Further, the predicted *iseR*, *iseU* and *iseJ* genes were found and sequenced in *P. denitrificans* NKNIS. Whereas IseJ is widespread, there is obviously considerable biodiversity in isethionate transport.

INTRODUCTION

The importance of isethionate (2-hydroxyethanesulfonate) was realized when the compound was shown to be the major anion (about 230 mM) in the squid giant axon (Koechlin 1954); it was then found to be a normal component in mammalian tissue (Kumpulainen *et al.* 1982) and in human urine (Jacobsen *et al.* 1967). The source of this isethionate was considered to be the gut flora (Fellman *et al.* 1980), and a mechanism of isethionate formation from taurine (2-aminoethanesulfonate) in bacteria was established recently (Krejčík *et al.* 2009; Styp von Rekowski *et al.* 2005). The compound is found in large amounts (at about 250 mM) in red algae (Barrow *et al.* 1993; Hellio *et al.* 2004; Holst *et al.* 1994), but the very large amounts (we calculated concentrations of about 2 M) on orb spiders' webs have been recognized only recently (Townley *et al.* 2006). Isethionate is widely used in commerce as a counter-ion in the formulation of cationic pharmaceuticals and, in derivatized form, in shampoos and soap replacements (e.g. Delobel and Pradinaud 2003; Sun *et al.* 2003).

We presume that the presence of isethionate in urine, on spiders' webs and from marine sources explains the widespread bacterial dissimilation of the compound indicated in reviews (Cook and Denger 2002; Lie *et al.* 1998). The degradative pathway of isethionate requires an unknown uptake system (see Cook and Denger 2002) and, in *Paracoccus denitrificans* NKNIS, it apparently involves an inducible, 62-kDa, cytochrome *c*-dependent, membrane-bound isethionate dehydrogenase [EC 1.1.2.-], whose product, sulfoacetaldehyde, is desulfonated to acetyl phosphate and sulfite by sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15.] (Brüggemann *et al.* 2004; Cook and Denger 2002; Ruff *et al.* 2003; Weinitschke *et al.* 2007). One *xsc* gene is known in strain NKNIS: however, it is located in a gene cluster that encodes taurine degradation but not isethionate degradation, for which the genes were unknown (Brüggemann *et al.* 2004).

The dissimilation of compounds related to taurine (2-aminoethanesulfonate) and isethionate is believed to converge at a single *xsc* in many organisms (Cook and Denger 2002; Weinitschke *et al.* 2007). Some organisms, however, have *xsc* paralogues and we thought that the presence of two *xsc* genes in a genome might represent different complete pathways for different substrates. *Ruegeria* [formerly *Silicibacter*] *pomeroyi* DSS-3 (Yi *et al.* 2007) contains the *xsc* gene (SPO3561), which is expressed during growth with taurine (Gorzynska *et al.* 2006). Additionally, a gene is present, annotated as *xsc* pseudogene (SPO2360) due to its lack of key amino acids in the active site. The presence of a second *xsc* (SPO2360) and the knowledge that the organism utilizes isethionate (Denger *et al.* 2006a), led us to the hypothesis that the flanking genes could encode isethionate uptake and degradation genes (see below). Orthologues of the putative isethionate dehydrogenase (IseJ) were found in many other organisms, several of them known isethionate degraders, whereas *iseJ* was absent in the genomes of isethionate non-utilizers. The hypothesis was tested in the terrestrial isethionate degrader *C. necator* HI6 and in the marine isethionate degrader *R. pomeroyi* DSS-3, where data from growth kinetics, enzymology and reverse transcription PCR largely confirmed the initial hypothesis.

MATERIALS AND METHODS

Materials

Isethionate (>98 %) was purchased from Fluka (Buchs, Switzerland). Other commercial chemicals (about 99 %) were from Fluka, Merck (München, Germany) or Sigma-Aldrich (Deisenhofen, Germany). Solutions of isethionate, taurine and acetate were sterilised by autoclaving, which was not detrimental to these stable compounds. Oligonucleotides were

synthesized by Microsynth (Balgach, Switzerland). Taq DNA polymerase, M-MuLV reverse transcriptase and RNase-free DNase were purchased from Fermentas GmbH (St Leon-Rot, Germany) and they were used as provided by the supplier. Total RNA was isolated using the E.Z.N.A. bacterial kit purchased from Omega Bio-Tek (Doraville, USA). A 50 bp DNA ladder was obtained from Fermentas. PCR products were purified with the E.Z.N.A. Cycle-Pure Kit from Omega-Biotek (Doraville, USA).

Organisms, growth media and preparation of cell-free extracts

C. necator H16 (DSM 428) (Pohlmann *et al.* 2006) was kindly provided by B. Bowien, *R. nubinhibens* ISM (González *et al.* 2003) by M. A. Moran and *B. xenovorans* LB400 (Chain *et al.* 2006) by J. M. Tiedje; *R. pomeroyi* DSS-3 (DSM 15171^T), *Burkholderia phymatum* STM815 (DSM 17167), *Burkholderia phytofirmans* PsJN (DSM 17436) and *Oligotropha carboxidovorans* OM5 (DSM 1227^T) were obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) and *D. acidovorans* SPH-1 (DSM 12586) was isolated in this laboratory (Schleheck *et al.* 2004). *Rhodobacterales* sp. HTCC2150, representing a major clade of the Rhodobacterales discovered elsewhere (Selje *et al.* 2004), was isolated by high throughput culturing (HTC) (Connon and Giovannoni 2002).

Organisms in Konstanz were grown aerobically at 30 °C in batch culture. Cells of *R. pomeroyi* DSS-3 and *R. nubinhibens* ISM were cultivated using modified SBM-M (*Silicibacter* basal medium) as described elsewhere (Denger *et al.* 2006a), whereas *C. necator* H16, *B. xenovorans* LB400, *B. phymatum* STM815, *B. phytofirmans* PsJN and *D. acidovorans* SPH-1 were grown in fresh-water, mineral-salts medium (Thurnheer *et al.* 1986). *O. carboxidovorans* OM5 was cultivated in the mineral medium recommended by DSMZ (medium 133). Taurine, isethionate or acetate (10-20 mM) was used as the sole added source of carbon and energy for growth. When isethionate concentration was determined during growth of *C. necator* H16, ammonium chloride in the medium was replaced by ammonium nitrate to avoid interference in the determination of isethionate.

Precultures and cultures (3-5 ml) for the determination of the substrate range were grown in 30-ml screw-cap 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 optical density at 580 nm, to assay protein, and to determine the concentrations of substrate and products. Cell-free extracts were obtained from organisms grown on the 1 l scale in 5-l Erlenmeyer flasks. Cells were harvested by centrifugation (30 000 g, 15 min, 4 °C) in the mid-exponential growth phase, washed in 50 mM potassium phosphate buffer, pH 7.2 (containing 5 mM MgCl₂) and resuspended in a small volume (2 to 5 ml) of the same buffer. Cells were

disrupted by four passages through a chilled French press set at 138 MPa, and cell debris were removed by centrifugation (20 000 g, 3 min, 4 °C). Membrane and soluble fractions were obtained by ultracentrifugation (200 000 g, 30 min, 4 °C). The membrane fraction was washed in 50 mM potassium phosphate buffer, pH 7.2 (containing 5 mM MgCl₂) and subjected to ultracentrifugation (see above) and resuspended in the same volume of the above mentioned buffer.

In Oregon, cultures of *Rhodobacterales* sp. HTCC2150 were grown at 16 °C in the dark in polycarbonate flasks containing 50 ml of sterilized seawater medium amended with a vitamin mixture, NH₄Cl and KH₂PO₄ ('LNHM') (Rappé *et al.* 2002). Isethionate (2, 5, 10, 20 µM) or glucose (1, 10 µM) was added to LNHM. All experiments were done in duplicate. Increase in cell densities and final cell yields were determined by flow cytometry on a Guava EasyCyte cell counter (Guava Technologies, Hayward, CA, USA) (Stingl *et al.* 2007).

Enzyme assays

Sulfoacetaldehyde acetyltransferase (Xsc) was assayed routinely by the colorimetric determination of acetyl phosphate (Ruff *et al.* 2003). Isethionate dehydrogenase (IseJ) was assayed spectrophotometrically as the isethionate-dependent reduction of beef-heart cytochrome *c* at 550 nm. The reaction mixture contained (in a final volume of 1 ml): 50 µmol potassium phosphate buffer, pH 7.2 (containing 5 mM MgCl₂), 12.5 µmol isethionate, 50 nmol cytochrome *c* and 0.1-0.5 mg protein. The reaction was linear for at least 2 min.

Analytical methods

Isethionate was determined by ion chromatography (Styp von Rekowski *et al.* 2005). Sulfate was quantified turbidimetrically as an insoluble suspension of BaSO₄ (Sörbo 1987). Sulfite was quantified as a fuchsin adduct as described elsewhere (Denger *et al.* 2001). Growth was followed turbidimetrically at 580 nm or determined as protein in whole cells, quantified by a Lowry-type-reaction (Kennedy and Fewson 1968). Protein content of crude cell extracts was assayed by protein-dye binding (Bradford 1976). Chromosomal DNA was isolated as described elsewhere (Desomer *et al.* 1991). The PCR primers listed in Table S1 were used to amplify gene fragments. Sequencing was done at MWG-Biotech AG (Ebersberg, Germany), and the sequences were analyzed using the Lasergene package (DNASTAR).

RT (Reverse transcription)-PCR

The primers listed in Table S1 were used for the RT-PCR reactions. PCR was performed as described elsewhere (Innis *et al.* 1990). Absence of DNA after RNA isolation was tested by PCR using *xsc* primers. Positive controls for success of RNA isolation were done after reverse

transcription using 16S rRNA-specific primers 16S-27F and 16S-533R (Weisburg *et al.* 1991). PCR products were visualized on 1.5 % agarose gels (Sambrook *et al.* 1989).

Sequence analyses and accession numbers

Sequences of the genomes of *C. necator* H16 (accession numbers NC_008313 and NC_008314) and *R. pomeroyi* DSS-3 (accession numbers NC_003911 and NC_006569) were obtained from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>), whose BLAST server was also used. Sequence data were analyzed using the different subroutines of the LASERGENE software package (DNASTAR) and with programmes SignalP and TMHMM (Bendtsen *et al.* 2004) available from the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk./services/>), as well as PROSITE, on the ExPASy Proteomics Server (<http://www.expasy.org/>).

RESULTS

Gene candidates for isethionate biodegradation

The flanking regions of the *xsc* pseudogene (SPO2360) in *R. pomeroyi* DSS-3 drew our attention (see Introduction). The upstream region contained five genes of potential interest. They could encode a dehydrogenase (SPO2359) of the 'BetA' family (glucose-methanol-choline (GMC) oxidoreductases; COG2303), particulate enzymes of 60-64 kDa (http://www.brenda-enzymes.info/php/result_flat.php4?ecno=1.1.99.1&organism=), and, divergently transcribed, a tripartite ATP-independent (TRAP) transporter (SPO2358-2356) [TC 2.A.56.-.-] and an IclR-type transcriptional regulator (SPO2355). This allowed the hypothesis, that an orthologue of the membrane-associated, 62-kDa isethionate dehydrogenase found by Brüggemann *et al.* (Brüggemann *et al.* 2004) was encoded by the BetA-type gene, that isethionate was transported into the cell via a TRAP transporter, and that both moieties were under the control of the IclR-type transcriptional regulator. The degradation of sulfoacetaldehyde would then proceed via the Xsc (SPO3561) and Pta (SPO3560) known from previous work (Fig. 1ab; Gorzynska *et al.* 2006).

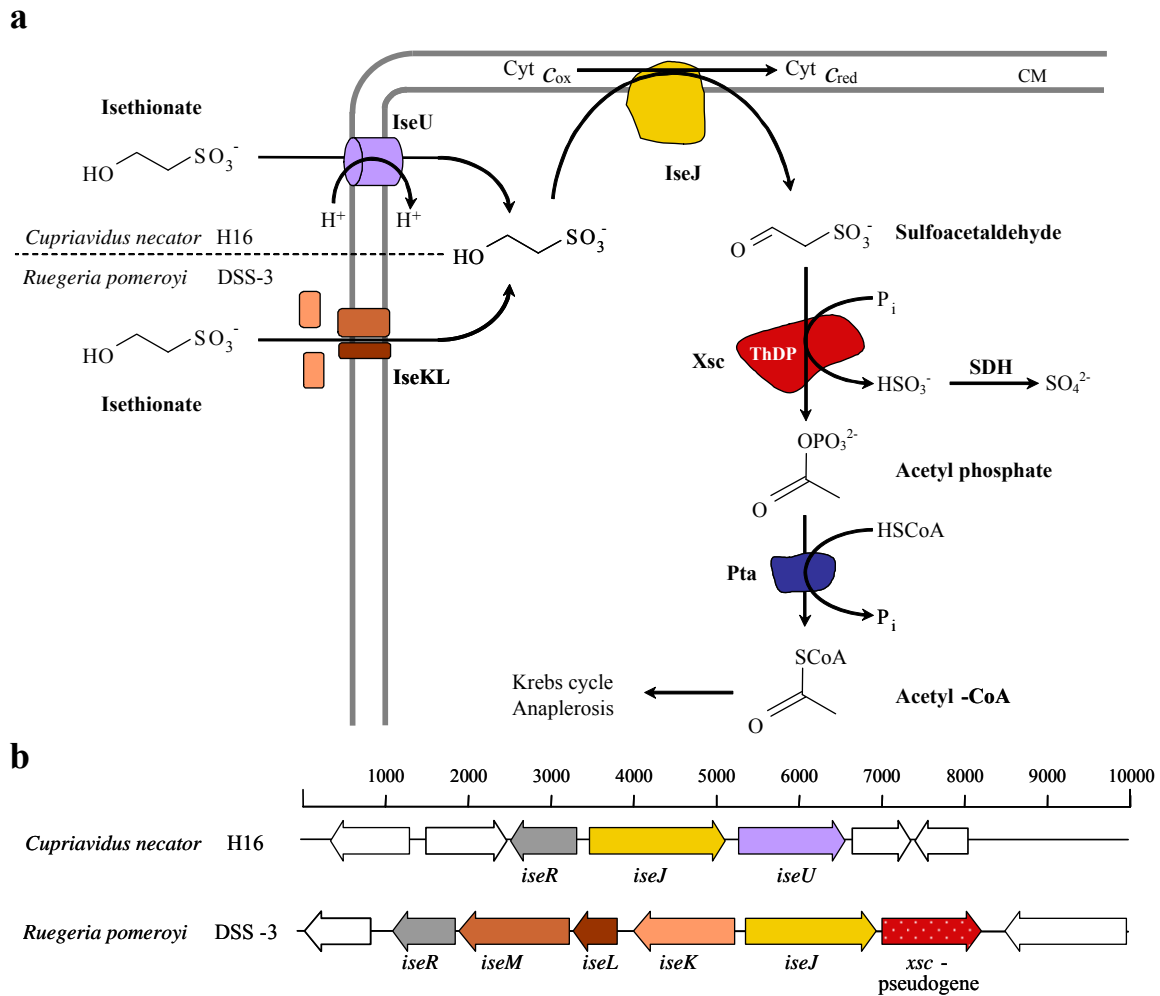


Fig. 1. Initial steps in the catabolism of isethionate in *Cupriavidus necator* H16 and *Ruegeria pomeroyi* DSS-3 (a) with the corresponding gene clusters (b). Abbreviations: CM, cytoplasmic membrane; SDH, sulfite dehydrogenase; ThDP, thiamine diphosphate. In strain H16, where data are available, SDH is periplasmic (Denger *et al.* 2008).

The putative gene products (SPO2355-SPO2359; IseJKLMR) were compared individually with protein sequences in the NCBI database using the BLAST algorithm. Many orthologues of IseJ were found and a dendrogram showed three distinct clusters (Fig. 2). About 30 organisms (Table 1, Table S2) from Fig. 2 contained at least two of the hypothetical gene products of interest, as well as Xsc, and these organisms were, thus, candidates to dissimilate isethionate: those organisms available to us, or described elsewhere, were all able to grow with isethionate (Baldock *et al.* 2007; Brüggemann *et al.* 2004; Denger *et al.* 2006a; Lie *et al.* 1998). The terrestrial isolates in Table 1, e.g. *Cupriavidus necator* H16, were predicted to use a major facilitator superfamily (MFS) isethionate transporter (IseU) instead of the TRAP system (Fig. 1ab, Table 1).

Table 1. Bacteria with orthologues of genes presumably encoding proteins involved in the dissimilation of isethionate, with the general source of the organism and its ability to grow with isethionate as a sole source of carbon and energy for growth.

Organism (locus tag abbreviation)	<i>iseJ</i>	<i>iseK</i>	<i>iseL</i>	<i>iseM</i>	<i>iseU</i>	<i>iseR</i>	Phylogeny	Source	Growth
<i>Paracoccus denitrificans</i> PD1222	(Pden_) 4278	-	-	-	4279	4280	alpha	terrestrial	+
<i>Paracoccus denitrificans</i> NKNIS	+ ^a	-	-	-	+ ^a	+ ^a	alpha	terrestrial	+
<i>Oligotropha carboxidovorans</i> OM5	(OCAR_) 6748	-	-	-	6747	6749	alpha	terrestrial	+
<i>Burkholderia phymatum</i> STM815	(Bphy_) 6221	-	-	-	6220	6222	beta	terrestrial	+
<i>Burkholderia phytofirmans</i> PsJN	(Bphyt_) 4377	-	-	-	4378	4376	beta	terrestrial	+
<i>Burkholderia xenovorans</i> LB400	(Bxe_) B0698	-	-	-	B0699	B0697	beta	terrestrial	+
<i>Cupriavidus necator</i> H16	(H16_) B1851	-	-	-	B1852	B1850	beta	terrestrial	+
<i>Delftia acidovorans</i> SPH-1	(Daci_) 5947	-	-	-	5948	5946	beta	terrestrial	+
<i>Desulfitobacterium hafniense</i> DCB-2	(Dhaf_) -	-	-	-	-	-	firmicutes	terrestrial	+
<i>Ruegeria pomeroyi</i> DSS-3	(SPO) 2359	2358	2357	2356	-	2355	alpha	marine	+
<i>Roseovarius nubinhibens</i> ISM	(ISM_) 12540	12545	12550	12555	-	12560	alpha	marine	+
<i>Roseovarius</i> sp. strain 217	(ROS217_) 05299	05294	05289	05284	-	05279	alpha	marine	+
<i>Rhodobacterales</i> sp. HTCC2150 ^b	(RB2150_) 10786	-	-	-	-	10791	alpha	marine	+
<i>Alphaproteobacterium</i> sp. HTCC2255 ^b	(OM2255_) 05990	-	-	-	-	05985	alpha	marine	n.t. ^c
<i>Pelagibacter ubique</i> HTCC1002 ^b	(PU1002_) 02111	-	-	-	-	-	alpha	marine	n.t.

^a Data generated in this paper. ^b Neither IseKLM nor IseU is present, but a TerC-like transporter and a possible TRAP transporter can be suggested for these bacteria. ^c n.t., not tested.

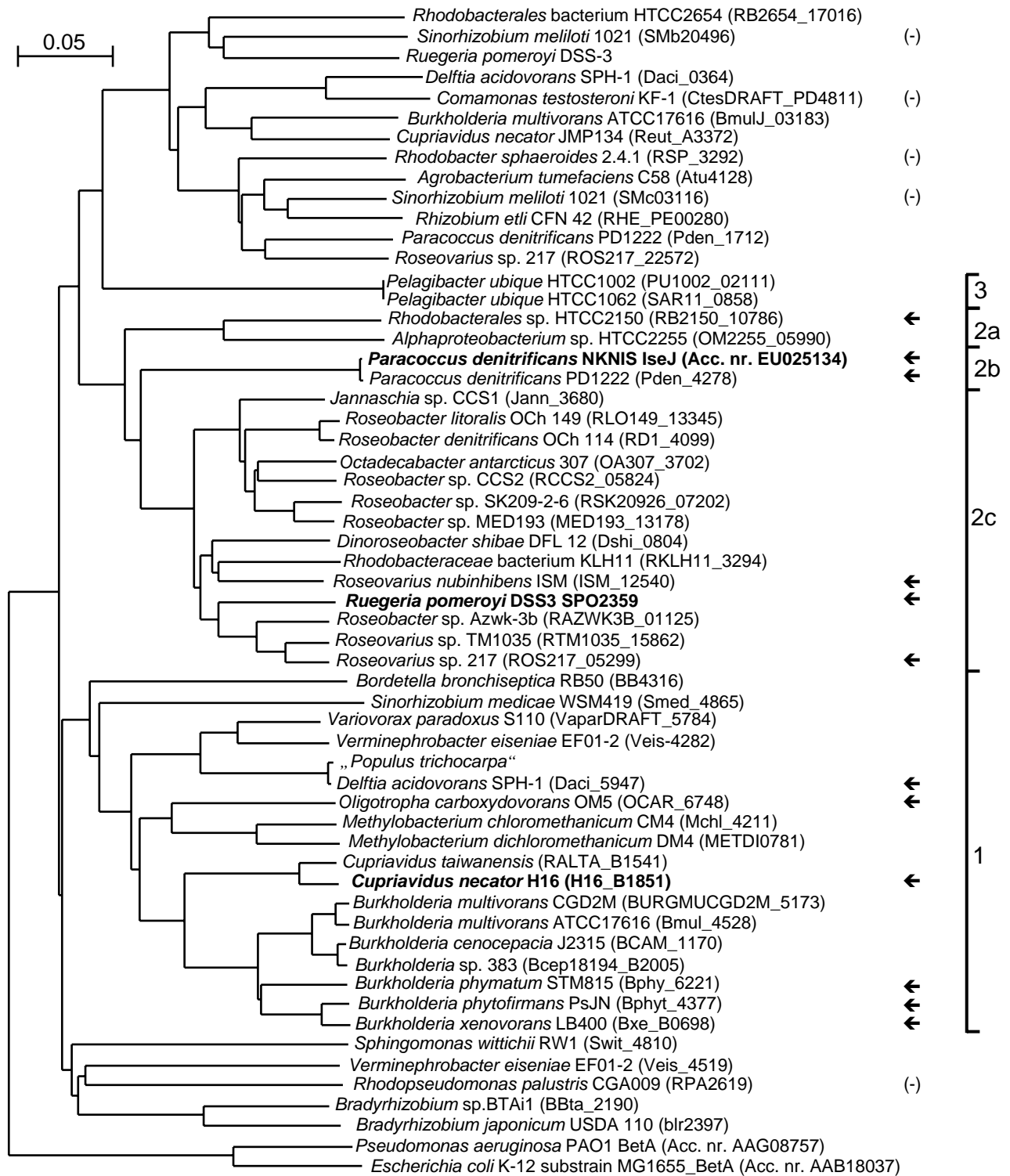
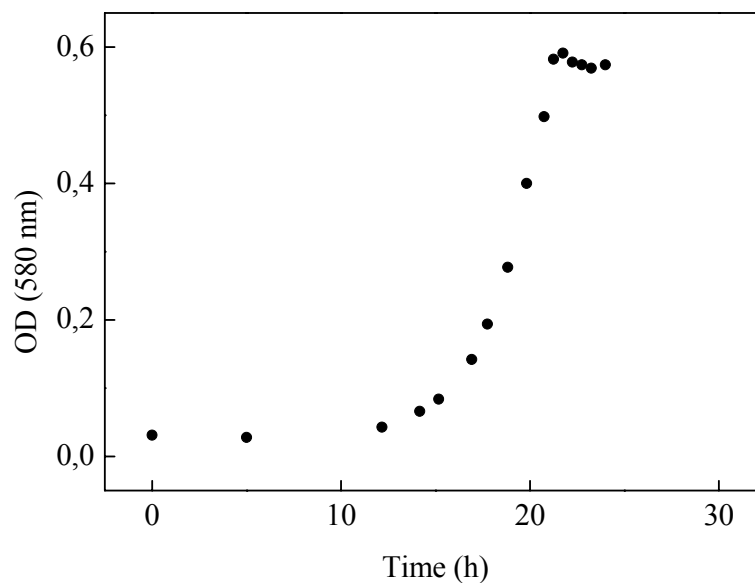


Fig. 2. Dendrogram of orthologues of putative isethionate dehydrogenases (IseJ). The three organisms given in boldface were used in this work; the outgroups represent characterized authentic BetA enzymes. Organisms indicated by bold arrows utilize isethionate as a source of carbon and energy for growth, those marked by minus sign (-) do not. GenBank accession numbers or locus tags are given in parentheses. The draft genome of *Populus trichocarpa* (poplar tree) apparently encodes IseJ, but the gene does not involve splicing and shares 99 % identity with the *Delftia acidovorans* sequence. We assume that bacterial DNA was sequenced. IseJ orthologues of *P. aeruginosa* PAO1 and *E. coli* K12 substrain MG1655 were taken as outgroups.

Growth physiology

The terrestrial organism, *C. necator* H16, could be grown in chloride-free medium, which allowed for the first time the determination of isethionate during its degradation by a micro-organism (the chloride ion interferes with the assay of isethionate by ion chromatography (IC); Styp von Rekowski *et al.* 2005). Growth with isethionate (Fig. 3a) as sole source of carbon and energy was rapid ($\mu = 0.35 \text{ h}^{-1}$) and concomitant with both the release of sulfate into the medium and with the utilization of isethionate, which was quantitative (Fig. 3b). No sulfite was detected in the medium (Fig. 3b). The growth yield was $6.6 \text{ g protein (mol C)}^{-1}$, which confirmed the quantitative utilization of the substrate (see Cook 1987). The marine organism *R. pomeroyi* DSS-3 was shown to utilize isethionate with quantitative recovery of sulfate (Denger *et al.* 2006a), which, with the same molar growth yield as taurine, confirms the extensive utilization of isethionate as a source of carbon and energy for growth. The growth rate of *R. pomeroyi* DSS-3 with isethionate was about 0.06 h^{-1} .

a



b

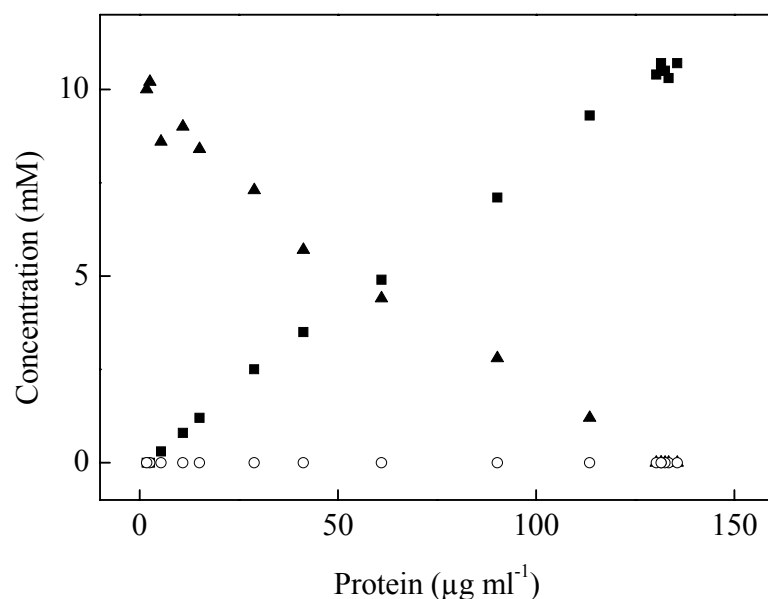


Fig. 3. Growth of *Cupriavidus necator* H16 (a) in chloride-free mineral-salts medium containing 10 mM isethionate as a sole source of carbon and energy. Concentrations of substrate and products (b) during growth of *C. necator* H16 with isethionate, plotted as a function of the protein concentration. Filled triangles show isethionate concentrations, filled squares sulfate concentrations and open circles sulfite concentrations.

Growth of the marine *Rhodobacterales* sp. HTCC2150 was followed in salts-supplemented sterilized sea water (LNHM, see Methods), because it is not culturable in defined media. Growth was carbon-limited as shown by comparing counts of cells in outgrown medium which had contained 0.0, 1.0 and 10.0 μM glucose: replicate flasks without the addition of glucose yielded $1.1 \cdot 10^6$ cells ml^{-1} , and additions of glucose resulted in a linear increase of about $5.5 \cdot 10^4$ cells $(\text{nmol glucose-carbon})^{-1}$. Growth with isethionate (2 – 20 μM) resulted in a linear increase in cell yield of about $1.3 \cdot 10^5$ cells $(\text{nmol isethionate-carbon})^{-1}$, about twice the value for glucose. Whatever the reason for the different yield coefficients, the key fact was that the organism grew with isethionate, and that growth was proportional to the concentration of substrate added.

Enzyme activities

Crude cell extracts of *C. necator* H16 were prepared from cells grown with isethionate or with acetate as the sole source of carbon and energy. The extracts of the isethionate-grown cells were shown to contain activities of both isethionate dehydrogenase and Xsc (Table 2), whereas the extracts of acetate-grown cells did not (not shown); the enzymes were considered

to be inducible. Soluble extract and membrane fractions were prepared from the induced cells. The activity of Xsc was found solely in the soluble fraction, whereas isethionate dehydrogenase was active in the membrane fraction (Table 2), as predicted (Brüggemann *et al.* 2004), and an inducible membrane protein of 60-62 kDa was detected in the membrane fraction by SDS-PAGE (not shown). We infer that this protein is IseJ.

Table 2. Specific activities of isethionate dehydrogenase and sulfoacetaldehyde acetyltransferase in soluble and particulate fractions of *C. necator* H16. Cell extracts were prepared from isethionate-grown cells. n.d., not detected.

	Specific activity (mkat (kg protein) ⁻¹)	
	IseJ	Xsc
Crude cell extract	0.3	2.8
Soluble fraction	n.d.	3.4
Particulate fraction	0.1	n.d.

Reverse transcription PCR

The hypothesis that gene candidates *iseU* and *iseJ* in *C. necator* H16 would encode uptake of isethionate and isethionate dehydrogenase, respectively (see Introduction), was tested by reverse transcription (RT-) PCR. No transcript of either gene was detected in acetate-grown cells (Fig. 4). In contrast, a transcript of each gene was detected in induced cells (Fig. 4). We interpret these data as support for our hypothesis that the proteins, IseU and IseJ, are synthesized inducibly during growth with isethionate. The genes *iseJ* and *iseU* are transcribed monocistronically.

The candidate genes *iseKLM* and *iseJ* of *R. pomeroyi* DSS-3 were hypothesized to encode a TRAP transporter for isethionate and isethionate dehydrogenase, respectively (see Introduction). None of those genes was transcribed during growth with acetate (not shown). In contrast, a transcript of each gene was detected in induced cells (not shown). We interpret these data as evidence for our hypothesis that the proteins, IseKLM and IseJ, are synthesized inducibly during growth with isethionate.

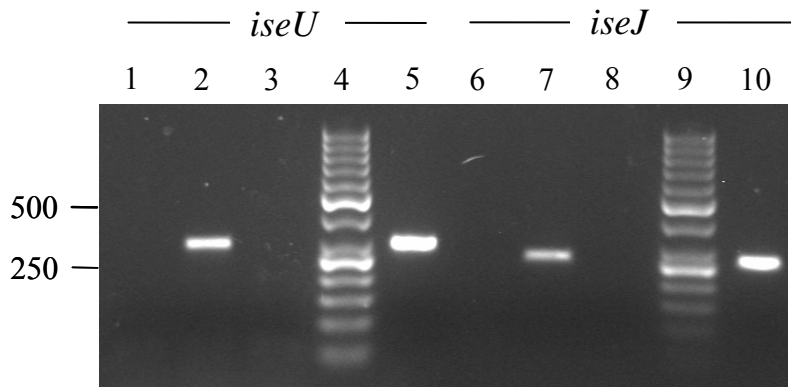


Fig. 4. Transcription of *iseU* and *iseJ* in *Cupriavidus necator* H16 during growth with isethionate. Transcripts from a representative RT-PCR experiment with a predicted length of 293 bp (for primer pair H16iseUF/H16iseUR) or 254 bp (for primer pair H16iseJF/H16iseJR). Lanes 1 and 6, negative control (no DNA); lane 2 and 7, RT-PCR products from RNA of isethionate-grown cells; lane 3 and 8, RT-PCR products from RNA of acetate-grown cells; lanes 4 and 9, 50 bp DNA ladder; lanes 5 and 10, positive control (*C. necator* H16 chromosomal DNA as template).

Data from genome sequencing projects

The gene cluster identified in *C. necator* H16 (*iseRJU*) was found in ten other terrestrial isolates whose genomes have been sequenced, and this allowed us to predict utilization of isethionate by these organisms which we confirmed by growth experiments with five organisms that were available to us (*Burkholderia xenovorans* LB400, *Burkholderia phymatum* STM815, *Burkholderia phytofirmans* PsJN, *Oligotropha carboxidovorans* OM5 and *Delftia acidovorans* SPH-1; Table 1, Table S2 in the Supplemental Material). Some of these organisms encode the subsequent enzyme in the degradative pathway (Xsc) at a different locus, as is the case in *C. necator* H16 (Weinitschke *et al.* 2007). One terrestrial isolate, *P. denitrificans* PD1222, shows a different order of genes, *iseRUJ*, followed by *xsc-tauZ-ptA*, which presumably encodes a complete and independent isethionate degradative pathway. The same phenomenon can be attributed to *Sinorhizobium medicae* WSM419 (Table S2), where the *iseRJU* is located in a gene cluster with taurine degradative genes. In the genome sequence of *Dinoroseobacter shibae* DFL 12 also, an *xsc* gene was found directly downstream of *iseJ*.

The gene cluster identified in *R. pomeroyi* DSS-3, *iseJKLMR*, was found in 13 other marine organisms whose genomes have been sequenced, and this allowed us to predict dissimilation of isethionate in *Roseovarius nubinhibens* ISM and *Roseovarius* sp. strain 217, the only organisms additionally available to us. The organisms did grow with isethionate. We regard this as confirmation of the function of IseJKLM.

Two more marine isolates (*Alphaproteobacterium* sp. HTCC2255 and *Rhodobacterales* sp. HTCC2150; Table 1) contain the *iseRJ* genes (as well as *xsc* and *pta*), but neither the *iseKLM* nor *iseU* genes. *Rhodobacterales* sp. HTCC2150 does, indeed, utilize isethionate (see above). Adjacent to *iseJ*, and overlapping by one bp, a TerC-family (possibly involved in tellurium transport) [TC 9.A.30.-.-] hypothetical protein with 6 predicted trans-membrane helices (TMH) is encoded which may represent at least part of an isethionate transporter (RB2150_10781). About 100 bp further away, a possible TRAP transporter (RB2150_10776, predicted periplasmic protein, and RB2150_10771, fused large and small permease subunit) was encoded. These three genes are conserved upstream of *iseJ* in strain HTCC2255 and in two sequences from a metagenome project (Sabehi *et al.* 2005). The genome sequences of *Pelagibacter ubique* strains HTCC1002 and HTCC1062 contain the *iseJ-terC* gene-pair (as well as *xsc* and *pta*), but the TRAP transporter consists of three subunits and is located 4 kb away from *iseJ*. We tentatively presume that *P. ubique* also utilizes isethionate.

One organism, which has been known for many years to utilize isethionate and which contains two orthologues of *xsc*, is the anaerobic firmicute *Desulfitobacterium hafniense* DCB-2 (Lie *et al.* 1998; Ruff *et al.* 2003). It does not contain any orthologue of *iseR*, *iseJ* or any relevant transporter gene. There is obviously an unknown set of genes to encode at least utilization of isethionate as electron acceptor in *D. hafniense*.

Sequence prediction in *P. denitrificans* NKNIS

The biochemical background to the present paper is the presence in *P. denitrificans* NKNIS of an inducible, 62-kDa, membrane-bound protein, assumed to be isethionate dehydrogenase (IseJ) (Brüggemann *et al.* 2004). The genome of a different strain of *P. denitrificans*, PD1222, has now been sequenced (NC_010355), and its 9-gene taurine cluster is essentially identical with the corresponding sequence in strain NKNIS (not shown). So we predicted that highly identical orthologues of the isethionate gene cluster in strain PD1222 (e.g. *iseRUJ-xsc*) would be found in strain NKNIS. We confirmed this hypothesis by amplifying and sequencing of overlapping fragments of *iseRU*, *iseUJ*, *iseJ* and *iseJ-xsc* yielding a sequenced region of 3.5 kb which shared 99 % identity with the corresponding region in the genome of strain PD1222.

DISCUSSION

The facile, routine determination of isethionate in growth medium has only recently become available (see also Ianniello 1988; Styp von Rekowski *et al.* 2005). Data from this and other methods allowed the physiology of growth of *C. necator* H16 with isethionate to be defined

(Fig. 3). Growth was quantitative, there were no detectable transient intermediates, and substrate utilization was concomitant with growth (Fig. 3b). The enzyme activities (Table 2) showed the inducible presence of the pathway shown in Fig. 1a, which was first indicated by Kondo *et al.* and King *et al.* (King *et al.* 1997; Kondo *et al.* 1977), and then given biochemical character by Brüggemann *et al.* (Brüggemann *et al.* 2004).

The inference from the latter work (Brüggemann *et al.* 2004), that an inducible, 62-kDa, membrane-bound isethionate dehydrogenase would be found in other organisms (Introduction), was confirmed in *C. necator* H16 (Table 2). Here, inducible transcription of the 1656-bp candidate *iseJ*, h16_B1851 was observed (Fig. 4). Similarly, the 1608-bp candidate *iseJ*, SPO2359, was inducibly transcribed in *R. pomeroyi* DSS-3 (not shown). Correspondingly, the isethionate⁺-phenotype of *P. denitrificans* NKNIS was confirmed to be accompanied by the *iseRUIJ-xsc* genotype. The power of prediction, that the presence of *iseJ* indicates isethionate utilization in an organism on the one hand, and that membrane-bound isethionate dehydrogenase indicates the presence of *iseJ* in the organism's genome on the other hand, is surely sufficient to establish that *iseJ* encodes isethionate dehydrogenase [EC 1.1.2.-].

Analysis of the sequence of IseJ in PROSITE confirms the preliminary data from BLAST searches (Fig. 2) that the enzyme is a member of the GMC oxidoreductases, a group of FAD-containing flavoproteins. The closest defined orthologue of IseJ is choline dehydrogenase [EC 1.1.99.1]; the '99' in the EC number indicates an uncertain reaction mechanism. In future, it will hopefully be possible to purify IseJ and to identify any cofactors. Alternatively, heterologous expression of *iseJ* might allow the confirmation of function, assuming that there is no problem with the specificity of the enzyme for cytochrome *c*, which we experienced with taurine dehydrogenase [EC 1.4.2.-] (e.g. Weinitschke *et al.* 2006). As an alternative to purifying IseJ, we tried to construct an *iseJ*⁻ deletion mutant, but we did not succeed despite being able to delete other genes in the same organism (S. Weinitschke, unpublished data). Membrane-bound isethionate dehydrogenase [EC 1.1.2.-] should not be confused with soluble sulfoacetaldehyde reductase [EC 1.1.1.-] (Styp von Rekowski *et al.* 2005), which has been characterized (Krejčík *et al.* 2009).

An isethionate TRAP transporter, IseKLM [TC 2.A.56.-], is present in many marine isolates, whereas an MFS-type isethionate transporter, IseU [TC 2.A.1.-], is present in terrestrial isolates (Table 1, Table S2, Fig. 4). Our rationale for this is that we predict much higher dilution of isethionate in the marine environment than in the terrestrial environment, where isethionate in the spiders's viscid fluid is initially present at molar concentrations, whereas

lower initial concentrations of isethionate in red algae are predicted (see Introduction). Thus, a powerful pump is needed by marine bacteria, whereas a facilitator suffices in terrestrial organisms. This observation is consistent with recent data suggesting a favoured use of TRAP transporters in saline environments and a more frequent occurrence of TRAP transporter genes in bacteria adapted to these conditions (Mulligan *et al.* 2007).

A third isethionate transporter was proposed above, but apart from the ‘TerC-family’ candidate, it is undefined. Its use here is to expand the likely range of isethionate utilizers to the cosmopolitan *P. ubique* (Table 1); presumably no regulator is present because the organism encodes almost none (Giovannoni *et al.* 2005).

Given the likely range of presumptive isethionate-utilizers, we can now consider the phylogeny of IseJ orthologues in Fig. 2. Cluster 2c represents the original hypothesis in marine alphaproteobacteria represented by *R. pomeroyi* DSS-3 (see Introduction), while Cluster 1 represents the terrestrial betaproteobacteria also mentioned in the Introduction and represented by *C. necator* H16. Cluster 2b represents two strains of a terrestrial alphaproteobacterium, with an MFS transporter. Cluster 2a represents marine alphaproteobacteria, with the TerC-family transporter. The latter is also found in two sequenced genomes of *P. ubique* (Cluster 3) and in metagenomic sequences from the Sargasso Sea sequencing project (Venter *et al.* 2004). Most clusters of orthologues of IseJ are distinct and include only potential isethionate utilizers (Fig. 2). The exception is in Cluster 1, which includes *Bordetella brontiseptica* RB50, an organism lacking the *xsc* gene. We wonder whether this candidate IseJ, if active, might be (i) involved in sulfur assimilation, presumably via a transaminase and taurine dioxygenase (BB3661) (Eichhorn *et al.* 1997) or (ii) whether *B. brontiseptica* has an unrecognized Xsc, as in *Bilophila wadsworthia* (Cook and Denger 2002).

Brüggemann *et al.* (Brüggemann *et al.* 2004) predicted the presence of a regulator to control induction of isethionate dissimilation. We now presume that IseR, an IclR-type protein, fulfils this function, because it is almost always co-localized with the genes encoding the degradative pathway (Table 1, Table S2, Fig. 1b). The biodiversity of the isethionate pathway, with its unknown pathway in *D. hafniense* DCB-2 (Table S2), and with at least three types of transporters concurs with the widespread occurrence of isethionate in the environment (see Introduction).

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SUPPLEMENTAL MATERIAL

Table S1. Oligonucleotides used in this work. Many oligonucleotides were developed for this project. The 16S-rRNA primers (Weisburg *et al.* 1991), those for *xsc* and *pta* in *C. necator* H16 (Weinitschke *et al.* 2007), and those for *xsc* and *pta* in *R. pomeroyi* DSS-3 (Gorzynska *et al.* 2006), were described elsewhere.

Organism	Target	Name	Sequence (5' → 3')	
Bacteria	16S	16S-27F	CAG AGT TTG ATC CTG GCT CAG	
		16S-533R	TTA CCG CGG CTG CTG GCA C	
<i>C. necator</i> H16	<i>iseJ</i>	H16iseJF	CAG CGA CGA CGA GAT CCT GCA TT	
		H16iseJR	CGC CGT GCG TCT TCG CGG ATG AAG	
	<i>iseU</i>	H16iseUF	CCG TGA TCG GCG CCA CCA TCG AAT	
		H16iseUR	CCA GGA TGC CGA TGC TGT CAT A	
	<i>xsc</i>	H16xscF	ACC GAC ATC GGC AAC ATC AAC TC	
		H16xscR	GGT TGT AGA AGT CCA CCT GGT TCT	
	<i>pta</i>	H16ptaF	TGG TGT CGA GCT TCT TCC TGA T	
		H16ptaR	GCC GGC TTC CAG GCT GGG AAA C	
	<i>R. pomeroyi</i> DSS-3	<i>iseJ</i>	SpIseJF	CTG GCA AAT CGC CTA AGC AAG
			SpIseJR	GGT TGA ACG GGT AGC CTG CAT TC
<i>iseK</i>		SpIseKF	CCT TCA GGA CGC AGT GAT GGA ATC	
		SpIseKR	CTT CAT GTC GGC GGG CAC TTC A	
<i>iseL</i>		SpIseLF	TCT TCC GGC GCT TTG TCC TGA A	
		SpIseLR	GAG ACC AGG AAC CAC CAT TGC AG	
<i>iseM</i>		SpIseMF	GGA AAC TGG ACG ATC CTC ATC T	
		SpIseMR	CCA AGG CAG GCA TAG GCG AAA TCC	
<i>xsc</i>		SpXscF	AAC ATC CCG CGT GAC ATG TGG AC	

Organism	Target	Name	Sequence (5'→3')
		SpXscR	GGC CAG TAT TCC ATG CCA TAA CC
	<i>pta</i>	SpPtaF	GTC TGA CCA TCC ACG ATC CGG ACA
		SpPtaR	CGG CCT CGG GCG GAT ACA TCA
<i>P. denitrificans</i> NKNIS	<i>xsc-iseJ</i>	PD1222xscF	CCC GTC GAG CGC GTG AAA CCG TC
		PD1222iseJR1	ACC TGC AGG CGC GGC TGG TGT TCA A
	<i>iseJ</i>	PD1222iseJF1	ACT GGC AGA CCG ATT GCG TGA AG
		PD1222iseJR2	GGC TAT TTC CAG ACC ATG CAC AAC
	<i>iseJ-iseU</i>	PD1222iseJF2	CTG CGC CCA GCG GTC GTA ATC CT
		PD1222iseUR	CTA TAT CGA CGG CGT GTT CTT CAA
	<i>iseU-iseR</i>	PD1222iseUF	GCG GAC GCC CGA TCC TGT CCG AA
		PD1222iseRR	GCA AGA ACC TCA AGA ATC TGA AGA

Table S2. Bacteria with orthologues of genes presumably able to encode proteins involved in the dissimilation of isethionate, with the general source of the organism and its ability to grow with isethionate as a sole source of carbon and energy for growth.

Organism (locus tag abbreviation)		<i>iseJ</i>	<i>iseK</i>	<i>iseL</i>	<i>iseM</i>	<i>iseU</i>	<i>iseR</i>	Phylogeny	Source	Growth
<i>Ruegeria pomeroyi</i> DSS-3	(SPO)	2359	2358	2357	2356	-	2355	alpha	marine	+
<i>Dinoroseobacter shibae</i> DFL 12	(Dshi_)	0804	0803	0802	0801	-	0800	alpha	marine	n.t. ^a
<i>Jannaschia</i> sp. strain CCS1	(Jann_)	3680	3679	3678	3677	-	3676	alpha	marine	n.t.
<i>Roseobacter denitrificans</i> OCh 114	(RD1_)	4099	4100	4101	4102	-	4103	alpha	marine	n.t.
<i>Roseobacter litoralis</i> OCh 149	(RLO149_)	13345	13340	13335	13330	-	13325	alpha	marine	n.t.
<i>Roseobacter</i> sp. strain CCS2	(RCCS2_)	05824	05819	05814	05809	-	05804	alpha	marine	n.t.
<i>Roseobacter</i> sp. strain MED193	(MED193_)	13178	13183	13188	13193	-	13198	alpha	marine	n.t.
<i>Roseobacter</i> sp. strain SK209-2-6	(RSK20926_)	07202	07207	07212	07217	-	07222	alpha	marine	n.t.
<i>Roseovarius nubinhibens</i> ISM	(ISM_)	12540	12545	12550	12555	-	12560	alpha	marine	+
<i>Roseovarius</i> sp. strain 217	(ROS217_)	05299	05294	05289	05284	-	05279	alpha	marine	+
<i>Roseovarius</i> sp. TM1035	(RTM1035_)	15862	15857	15852	15847	-	15842	alpha	marine	n.t.
<i>Roseovarius</i> sp. Azwk-3b	(RAZWK3B_)	01125	01120	01115	01110	-	01105	alpha	marine	n.t.
<i>Rhodobacteraceae</i> bacterium KLH11	(RKLH11_)	3294 ^b	3142 ^b	3332 ^b	3708 ^b	-	3151 ^b	alpha	marine	n.t.
<i>Octadecabacter antarcticus</i> 307	(OA307_)	3702 ^b	4101 ^b	1989 ^b	2940 ^b	-	2469 ^b	alpha	marine	n.t.
<i>Alphaproteobacterium</i> sp. HTCC2255 ^c	(OM2255_)	05990	-	-	-	-	05985	alpha	marine	n.t.
<i>Rhodobacterales</i> sp. HTCC2150 ^c	(RB2150_)	10786	-	-	-	-	10791	alpha	marine	+
<i>Pelagibacter ubique</i> HTCC1002 ^c	(PU1002)	02111	-	-	-	-	-	alpha	marine	n.t.
<i>Pelagibacter ubique</i> HTCC1062 ^c	(SAR11_)	0858	-	-	-	-	-	alpha	marine	n.t.
<i>Paracoccus denitrificans</i> PD1222	(Pden_)	4278	-	-	-	4279	4280	alpha	terrestrial	+

Organism (locus tag abbreviation)	<i>iseJ</i>	<i>iseK</i>	<i>iseL</i>	<i>iseM</i>	<i>iseU</i>	<i>iseR</i>	Phylogeny	Source	Growth
<i>Paracoccus denitrificans</i> NKNIS	+ ^d	-	-	-	+ ^d	+ ^d	alpha	terrestrial	+
<i>Sinorhizobium medicae</i> WSM419 (Smed_)	4865	-	-	-	4866	4864	alpha	terrestrial	n.t.
<i>Oligotropha carboxidovorans</i> OM5 (OCAR_)	6748	-	-	-	6747	6749	alpha	terrestrial	+
<i>Methylobacterium extorquens</i> CM4 (Mchl_)	4211	-	-	-	4212	-	alpha	terrestrial	-
<i>Burkholderia multivorans</i> ATCC 17616 (Bmul_)	4528	-	-	-	4527	4529	beta	terrestrial	n.t.
<i>Burkholderia phymatum</i> STM815 (Bphy_)	6221	-	-	-	6220	6222	beta	terrestrial	+
<i>Burkholderia phytofirmans</i> PsJN (Bphyt_)	4377	-	-	-	4378	4376	beta	terrestrial	+
<i>Burkholderia xenovorans</i> LB400 (Bxe_)	B0698	-	-	-	B0699	B0697	beta	terrestrial	+
<i>Burkholderia</i> sp. strain 383 (Bcep18194_)	B2005	-	-	-	B2004	B6006	beta	terrestrial	n.t.
<i>Burkholderia multivorans</i> CGD2M (BURGMUCGD2M_)	5173	-	-	-	-	5174	beta	terrestrial	n.t.
<i>Burkholderia cenocepacia</i> J2315 (BCAM)	1170	-	-	-	1171	1169	beta	terrestrial	n.t.
<i>Cupriavidus necator</i> H16 (H16_)	B1851	-	-	-	B1852	B1850	beta	terrestrial	+
<i>Cupriavidus taiwanensis</i> (RALTA_)	B1541	-	-	-	B1542	B1540	beta	terrestrial	n.t.
<i>Delftia acidovorans</i> SPH-1 (Daci_)	5947	-	-	-	5948	5946	beta	terrestrial	+
<i>Verminephrobacter eiseniae</i> EF01-2 (Veis_)	4282	-	-	-	4283	-	beta	terrestrial	n.t.
<i>Variovorax paradoxus</i> S110 (Vapar_)	3836	-	-	-	3837	3835	beta	terrestrial	n.t.
<i>Desulfitobacterium hafniense</i> DCB-2 (Dhaf_)	-	-	-	-	-	-	firmicutes	terrestrial	+

^a n.t., not tested. ^b Contiguous genes are represented by these numbers. ^c Neither IseKLM nor IseU is present, but a TerC-like transporter and a possible TRAP transporter can be suggested for these bacteria. ^d Data generated in this paper.

CHAPTER 3

Sulfoacetate degraded via novel sulfoacetyl-CoA and sulfoacetaldehyde in *Cupriavidus necator* H16

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SUMMARY

Sulfoacetate is a widespread natural product. Its biodegradation in currently cultured bacteria proceeds with considerable energy input via sulfoacetaldehyde. Whereas the fate of sulfoacetaldehyde in e.g. *Cupriavidus necator* (*Ralstonia eutropha*) H16 is known, the pathway from sulfoacetate to sulfoacetaldehyde is not. Data from genome sequences enabled us to hypothesize that the unknown, inducible pathway, which initiates sulfoacetate utilization (*sau*), involved a 4-gene cluster (*sauRSTU*) on the *C. necator* genome (H16_A2746 to H16_A2749). SauR would be the transcriptional regulator. SauU was deduced to be a transporter of the major facilitator superfamily; *sauU* was subject to inducible transcription and an in-frame knock-out in *sauU* prevented growth with sulfoacetate. SauT was deduced to be a sulfoacetate-CoA ligase; *sauT* was subject to inducible transcription and an in-frame knock-out in *sauT* prevented growth with sulfoacetate. SauT was labile, but could be separated and shown to generate AMP and an unknown, labile CoA-derivative from sulfoacetate, CoA and ATP. This unknown compound, analyzed by MALDI-TOF-MS in the negative-ion mode, had a relative molecular mass of 889.7, which identified it as protonated sulfoacetyl-CoA (calcd. 889.6). SauS was deduced to be sulfoacetaldehyde dehydrogenase (acylating); *sauS* was subject to inducible transcription and an in-frame knock-out in *sauS* prevented growth with sulfoacetate. The enzyme was purified 175-fold to homogeneity and characterized. Peptide-mass fingerprinting confirmed the *sauS* locus (H16_A2747). SauS converted sulfoacetyl-CoA and NADPH to sulfoacetaldehyde, CoA and NADP⁺. In different bacteria, evidence for different regulators, transporters and ligases was found. There is considerable biodiversity in the pathway.

INTRODUCTION

Sulfoacetic acid, as the sulfonate ester, was first recognized as a natural product in plant alkaloids (Folkers *et al.* 1944). Free sulfoacetate was then found to be widespread in plants and algae (Gupta and Sastry 1988; Lee and Benson 1972; Shibuya *et al.* 1963). The compound was also found to be an intracellular intermediate of the bacterial degradation of the plant sulfolipid (sulfoquinovosyldiacylglycerol), specifically from its polar head-group, sulfoquinovose (Martelli and Benson 1964). The plant sulfolipid is ubiquitous in plants and algae, and widespread in phototrophic bacteria (e.g. Harwood 1980), and can represent half of the total lipid content in some marine algae (Dembitsky *et al.* 1990). The sulfur content of leaves is comprised mainly of sulfolipid and proteins, so senescence of deciduous plants introduces significant amounts of sulfoquinovose to the soil (Harwood and Nicholls 1979). So this precursor of sulfoacetate is widespread in nature. Sulfoacetate can also be the product of the bacterial assimilation of

taurine-nitrogen (Denger *et al.* 2004b; Krejčík *et al.* 2008). The latter sulfoacetate-precursor is a major organic solute in marine creatures (Allen and Garrett 1971; Yin *et al.* 2000) and in mammals (Huxtable 1992), which excrete it in urine (Stipanuk 2004a). Sulfoacetate is also introduced into the environment in households: sodium lauryl sulfoacetate is widespread in cosmetics and personal care products, covered by e.g. the EU Cosmetics Directive.

The biodegradation of sulfoacetate was first observed by Martelli and Benson (Martelli and Benson 1964), and evidence was presented for the hydrolytic cleavage of sulfoacetate to glycolate, but the organism was lost (Cook and Denger 2002; Martelli and Souza 1970). King and Quinn (King and Quinn 1997a) isolated aerobic Gram-positive and Gram-negative bacteria which degraded sulfoacetate by a different pathway, which involved desulfonation via inducible sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15]. Different bacteria under strictly anoxic conditions also utilize sulfoacetate via Xsc (Cook and Denger 2002). Aerobic *Ralstonia* sp. strain EDS1 dissimilates a range of organosulfonates, including sulfoacetate, via Xsc, and the low molar growth yield with sulfoacetate (60 % of the value for all other carbon sources) led to the conclusion that the reduction of sulfoacetate to sulfoacetaldehyde, the substrate for Xsc, could be metabolically expensive (Denger and Cook 2001). The inducible involvement of Xsc in the degradation of sulfoacetate, taurine and isethionate was confirmed in *Cupriavidus necator* (*Ralstonia eutropha*) H16, where the common pathway also included phosphate acetyltransferase (Pta) [EC 2.3.1.8], a sulfite exporter TauE [TC 9.A.29.2.1] and sulfite dehydrogenase (SorAB) [EC 1.8.2.1] (Fig. 1; see also Supplemental Material) (Denger *et al.* 2008; Weinitschke *et al.* 2007). Genes encoding the same enzymes, or their equivalents, were found in sulfoacetate-utilizing *Burkholderia xenovorans* LB400, *Roseovarius nubinhibens* ISM or *Oligotropha carboxidovorans* OM5 (see Supplemental Material), but it was unclear which proteins led to the formation of sulfoacetaldehyde from sulfoacetate.

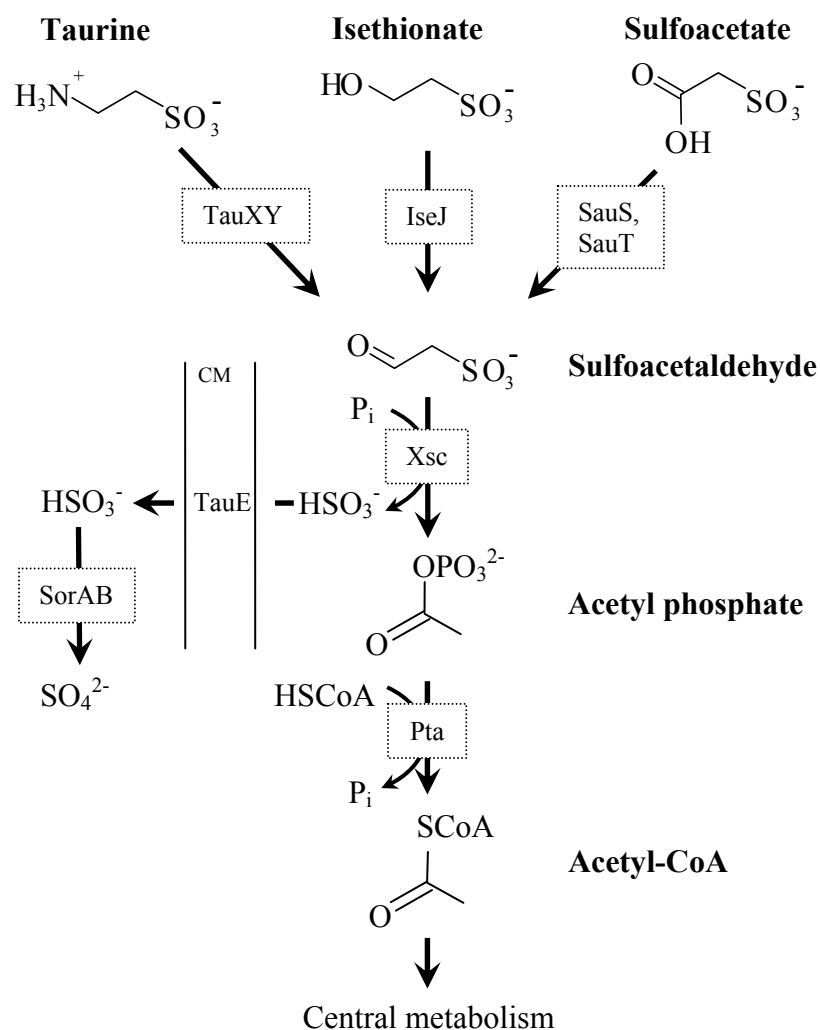


Fig. 1. Sulfoacetaldehyde as the point of convergence of the degradative pathways for sulfoacetate, isethionate and taurine, and the degradative pathway of sulfoacetaldehyde in *C. necator* H16. Taurine dehydrogenase (TauXY) and isethionate dehydrogenase (IseJ) are inducible, membrane-bound, cytochrome *c*-coupled enzymes which have not been purified (Brüggemann *et al.* 2004; Denger *et al.* 2008; Weinitschke *et al.* 2007; Weinitschke *et al.* 2009). TauE (Weinitschke *et al.* 2007) is a sulfite exporter, located in the cytoplasmic membrane (CM). SorAB is a periplasmic sulfite dehydrogenase described elsewhere (Denger *et al.* 2008).

The convergence of metabolism of C₂ sulfonates at one Xsc (Fig. 1) is widespread (Cook and Denger 2002), but some organisms contain paralogues of *xsc*, e.g. *Desulfitobacterium hafniense* DCB-2 (Fig. 2E). Here, the gene cluster upstream of one *xsc* (Dhaf_0189) was annotated as encoding a two-subunit acyl-CoA ligase and a NAD(P)-coupled aldehyde dehydrogenase (Dhaf_0190); this combination of reactions could convert sulfoacetate to sulfoacetaldehyde via hypothetical sulfoacetyl-CoA. Orthologues of the presumptive sulfoacetyl-CoA dehydrogenase (deacylating) (SauS: sulfoacetate ut*l*ization) are widespread, often with a neighbouring gene encoding either a two-subunit presumptive sulfoacetate-CoA ligase (SauPQ) as described above,

or a single-subunit presumptive sulfoacetate-CoA ligase (SauT) (Fig. 2B). Just as an alternative sulfoacetate-CoA ligase (SauPQ; Fig. 2D) can be suggested, there are different potential transport systems (SauU, SauFGH; Fig. 2B,C), and different potential regulators (SauR, SauI, SauV; Fig. 2B,C,D) in different organisms.

We chose to explore primarily the *sauRSTU* cluster in *C. necator* H16, which grew fast and has an established protocol to generate in-frame deletions. In the genome of *C. necator* H16, genes encoding a putative regulator (SauR, H16_A2746), a transporter (SauU, H16_2749), a single-subunit ligase (SauT, H16_A2748) and a putative sulfoacetaldehyde dehydrogenase (SauS, H16_A2747) were found in a cluster and could encode the above hypothesized pathway leading from sulfoacetate to sulfoacetaldehyde.

The biodiversity of different potential mechanisms for ligation and transport, represented by *Roseovarius nubinhibens* ISM (SauFGH) and *O. carboxidovorans* OM5 (SauPQ), was included in the study.

MATERIAL AND METHODS

Materials

Sulfoacetate (99 %) was purchased from Acros Organics (Geel, Belgium). Other commercial chemicals (about 99 %) were from Fluka (Seelze, Germany), Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany). Sulfoacetyl-CoA is not available commercially, and we failed to synthesize it chemically using protocols for the synthesis of 3-hydroxybutyryl-CoA (Chohan and Copeland 1998; Stadtman 1957). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Materials for (RT-)PCR and cloning were purchased from Fermentas GmbH (St Leon-Rot, Germany) and used as provided by the supplier. Phusion DNA Polymerase from NEB (Ipswich, USA) was used for construction of deletion mutants. Chromosomal DNA was isolated as described elsewhere (Desomer *et al.* 1991). Total RNA was isolated using the E.Z.N.A. bacterial kit (Omega Bio-Tek, Doraville, USA). PCR products were purified using QIAquick[®] Spin kit (Qiagen, Hilden; Germany).

Bacteria, growth media and growth conditions

C. necator H16 (DSM 428; e.g. Pohlmann *et al.* 2006) was isolated in Göttingen. *R. nubinhibens* ISM (González *et al.* 2003) was kindly provided by M. A. Moran, *B. xenovorans* LB400 (e.g. Chain *et al.* 2006) by J. M. Tiedje, *Methylobacterium extorquens* CM4 (formerly *M. chloromethanicum*) and *Methylobacterium extorquens* DM4 (formerly *M. dichloromethanicum*) by S. Vuilleumier. *O. carboxidovorans* OM5 (DSM 1227) (e.g. Meyer *et al.* 1993) was purchased from the German Culture Collection (DSMZ,

Braunschweig, Germany) and cultivated according to the Collection's instructions. *C. necator* H16, *B. xenovorans* and *Methylobacterium* spp. were grown in fresh-water, mineral-salts medium (Thurnheer *et al.* 1986), whereas *R. nubinhibens* ISM was cultivated using modified *Silicibacter* basal medium (SBM-M) (Denger *et al.* 2006a). Sulfoacetate (10-20 mM) was used as the sole added source of carbon and energy for growth. For enzyme assays with the mutants which were unable to grow with sulfoacetate, 10 mM acetate served as a growth substrate in the presence of 10 mM sulfoacetate to induce the sulfoacetate degradative enzymes.

Precultures and cultures (5 ml) for the determination of the substrate range were grown in 50-ml screw-cap 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 optical density at 580 nm, to assay protein, and to determine the concentrations of substrate and products.

Escherichia coli strains used for site-directed mutagenesis (see Table S3) were grown in LB-medium at 37 °C. Appropriate antibiotics were added to media in the following concentrations: 50 µg ml⁻¹ ampicillin, 20 µg ml⁻¹ tetracycline, and 5 µg ml⁻¹ trimethoprim.

Preparation of cell-free extracts

Cell-free extracts were obtained from organisms grown on the 1 l scale in 5-l Erlenmeyer flasks. 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 resuspended in a small volume (2 to 5 ml) of the same buffer. Cells were disrupted by four passages through a chilled French press set at 138 MPa, and whole cells and cell debris were removed by centrifugation (20 000 g, 3 min, 4 °C). The membrane and soluble fractions were obtained by ultracentrifugation (200 000 g, 30 min, 4 °C). DNA was removed by addition of DNase prior to disruption.

Enzyme assays

Sulfoacetate-CoA ligase (SauT) was assayed discontinuously by HPLC as the formation of sulfoacetyl-CoA. The reaction mixture contained (in a final volume of 1 ml): 50 µmol Tris/HCl buffer, pH 8.0 or 9.0 (containing 5 mM MgCl₂), 1 µmol ATP, 2 µmol sulfoacetate, 0.5 µmol CoA and 0.1-1 mg protein. SauT activity was estimated through decrease of CoA, since no reference material of sulfoacetyl-CoA was available for quantification of the novel compound. Sulfoacetaldehyde dehydrogenase (acetylating) (SauS) was assayed spectrophotometrically as the sulfoacetaldehyde-dependent reduction of NADP⁺ at 365 nm, which is the reverse reaction. The reaction mixture contained (in a final volume of 1 ml): 50 µmol Tris/HCl buffer, pH 9.0 (containing 5 mM MgCl₂), 1 µmol NADP⁺, 3 µmol sulfoacetaldehyde, 0.5 µmol CoA and

protein concentrations ranging from 1-100 μg of protein. The reaction was linear for at least 1 min.

Enzyme purification of SauS and identification of the corresponding gene

For purification of SauS, anion exchange chromatography (MonoQ, HR 10/10, Pharmacia) was performed with soluble fraction at a flow rate of 1 ml min^{-1} . An increasing gradient of sodium sulfate in 50 mM Tris/HCl buffer, pH 8.7, was applied, and SauS eluted at 85 mM sodium sulfate. Active fractions were combined, rebuffed on PD10 columns with 50 mM potassium phosphate buffer, pH 6.5, and loaded on to a cation exchange column (MonoS, HR 5/5, Pharmacia). Again applying in increasing gradient, SauS eluted at 95 mM sodium sulfate. Samples for the determination of the *N*-terminal amino acid sequence or for peptide mass fingerprinting were excised bands from SDS-PAGE gels, which were analyzed by Toplab (Martinsried, Germany).

Enzyme separation of SauT

For separation of SauT, anion exchange chromatography with a MonoQ column (see above) was performed with soluble fraction. In 50 mM potassium phosphate buffer, pH 6.5, an increasing gradient of sodium sulfate was applied, and SauT eluted at 120 mM sodium sulfate. Fractions were desalted, concentrated and subjected to a hydroxyapatite column. In 10 mM potassium phosphate buffer, pH 6.7, an increasing gradient of potassium phosphate was applied, and SauT eluted at about 100 mM potassium phosphate.

Analytical methods

Sulfoacetate was determined by ion chromatography (Denger *et al.* 2004b). Sulfate was quantified turbidimetrically as an insoluble suspension of BaSO_4 (Sörbo 1987). Sulfite was quantified as the fuchsin adduct (Ruff *et al.* 2003). Growth was followed turbidimetrically at 580 nm or assayed as Lowry-type protein in whole cells (Kennedy and Fewson 1968). Protein content of crude cell extracts was determined by protein-dye binding (Bradford 1976). Denatured proteins were analyzed on 13 % SDS-PAGE gels and stained with Coomassie Brilliant Blue R250 (Laemmli 1970). Native molecular weight of separated proteins was calculated after gel filtration by interpolation in a standard curve. Values K_m^{app} were derived by hyperbolic curve-fitting.

Identification of sulfoacetyl-CoA

Sulfoacetyl-CoA was visualized by reversed phase HPLC with diode array detection. The stationary phase was Nucleosil 5-C18 (125 x 3 mm). The mobile phase (0.5 ml min^{-1}) was a 100 mM potassium phosphate solution pH 5 with a gradient from 0 to 30 % methanol. The

identity of sulfoacetyl-CoA was confirmed by matrix-assisted, laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Samples of reaction mixtures containing putative sulfoacetyl-CoA generated in an assay of purified SauS were mixed with a matrix of saturated α -cyano-4-hydroxy-cinnamic acid in 50 % acetonitrile and 1 % trifluoroacetic acid (HCCA-matrix). The dried-droplet-method, with 0.8 μ l samples on a MALDI steel target, was used, and samples were analyzed in the negative-ion mode in a Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer (Hollemeier *et al.* 2007).

Cloning and sequencing

Standard DNA techniques (Ausubel *et al.* 1987; Sambrook *et al.* 1989) were used to isolate bacterial plasmid DNA, to transform plasmid DNA into *E. coli*, and for general DNA handling. Low-throughput plasmid DNA was isolated using the QIAprep[®] Miniprep kit, high-throughput isolation of plasmid DNA was done using a boiling method (Holmes and Quigley 1981). Colony PCR was done as described elsewhere (Coenye *et al.* 2002). Sequencing was performed at GATC (Konstanz, Germany) or Microsynth, and the sequences were analyzed using the Lasergene package of DNASTAR (Madison, WI, USA).

Reverse transcription (RT-)PCR

The primers listed in Table S1 were used for RT-PCR reactions. PCR was done as described elsewhere (Innis *et al.* 1990). Absence of DNA after isolation of RNA was tested by PCR using primers for *xsc*. Positive controls for the success of RNA isolation were done after reverse transcription using 16S rRNA-specific primers 16S-27F and 16S-533R (Table S1; Weisburg *et al.* 1991). PCR products were visualized after separation on 1.5 % agarose gels.

Construction of deletion mutants in individual *sau*-genes

Mutants of *C. necator* H16 containing in-frame deletions in one of the *sauSTU* genes were constructed by gene replacement mutagenesis (for details see Supplemental Material). Thereby, the wild-type functional genes were replaced by an engineered defective small gene.

Sequence analyses and accession numbers

Sequences of the genomes of *C. necator* H16 (accession numbers NC_008313 and NC_008314), *Burkholderia xenovorans* LB400 (accession numbers NC_007951, NC_007952 and NC_007953), *R. nubinhibens* ISM (accession number NZ_AALY00000000) and *Oligotropha carboxidovorans* OM5 (NC_011386) were obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), whose BLAST (Altschul *et al.* 1997) server was also used. Sequence data were analyzed using different subroutines of the Lasergene software package (DNASTAR), with SignalP (Bendtsen *et al.* 2004), available from the Center for

Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/>), with PROSITE, on the ExPASy Proteomics Server (<http://www.expasy.org/>), with HMMTOP 2.0 (<http://www.enzim.hu/hmmtop/index.html>) (Tusnády and Simon 2001) and with the Blast server of the Transport Classification Database (<http://www.tcdb.org>). Neighbour-joining trees were generated using the program NJplot, based on ClustalX-alignments.

RESULTS

Growth kinetics

C. necator H16 grew exponentially ($\mu = 0.14 \text{ h}^{-1}$) with 10-20 mM sulfoacetate as the sole source of carbon and energy (Fig. S1A). Growth was concomitant with substrate utilization and with the stoichiometric recovery of the sulfonate moiety as sulfate (Fig. S1B). The molar growth yield was $5.1 \text{ g protein (mol C)}^{-1}$, somewhat lower than the $6.0\text{-}6.6 \text{ g protein (mol C)}^{-1}$ with taurine, isethionate or acetate. The specific utilization rate of sulfoacetate was calculated to be $7.6 \text{ mkat (kg protein)}^{-1}$. Similar data were obtained with cultures of *B. xenovorans* LB400 ($\mu = 0.04 \text{ h}^{-1}$), where the lower growth yield with sulfoacetate was more marked: $3.0 \text{ g protein (mol C)}^{-1}$ compared with $4.7\text{-}5.9 \text{ g protein (mol C)}^{-1}$ for taurine, isethionate or acetate. *R. rubinhibens* ISM also grew exponentially ($\mu = 0.05 \text{ h}^{-1}$) with quantitative utilization of sulfoacetate. *O. carboxidovorans* OM5 grew slowly with sulfoacetate ($\mu = 0.008 \text{ h}^{-1}$); acetate ($\mu = 0.017 \text{ h}^{-1}$) supported faster growth. The molar growth yield was $4.3 \text{ g protein (mol C)}^{-1}$ which was lower than with acetate ($5.2 \text{ g protein (mol C)}^{-1}$). There is, thus, mass balance for sulfoacetate utilization in all organisms tested, and a tendency to lower growth yields.

Enzyme activities and growth of mutants of *C. necator* H16

The first scalar enzyme in the postulated pathway (Fig. 2A) was SauT. Extracts of acetate- (Table 1), taurine- or isethionate-grown cells (not shown) showed no activity of this enzyme. Extracts of sulfoacetate-grown cells catalyzed the CoA- and ATP-dependent conversion of sulfoacetate to sulfoacetyl-CoA (see below) and AMP. This was interpreted as inducible activity of SauT, which was shown by ultracentrifugation to be soluble. The specific activity of the enzyme was low (Table 1), and difficult to quantify, because we could not stop the reaction without destroying the sulfoacetyl-CoA formed during the reaction.

The second scalar enzyme was SauS (Fig. 2A). Extracts of acetate- (Table 1), taurine- or isethionate-grown cells (not shown) showed no activity of this enzyme. Extracts of sulfoacetate-grown cells catalyzed the NADP⁺- and CoA-dependent conversion of sulfoacetaldehyde to sulfoacetyl-CoA (see below). This was interpreted as inducible activity of SauS, which was

found to be soluble. The enzyme was highly active (Table 1), which allowed enough sulfoacetyl-CoA to be collected from the HPLC to confirm that SauS was also active in the forwards reaction. SauS was identified as the gene product encoded at H16_A2747 (see below).

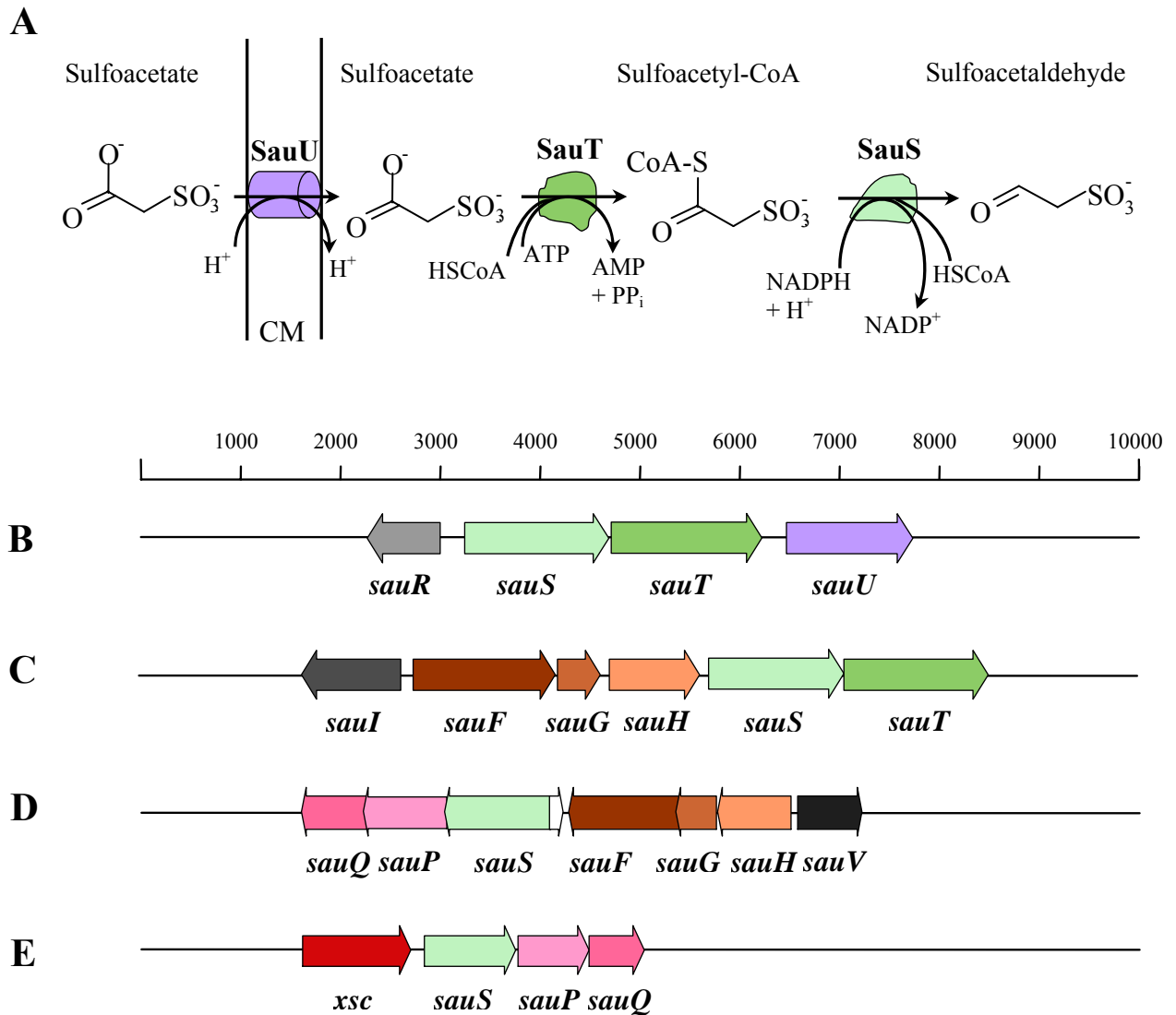


Fig. 2. Initial reactions in the pathway for the dissimilation of sulfoacetate in *C. necator* H16 (A), the cluster of genes encoding regulated expression of pathway proteins (B), and exemplary *sau*-gene clusters in genomes of other organisms belonging to different clades and living in diverse habitats (see Table 4): *Roseovarius nubinhibens* ISM (C), *Oligotropha carboxidovorans* OM5 (D), and *Desulfitobacterium hafniense* DCB-2 (E). Abbreviations: CM, cytoplasmic membrane; SauR, putative regulator of sulfoacetate utilization; SauS, sulfoacetaldehyde dehydrogenase (deacylating); SauT, single-subunit sulfoacetate-CoA ligase; SauU, MFS (major facilitator superfamily)-type transporter responsible for sulfoacetate uptake. SauI, putative LacI-type transcriptional regulator; SauFGH, putative TTT (tripartite tricarboxylate transporter)-family transporter for sulfoacetate uptake; SauPQ, heteromeric sulfoacetate-CoA ligase; SauV, putative LysR-type transcriptional regulator; Xsc, sulfoacetaldehyde acetyltransferase.

Table 1. Activities of enzymes and transcription of genes under different growth conditions of *C. necator* H16.

	Specific activity or transcription in cells grown with:	
	Sulfoacetate	Acetate
Sulfoacetaldehyde dehydrogenase (SauS)	10.4 ^a	n.d. ^b
Transcription of <i>sauS</i>	+ ^c	-
Sulfoacetate-CoA ligase (SauT)	0.2	n.d.
Transcription of <i>sauT</i>	+	-
Transcription of <i>sauU</i> (Sulfoacetate importer)	+	-

^a Specific enzyme activity is given in the SI unit mkat (kg protein)⁻¹; ^b n.d., not detected; ^c RNA transcript is scored as: -, absent; +, strong

Each of the candidate genes (Fig. 2B) to encode SauU (uptake (major facilitator superfamily (MFS) [TC 2.A.1.14.-]); H16_A2749), SauT (H16_A2748) and SauS (H16_A2747) was found to be transcribed inducibly (Fig. 3, Table 1), which corresponded to the inducible nature of the degradative pathway.

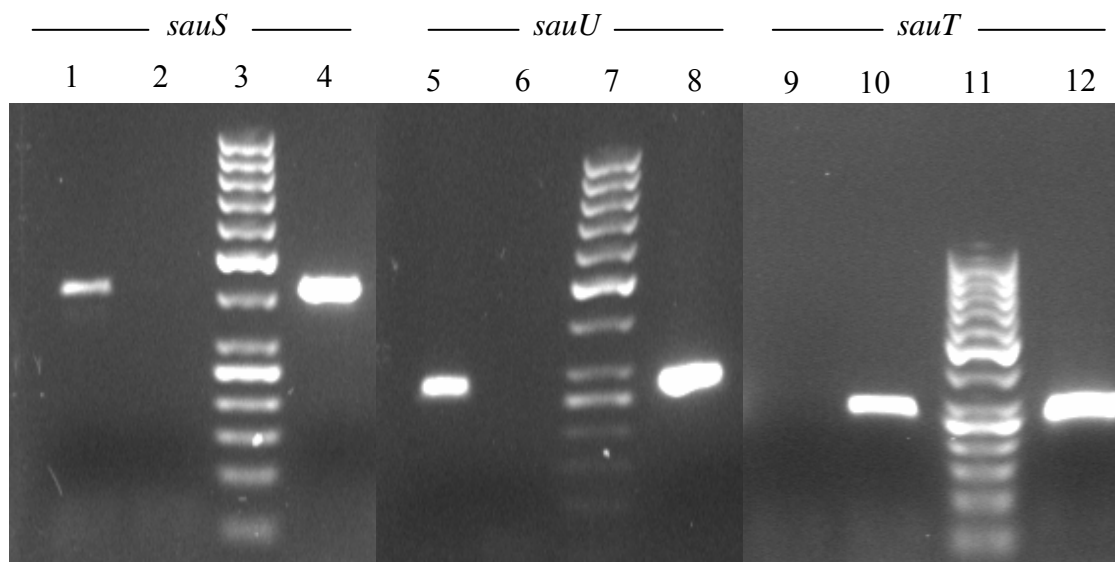


Fig. 3. Reverse transcription experiments with RNA of *C. necator* H16. Lanes 3, 7, and 11, marker (50bp DNA-ladder); lanes 4, 8, and 12, positive control (chromosomal DNA as template); lanes 1, 5, and 10, cDNA from sulfoacetate-grown cells as a template; lanes 2, 6, and 9, negative control (no DNA).

We were able to generate three deletion mutants, each with an in-frame mutation in *sauU* (H16 Δ *sauU*), *sauT* (H16 Δ *sauT*) or *sauS* (H16 Δ *sauS*), respectively. None of these mutants grew

with sulfoacetate, but each grew with acetate, taurine, isethionate or sulfoacetaldehyde, as the wild type did. The mutations were specific for sulfoacetate metabolism (see Fig. 1).

Mutant H16 Δ *sauU* synthesized neither SauS nor SauT (Table 2), so presumably no sulfoacetate entered the cell to enable induction to occur. In contrast, mutant H16 Δ *sauT* expressed SauS (Table 2) and mutant H16 Δ *sauS* expressed SauT (Table 2), so regulation of the gene cluster, presumably by SauR (Fig. 2B), was unaffected. No mutation in *sauR* was obtained. There appears to be irrefutable evidence for the functions of SauSTU.

Table 2. Phenotypes and enzyme activities of different (*C. necator* H16-) deletion mutants.

	H16 Δ <i>sauS</i>	H16 Δ <i>sauT</i>	H16 Δ <i>sauU</i> ^a
Growth with sulfoacetate	-	-	-
Sulfoacetaldehyde dehydrogenase (SauS) activity	bdl ^b	22.3 ^c	bdl
Sulfoacetate-CoA ligase (SauT) activity	0.03	bdl	bdl

^a H16 Δ *sauU* mutant strains could not be induced by growth with acetate and sulfoacetate, because *sauU* encodes the sulfoacetate transporter and thus, sulfoacetate could not enter the cells to induce the degradative enzyme systems.

^b bdl, below detection limit; ^c specific enzyme activity is given in the SI unit mkat (kg protein)⁻¹.

Identification of sulfoacetyl-CoA as the intermediate in sulfoacetate degradation

Putative sulfoacetyl-CoA was detected by HPLC as a novel peak generated during the activation of sulfoacetate by SauT, and during the oxidation of sulfoacetaldehyde by SauS (see above). The UV-spectrum of the unknown involved maxima at 212 and 257 nm and a minimum at 225 nm, similar to HSCoA but with a slightly shorter (0.7 min) retention time. The compound had a half-life of about 2 h under these conditions (pH 9.0). Samples, taken during the reaction of purified SauS with sulfoacetaldehyde, NADP⁺ and HSCoA, were analyzed by MALDI-TOF-MS in the negative-ion mode. The formation of a compound ($m/z = 888.7 = [M-1]^-$) was detected. The value of *M* (889.7) corresponds to that calculated (889.6) for protonated sulfoacetyl-CoA. This was taken as confirmation of the identity of sulfoacetyl-CoA.

Purification and characterization of SauS

SauS was purified 175-fold to apparent homogeneity in two steps (Fig. 4, Table 3). The sequence of six *N*-terminal amino acids was determined to be SVQILH. This corresponded to the derived sequence of only one ORF (H16_A2747) in the genome of *C. necator* H16, which confirmed our hypothetical locus for *sauS* (Fig. 2B). Peptide mass fingerprinting of SauS confirmed this conclusion. The derived molecular mass of mature SauS, 51.5 kDa, corresponds to the value (about 52 kDa) for the denatured protein (Fig. 4) interpolated into the standard curve. Separation of native SauS on a calibrated gel filtration column allowed a molecular mass of 115 kDa to be calculated. Given the errors in the method (le Maire *et al.* 1996), we tentatively postulate that native SauS is a dimer.

SauS was specific for NADP⁺ as a cofactor which could not be replaced by NAD⁺. The K_m^{app} -values for NADP⁺, CoA and sulfoacetaldehyde were 64 μM , 102 μM and 330 μM , respectively. The enzyme was specific for sulfoacetaldehyde; none of the ten tested compounds was a substrate: acetaldehyde, phosphonoacetaldehyde, glycolaldehyde, formaldehyde, propionaldehyde, succinic semialdehyde, glyoxylate, betaine aldehyde, glyceraldehyde, 2-oxobutanoic acid. None of these compounds inhibited the enzyme reaction. The optimal activity of the enzyme was obtained in 50 mM Tris/HCl buffer pH 9 containing 5 mM magnesium chloride. The enzyme could be stored at 4 °C or -18 °C but lost 30 % of its activity after one week.

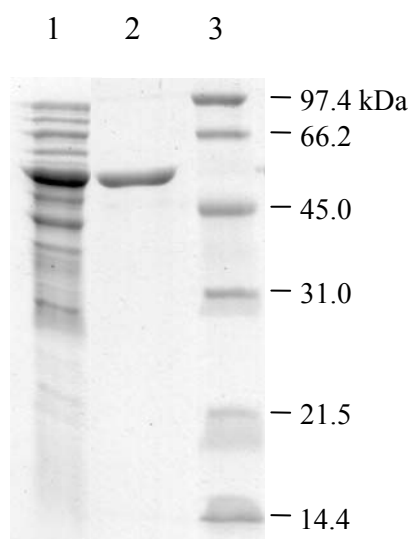


Fig. 4. Electropherogram of sulfoacetaldehyde dehydrogenase (SauS) after purification steps and of marker proteins. Lane 1, SauS after anion exchange chromatography; lane 2, SauS after cation exchange chromatography; lane 3, molecular weight markers.

Table 3. Purification of sulfoacetaldehyde dehydrogenase (SauS) from *C. necator* H16.

Purification Step	Specific activity (mkat kg ⁻¹)	Volume (ml)	Total protein (mg)	Recovery (%)	Purification (-fold)
Crude cell extract	7	2.1	53	100	1
Soluble fraction	9	2.0	37	94	1
Anion exchanger (Pool)	35	3.5	4	37	5
Cation exchanger	1191	0.9	0.002	1	175

Separation of SauT

Two separate steps with SauT were possible before activity was lost. This preparation showed sulfoacetate-dependent formation of sulfoacetyl-CoA and AMP, but SauT was insufficiently concentrated to be visible on SDS-PAGE gels.

Comparative genomics and experimental support for hypotheses

SauS was found to have 24 orthologues in bacteria from a range of phyla and habitats (Table 4). The locus was annotated ‘aldehyde dehydrogenase’, ‘acetaldehyde dehydrogenase’ or ‘succinate semialdehyde dehydrogenase’, but the SauS orthologues of sulfoacetate-utilizing bacteria and of bacteria containing further *sau*-genes in a cluster with *sauS* formed a single clade in a dendrogram (Fig. S2). This clade was separate from SauS orthologues derived from known sulfoacetate nonutilizers and bacteria lacking any additional *sau*-gene.

There were 7 different patterns of genes clustered around *sauS* (Table 4). Four other terrestrial betaproteobacteria share with *C. necator* H16 the cluster *sauRSTU* and the ability to utilize sulfoacetate (Table 4): where tested (*B. xenovorans* LB400), inducible activities of SauS and SauT were detected. Two other terrestrial betaproteobacteria contain *sauST*, but the rest of any pathway is unknown (Table 4).

The gene cluster in the strains of *Desulfitobacterium hafniense* (Table 4 and Introduction) and *Rhodopseudomonas palustris* BisA53, though inadequate for a complete pathway (Fig. 2E), suggests the presence of a two-subunit sulfoacetate CoA ligase (SauPQ), which could be explored more easily in the terrestrial alphaproteobacterium *O. carboxidovorans* OM5 (Fig. 2D). This organism utilized sulfoacetate (see above), and showed inducible activity of SauS and of sulfoacetate-CoA ligase, which we presume to be the gene product of *sauPQ*. The inducibility of SauPQS was attributed to the putative transcriptional regulator, SauV, which was solely found in combination with heteromeric ligase SauPQ. It was also presumed to be active in the two strains

of *Methylobacterium extorquens* harbouring SauPQ (Table 4). These organisms do not utilize sulfoacetate as a sole source of carbon (and energy) for growth, but do degrade the compound if a further energy source is supplied (Table 4). We attribute this to the generally poor energy yield from sulfoacetate (see above) being in this case additionally reduced, when the standard degradative pathway of sulfoacetaldehyde (via Xsc and Pta; see Introduction) is replaced by a lower-yield alternative: instead of phosphotransacetylase (Pta), a combination of acetate kinase (Ack) and energy-consuming acetate-CoA ligase (ACS) is used to generate acetyl-CoA from acetyl phosphate (for details see Supplemental Material).

O. carboxidovorans OM5 also introduces a different transporter, SauFGH (Table 4), which was more suitably examined in *R. nubinhibens* ISM (see Fig 2C), with which RT-PCR was successful. SauFGH was a candidate tripartite tricarboxylate transporter (TTT) [TC 2.A.80.-.-], whose characterized representatives (TctABC) are 504, 144 and 325 residues long, with 12, 4 and 0 predicted transmembrane helices (TMH), respectively (Winnen *et al.* 2003). *R. nubinhibens* ISM was shown to express SauST inducibly (see above) and RT-PCR showed that *sauFGH* were transcribed inducibly (Fig. S3), and presumably expressed: the derived proteins, SauFGH are 495 (14 TMH), 157 (4 TMH) and 320 residues long (0 TMH), respectively. The induction was presumed to be under the control of SauI, present in 12 alphaproteobacteria (Table 4).

Table 4. Bacteria with orthologues of *sau* (sulfoacetate utilization)-genes: phylum, habitat and growth.

Organism (locus tag abbreviation)	<i>sauFGH</i>	<i>sauI</i>	<i>sauV</i>	<i>sauPQ</i>	<i>sauR</i>	<i>sauS</i>	<i>sauT</i>	<i>sauU</i>	Phylum	Habitat	Growth
<i>Burkholderia xenovorans</i> LB400 (Bxe_)	-	-	-	-	B0700	B0701	B0702	B0703	beta	terrestrial	+
<i>Cupriavidus necator</i> H16 (H16_)	-	-	-	-	A2746	A2747	A2748	A2749	beta	terrestrial	+
<i>Cupriavidus necator</i> JMP134 (Reut_)	-	-	-	-	A0868	A0867	A0866	A0865	beta	terrestrial	+
<i>Burkholderia phymatum</i> STM815 (Bphy_)	-	-	-	-	6219	6218	6217	6216	beta	terrestrial	+
<i>Burkholderia phytofirmans</i> PsJN (Bphyt_)	-	-	-	-	4381	4382	4383	4384	beta	terrestrial	+
<i>Verminephrobacter eiseniae</i> EF01-2 (Veis_)	- ^a	- ^b	-	-	-	4280	4281	-	beta	terrestrial	n.t. ^c
<i>Variovorax paradoxus</i> S110 (Vapar_)	- ^a	- ^b	-	-	-	3831	3832	-	beta	terrestrial	n.t.
<i>Desulfitobacterium hafniense</i> Y51 (DSY)	- ^a	- ^b	-	0246-0245	-	0244	-	-	Firmicutes	terrestrial	n.t.
<i>Desulfitobacterium hafniense</i> DCB-2 (Dhaf_)	- ^a	- ^b	-	0191-0192	-	0190	-	-	Firmicutes	terrestrial	n.t.
<i>Methylobacterium extorquens</i> CM4 (Mchl_)	-	-	4222	4206-4207	-	4208	-	4209	alpha	terrestrial	+ ^{d,e}
<i>Methylobacterium extorquens</i> DM4 (METDI)	-	-	0779	0775-0776	-	0777	-	0778	alpha	terrestrial	+ ^{d,e}
<i>Oligotropha carboxidovorans</i> OM5 (OCAR_)	6740-6742	-	6743	6736-6737	-	6738	-	-	alpha	terrestrial	+
<i>Rhodopseudomonas palustris</i> BisA53 (RPE_)	- ^a	- ^b	-	3202-3203	-	3204	-	-	alpha	terrestrial	n.t.

Organism (locus tag abbreviation)		<i>sauFGH</i>	<i>sauI</i>	<i>sauV</i>	<i>sauPQ</i>	<i>sauR</i>	<i>sauS</i>	<i>sauT</i>	<i>sauU</i>	Phylum	Habitat	Growth
<i>Roseobacter denitrificans</i> Och 114	(RD1_)	2922-2920	2923	-	-	-	2918	2917	-	alpha	marine	n.t.
<i>Jannaschia</i> sp. strain CCS1	(Jann_)	1563-1565	1562	-	-	-	1566	1567	-	alpha	marine	n.t.
<i>Roseobacter</i> sp. strain AzwK-3b	(RAZWK3B_)	05127-05117	05132	-	-	-	05112	05107	-	alpha	marine	n.t.
<i>Roseobacter litoralis</i> Och149	(RLO149_)	19174-19184	19169	-	-	-	19189	19194	-	alpha	marine	n.t.
<i>Roseovarius nubinhibens</i> ISM	(ISM_)	08295-08305	08290	-	-	-	08310	08315	-	alpha	marine	+
<i>Roseovarius</i> sp. strain 217	(ROS217_)	07190-07180	07195	-	-	-	07175	07170	-	alpha	marine	- ^d
<i>Roseovarius</i> sp. TM1035	(RTM1035_)	16722-16732	16717	-	-	-	16737	16742	-	alpha	marine	n.t.
<i>Alphaproteobacterium</i> sp. HTCC2255	(OM2255_)	04005-03995	04010	-	-	-	03990	03985	-	alpha	marine	n.t.
<i>Rhodobacterales</i> sp. HTCC2150	(RB2150_)	12706-12696	12711	-	-	-	12691	12686	-	alpha	marine	n.t.
<i>Rhodobacterales</i> sp. HTCC2083	(RB2083_)	1184, 2033, 2301 1550		-	-	-	2432	2223	-	alpha	marine	n.t.
<i>Thalassiosibium</i> sp. R2A62	(TR2A62_)	0970-0972	0973	-	-	-	0969	0968	-	alpha	marine	n.t.
<i>Paracoccus denitrificans</i> PD1222	(Pden_)	-	1014	-	-	-	1013	1012	1011	alpha	terrestrial	+

^a Neither *sauFGH* nor *sauU* is present in the cluster, transport unclear. ^b Neither *sauR* nor *sauI* is present in the cluster, regulation unclear. ^c n.t., not tested. ^d No *pta* present in the genome (see Discussion). ^e Organism degrades sulfoacetate only in presence of an additional energy source (e.g. 10 mM succinate).

DISCUSSION

The pathway in Figure 2 (and its equivalents in other organisms; see Table 4) shows a considerable input of energy due to the initial activation of sulfoacetate. This input is necessary to reduce the growth substrate (a carboxylic acid) to the corresponding aldehyde, which is the substrate for the only known, high-turnover desulfonative enzyme with a two-carbon substrate, Xsc (Brüggemann *et al.* 2004; Denger *et al.* 2001; Ruff *et al.* 2003). One consequence of this unusual energy input into a degradative pathway is obviously that the molar growth yield is reduced, sometimes by only about 20 %, as in *C. necator* H16 (see above), sometimes up to 40 %, as in *B. xenovorans* LB400 (see above) and in *Ralstonia* spp. (Denger and Cook 2001). We suspect that this lowering of the growth yield can be extreme, such that *M. extorquens* spp. do not grow with sulfoacetate as sole source of carbon and energy (Table 4), but they do degrade the compound only in the presence of a second carbon source (see above). An even more extreme effect may be seen in *Roseovarius* sp. strain 217 (Table 4) which failed to degrade sulfoacetate under any condition tested. In both cases, the organisms show a modified pathway of sulfoacetaldehyde degradation (Baldock *et al.* 2007), whose effect is to reduce the energy output by one ATP-equivalent (see Supplemental Material).

When the lowered growth yield of *Ralstonia* spp. was discovered (Denger and Cook 2001), we suspected that a phosphorylated intermediate would be involved, analogous to steps in the biosynthesis of e.g. lysine or proline. The reactions we have established (Fig. 2) seem to be novel in degradative pathways.

The pathway contains three steps, one vectorial and two scalar, and a novel metabolic intermediate, sulfoacetyl-CoA (Fig. 2A). This compound must be as ubiquitous as sulfoacetate, but it will be almost undetectable in the environment, partly because it will be intracellular and partly because it is so labile. We suggested that sulfoacetate is a ubiquitous natural as well as xenobiotic component of the biosphere (see Introduction), although there are no direct determinations of this. We consider the biodiversity of sulfoacetate degradation (depicted in Fig. 2A and Table 4) to be an indicator to support this hypothesis, because organisms have evolved and sustained so many different variants of the degradative pathway. The first step in the pathway is transport, which involves SauU in *C. necator* H16 (Fig. 2). The cytoplasmic membrane is apparently impermeable for sulfoacetate unless a transporter is present, because deletion mutant H16 Δ *sauU* is obviously unable to induce expression of the *sau*-gene cluster (Table 3). This emphasizes the impermeability of membranes to sulfonates

pointed out elsewhere (Graham *et al.* 2002; Mampel *et al.* 2004) and supports the idea that nature uses the sulfonate substituent to prevent a molecule crossing a membrane. The sequence of SauU indicates that it belongs to the major facilitator superfamily [TC 2.A.1.-.-] of transporters. The closest orthologues (subgroup 14) have ~ 30 % sequence identity, but do share the predicted structure of 12 transmembrane helices. There seems to be some rationale in the distribution of transport systems; the low energy input to the MFS uptake system in terrestrial organisms, where higher concentrations of sulfoacetate may be expected, contrasts with the multi-component system (SauFGH), presumably a pump, in marine organisms, whose environment readily dilutes any excreted sulfoacetate (Krejčík *et al.* 2008). This is consistent with the finding that secondary transport systems containing extracytoplasmic solute receptors with high ligand affinity are widespread in marine organisms (Mulligan *et al.* 2007). There are also organisms (Table 4) which do not feature any genes encoding known transport proteins adjacent to the sulfoacetate degradative gene cluster. This implies even higher diversity in transport mechanisms for sulfoacetate.

Sulfoacetaldehyde dehydrogenase (SauS), apparently the marker enzyme for the pathway (Table 4), has been purified in this study. It shows highest similarity to aldehyde dehydrogenases (acetylating) [EC 1.2.1.10]. (Acet)aldehyde dehydrogenases [EC 1.2.1.-] (Powlowski *et al.* 1993) are thought to be NAD⁺-dependent enzymes (also reacting with NADP⁺ but at a lower rate), existing as dimers (Söhling and Gottschalk 1993), tetramers or polymers. Purified SauS from *C. necator* H16 is presumed to form a dimer in solution and acts solely with NADP⁺ as a cofactor and sulfoacetaldehyde as a substrate.

In most organisms degrading sulfoacetate, a gene encoding a putative transcriptional regulator is present directly adjacent to one of the *sau*-genes (see Table 4). We postulate it to represent the regulator responsible for transcription of the *sau*-genes. Three types of regulators were found in association with *sau*-genes: an IclR-type (SauR), a LacI-type (SauI) and a LysR-type (SauV) regulator. It seems that SauI occurs mainly in alphaproteobacteria whereas SauR occurs mainly in betaproteobacteria (except *O. carboxidovorans* OM5). SauV was exclusively observed in bacteria harbouring a heteromeric sulfoacetate-CoA ligase (SauPQ).

The two different ligases, SauT and SauPQ, are known, but currently uncharacterized. This novel pathway warrants much more research.

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Karin Denger for discussions, and to Stéphane Vuilleumier for kindly providing *Methylobacterium* spp. We also thank Tobias Erb (Freiburg, Germany) for sharing his experience on synthesizing CoA-esters and to the late Hal Dixon (Cambridge, England) for phosphonoacetaldehyde. S. W. was supported by a grant (Co 206/6-1) from the Deutsche Forschungsgemeinschaft to A. M. C and T. H. M. S. M. B. was supported by the University of Konstanz. B. K. and B. B. were supported by the competence network “BiotechGenoMik” financed by the German Federal Ministry of Education and Research (BMBF).

SUPPLEMENTAL MATERIAL

Table S1. Primers used in this study for RT-PCR with *C. necator* H16 and *R. nubinhibens* ISM.

Organism	Target	Name	Sequence (5'→3')
Bacteria	16S	16S-27F	CAGAGTTTGATCCTGGCTCAG
		16S-533R	TTACCGCGGCTGCTGGCAC
<i>C. necator</i> H16	<i>sauS</i>	H16sauSF	ATTCGGTCAGCCTGCATTCGTCC
		H16sauSR	GTAGTCGCCCAGCAGGTCCGCTTCA
	<i>sauT</i>	H16sauTF	TGCTGCTGACGCAGCGCAACCT
		H16sauTR	GATCGTTCAGCAGGTAGGCGATGAT
	<i>sauU</i>	H16sauUF	CTATGCCGCCATGCAGATTCTC
		H16sauUR	CTGTGCTTGTCGGGCGAATCCTTGA
<i>R. nubinhibens</i> ISM	<i>sauS</i>	ISM_sauSF	GACAAGATCACCAAGAACCAT
		ISM_sauSR	TCGGCATGGATATACTCGAGCAG
	<i>sauT</i>	ISM_sauTF	TCTGTTCTTCGACGGAGGCGATCCGCT
		ISM_sauTR	TGGTGCCGGAGGTGTACATCAA
	<i>sauF</i>	ISM_sauFF	ACCACGCTCGACGGTCACACCA
		ISM_sauFR	GAACCGCTGCGCACCCATGATGT
	<i>sauG</i>	ISM_sauGF	TCCGCGGCTGATCTCGACCGTCTTC
		ISM_sauGR	GACGGCGGTCATCACGGCGCGT
	<i>sauH</i>	ISM_sauHF	TTCTCGGGCGCGGGCCTCTTTGT
		ISM_sauHR	CACACCGCGAAAGTTCACCGAGGCGT

Table S2. Primers used in this study for site-directed in-frame mutagenesis in *C. necator* H16. D, primer was used for construction of the small defective gene by which the wild-type gene was replaced during mutagenesis. S, primer was used for PCR and sequencing of the correspondent gene and its surrounding in the constructed deletion mutants. Introduced restriction sites are underlined.

Target	Aim	Name	Sequence (5' → 3')
<i>sauS</i>	D	h16_A2747_Xf	GCGGACAGGAT <u>CTAGAGA</u> ATGCGGAAG
	D	h16_A2747_Hr	GCAAATCGGAAGCTTTGGACTGCC
	D	h16_A2747_Hf	GCCCGATGAAGCTT <u>ACCTGCTGGG</u>
	D	h16_A2747_Br	GCAGGTTGATCGGATCCACCACC
	S	h16_sauSproof_f2	GAGCACTCGTAGTGGCGGTT
	S	h16_sauSproof_r2	GGTGCCGGACGTGTACATGAGCA
<i>sauT</i>	D	h16_A2748_Xf	GCAGCTTGGTCTAGAGCTGCCGGTG
	D	h16_A2748_Er	GCGTGTCGAAGAATTCGGGTTCCG
	D	h16_A2748_Ef	AAGACGCCGAAGGAATTCGCTTTATC
	D	h16_A2748_Br	TGCTTCATAGCCGGATCCTATGCGC
	S	h16_sauTproof_f2	ACAATGCCACCAGTTGCTCTTCGG
	S	h16_sauTproof_r1	GGTAGGCAAAGGCCGAGAACACCA
<i>sauU</i>	D	h16_A2749_Xf	CTGGGCCGCTTCTAGACGCCG
	D	h16_A2749_Er	GATCATGTGCCGGGAATTCATACGTTG
	D	h16_A2749_Ef	GCTGTTCATGAATTCGCTGCTGACG
	D	h16_A2749_Br	GCTATGCGGATCCCAAGCGCG
	S	h16_sauUproof_f2	ATGGCTATTTCTACATCAGCGG
	S	h16_sauUproof_r1	TCTACATGTCGTTTCGCGCAA
pUC19	D	ABI_for	ACGACGTTGTAAAACGACGGCCAG
	D	ABI_rev	TTCACACAGGAAACAGCTATGACC

Table S3. Strains used for and gained by deletion mutagenesis.

Strain	Description	Reference
<i>E. coli</i> JW1	-	(Kolmar <i>et al.</i> 1990)
<i>E. coli</i> S17-1	Integrated RP4, T _p ^R , Sm ^R	(Simon <i>et al.</i> 1983)
<i>C. necator</i> H16	Wildtype	(Pohlmann <i>et al.</i> 2006)
<i>C. necator</i> H16Δ <i>sauS</i>	Deletion mutant of H16 in <i>sauS</i>	This study
<i>C. necator</i> H16Δ <i>sauT</i>	Deletion mutant of H16 in <i>sauT</i>	This study
<i>C. necator</i> H16Δ <i>sauU</i>	Deletion mutant of H16 in <i>sauU</i>	This study

Plasmid	Description	Reference
pUC19	ColE1, Ap ^R	(Yanisch-Perron <i>et al.</i> 1985)
pUC19Δ <i>sauS</i>	pUC19 with flanking regions of <i>sauS</i>	This study
pUC19Δ <i>sauT</i>	pUC19 with flanking regions of <i>sauT</i>	This study
pUC19Δ <i>sauU</i>	pUC19 with flanking regions of <i>sauU</i>	This study
pNHG1	ColE1, RP4- <i>oriT</i> , Tc ^R , Km ^R , <i>sacB</i>	(Jeffke <i>et al.</i> 1999)
pNHG1Δ <i>sauS</i>	pNHG1 with insert of pUC19Δ <i>sauS</i>	This study
pNHG1Δ <i>sauT</i>	pNHG1 with insert of pUC19Δ <i>sauT</i>	This study
pNHG1Δ <i>sauU</i>	pNHG1 with insert of pUC19Δ <i>sauU</i>	This study

Biodiversity in sulfonate degradation

Analogous to *C. necator* H16 (Fig. 1), *Roseovarius nubinhibens* ISM also utilizes taurine, isethionate and sulfoacetate, but in taurine-degradation, taurine:pyruvate aminotransferase (Tpa, [EC 2.6.1.77]) replaces taurine dehydrogenase. In isethionate degradation, different isethionate transporters are involved, and a different sulfite exporter [TC 9.B.63.1.2] is involved in sulfonate metabolism (Denger *et al.* 2009; Weinitzschke *et al.* 2009). Strains H16 and ISM have Xsc's from different subgroups (Brüggemann *et al.* 2004). The major difference concerning sulfoacetate metabolism is the putative tripartite tricarboxylate transporter (TTT; [TC 2.A.80.-.-]) SauFGH, which is presumed to pump sulfoacetate into the cell (Fig. 2C), in contrast to the MFS transporter (SauU) in *C. necator* H16 (Fig. 2B).

Oligotropha carboxidovorans OM5 utilizes sulfoacetate and isethionate but lacks any gene encoding for enzymes of taurine deamination (TauXY or Tpa, respectively) and thus does not utilize the compound. Concerning sulfoacetate degradation, a potential alternative to SauT (Fig 2B) was found in the *sau*-gene cluster: a two-subunit ligase, SauPQ (Fig. 2D). The organism also potentially encodes SauFGH.

Each of the three major organisms in the study has a different potential regulator of sulfoacetate, SauR in *C. necator* H16 (Fig. 2B), SauI in *R. nubinhibens* ISM (Fig. 2C) and SauV in *O. carboxidovorans* OM5 (Fig. 2D).

Biodiversity also involves Pta, which is replaced by acetate kinase and acetate-CoA ligase in e.g. *Roseovarius* sp. strain 217 (Baldock *et al.* 2007) and *Methylobacterium* spp. (see Table 4). The replacement of Pta by an ATP-consuming acetate-CoA ligase may influence the ability of utilization of sulfoacetate as an energy source in those organisms (see Discussion).

Construction of deletion mutants in individual *sau*-genes

A small fragment at the 3-prime and another at the 5-prime end of the appropriate wild-type gene were amplified using primers with engineered restriction sites (Table S2). Primers were chosen such that in-frame deletions were generated. The size of the respective deletion was 1404 bp for *sauS*, 1431 bp for *sauT*, and 882 bp for *sauU*. The two amplicons were ligated into pUC19 (Yanisch-Perron *et al.* 1985) yielding plasmids pUC19 Δ *sauS*, pUC19 Δ *sauT* and pUC19 Δ *sauU* (Table S3). Subsequently, resulting clones for each of the constructs were controlled by sequencing with primers ABI_for and ABI_rev (Table S2) to exclude clones with undesired point mutations occasionally occurring during PCR amplification. The inserts were then subcloned into suicide vector pNHG1 (Jeffke *et al.* 1999) containing tetracycline resistance gene *tet* and *sacB* (encoding levansucrase) as selection markers (Kaniga *et al.* 1991), yielding plasmids pNHG1 Δ *sauS*, pNHG1 Δ *sauT* and pNHG1 Δ *sauU* (Table S3). These

plasmids were transferred from *E. coli* S17-1 into *C. necator* H16 by conjugative biparental mating (Srivastava *et al.* 1982) and doubly recombined mutants were selected as described elsewhere (Jeffke *et al.* 1999; Lenz *et al.* 1994). The mutant genotype of selected clones of each of the mutants was verified by PCR, using primers upstream and downstream of the mutation site (Table S2, Fig. S4), and by subsequent sequencing of the amplicons.

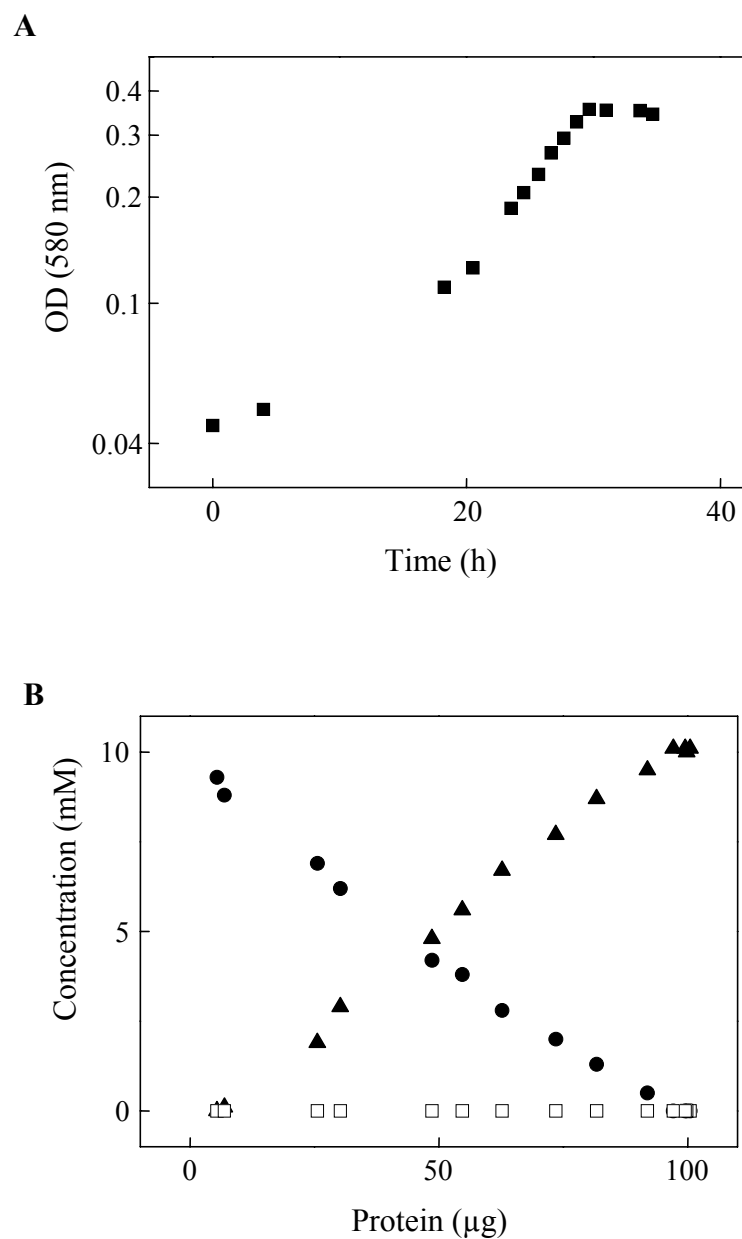
Quantification of growth of *C. necator* H16

Fig. S1. Semi-log growth curve (A) of *C. necator* H16 with 10 mM sulfoacetate as sole source of carbon and energy. Concentrations of substrate and products (B) are plotted as a function of protein concentration. Filled circles, sulfoacetate; filled triangles, sulfate; open squares, sulfite.

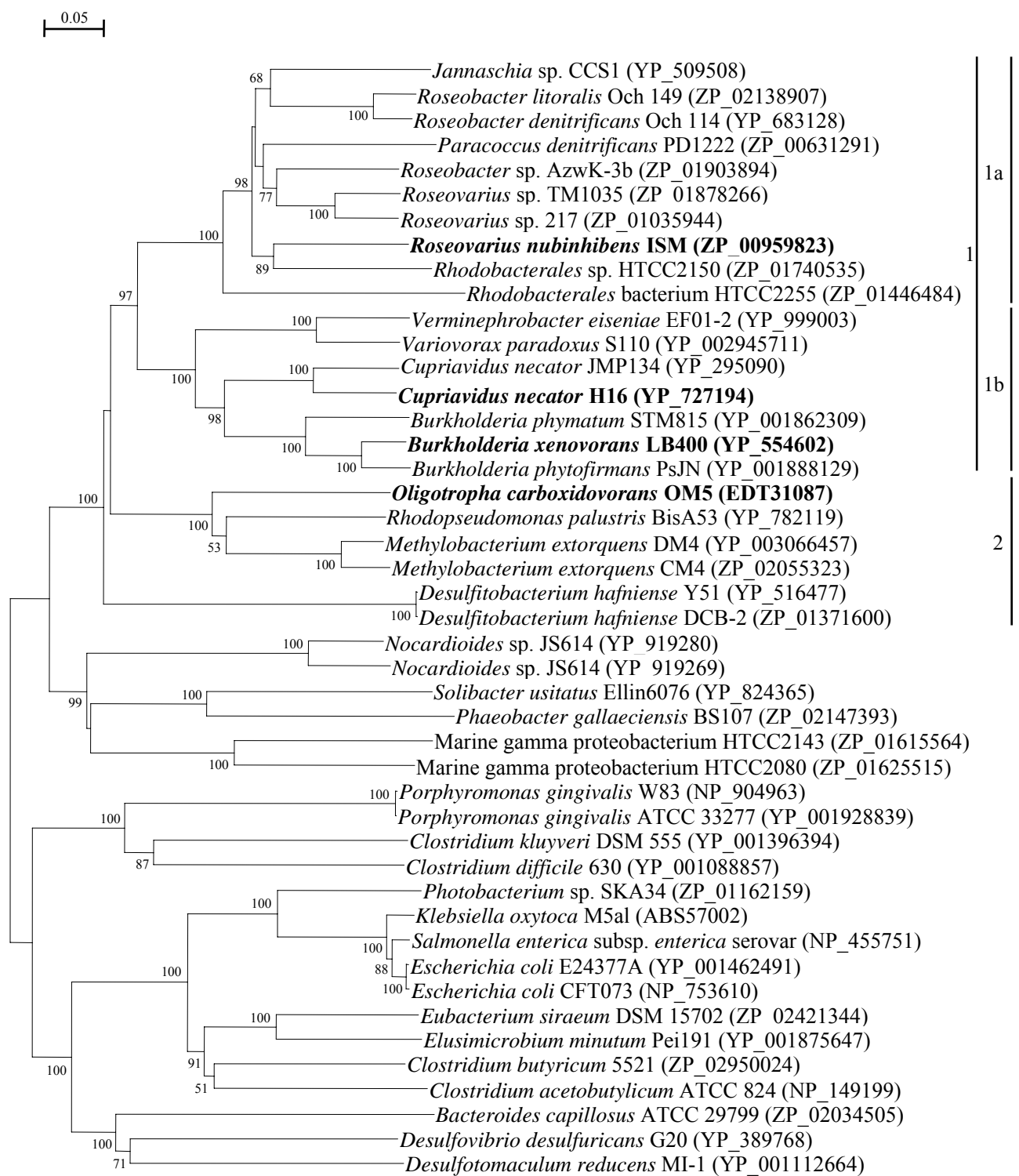


Fig. S2. Phylogenetic tree (generated with NJ plot based on a Clustal X alignment) of sulfoacetaldehyde dehydrogenase SauS and orthologues. Bacteria containing orthologues of SauS which were tested for enzyme activity in this study are given in bold face. Accession numbers are given in parentheses. Scale bar, 5 % sequence divergence. The bold vertical line indicates organisms containing *sau*-gene clusters, the group of organisms indicated by '1' contains orthologues of SauT (homomeric sulfoacetate-CoA ligase), '1a' indicates the alphaproteobacteria in the latter group, '1b' the betaproteobacteria. Group '2' includes organisms containing SauPQ (heteromeric sulfoacetate-CoA ligase).

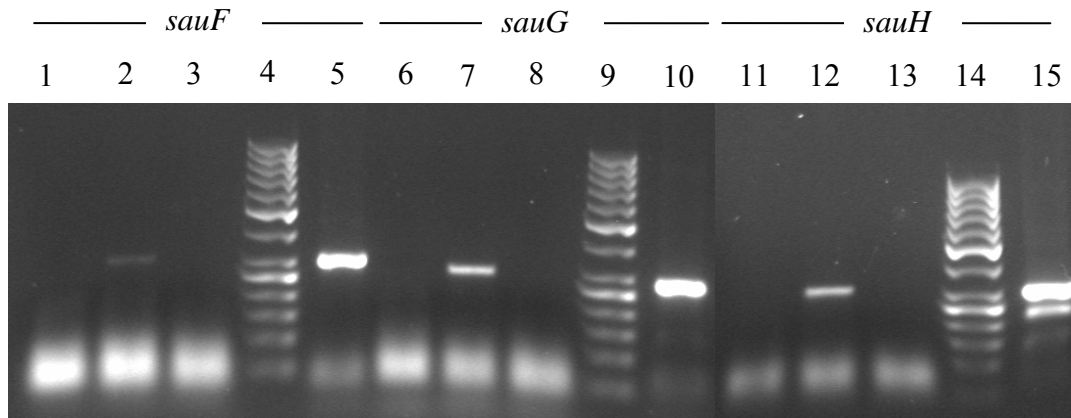


Fig. S3. Reverse transcription PCR with RNA of *R. nubinhibens* ISM. Lanes 4, 9, and 14, marker (50 bp DNA-ladder); lanes 5, 10, and 15, positive control (chromosomal DNA as template); lanes 1, 6, and 11, negative control (no DNA); lanes 3, 8, and 13, cDNA from acetate-grown cells as a template; lanes 2, 7, and 12, cDNA from sulfoacetate-grown cells as a template.

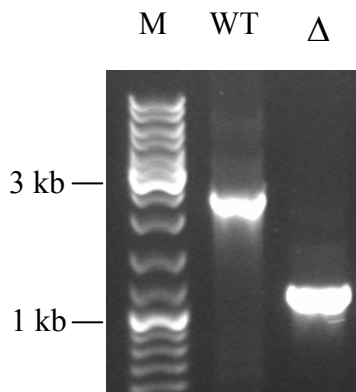


Fig. S4. Typical confirmation (by PCR) that a deletion mutant had been successfully generated. Amplicons of chromosomal DNA from strains H16 (WT) and H16Δ*sauU* (Δ) were obtained using primers h16_sauUproof_f2 and h16_sauUproof_r1 (see Table S2). M, marker (GeneRuler DNA ladder mix).

GENERAL DISCUSSION

Ubiquity of sulfonates in nature

Sulfonates are widespread in nature. Although there are no exact data of the amounts in soil, freshwater or ocean samples, their ubiquitous distribution can be assessed by the diverse – both natural and antropogenic – sources for sulfonates (see General Introduction). Another argument for the ubiquity of sulfonates was investigated during this study, namely the widespread ability of bacteria to degrade sulfonates in terrestrial and marine habitats. Several pure cultures of sulfonate utilizing bacteria as well as successful enrichment cultures of sulfonate degrading organisms were analyzed. By comparative genomics, 25 presumably sulfoacetate degrading (see Chapter 3), and 35 presumably isethionate degrading bacteria (see Chapter 2) were identified. In both cases, marine and terrestrial organisms were found. The list of organisms containing taurine degradative gene clusters is even longer (> 50) and also includes isolates from soil, freshwater and oceans. BLAST search in metagenomic databases confirmed the widespread occurrence of e.g. sulfoacetate degradative genes. Obviously, the potential to utilize sulfonates – as a source of carbon and energy, sulfur, nitrogen (where applicable), as electron donor or acceptor – is present and is retained in a wide range of bacteria. This argues for the presence of sulfonates in the biosphere in sufficient amounts to justify the maintenance of the gene clusters encoding this biodegradative potential.

As different sulfonates can be transformed amongst each other (Fig. 1), a variety of organisms in a habitat has the chance to benefit from the presence of one specific sulfonate (e.g. taurine) even if they do not possess the enzymes of the initial degradation steps (e.g. taurine dehydrogenase or taurine:pyruvate aminotransferase). They can still profit by utilizing intermediates of taurine metabolism (e.g. sulfoacetaldehyde, sulfoacetate, isethionate) which are excreted by other organisms (e.g. *Acinetobacter calcoaceticus* SW1, *Rhodopseudomonas palustris* CGA009, *Neptuniibacter caesariensis* MED92, *Klebsiella oxytoca* TauN1, *Chromohalobacter salexigens* DSM 3043; see General Introduction).

Thus, sulfonates can represent important sources of sulfur, carbon (and sometimes nitrogen) for a whole network of different bacteria.

Open questions in sulfoacetate degradation

Different pathways for sulfoacetate degradation?

In this study and others (e.g. King and Quinn 1997a), sulfoacetate degradation was observed to proceed via sulfoacetaldehyde (SAA). In contrast, 30 years before, degradation of sulfoacetate via glycolate by a putative hydrolytic cleavage was discovered (Martelli and Souza 1970). As the organism was then lost and not available for further investigations, we were interested in finding organisms by enrichment cultures which could exhibit the same pathway. Chromosomal DNA of ten sulfoacetate degrading enrichment cultures (from different soil samples) was subjected to PCR with degenerate primers for *xsc* (sulfoacetaldehyde acetyltransferase), representing the marker gene for the pathway involving SAA. All organisms showed the presence of an *xsc* gene (Weinitschke, unpublished data), thus indicating the presence of a degradative pathway for sulfoacetate proceeding via SAA. It is not yet clear if the pathway including desulfonation by Xsc is in fact prevailing in nature, the investigation of more samples would be needed to obtain statistically relevant data. But as at least 25 genome-sequenced organisms harbor the *sau*-genes identified in this study (see Chapter 3), including considerable diversity in transport and regulation mechanisms, it can be assumed that this pathway is of ecological relevance.

Regulation of sulfoacetate degradation

In the present study (Chapter 3), it was postulated that sulfoacetate degradation is regulated. This was concluded from inducible transcription of the responsible genes as well as inducibility of the corresponding degradative enzymes. In *C. necator* H16, adjacent to the genes encoding transport and degradation of sulfoacetate (*sauSTU*), there is a gene which is predicted to encode a transcriptional regulator (IcIR-family). We did not succeed in constructing a deletion mutant defective in *sauR*, which could have confirmed the function of the gene, but we nevertheless hypothesize *sauR* to encode a transcriptional regulator for the degradation of sulfoacetate. The gene *sauR* is present not only in *C. necator* H16, but also in most of the terrestrial betaproteobacteria, (see Chapter 3). In genomes of terrestrial alphaproteobacteria, another type of regulator is present: a gene encoding a transcriptional regulator of the LysR-family was found beside the degradative genes *sauSPQU*. And a third putative type of sulfoacetate regulator (belonging to the LacI-family) was observed in sulfoacetate degradative gene clusters of marine alphaproteobacteria. The function of these regulatory genes could be conclusively proven in future, carrying out further attempts of deletion mutagenesis.

Transport phenomena involved in sulfonate degradation

Export of sulfonates

In *C. necator* H16, the DUF81 family protein TauE was found to be involved in sulfite export after desulfonation of three sulfonates – taurine, isethionate or sulfoacetate – which was supported by inducible transcription of the gene during growth with each of the three compounds (see Chapter 1). Since TauE became of growing interest as a potential exporter of not only sulfite but also sulfonates during assimilation of taurine-nitrogen (see General Introduction), the involvement of the protein is currently investigated more intensively by mutational analysis.

Import of taurine

Two known transport mechanisms for taurine (TauABC, TauKLM) were mentioned in the General Introduction. In addition, a third putative transport protein (TauP) for taurine is currently under investigation. This potential alternative to TauABC and TauKLM was discovered in *Delftia acidovorans* SPH-1, where orthologues of several taurine degradative genes were found, but neither *tauABC* genes nor *tauKLM* genes could be identified in the genome sequence (David Schleheck, personal communication). Instead, between *tauR* and *tauX*, a gene is located encoding a permease. This permease represents an orthologue of the GABA (γ -aminobutyrate) permease GabP ([TC 2.A.3.1.4]; Hu and King 1998) which is a member of the amine-polyamine-choline (APC) transporters [TC 2.A.3.1.-]. This orthologue, which we tentatively termed TauP, was also found to be encoded adjacent to *tauR* in *C. necator* H16 suggesting a possible involvement in sulfonate utilization. Until now, this hypothesis is still largely unexplored except some experimental support: in *C. necator* H16, reverse transcription experiments showed constitutive transcription of *tauP* during growth with taurine or acetate as a carbon source (Vongrad and Weinitschke unpublished data). Furthermore, TauP was also considered to be responsible for transport of other sulfonates: when the C₃ sulfonate homotaurine served as a nitrogen source for *C. necator* H16, *tauP* was also transcribed (Mayer and Cook 2009). In contrast to *Delftia acidovorans* SPH-1, both *tauABC* and *tauP* are present in *C. necator* H16. As these *tauABC* genes were also found to be transcribed during growth with taurine as a carbon source (Vongrad & Weinitschke unpublished data), it remains unclear if *tauABC* or *tauP* is involved in taurine transport. It is also possible that both TauP and TauABC are responsible for taurine import, acting in different growth phases, i.e. at different taurine concentrations. Because of the inconclusive RT-PCR data, the role of TauP should be investigated by mutational analysis.

Import of isethionate

In association with isethionate degradation, an MFS family importer (IseU) for the compound was proposed in *C. necator* H16 (see Chapter 2). The involvement of the gene *iseU* was supported by its inducible transcription during growth of the organism with isethionate. A mutant defective in *iseU*, which could be constructed in future studies, would have the potential to unambiguously prove function of IseU.

Import of sulfoacetate

During sulfoacetate degradation in *C. necator* H16, the gene *sauU*, which encodes an MFS family transporter, was inducibly transcribed (see Chapter 3). Deletion mutagenesis was performed, and the resulting mutant (*C. necator* H16 Δ *sauU*) defective in *sauU* was not able to grow with sulfoacetate, whereas it was still able to utilize other sulfonates. Furthermore, in *C. necator* H16 Δ *sauU*, the enzymes for sulfoacetate degradation could not be induced by adding sulfoacetate to the growth medium which is in contrast to mutants defective in other *sau*-genes (*sauS* and *sauT*, respectively). This indicates that sulfoacetate is not able to enter the cell in *C. necator* H16 Δ *sauU*, providing conclusive proof for the identity of SauU as the importer for sulfoacetate in *C. necator* H16.

Genes involved in anaerobic degradation of sulfonates?

The anaerobe *Desulfitobacterium hafniense* DCB-2 is able to utilize isethionate and other sulfonates as an electron acceptor (Lie *et al.* 1999) in which acetate and sulfide are the end products. This process is presumed to proceed via sulfoacetaldehyde (SAA) with subsequent formation of acetate (Lie *et al.* 1996). However, in the sequenced genome of the organism, we did not find any of the gene candidates (*iseJUR* or *iseJKLMR*) for conversion of isethionate to SAA described in this study (Chapter 2). *D. hafniense* DCB-2 harbours two *xsc* genes (Dhaf_4634 and Dhaf_0189), which is also the case in some other organisms. In *P. denitrificans* PD1222, 3 *xsc* genes were found: *xsc1* is located in a cluster containing taurine-degradative genes (*tauRKLMXYZ*, *pta*), *xsc2* in a cluster with *sau*-genes (*sauISTU*), and *xsc3* in a cluster with *ise*-genes (*iseRUJ*). This gene organization implies that in some organisms, three *xsc* genes are present in independently regulated gene clusters responsible for utilization of different C₂ sulfonates, whereas other organisms only possess one *xsc* gene for the same three degradation pathways.

In *D. hafniense* DCB-2, the situation is still unclear: one of the *xsc* genes ('*xsc1*') is located near putative *sau*-genes (Fig. 2), upstream of which a dehydrogenase (annotated as

diaminopimelate dehydrogenase) is encoded followed by a DMT (drug metabolite) transporter. The other *xsc* (*xsc2*) is adjacent to a range of genes encoding putative MFS (major facilitator superfamily) transporters and a transcriptional regulator (GntR-family). Upstream of this second *xsc*, several transport systems (TRAP as well as ABC transporters) are encoded and a hypothetical protein with unknown function.

As no orthologues of IseJ, IseU and IseR were found to be encoded in the genome of *D. hafniense* DCB-2, it is probable that under anoxic conditions, another not yet characterized type of dehydrogenase is responsible for conversion of isethionate to SAA. The genes encoding this pathway for utilization of isethionate as electron acceptor could be encoded by genes adjacent to one of the *xsc* genes, or the genes could also be scattered over the genome.

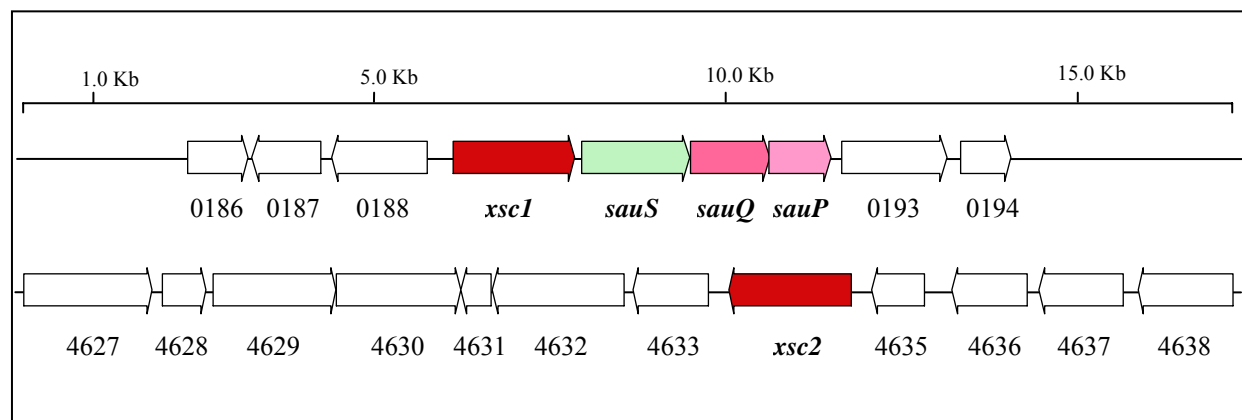


Fig. 2. The two *xsc* genes of *D. hafniense* DCB-2 and their flanking regions. In the following, the locus tag numbers of the genes and their respective annotation in the genome are listed (locus tag abbreviation is Dhaf_): 0186, protein of unknown function DUF6 transmembrane; 0187, diaminopimelate dehydrogenase; 0188, transposase family protein; 0189, sulfoacetaldehyde acetyltransferase (= Xsc1); 0190, hypothetical protein (= SauS); 0191, succinate-CoA ligase ADP-forming (= SauQ); 0192, CoA-binding domain protein (= SauP); 0193, anion transporter; 0194, transcriptional regulator, GntR family; 4627, amino acid permease; 4628, TetR family transcriptional regulator; 4629, ABC transporter (ATP-binding / permease protein); 4630, predicted transport protein (ATPase and permease component); 4631, hypothetical protein; 4632, TRAP dicarboxylate transporter, DctM subunit; 4633, TRAP dicarboxylate transporter, DctP subunit; 4634, sulfoacetaldehyde acetyltransferase (= Xsc2); 4635, transcriptional regulator, GntR family; 4636, methyl-accepting chemotaxis sensory transducer; 4637, pseudogene; 4638, major facilitator superfamily MFS_1.

As one of the two *xsc* genes is located near the genes encoding sulfoacetaldehyde dehydrogenase (SauS) and a heteromeric sulfoacetate-CoA ligase (SauPQ) (Fig. 2) which are probably responsible for conversion of sulfoacetate to SAA (see Chapter 3), we predict that *D. hafniense* DCB-2 is also able to use sulfoacetate as an electron acceptor for growth under anoxic conditions. The function of SauS was proven by enzyme purification and deletion mutagenesis of the orthologous gene in *C. necator* H16, and SauPQ was found in sulfoacetate-degrading organisms showing sulfoacetate-CoA ligase activity. There are no genes encoding orthologues of sulfoacetate importer (SauU, SauFGH) or putative regulators for sulfoacetate metabolism (SauI, SauR, SauV) in the genome of *D. hafniense* DCB-2. But downstream of *sauSPQ*, a putative transporter (annotated as anion transporter) and also a regulator (annotated as GntR-family transcriptional regulator) are encoded (Fig. 2) which could serve as sulfoacetate transporter and regulator, respectively.

To identify the genes and enzymes responsible for isethionate and sulfoacetate degradation under anoxic conditions, further experiments will have to be done in this organism.

Concluding remarks

In this study, the degradation of three C₂ sulfonates was investigated thoroughly, and the predicted convergence of these degradative pathways at the common intermediate sulfoacetaldehyde was confirmed by physiological, biochemical and molecular means.

Genes probably encoding isethionate degradative enzymes were identified, and the hypothesis was given experimental support.

The nature of the initial reactions leading from sulfoacetate to sulfoacetaldehyde was elucidated, and thereby a novel intermediate (sulfoacetyl-CoA) was identified.

Transport phenomena linked to C₂ sulfonate degradation were clarified by proposing a novel sulfite exporter (TauE), and by proving function of the predicted MFS-transport protein SauU as the importer of sulfoacetate. Genes and enzymes for import of isethionate (IseU, IseKLM) and taurine (TauP, TauABC) in *C. necator* H16 are supposed but remain to be confirmed.

Remarkable biodiversity of the genes involved in the isethionate and sulfoacetate degradative pathways was found, particularly in transport and regulation.

APPENDIX

Abbreviations

ABC	ATP-binding cassette
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
CoA	coenzyme A
DMS	dimethylsulfide
DSM	Deutsche Sammlung für Mikroorganismen
GABA	γ -aminobutyrate
HPLC	high performance liquid chromatography
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MFS	major facilitator superfamily
MS	mass spectrometry
NAD(P) ⁺	nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PLP	pyridoxal 5'-phosphate
PMF	peptide mass fingerprinting
Pta	phosphate acetyltransferase
RT-PCR	reverse transcription PCR
SAA	sulfoacetaldehyde
SDH	sulfite dehydrogenase
SDS	sodium dodecylsulfate
SQDG	sulfoquinovosyldiacylglycerol
TDH / TauXY	taurine dehydrogenase
TMH	transmembrane helix

Tpa	taurine:pyruvate aminotransferase
TRAP	tripartite ATP-independent periplasmic
Tris	tris(hydroxymethyl)aminomethane
TTT	tripartite tricarboxylate transporter
Xsc	sulfoacetaldehyde acetyltransferase

Record of contributions

Chapter 1:

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

I participated in writing the paper and performed all experiments except for some enzyme assays (Stefanie Imminger, Matthias Buhmann) and RT-PCR (Nicole Bohnenberger) which were done by students during their Vertiefungskurs under supervision by K. Denger and myself.

This chapter was published in *Microbiology* (2007) **193**: 3055-3060.

Chapter 2:

Gene clusters involved in isethionate degradation in terrestrial and marine bacteria.

I participated in writing the paper and performed the experiments with the following constraints: P. I. Sharma helped (as a student assistant) with performance of some of the RT-PCR experiments in *R. pomeroyi* DSS-3, and U. Stingl performed the growth experiment with *Rhodobacterales* sp. HTCC2150.

This chapter was accepted for publication in *Applied and Environmental Microbiology*.

Chapter 3:

Sulfoacetate degraded via novel sulfoacetyl-CoA and sulfoacetaldehyde in *Cupriavidus necator* (*Ralstonia eutropha*) H16.

I wrote the paper and performed the experiments with the following exceptions: M. Buhmann helped (as a Vertiefungskurs student / student assistant) with performance of the RT-PCR experiments and part of the enzyme activity measurements in *R. nubinhibens* ISM. K. Hollemeyer performed the MALDI-TOF-MS measurements for identification of sulfoacetyl-CoA.

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Conference attendance and poster presentations

Annual Meetings of the Association for General and Applied Microbiology (VAAM):

March 2008, Frankfurt

April 2007, Osnabrueck

March 2006, Jena

September 2005, Göttingen

Workshop on Microbial Sulfur Metabolism of the European Molecular Biology Organisation (EMBO) and the Federation of European Microbiological Societies (FEMS):

March 2009, Tomar, Portugal (Awardee of a Young Scientist Meeting Grant).

The 12th International Symposium on Microbial Ecology of the International Society for Microbial Ecology (ISME):

August 2008, Cairns, Australia.

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