Sulfite dehydrogenases in organotrophic bacteria: enzymes, genes and regulation.

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So eine Arbeit wird eigentlich nie fertig,

man muss sie für fertig erklären,

wenn man nach Zeit und Umständen das möglichste getan hat.

(Johann Wolfgang von Goethe, Italienische Reise, 1787)

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TABLE OF CONTENTS

Summary

Sulfite is the product of desulfonation in organotrophic bacteria. Due to its high reactivity with biomolecules (DNA, proteins) it has to be detoxified by oxidation to sulfate catalyzed by sulfite-oxidizing enzymes (SOEs). In prokaryotes the responsible enzymes are sulfite dehydrogenases (SDHs) which are molybdopterin cofactor (Moco)-binding enzymes belonging to the DMSO reductase family. SDHs are highly diverse in their overall structures and up to date two bacterial SDHs have been characterized: SorA and/or SorT. About 50% of the organisms which are known to degrade organosulfonates excrete the sulfonate moiety as sulfate but do not share any sequence similarities to SorA or SorT. Therefore, the focus of this study was filling up the gap of knowledge about sulfite dehydrogenases in organotrophic bacteria.

Physiological studies with taurine and cysteate as sole carbon and energy source in *Ruegeria pomeroyi* DSS-3 revealed that taurine is completely utilized concomitant with the excretion of sulfate. However, negligible sulfite-oxidizing activity was measured. In contrast, cysteate was not quantitatively utilized and sulfite was excreted. The highest SDH activity was found in these cell extracts using a previously described enzyme assay. The activity was purified and it turned out that a homotrimer, consisting of three monomers of 11 kDa, is responsible for this enzyme activity. This 11 kDa protein is a soluble protein located in the periplasm which is encoded by a gene (SPO3124 for *R. pomeroyi* DSS-3) within a three-gene operon with genes encoding for an ECF26-type RNA polymerase σ-factor (SPO3125) and an anti σ-factor (SPO3126). Reverse transcription (RT)-PCR experiments and regulator studies using *lacZ*-fusion plasmids revealed that the SPO3124-26 gene operon is induced by sulfite. The discovered 11 kDa protein was interpreted to represent a periplasmic sensor protein for an ECF-type signal transduction through the cytoplasmatic membrane, e. g. for sensing of sulfite stress or of other environmental stress, and that the apparent SDH activity measurable for this protein is a reflection of its ‘sensory domain’.

Bioinformatic analyses revealed that two-thirds of the organisms with an unknown SDH possess a three-gene cluster: *soeABC*, which is in close neighbourhood of genes involved in desulfonation. A hypothesis for the function of SoeABC was developed in which SoeA, annotated as a Moco containing oxidoreductase, oxidizes sulfite while transferring the

electrons to SoeB, a [4Fe-4S]-cluster binding protein which itself transfers the electrons to SoeC, a transmembrane protein, for which it would be conceivable that the electrons are shuttled to the respiratory chain. First hints for the involvement of SoeABC in sulfite oxidation were obtained by orbitrap analyses and gradient SDS-PAGE indicating that SoeA is inducibly expressed in taurine-grown cell extracts of *R. pomeroyi* DSS-3 (SPO3559), *R. nubinhibens* ISM (ISM_10700) and *Roseovarius* sp. strain 217 (ROS217_11926). Further, Clark-type electrode experiments revealed that the enzyme is located in the membrane supporting the hypothesis that SoeABC is attached to the membrane. An *soeA* knockout mutant of *R. pomeroyi* DSS-3 was created by insertional mutation. The mutant was not able to grow with taurine or isethionate within the first two days of growth. However, revertants occurred within six days of growth. It was shown that the *soeABC* gene cluster is co-transcribed and also co-transcribed with the upstream genes *xsc* and *pta*. These findings led to the assumption that TauR is induced by sulfoacetaldehyde (SAA), which is an intermediate in taurine- and in isethionate-degradation. To test this hypothesis, SAA was supplied intracellularly to cysteate-grown *R. pomeroyi* DSS-3 cells. These cells were now able to quantitatively utilize cysteate while sulfate was excreted; only traces of sulfite were detected. Phylogenetic analyses of SoeA (*R. pomeroyi* DSS-3) within the DMSO reductase family revealed a close relationship with PsrA which is required for sulfide oxidation. This protein shares 24% sequence identity to the PsrLC complex which was previously described as a novel sulfite dehydrogenase in some green sulfur bacteria.

In this study, an 11 kDa protein induced by sulfite stress was discovered. In the absence of an active sulfite-oxidizing enzyme, the 11 kDa protein presumably acts as sensor protein in *R. pomeroyi* DSS-3. However in this bacterium as well as in members of the order of *Rhodobacterales*, a novel type of sulfite dehydrogenase was discovered, which was termed SoeABC.

Zusammenfassung

Sulfit, ein Spaltprodukt von Desulfonierungsreaktionen in organotrophen Bakterien, ist aufgrund seiner Nukleophilie und seines hohen Reduktionspotentials toxisch, da es mit Biomolekülen (DNA, Proteinen) in der Zelle reagieren kann. Daher ist eine Detoxifizierung in Form einer Sulfitoxidation, katalysiert durch sulfitoxidierende Enzyme (SOEs), notwendig. Diese Funktion übernehmen in Prokaryoten die strukturell sehr unterschiedlichen Sulfitdehydrogenasen (SDH), die einen Molybdopterin-Kofaktor (Moco) binden und zu der DMSO Reduktase Familie gehören. Bislang wurden zwei bakterielle SDHs beschrieben: SorA und SorT. Jedoch weisen nur 50% der desulfonierenden Bakterien Sequenzhomologien zu SorA und/oder SorT auf, obwohl in den meisten Fällen der Organosulfonat-Schwefel in Form von Sulfat ausgeschieden wird. Daher ist anzunehmen, dass es noch weitere, bislang unbekannte SDHs in diesen Bakterien gibt. Deren Identifizierung und Charakterisierung war das Ziel dieser Doktorarbeit.

Physiologische Experimente in *Ruegeria pomeroyi* DSS-3 mit Taurin oder Cysteat als alleiniger Kohlenstoff- und Energiequelle zeigten, dass Taurin vollständig unter Ausscheidung von Sulfat verwertet wurde, während Cysteat nur zum Teil verstoffwechselt und der Schwefel in Form von Sulfit ausgeschieden wurde. Anschließende SDH Aktivitätsmessungen, die mit einem für SDHs etablierten Enzymassay durchgeführt wurden, zeigten, dass in Extrakten aus mit Taurin gewachsenen Zellen nur eine geringe Enzymaktivität vorlag, hingegen in Extrakten aus mit Cysteat gewachsenen Zellen die höchste Aktivität gemessen wurde. Dieses Enzym wurde gereinigt und es stellte sich heraus, dass ein Homotrimer, bestehend aus drei Monomeren zu je 11 kDa, für diese Aktivität verantwortlich war. Das Gen für dieses periplasmatische, lösliche 11 kDa Protein liegt in einem drei Gene umfassenden Operon, welches für das 11 kDa Protein kodiert (SPO3124), einem RNA Polymerase σ-Faktor (SPO3125) vom ECF26-Typ und einem Anti σ-Faktor (SPO3126). Transkriptions-analysen und die Verwendung von *lacZ*-Fusionsplasmiden zeigten, dass Sulfit der Induktor für das Operon SPO3124-26 ist. Aufgrund der gewonnenen Daten wird angenommen, dass das neu entdeckte 11 kDa Protein als periplasmatisches Sensorprotein agiert und somit eine Rolle in der Signaltransduktion, gesteuert über ECF σ-Faktoren, einnimmt.

Aufgrund von bioinformatischen Analysen wurde ein aus drei Genen bestehendes Gencluster gefunden, welches sich in unmittelbarer Nachbarschaft von Genen befindet, die für desulfonierende Enzyme kodieren. Dieses Gencluster, welches in dieser Arbeit als *soeABC* bezeichnet wurde, tritt bei zwei Drittel der desulfonierenden Organismen auf, deren SDH unbekannt ist. Die daraus abgeleitete Hypothese über die Funktion von SoeABC besagt, dass SoeA, welches als Molybdopterin-Kofaktor (Moco) bindende Oxidoreduktase annotiert ist, die Sulfitoxidation katalysiert und die beiden Elektronen vom Moco auf SoeB überträgt, welches als [4Fe-4S]-Komplex Bindeprotein annotiert ist. Dieses überträgt die Elektronen auf SoeC, ein Transmembranprotein, welches die Elektronen letztendlich auf eine Komponente der Atmungskette übertragen könnte. Erste Hinweise für diese Hypothese lieferten Orbitrap-Analysen und Gradientengele, welche zeigten, dass SoeA in Taurin-gewachsenen Zellextrakten von *R. pomeroyi* DSS-3 (SPO3559), *R. nubinhibens* ISM (ISM_10700) und *Roseovarius* sp. strain 217 (ROS217_11926) induziert exprimiert wurde. Mit Hilfe von Sauerstoffelektrodenmessungen konnte gezeigt werden, dass sich die Enzymaktivität in der Membranfraktion befindet, welches ebenfalls für die Hypothese einer membrangebundenen SDH spricht. Eine *soeA*-knockout Mutante von *R. pomeroyi* DSS-3, konnte innerhald der ersten beiden Inkubationstage weder Taurin noch Isethionat verwerten. Jedoch zeigte sich nach sechstägiger Inkubation eine Reversion der Mutation, welche ein Wachstum mit Taurin und Isethionat ermöglichte. Transkriptionsanalysen zeigten, dass das *soeABC* Gencluster und die davor geschalteten Gene (*xsc*, *pta*) ko-transkribiert wurden, welches die Vermutung eines gemeinsamen Regulators (TauR) nahelegt, der durch Sulfoacetaldehyd (SAA), ein Zwischenprodukt des Taurin- und Isethionatabbaus, induziert wird. Als Beweis wurde zu Cysteat verstoffwechselnden *R. pomeroyi* DSS-3 Zellen intrazellulär gebildetes SAA gegeben. Unter diesen Bedingungen waren die Zellen nun in der Lage, Cysteat vollständig zu verwerten und den Sulfonat-Schwefel hauptsächlich in Form von Sulfat auszuscheiden. Phylogenetische Analysen von SoeA (*R. pomeroyi* DSS-3) mit anderen Proteinen der DMSO-Reduktase Familie zeigten eine nahe Verwandtschaft von SoeA mit PrsA, welches eine Rolle bei der Sulfidoxidation einnimmt und eine 24%-ige Sequenzidentität zu PrsLC aufweist, welches als neue SDH in einigen grünen Schwefelbakterien beschrieben wurde.

In der vorliegenden Doktorarbeit wurde ein periplasmatisches, über ECF σ-Faktoren reguliertes Sensorprotein (z. Bsp. für Sulfitstress) und eine neue, vor allem in der Ordnung *Rhodobacterales* vorkommende SDH (SoeABC) beschrieben.

1. Introduction

1.1. Organosulfonates-an overview

Organosulfonates are sulfonated organic compounds containing the $-SO_3^-$ moiety. The sulfonate moiety can be linked to oxygen ($R\text{-}O\text{-}SO_3^-$, sulfate esters), to nitrogen ($R\text{-}N\text{-}SO_3^-$) or to carbon atoms ($R\text{-}C\text{-}SO_3^-$). The latter type of compounds (*C*-sulfonates) is at the focus of this work and will subsequently be termed “sulfonates” or “organosulfonates” *sensu stricto*. While sulfate esters and *N*-sulfonates can be easily hydrolyzed, *C*-sulfonates are highly stable and resistant to alkaline and acid hydrolysis (Wagner & Reid, 1931). To our current knowledge, only bacteria and fungi are capable to cleave the *C*-sulfonate bond (Huxtable, 1992). The sulfonate group is a strong acid and is negatively charged over the physiological pH range and therefore requires active transport across cell membranes. Both aliphatic and aromatic sulfonates occur naturally or as xenobiotics. An overview about various sulfonates is given in Fig. 1 and is described in more detail in the following chapter.

1.1.1. Natural and xenobiotic organosulfonates

Naturally occurring sulfonates are widespread and ubiquitous and can be found in terrestrial (Huxtable, 1986; Autry & Fitzgerald, 1990) and in marine environments (Vairavamurthy *et al.*, 1997), but also in the atmosphere (Baker *et al.*, 1991).

The smallest organosulfonate is methanesulfonate which arises from the oxidation of atmospheric dimethylsulfide (DMS). The compound precipitates on the land and sea surface by rain, snow and dry deposition (Kelly & Murrell, 1999). The precursor of DMS is dimethylsulfoniopropionate (DMSP), which acts as osmolyte in marine algae and which is the major source of sulfur in the atmosphere. The annual production of these compounds is astonishing. It has been reported that the annual methanesulfonate formation is about 20 million tonnes and the annual input of DMSP is 40 to 70 million tonnes (Kiene *et al.*, 1996; Kelly & Murrell, 1999).

The most prominent aliphatic organosulfonate is taurine. The compound was first isolated from the bile of the ox, *Bos taurus*, from which the name is derived (Demarçay, 1838).

Taurine is often referred to as a non-proteinogenic amino acid, even though the compound lacks a carboxylic acid group. However, taurine is a water-soluble β-aminosulfonic acid which is zwitterionic at pH 7 and thus very polar and hydrophilic (Brosnan & Brosnan, 2006). In mammals, it is one of the most abundant low-molecular weight organic constituents, occurring at 1 g taurine per kg body weight, predominantly in muscle, brain and blood. In addition to its role in the synthesis of the bile salt taurocholate, it has been proposed to act as an antioxidant, an intracellular osmolyte, a membrane stabilizer, and as neurotransmitter (Huxtable, 1992). It also appears that taurine promotes the generation of photoreceptor cells from retinal progenitor cells (Young & Cepko, 2004). Regarding these vast amounts of taurine in mammals, it seems suprising that eukaryotes are not able to cleave the carbon-sulfur bond. Thus, to maintain homeostasis, taurine has to be excreted, mainly in urine but also faeces, semen, breast milk or tears, either unmodified, or in the form of taurocholate, or related bile salts (Irving *et al.*, 1986; Huxtable, 1992; Gowda *et al.*, 2009). Therefore, taurine is released into the environment, ranging from 150 to 800 µmol per day in human urine (Irving *et al.*, 1986). In the plant kingdom, with the exception of algae, taurine is either absent or found only sporadically in low concentrations (Huxtable, 1992). In bacteria, taurine occurs as a component of bacterial sulfolipids (Corcelli *et al.*, 2004).

The buffers PIPES (1,4-piperazinediethanesulfonic acid) and HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid) are further taurine derivatives, which are commonly used in laboratories. Homotaurine (3-amino-propanesulfonate, APS), a C_3-sulfonate, is a homologue of taurine and an analogue of the neurotransmitter 4-aminobutyrate (GABA) which is the reason why it has come into the focus of the pharmaceutical industry in the treatment of Alzheimer's disease (Aisen, 2005).

Isethionate (2-hydroxyethansulfonate) 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), occurrs in red algae (up to 250 mM) (Barrow *et al.*, 1993; Holst *et al.*, 1994; Hellio *et al.*, 2004), and in the sticky droplets of spider's webs (about 2 M) (Townley *et al.*, 2006). Furthermore, the non-toxic and water-soluble isethionate and its derivatives serve as counter-ion in parmaceuticals and are used in hair shampoos and as soap replacement (Korting *et al.*, 1992; Delobel & Pradinaud, 2003; Nguewa *et al.*, 2005; Ghosh & Blankschtein, 2007).

Coenzyme M (2-mercaptoethanesulfonate) is required for methyl-transfer reactions in methanogenesis (Taylor *et al.*, 1974; White, 1988).

Sulfoacetaldehyde (SAA) is a key intermediate in C_2- and C_3-sulfonate degradation, including *N*-methyltaurine, taurine, isethionate, sulfolactate and sulfoacetate (Cook & Denger, 2002; Weinitschke *et al.*, 2006; Denger *et al.*, 2009). It is desulfonated by sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15], forming sulfite and acetyl phosphate (chapter 1.1.2).

N-acetyltaurine was found in spiders's webs as well as in red algae (Lindberg, 1955; Vollrath *et al.*, 1990).

The amino acid cysteate (2-amino-3-sulfopropionate) can be found in hydrolysates of wool, where it was formed by weathering of cystine (Consden *et al.*, 1946), and in human hair (Zahn & Gattner, 1997). Like other sulfonates, cysteate was also detected in marine algae (Miyazawa *et al.*, 1970; Ito *et al.*, 1977) as well as in spiders's webs (Fischer & Brander, 1960).

Sulfoquinovose (SQ) is the polar head group of the plant and algal sulfolipid (sulfoquinovosyl diacylglycerol, SQDG), which plays a major role in the biological sulfur cycle where it can represent up to 50% of the sulfur in plants (and algae). Especially in photosynthetic plant tissues SQDG concentrations are observed in the range of 1-6 mM (Benson *et al.*, 1959; Daniel *et al.*, 1961; Benson, 1963). The global biosynthesis of SQDG is about $3.6*10^{10}$ tons per year (Harwood & Nicholls, 1979). First, it was thought that SQDG plays a role in photosynthesis (Harwood & Nicholls, 1979), however, many photosynthetic bacteria almost or completely lack the compound. Instead, several non-photosynthetic organisms harbour the compound (e. g. *Sinorhizobium meliloti* (Cedergren & Hollingsworth, 1994)). But also in eggs and sperm cells of the sea urchin *Pseudocentrotus depressus* SQDG was found (Isono *et al.*, 1967). Currently it is believed that SQDG is required for chloroplast stabilization and function (Yu & Benning, 2003). Based on these huge amounts of SQDG biosynthesis, the question arises how the sulfolipid, or SQ respectively, is degraded. Roy and colleagues proposed a SQ degradation pathway based on the consideration that SQ is an analogon of glucose 6-phosphate (Roy *et al.*, 2003). The hypothesized sulfoglycolytic pathway is supposed to be a source of several sulfonates, such as sulfolactate, Dhps, sulfolactaldehyde and presumably cysteate (Shibuya *et al.*, 1963).

Aeruginosin B is one of a few naturally occurring aromatic sulfonates and was found as a red pigment in *Pseudomonas aeruginosa* (Herbert & Holliman, 1964).

Other examples are the siderophore petrobactin-sulfonate from an oil-degrading marine bacterium (Hickford *et al.*, 2004) and phorbasin D, a diterpenyl-taurine derivate, which belongs to a recently found new class of terpenes and which was isolated from *Phorbas* sp., an Australian marine sponge (Lee *et al.*, 2008; Zhang & Capon, 2008).

Xenobiotic sulfonates are used for a wide variety of applications, such as dyestuff and brighteners for clothes, as detergents amd cement additives or as industrial chemical intermediates in pharmaceutical industry. The most widespread aromatic sulfonates are the linear alkylbenzene sulfonates (LAS, (Cook, 1998)). Their annual use, mainly as laundry detergent, amounts to 430,000 tons in Western Europe (Jensen *et al.*, 2007). Although LAS is not toxic for higher organisms, it may have toxic effects on algae and invertebrates at the concentrations found in polluted water (reviewed in Kertesz, 2000).

Another detergent is *p*-toluenesulfonate, an hydrotropic agent, which is extensively used in the detergent industry to obtain clear liquid formulations (González *et al.*, 2000). Frequently used textile, food and drug dyes are sulfonated azo dyes, such as Congo red (Namasivayam & Kavitha, 2002).

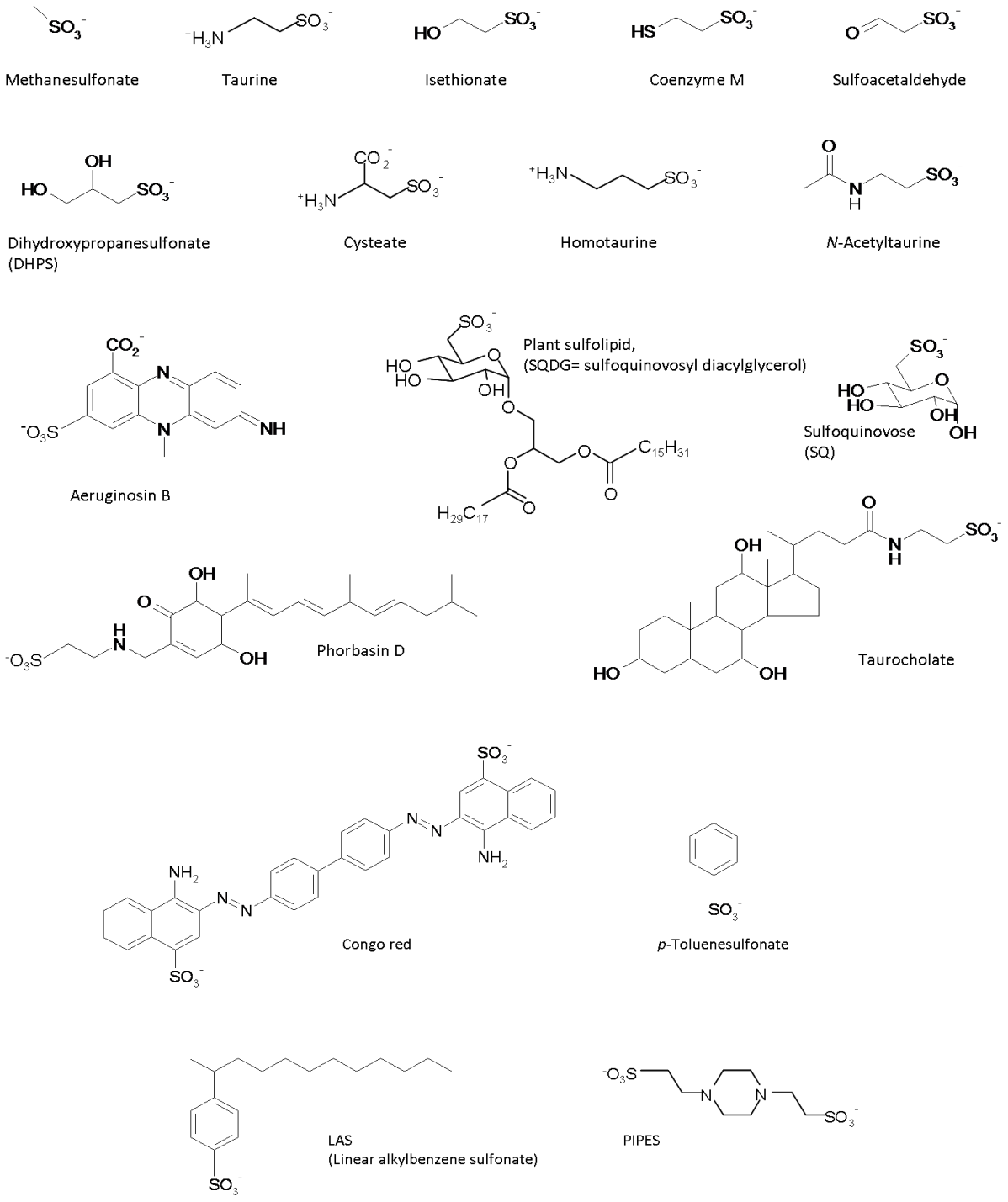


Fig. 1 Naturally occurring and xenobiotic organosulfonates.

1.1.2. Microbial aerobic dissimilation of organosulfonates

The previous chapter showed that organosulfonates are widespread and ubiquitous. Given the enormous quantities of sulfonates released into the environment, it is obvious that these compounds must be degraded somehow.

1.1.2.1. Degradation pathways and desulfonating enzymes

This chapter will focus only on the dissimilative degradation pathways of organosulfonates which are relevant for this work. Fig. 2 is a simplified scheme illustrating the three different types of desulfonative enzymes (Xsc (Ruff *et al.*, 2003), CuyA (Denger *et al.*, 2006), SuyAB (Rein *et al.*, 2005)), which are known to be involved in the dissimilation of C_2- and C_3-sulfonates.

One of the key intermediate in the degradation of C_2-sulfonates (taurine, isethionate, sulfolactate) is sulfoacetaldehyde (SAA), which is desulfonated by sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15] forming acetylphosphate and sulfite (Cook & Denger, 2002, Ruff *et al.*, 2003). This phosphatolytic and soluble enzyme is located in the cytoplasm and requires thiamine diphosphate (ThDP) as cofactor. The *xsc* gene occur in Proteobacteria, and in Gram-positive bacteria, and at least three subgroups were identified based on amino acid sequences in different organisms (Cook & Denger, 2002; Ruff *et al.*, 2003). Some organisms (e. g. *Ruegeria pomeroyi* DSS-3) harbor a *xsc*-pseudogene, which lack key amino acids in the active site (Weinitschke *et al.*, 2010).

The carbon-sulfur bond of cysteate is directly desulfonated by (*R*)-cysteate sulfo-lyase (CuyA) [EC 4.4.1.25] (Denger *et al.,* 2006). The enzyme uses pyridoxal 5'-phosphate (PLP) as cofactor and catalyzes the formation of pyruvate, ammonium and sulfite. CuyA is widely distributed, not only in terrestrial organisms, but also in marine bacteria (Denger *et al.*, 2009).

The third desulfonating enzyme is an Fe^{2+}-dependent altronate dehydratase-like enzyme named 3-sulfolactate sulfo-lyase (SuyAB) [EC 4.4.1.24], which consists of a small (8 kDa) and a large (42 kDa) subunit. The enzyme acts only on 3-sulfolactate, which is directly desulfonated to pyruvate and sulfite (Rein *et al.*, 2005). In 2010, it turned out that the substrate for SuyAB is (*R*)-sulfolactate, thus SuyAB is a (*R*)-sulfolactate sulfo-lyase (Denger & Cook, 2010).

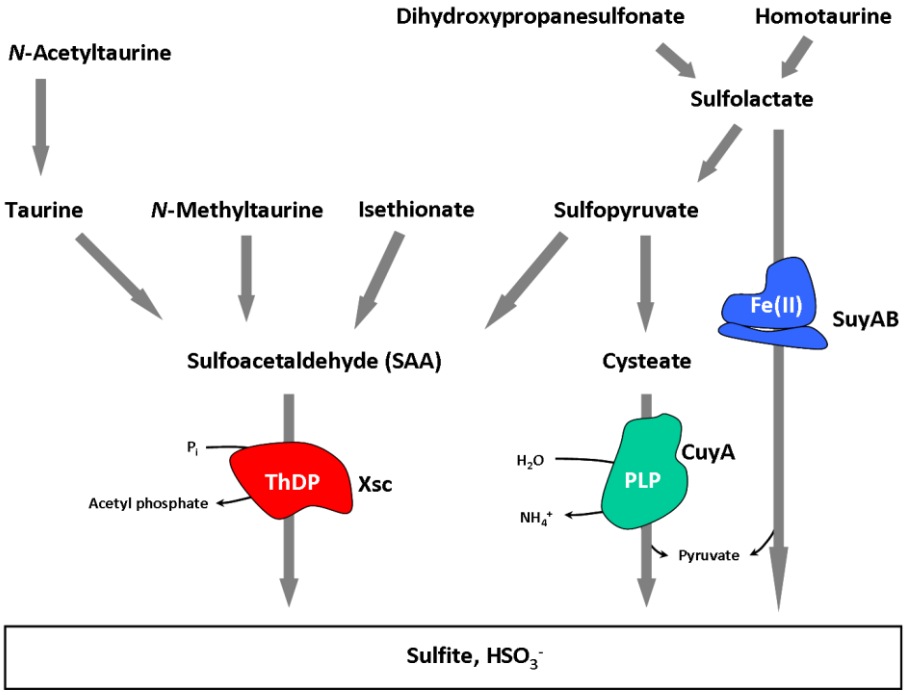


Fig. 2 Overview of the pathways catalyzed by the three desulfonating enzymes involved in the dissimilative degradation of organosulfonates. Description and abbreviations are written in the main text.

The main work of this thesis was done in *Ruegeria pomeroyi* DSS-3 (formerly *Silicibacter pomeroyi* DSS-3) with taurine, isethionate, or cysteate as sole carbon and engery source. An overview of the degradation pathways of these sulfonates in this marine α-Proteobacterium as well as the corresponding gene maps and gene tags are depicted in Fig. 3. The inducible transcription of genes involved in taurine uptake and dissimilation was tested and confirmed by Gorzynska and collegues (Gorzynska *et al.*, 2006). Taurine uptake is attributed to an ATP binding-cassette transporter (ABC-transporter, TauABC) [TC 3.A.1.17.1] consisting of three subunits: TauA, an extracytoplasmic taurine binding protein, TauB, an ATPase, and TauC, a permease (Eichhorn *et al.*, 2000). The first step involves a transamination reaction of taurine and pyruvate forming sulfoacetaldehyde (SAA) and alanine. This reaction is catalyzed by taurine:pyruvate aminotransferase (Tpa) [EC 2.6.1.77], a pyridoxal 5'-phosphate (PLP) dependent and widespread enzyme found in many bacteria (e. g. *Rhodococcus*, *Roseovarius* or *Roseobacter*) (Laue & Cook, 2000a). Alanine is deaminated to pyruvate by NAD^+-dependent alanine dehydrogenase (Ald) [EC 1.4.1.1] (Laue & Cook, 2000b). It is assumed that ammonia is exported by an ammonia-methylammonia transporter (Amt-1) belonging to the ammonia transporting and sensory proteins [TC 2.A.49.1.1] (Gorzynska *et al.*, 2006). The corresponding genes are located in the same gene cluster (SPO0673-SPO0676, *tpa*, *tauABC*), whereas, *ald* (SPO0222) and *amt-1* (SPO2093) are located elsewhere on the chromosome. As described previously, SAA is a key intermediate in the degradation of various sulfonates and

is desulfonated by Xsc (see Fig. 2) forming sulfite and acetyl phosphate whose acetyl moiety is transferred to coenzyme A (CoA) by phosphate acetyltransferase (Pta) [EC 2.3.1.8] (Stadtman *et al.*, 1951; Ruff *et al.*, 2003). Acetyl-CoA can be metabolized in the Krebs cycle or can be used for anaplerotic reactions. The genes for *xsc* and *pta* are located in the same operon (SPO3561/*xsc*, and SPO3560/*pta*) and are accompanied by a transcriptional regulator, termed *tauR* (SPO3562). TauR was classified to the MocR-subfamily of the GntR-type regulators (Rigali *et al.*, 2002) and controls taurine dissimilation (Wiethaus *et al.*, 2008; Krejčík *et al.*, 2010).

Isethionate degradation in marine and terrestrial bacteria was investigated by Weinitschke and coworkers (Weinitschke *et al.*, 2010). Isethionate is imported by a TRAP transporter (tripartite ATP-independent periplasmic transporter, IseKLM) [TC 2.A.56.-.-] which is present in many marine isolates. An inducible, cytochrome *c*-dependent and membrane-bound isethionate dehydrogenase (IseJ) [EC 1.1.2.-.-] oxidizes isethionate to SAA which is metabolized in a pathway analogous to taurine degradation involving the same genes. The corresponding gene cluster for isethionate degradation is controlled by the IclR-type transcriptional regulator (IseR, SPO2355). Interestingly, a second *xsc* gene (SPO2360) is present in the *iseJ* gene cluster, however, it lacks key amino acids in the active site and therefore it is not functional and designated as pseudogene.

The genes coding for cysteate degradation are located on the megaplasmid (SPOA0158-SPOA0159) in an operon which is assumed to be controlled by the LysR-type transcriptional regulator CuyR (Denger *et al.*, 2006). The nature of cysteate uptake is still unknown but once in the cytoplasm, cysteate gets desulfonated by CuyA to pyruvate (see Fig. 2), which is further metabolized in amphibolic pathways. The released ammonia is excreted by Amt-1 and the fate of sulfite will be the focus of the following chapters.

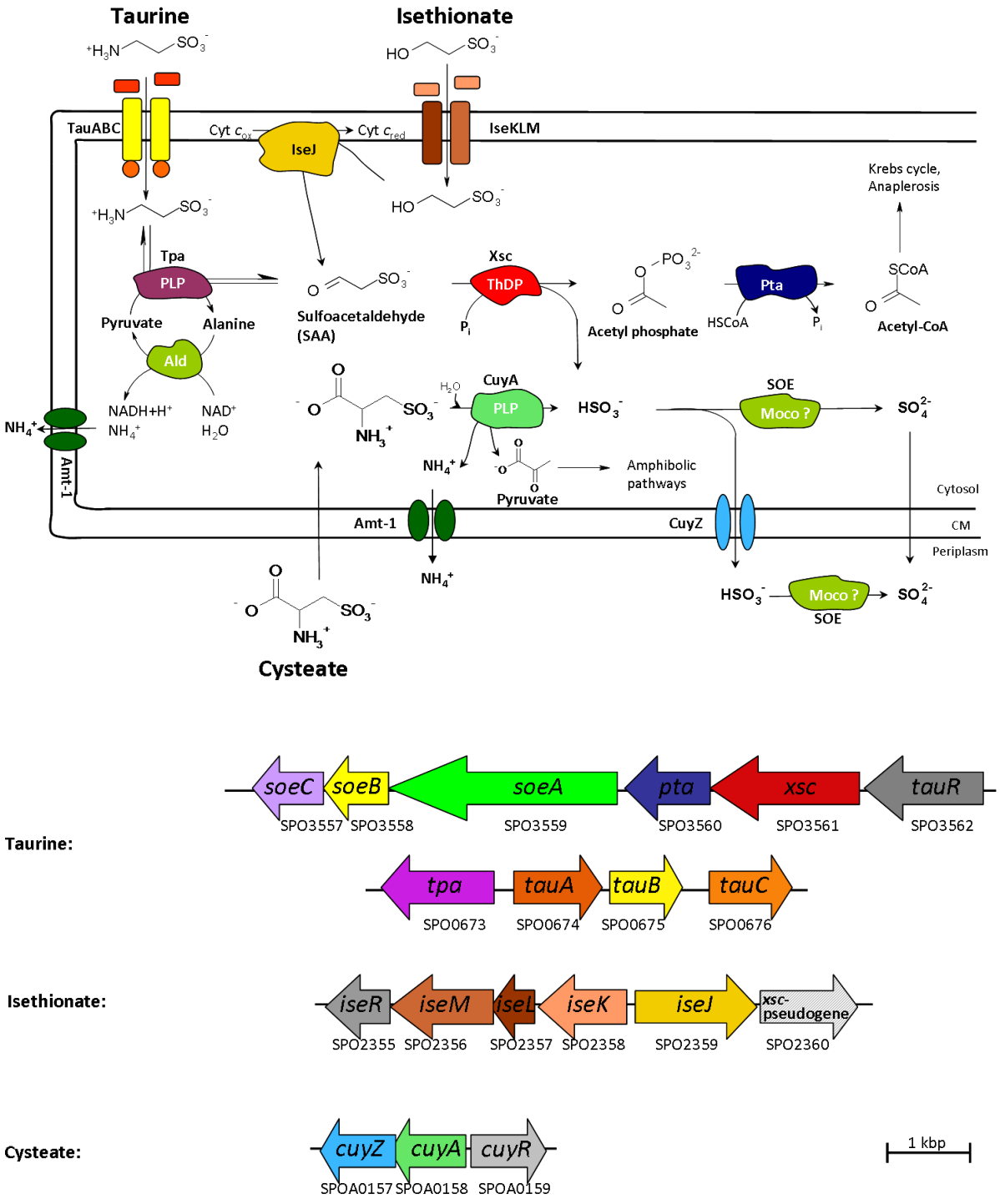


Fig. 3 Initial steps of cysteate, taurine and isethionate degradation in *Ruegeria pomeroyi* DSS-3. CM, cytoplasmic membrane, SOE, sulfite-oxidizing enzyme. Further abbreviations are explained in the main text (**upper panel**). Corresponding gene maps and gene tags encoding the relevant enzymes in *Ruegeria pomeroyi* DSS-3 (**lower panel**).

1.1.2.2. Sulfite: an useful but fateful product

The sulfite anion is a strong reducing agent, whose redox potential (E_0' (SO_4^{2-}/SO_3^{2-})) is -516 mV (Thauer *et al.*, 1977) but also a strong nucleophil (Beyer & Walter, 1998). Due to these properties, sulfite and its derivatives are used in various industrial applications (e. g. bleaching of paper (Biermann, 1993), leather tanning (Robinson, 1946), antioxidant and colour stabilizer (Le Tien *et al.*, 2006). However, the high reactivity of sulfite can potentially lead to a number of mostly undesirable reactions in cells: addition to aldehydes and ketones resulting in an irreversible attachment to biomolecules (e. g. DNA and proteins) (Schroeter, 1966; Petering & Shih, 1975), reversible addition to uracil and cytosine resulting in deamination- and transamination reactions (leading to mutations) (Shapiro *et al.*, 1974; Hayatsu, 1976; Shapiro & Gazit, 1977), oxidative sulfitolytic reactions of thiamine (Williams *et al.*, 1935) and disulfide bonds (Cecil, 1963), and generation of sulfur trioxide anion radicals ($SO_3^{\cdot-}$), which lead to an increase in reactive oxygen species (ROS) (Gunnison, 1981; Sun *et al.*, 1992; Gould & Russell, 1991; Vincent *et al.*, 2004). Hence, in eukaryotic and bacterial cells, sulfite causes oxidative stress and can be toxic. The resulting advantage is that since the 17th century sulfite additives are used as preservatives and antioxidants in foods (dried fruits, tinned crabmeat, sausage meat, quick frozen chips, and jams), beverages (beer, wine), and pharmaceuticals. Although, the usage of sulfite additives (sulfites, bisulfites and sulfur dioxides (SO_2)) has been approved in the United States as early as the early 1800s (Gould & Russell, 1991), it remains controversal due to known hypersensitivic allergic reactions in humans. Therefore, products which contain sulfites (derivatives) must be marked and must be indicated by the E-numbers E220-228 (EUFIC, 2009). Interestingly, sulfite has not just a much more pronounced antibacterial and antifungal effect than other food preservatives (Mecteau *et al.*, 2002), it was already known to the Romans, ancient Greeks and Egyptians who used SO_2 to sterilze wine barrels and amphoras (Estreicher, 2004). However, several microorganisms, e. g. some purple sulfur bacteria (Brune, 1995) and members of the genus *Sulfitobacter* (Sorokin, 1995) are able to use sulfite as sole electron source during lithotrophic growth and the human pathogen *Campylobacter jejuni* is resistant against sulfite due to the activity of a sulfite-oxidizing enzyme (see chapter 1.3.3.2).

Another exogenous source of sulfite (as sulfur dioxide) is fossil fuel combustion. It is noteworthy that sulfur dioxide is a major air pollutant causing acid rain (EPA, 2012).

As mentioned before, sulfite is generated during dissimilation reactions (e. g. sulfonate degradation, chapter 1.1.2.1), or from the metabolism of sulfur-containing amino acids (methionine and cysteine) (Shih *et al.*, 1977; Griffith, 1987). But also during assimilation reactions, such as the synthesis of sulfur-containing amino acids and coenzyme M, or as 'active sulfate' which is formed by 3'-phosphoadenosine 5'-phosphosulfate (PAPS) sulfite is produced. The antimicrobial effect of sulfite is also shown by the fact that neutrophils release sulfite during the oxidative burst (a reaction of the non-specific immune system) in response to lipopolysaccharides (LPS, 1*10 nmol sulfite*h^{-1} per 10^7 neutrophils) (Mitsuhashi *et al.*, 1998).

The importance of an efficient detoxification of sulfite was shown in humans, in which a sulfite oxidase (see chapter 1.3.3.1) deficiency is known to lead to early death in infancy due to neurological abnormalities, seizures, and mental retardation (e. g. Irreverre *et al.*, 1967; Mudd *et al.*, 1967). This illustrates the potential toxicity not just for humans but also for microbes, fungi and yeast, and makes a detoxification or an export system essential.

1.2. Microbial mechanisms for sulfite elimination

As described in the previous chapter, sulfite is toxic due to damage and modification of biomolecules. However, eukaryotic and prokaryotic cells are constantly exposed to sulfite which is either formed endogenously or exogenously. Therefore, cells need a protection system for the highly reactive sulfite. Possibilities for the cell to cope with sulfite are depicted in Fig. 4. These comprise sulfite export, sulfite reduction, and the direct and indirect sulfite oxidation and will be the main focus in the following chapters.

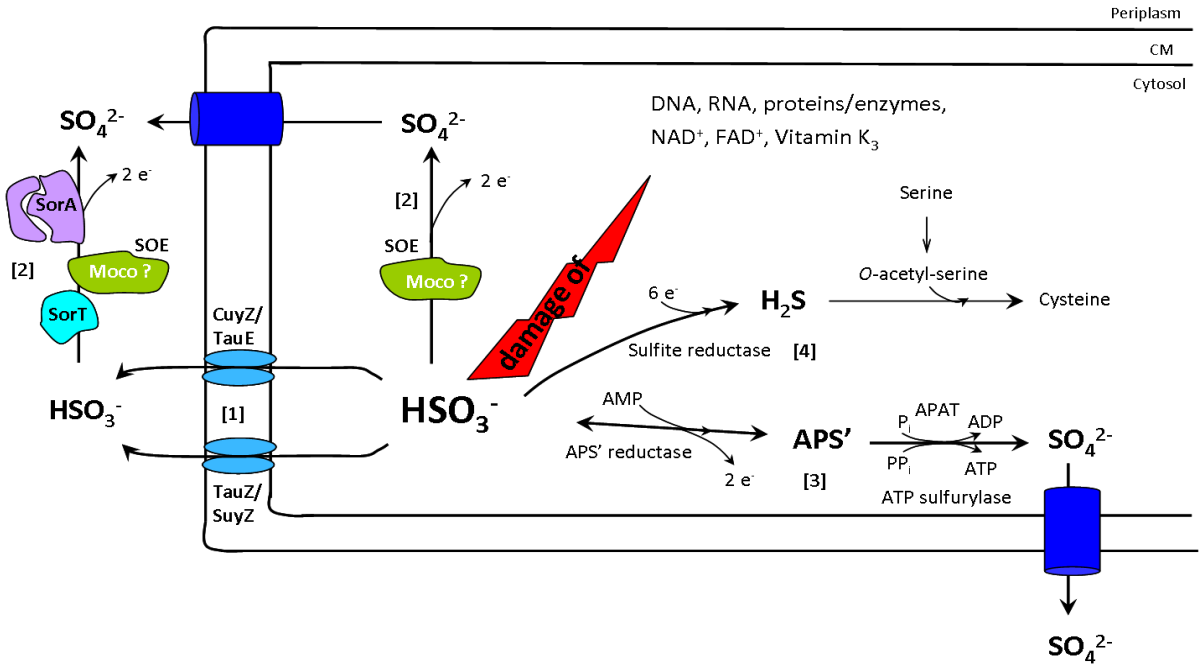


Fig. 4 Overview of the microbial strategies to cope with sulfite. It might be possible to excrete sulfite into the periplasm [1], where it might get directly oxidized to sulfate by sulfite-oxidizing enzymes (SOEs) [2]. Although all known SOEs (SorA, SorT) are located in the periplasm, sulfite oxidation would also be possible in the cytoplasm analogue to indirect sulfite oxidation *via* the intermediate APS' [3]. Further, sulfite might get reduced to sulfide by sulfite reductase [4]. CM, cytoplasmic membrane. See text for further abbreviations and details.

1.2.1. Sulfite exporters

Export of sulfite/sulfate is required to maintain homeostasis but also for protection against toxic sulfite.

A first candidate gene for an exporter was hypothesized in *Paracoccus denitrificans* NKNIS, which is located between the *xsc* and *pta* genes and which was termed *tauZ* (Brüggemann *et al.*, 2004). TauZ [TC 9.B.63.1.1] is widespread and two orthologues were discovered in *Paracoccus pantotrophus* NKNCYSA, one of which is located in the taurine-degradative gene cluster, and the other which is adjacent to *suyAB* genes, which are required for cysteate degradation, termed *suyZ* [TC 2.A.98.1.1] (Rein *et al.*, 2005). Initially it was assumed that both exporters are sulfate exporters due to the high levels of sulfite dehydrogenase activity in crude extracts and high concentrations of sulfate in the culture supernatant of *Paracoccus pantotrophus* NKNCYSA (Rein *et al.*, 2005). Both genes are inducibly (co-) transcribed during growth with taurine (*tauZ*) or cysteate (*suyZ*) (Brüggemann *et al.*, 2004; Rein *et al.*, 2005). So far, all known sulfite dehydrogenases (SorA, SorT) are located in the periplasm (Kappler *et al.*, 2000; Denger *et al.*, 2008; Wilson & Kappler, 2009), thus TauZ and SuyZ are supposed to be exporters for sulfite rather than sulfate.

Another orthologue of *tauZ* is *cuyZ*, which is inducibly (co-) transcribed with *cuyA* in *Ruegeria pomeroyi* DSS-3 during growth with cysteate (Denger *et al.*, 2006) and in *Roseovarius nubinhibens* ISM during growth with sulfolactate (Denger *et al.*, 2009). Therefore, CuyZ [TC 9.B.7.1.1] is also attributed to function as sulfite exporter.

A fourth type of sulfite exporter is TauE [TC 9.A.29.2.1], which was discovered in *Cupriavidus necator* H16 (formerly *Ralstonia eutropha* H16) and which belongs to the DUF (domain of unknown function) 81 family. Like the other sulfite exporters, *tauE* is located downstream of the *xsc* and *pta* genes and is inducibly transcribed during growth with various sulfonates (Weinitschke *et al.*, 2007).

1.2.2. Sulfite reduction

Typical cytoplasmic sulfite reductases [EC 1.8.99.1] contain a siroheme cofactor, which is covalently coupled to an iron-sulfur ([4Fe-4S]) cluster in their active centre (Crane & Getzoff, 1996). This double cofactor delivers six electrons for the energy-consuming reduction of sulfite to sulfide (Fig. 4, step 4). The cofactors can be reduced by two electrons at a time, therefore sulfite reduction involves three sequential transfers of two electrons (Tan & Cowan 1991; Lui *et al.*, 1993).

Sulfite reductases are grouped into two classes: assimilatory (aSir) or dissimilatory sulfite reductases (dSir), depending on their biological function, spectroscopic characteristics, and catalytic properties (Siegel, 1975; Peck Jr & Lissolo, 1988).

aSir, found in bacteria, fungi and plants but not in animals, catalyzes the direct reduction of sulfite. The generated sulfide is immediately converted into sulfur-containing amino acids, or coenzyme M (Siegel & Kamin, 1968).

dSir, found primarily in sulfate-reducing bacteria and archaea (e. g. *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus* (Rabus *et al.*, 2006)), reduces sulfite in the terminal redox couple of a respiratory electron transfer chain with the generated sulfide subsequently being excreted into the environment (Postgate, 1979; Odom *et al.*, 1993). In addition, dSIR primarily produce incompletely reduced sulfur in the forms of trithionate ($S_3O_6^{2-}$) and, to a less extent, thiosulfate ($S_2O_3^{2-}$) (Kobayashi *et al.*, 1969). Several types of dSir have been isolated and characterized from *Desulfovibrio* and *Archaeoglobus*. The isolated enzyme of these organisms is a DsrAB complex receiving electrons from membrane-bound quinol molecules. Interestingly, similar enzymes were also found in the phototroph *Chromatium vinosum* and the sulfur oxidizer *Thiobacillus denitrificans*, where they act as reverse sulfite reductases to generate reducing equivalents for oxidative and photo-phosphorylation (Schedel *et al.*, 1979; Schedel & Trüper, 1979). Non-DsrAB reductases have also been described: the anaerobic sulfite reductase AsrABC from *Salmonella enterica* and the coenzyme F_{420}-dependent sulfite reductase Fsr from *Methanocaldococcus jannaschii* (Huang & Barrett, 1990; Johnson & Mukhopadhyay, 2005). A novel type of cytochrome *c* sulfite reduction system was just recently discovered in the ε-Proteobacterium *Wolinella succinogenes* and δ-Proteobacterium *Shewanella oneidensis* MR-1. Thereby, sulfite is

reduced by MccA (multiheme cytochromes *c*, formerly named octaheme SirA), a periplasmic, octaheme cytochrome *c* sulfite reductase (Kern *et al.*, 2011; Shirodkar *et al.*, 2011).
The respiratory cytochrome *c* nitrate reductase (NrfA) from several γ-, δ-, and ε-Proteobacteria has been described to possess sulfite reductase activity although the physiological significance of this activity is not clear (Pereira *et al.*, 1996; Stach *et al.*, 2000; Lukat *et al.*, 2008; Kemp *et al.*, 2010; Pereira *et al.*, 2011; Simon *et al.*, 2011).

1.2.3. Sulfite oxidation

The third potential pathway for sulfite elimination is sulfite oxidation, which was discovered about 50 years ago (Charles & Suzuki, 1965), and which has been regarded as part of a bacterial energy-generating process. However, more recently sulfite oxidation has been shown to play a role in the degradation of organosulfur compounds and in the detoxification of sulfite (Reichenbecher *et al.*, 1999; Denger *et al.*, 2008; Kappler *et al.*, 2012).
Generally, two pathways of sulfite microbial oxidation have been described (Fig. 4): direct oxidation probably by a molybdopterin-cofactor (Moco)-containing sulfite-oxidizing enzyme (SOE); and an indirect, AMP-dependent oxidation *via* the intermediate adenosine-5'-phospho-sulfate (APS'). Both pathways can be distinguished by their cellular function and localization: in terms of sulfite detoxification, e. g. during the degradation of organosulfur compounds, it seems to occur exclusively *via* direct enzymatic oxidation by periplasmic SOEs (see chapter 1.3). In cases of energy conservation during chemolithotrophic oxidation of reduced sulfur compounds, sulfite oxidation proceeds *via* APS' in the cytoplasm. Indeed, the simultaneous presence of both pathways has been established for a number of chemo- and photolithotrophic sulfur oxidizers belonging to the β- and γ-Proteobacteria (Brune, 1995; Friedrich, 1997), the green sulfur bacteria (Trüper & Fischer, 1982), the Gram-positive bacteria (Krasil'nikova *et al.*, 1998), and the archaeon *Acidianus ambivalens* (Zimmermann *et al.*, 1999).
So far, there is no evidence for the indirect pathway in α-Proteobacteria or *Ectothiorhodospiraceae*. In contrast, in the sulfur-oxidizing, bacterial endosymbionts of invertebrates, only the indirect APS'-reductase pathway has been described (Nelson & Hagen, 1995). Overall, direct sulfite oxidation appears to be much more widespread than the indirect pathway. This seems surprising considering the advantage of energy conservation in

the indirect pathway. Actually, almost all sulfur oxidizing bacteria that do not posses both pathways have been shown to oxidize sulfite directly.

Although there is evidence for a linkage of direct sulfite oxidation and the respiratory chain at the cytochrome *b*/quinol level (Beffa *et al.*, 1993) recent studies have shown that there is no clear link between the distribution of particular types of SOEs and the presence of genes encoding the initial sulfur compound oxidative steps (Wilson & Kappler, 2009). Moreover, it was shown that SOEs are induced in response to sulfite (Kappler *et al.*, 2012).

Indirect AMP-dependent sulfite oxidation involves three enzymes: APS'-reductase [EC 1.8.99.2], ATP-sulfurylase [EC 2.7.7.4], and APAT (adenylylsulfate: phosphate adenylyl transferase). As mentioned before, APS' is formed from sulfite and AMP by APS' reductase acting in reverse. Sulfate is released from APS' in a second step and the AMP moiety of APS' is transferred either to pyrophosphate by ATP sulfurylase or to phosphate by APAT, resulting in the formation of ADP or ATP, respectively. ADP can further be phosphorylated to ATP by adenylate kinase, therefore both enzymes catalyze substrate phosphorylation, and further electron transport phosphorylation both of which can be of energetic importance, especially in chemolithotrophic bacteria (Dahl, 1996). Another function of the APS'-pathway is in the assimilatory and dissimilatory sulfate reduction. While the APS-reductases involved in dissimilaroty sulfate reduction resembles the same enzyme, the APS reductase functioning in assimilatory sulfate reduction are completely different enzymes (Bick *et al.*, 2000).

1.3. Sulfite-oxidizing enzymes (SOEs)

As described in the previous chapter, direct sulfite oxidation is essential for detoxification of sulfite, and is catalyzed by sulfite-oxidizing enzymes (SOEs). At present two types of enzymes catalyze the direct sulfite oxidation to sulfate: sulfite oxidases (SO) [EC 1.8.3.1], and sulfite dehydrogenases (SDH) [EC 1.8.2.1]. Both enzymes are metalloproteins harboring a molybdopterin-cofactor (Moco) as redox centre. The main difference between these enzymes is their electron acceptor: SOs transfer electrons to molecular oxygen, ferricyanide, or cytochrome *c*, while SDHs use either ferricyanide or cytochrome *c* but no oxygen.

1.3.1. Phylogeny

All sulfite oxidases (SO) and sulfite dehydrogenases (SDHs) characterized to date belong to the SO family, which contains three groups of enzymes distinguished by the size and structure of the molybdenum (Mo) domain (Fig. 5). All sequences contain a conserved cysteine residue that serves as a ligand to the Mo atom in this enzyme family (Kappler & Dahl, 2001). The sequences identified originate from many different bacterial phyla: Proteobacteria, the green non-sulfur bacteria, the high-GC Gram-positive bacteria, *Bacilli*, *Actinomycetes*, *Myxobacteria*, *Planctomycetes*, *Euryarchaeota*, *Crenarchaeota* (Kappler, 2008).

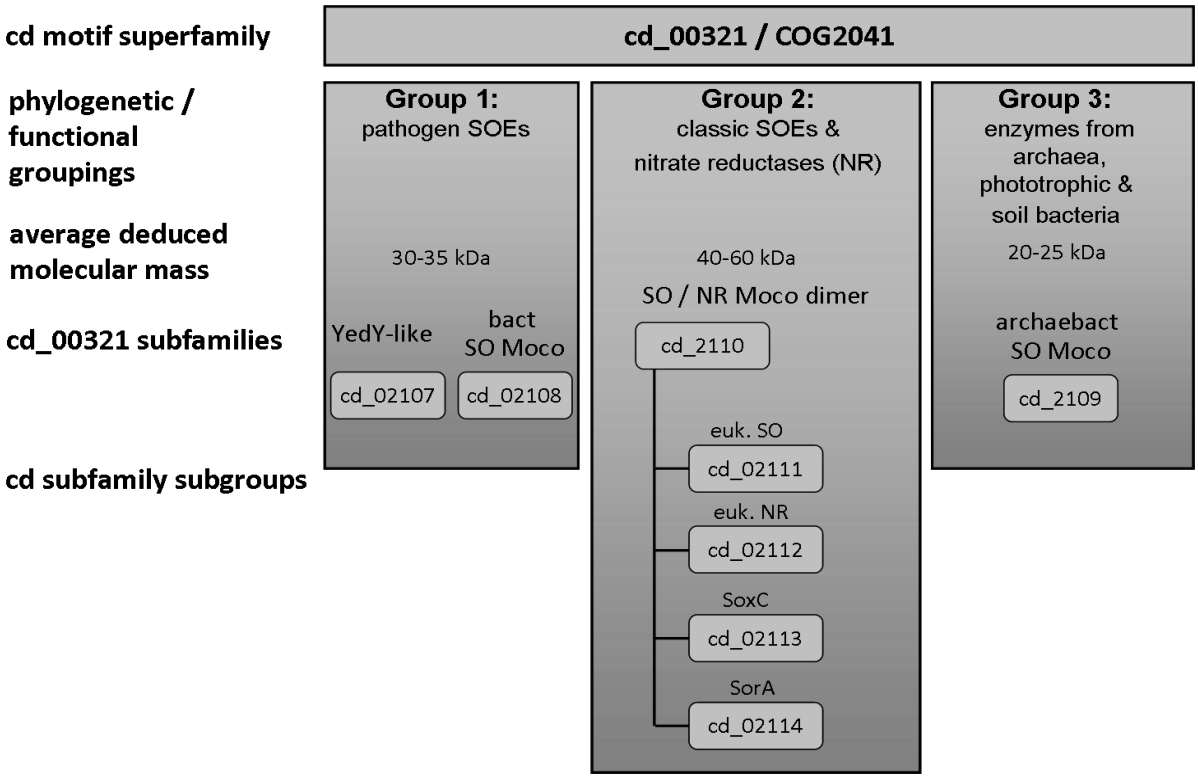


Fig. 5 Classification of the sulfite oxidase (SO) enzyme family in relation with conserved motifs (COG, cd) and phylogenetic/functional groupings. SOEs, sulfite-oxidizing enzymes. Further abbreviations are explained in the main text. Adapted and modified from (Wilson & Kappler, 2009).

SOEs in **group 1** are highly conserved and mainly originate from known bacterial pathogens such as *Pseudomonas aeruginosa*, pathogenic and non-pathogenic *E. coli* strains, *Salmonella*, *Yersinia*, *Shewanella*, *Burkholderia* and *Ralstonia* species (Kappler, 2008). On the basis of the structure of the Mo-binding domain, group one SOEs can be divided into two subgroups:

YedY-like enzymes, and proteins harboring a 30 kDa Mo-domain (cd_02108) (Wilson & Kappler, 2009). Genes encoding YedY-like proteins are associated with proteins characterized by binding of heme *b* and six transmembrane helices (Brokx *et al.*, 2005; Marchler-Bauer *et al.*, 2005). Genes of the second subgroup are found in association with genes encoding proteins of the COG4117 "thiosulfate reductase cytochrome *b* subunit" type, with four predicted transmembrane helices (Marchler-Bauer *et al.*, 2005).

The YedY protein from *E. coli* is the only enzyme within this group which has been characterized and crystallized to date (Loschi *et al.*, 2004). The protein fold of YedY is most similar to that of chicken SO (cSO) and to that of the bacterium *Starkeya novella* although the overall sequence identity is approximately 20% over a span of about 170 amino acids, in a region which is responsible for binding the Molybdenum cofactor, Moco (Loschi *et al.*, 2004). Despite the sequence homologies to known SOEs, YedY lacks analogous structures, such as the dimerization and heme-binding domain. But also the substrate-binding pockets of these enzymes show differences in their structures: the highly conserved Arg residues, which stabilize the binding of sulfite and sulfate, are not present in YedY (Loschi *et al.*, 2004). A further interesting point is that EPR (electron paramagnetic resonance) experiments revealed that only the Mo (IV) → Mo (V) transition is observed in this enzyme class (chapter 1.3.2) (Brokx *et al.*, 2005). As mentioned before, the *yedY* gene is part of the putative *yedYZ* operon in *E. coli* as well as of a wide variety of (primarily Gram-negative) bacteria. YedZ is a heme *b*-binding six transmembrane protein, probably interacting with the quinone pool in the cytoplasmic membrane and shuttling electrons to or from other membrane-associated oxidoreductases (Brokx *et al.*, 2005). Nevertheless, the *yedYZ* operon does not necessarily mean that YedY and YedZ build a complex. This remains unclear, as well as the *in vivo* function of these enzymes. It was shown that YedY does not show any detectable sulfite-oxidizing activity, in contrast, it may function as a reductase for sulfoxides and *N*-oxides (Loschi *et al.*, 2004).

The enzymes classified with cd_02108 differ from the YedY-like enzymes in terms of their association with a different type of membrane subunit and in the much lower degree of sequence identity within the Moco-binding domain (Kappler, 2008). These enzymes also do not show any sulfite-oxidizing activity and therefore are not considered in more detail in this thesis.

A unique characteristic of **group 2** SOEs is their Moco-binding and dimerization domain. Furthermore, most of the group 2 SOEs occur in extracellular compartments (Kappler, 2008). For more details of eukaryotic and prokaryotic sulfite dehydrogenases and sulfite oxidases see chapter 1.3.3.

Nitrate reductase (NR, [EC 1.6.6.1-3]) catalyzes NAD(P)H-dependent reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is the first step of nitrate assimilation in higher plants, algae and fungi. The NR monomer is composed of an approximately 100 kDa polypeptide and FAD^+, heme-iron, and a Moco, in which the nitrate-reducing active site is localized (Campbell, 1999).

The Sox (sulfur oxidizing enzyme) CD-like enzymes comprise a group harboring sequences which are related to the SoxC subunit of the SoxCD protein that was first described in *Paracoccus* species (Kelly *et al.*, 1997; Wodara *et al.*, 1997). The SoxCD protein is a periplasmic heterotetramer with SoxC harboring the Moco as first redox centre and SoxD a *c*-type cytochrome containing one or two heme groups as second redox centre, whereas a single heme group is sufficient for SoxCD activity (Quentmeier *et al.*, 2000; Kappler *et al.*, 2001; Bardischewsky *et al.*, 2005). SoxCD itself is part of a large protein complex, termed TOMES (thiosulfate oxidizing multi-enzyme system) which is encoded by the *sox* gene cluster consisting of 15 genes which are organized in three transcriptional units (Friedrich *et al.*, 2008). The TOMES enzyme complex oxidizes hydrogen sulfide, sulfur, thiosulfate, and sulfite with horse cytochrome *c* as the final electron acceptor (Friedrich *et al.*, 2005). However, the isolated SoxCD complex has a low affinity for sulfite rather it enhances the reaction rate of the TOMES complex (Quentmeier *et al.*, 2000; Bardischewsky *et al.*, 2005) and is therefore not considered as SOE.

Group 3 SOEs contain sulfite oxidases with the most reduced Moco-binding domain structures: these enzymes lack the dimerization domain and exhibit a reduced Mo-binding domain accompanied by the loss of some N-terminal parts. So far, none of these enzymes have ever been studied (Kappler, 2008). Most of the protein sequences derive from archaea species, such as *Crenarchaeota* and *Eurarchaeota*, and some high-GC Gram-positive bacteria, but also Firmicutes, and members of the *Thermus*/*Deinococcus* group. Due to a lack of an export signal and transmembrane helices, it could be assumed that theses proteins are cytoplasmic enzymes, in contrast to the described enzymes of the former groups (Kappler, 2008).

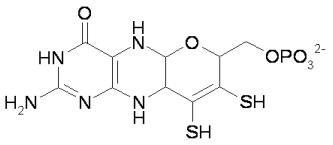
1.3.2. The molybdopterin cofactor (Moco)

The Moco-containing enzymes fall into four distinct groups: the xanthine dehydrogenase family, the sulfite oxidase (SO) family, the archaeal aldehyde:ferredoxin oxidoreductase, and the DMSO reductase family (McEwan *et al.*, 2002). The core component of these enzymes is their cofactor: molybdopterin (MPT)-cofactor (Moco), a tricyclic ring system composed of a pterin group fused to a pyran ring (Fig. 6). The pyran ring contains a phosphate group and a *cis*-dithiolene moiety that acts as a bidentate ligand for Mo or W (tungsten). A third thiolate is most likely from a cysteine (cys) residue, Cys-207 in rat and human liver SO, which is conserved in all known sequences of SOs and assimilatory nitrate reductases (Garrett & Rajagopalan, 1996). Furthermore, all available crystal structures of SO enzymes show a conserved fold, the SUOX (sulfite oxidases) –fold, of the molybdenum domain, which comprises a characteristic mixture of 10-12 α-helices and two to three β-sheets (Workun *et al.*, 2008).

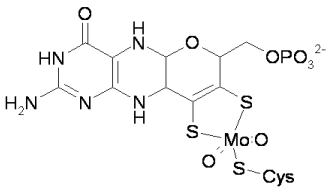
Mo is the only second-row transition metal that has known biological functions in all phylogenetic lineages of the Tree of Life: more than 50 molybdoenzymes are known to date (Hille, 1996). In several archaeal Moco-containing enzymes (such as the aldehyde:ferredoxin oxidoreductase) W, another group VIA element, appears to be preferred (Johnson *et al.*, 1996).

In SO and xanthine dehydrogenases there is one MPT per Mo (Kisker *et al.*, 1998). In contrast, there are two MPT molecules per Mo in enzymes of the DMSO reductase family. Furthermore, both MPT molecules are modified to form a dinucleotide (*bis*(molybdopterin guanine dinucleotide)) in DMSO reductases (Schindelin *et al.*, 1996) (Fig. 6). Most of the bacterial Moco-containing enzymes characterized to date contain the dinucleotide form with the exception of sulfite dehydrogenases which contain one MPT.

molybdopterin (MPT):



mononucleotide MPT-type
(e.g. sulfite oxidases)



bis(dinucleotide guanine MPT-type)
(e.g. *Rhodobacter* DMSO reductases)

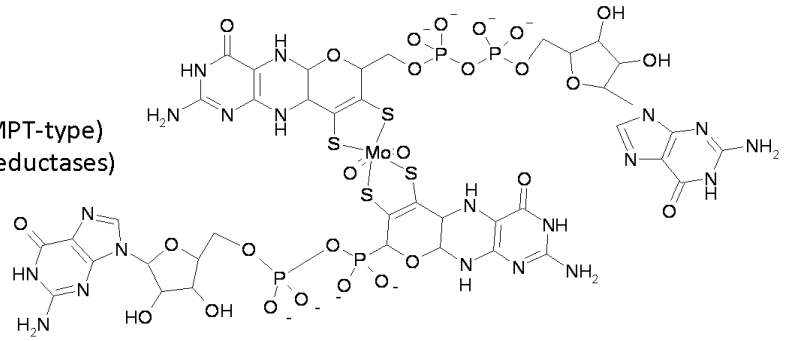


Fig. 6 Structure of the molybdopterin (MPT) and examples of the MPT-cofactor (Moco) in different Moco-containing enzymes. Cys, cysteine.

Based on the results from spectroscopic data and studies of model compounds, a catalytic mechanism for enzymatic sulfite oxidation has been proposed and is shown in Fig. 7 (Hille, 1994). The initial reaction step is a nucleophilic attack of the electron pair of sulfite on one of the Mo=O bonds resulting in a reduction of the molybdenum from the Mo(VI) to the Mo(IV) state. Subsequently, sulfate is released by hydrolysis in a coupled electron proton transfer reaction, whereas the Mo=O ligand is modified with a hydroxo- or water ligand. This reductive half reaction is followed by a subsequent transfer of the two electrons from the Moco to a second redox centre and finally to an external electron acceptor, such as oxygen or cytochromes from the respiratory chain, to regenerate the fully oxidized state of the Mo centre. The second redox centre is typically a one-electron redox group, normally a heme or an iron-sulfur cluster, resulting in the generation of the stable intermediate Mo(V) state (Hille, 1996). This second mechanism, the oxidative half reaction, requires a number of intramolecular electron transfer (IEF) processes (Feng *et al.*, 2007). If no additional redox centres are present in the enzyme, such as in the plant SO (Eilers *et al.*, 2001b), or as in SorT from *Sinorhizobium meliloti* (Wilson & Kappler, 2009), the two electrons are directly transferred from the Moco to an external electron acceptor. This reaction mechanism of sulfite oxidation is a typical hybrid ping-pong mechanism involving two reactive sites.

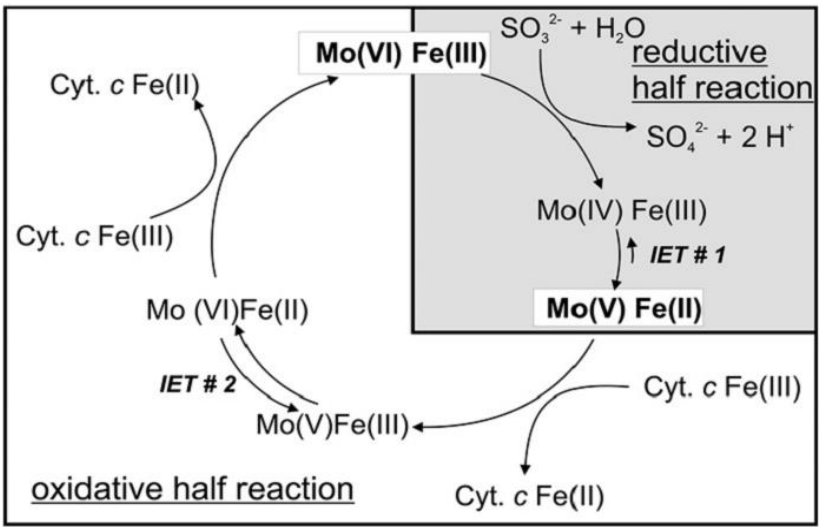


Fig. 7 Typical hybrid ping-pong mechanism of sulfite oxidation by heme-containing SOEs. IET, intramolecular electron transfer. More details are described in the main text. Adapted from (Schrader *et al.*, 2003; Kappler, 2011a).

As mentioned above, a sulfite oxidase deficiency in humans usually results in an early death, to severe neonatal neurological problems, such as dislocation of ocular lenses, mental retardation, and in an attenuated growth of the brain (Dublin *et al.*, 2002). Up to now, no effective therapies are known (Johnson, 2002). These symptoms result from either point mutations in the enzyme itself, or the inability to produce the Moco, which would affect all Mo-containing enzymes (Matthies *et al.*, 2004).

1.3.3. Characterized SOEs

As discussed in chapter 1.3.1, the eukaryotic and prokaryotic SOEs belong to the same enzyme family of sulfite oxidases, with a highly conserved SUOX-fold of the molybdenum domain. However, as shown in Table 1 there are significant structural differences, in terms of the enzyme localization, composition, molecular weight, and the amount and type of metals in the redox centres. Moreover, while the vertebrate and plant SOEs appear to have overall uniform architecture, bacterial SOEs are much more diverse.

Table 1. Four types of sulfite-oxidizing enzymes (SOEs). The amount of redox centres are per enzyme molecule. References: [1]= Kisker *et al.*, 1997, [2]= Eilers *et al.*, 2001a, [3]=Kappler *et al.*, 2000, [4]= Wilson & Kappler, 2009

SOEs	Localization	Type	Molecular weight	Metal centre	References
vertebrate	mitochondria, liver	α_2-homodimer	~ 110 kDa	1 x Mo, 1 x heme (b_5-type)	[1]
plant (*Arabidopsis thaliana*)	peroxisome	α_2-homodimer	~ 90 kDa	1 x Mo	[2]
SorA (*Starkeya novella*)	periplasm	α, β-heterodimer	α: ~ 41 kDa β: 8.8 kDa	1 x Mo, 1 x heme (c_{550}-type)	[3]
SorT (*Sinorhizobium meliloti*)	periplasm	α_2-homodimer	α: ~ 78 kDa	1 x Mo	[4]

Although there are differences in the localization of the SOEs, it is remarkable that all SOEs studied so far - with the exception of some acidophilic organisms (Pronk *et al.*, 1990) - seem to reside in cell compartments outside the cytoplasm and have a similar topological position with respect to the respiratory chain. Another unique feature is dimerization: sulfite oxidases either occur as homodimers or heterodimers. From animal systems, only homodimeric SOs have been purified and no evidence exists for a monomeric enzyme. Each monomer can be divided into two functional domains: the C-terminal Moco-binding domain mediating dimer formation, and an N-terminal domain constituting the second redox centre, such as a cytochrome b_5 domain (Johnson & Rajagopalan, 1977). Bacterial sulfite dehydrogenase (SDH), chicken and human liver SO show similar, high affinities for their substrate sulfite, and have nearly identical active site geometries that contain several conserved residues, including Cys-104, Tyr-236, His-57, and Arg-55 (all residues are given in SDH numbering (Kappler & Bailey, 2004; Kappler & Bailey, 2005; Doonan *et al.*, 2006)), whereas the latter is the most important one since it mediates substrate binding and product release as well as influencing turnover rates (Bailey *et al.*, 2009).

All characterized SOEs have $K_M^{sulfite}$ values in the low mircomolar range (approximately 4-100 µM) and turnover numbers between approximately 26 s^{-1} for vertebrate SOEs and approximately 350 s^{-1} for bacterial SOEs (Kappler *et al.*, 2006).

1.3.3.1. Eukaryotic sulfite oxidases

Arabidopsis thaliana sulfite oxidase (**pSO**) is the smallest eukaryotic Mo enzyme, consisting of a Moco-binding domain, but lacking the heme domain known from other plant SO sequences (Eilers *et al.*, 2001a). In Fig. 8 a ribbon diagram of the homodimer is shown with an N-terminal Moco-binding domain (green) and a C-terminal dimerization domain (orange), which interacts with the second monomer (Schrader *et al.*, 2003).

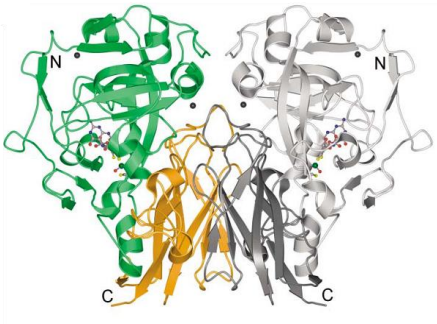


Fig. 8 A ribbon diagram of the homodimer of plant sulfite oxidase (pSO) of *Arabidopsis thaliana*. The two domains of one monomer are colour coded, showing the Moco-binding domain in green and the dimerization domain in orange. The second monomer is shown in shades of grey.
Adapted from (Schrader *et al.*, 2003).

The first atomic structure of a eukaryotic Mo-containing enzyme was solved for chicken SO (**cSO**) (Kisker *et al.*, 1997). However, no crystal structure for the human sulfite oxidase is yet available. The primary sequences of pSO and cSO share 46% identity with the main difference of lacking the heme binding domain in pSO (Schrader *et al.*, 2003).

In general, sequence homologies among eukaryotic SOs are very high, with 68% identity observed between cSO and hSO enzymes, and 88% between the rat SO and human SO (Kisker *et al.*, 1997).

A ribbon diagramm of a vertebrate enzyme is shown in Fig. 9. The enzyme is a homodimer located in the mitochondrial intermembrane space. Each subunit contains a small N-terminal b_5-type cytochrome domain, a large central Mo-binding domain, and a large C-terminal interface domain. In each subunit the Mo domain and the b_5-type heme domain are linked by a flexible peptide loop of 10 amino acids resulting in a relatively large distance between the two redox centres (Mo-Fe distance 32.3 Å, in contrast to bacterial SorAB: 16.6 Å) (Kisker *et al.*, 1997; Pacheco *et al.*, 1999; Feng *et al.*, 2003a). Despite the large distance between the two redox centres, the intramolecular electron transfer (IET) rates (> 1000 s^{-1}) are high due to conformational changes within the enzyme which bring the two subunits closer together during catalysis (Codd *et al.*, 2002; Feng *et al.*, 2002 ; Feng *et al.*, 2003b).

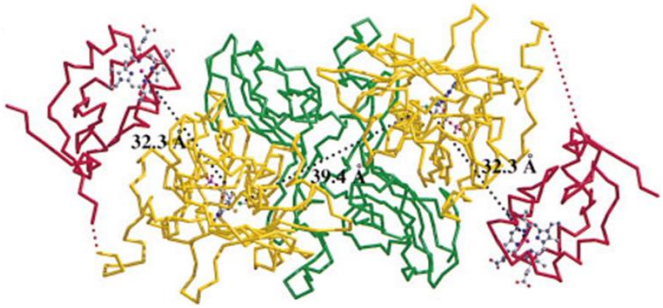


Fig. 9 Ribbon diagramm of eukaryotic homodimeric sulfite oxidase. The N-terminal heme-binding domain (red) is connected to the Moco-binding domain (yellow) and the C-terminal dimerization domain (green). The Moco and the heme are shown in ball-stick representation. The red dotted line indicated the loop linker region, and the grey dotted lines connect the metal centres and the cofactors and indicate the distance between the Mo and the Fe. Adapted from (Kisker *et al.*, 1997)

Eukaryotic SOs have also been purified from bovine, chicken, rat, avian and human livers (Rajagopalan, 1980), whereas the rat SO was the first Moco-containing enzyme which was cloned and expressed in an active form (Garrett & Rajagopalan, 1994).

1.3.3.2. Prokaryotic sulfite dehydrogenases (SDHs)

The best characterized bacterial sulfite dehydrogenase is **SorAB** from *Starkeya novella* (formerly: *Thiobacillus novellus*) for which a crystal structure has been obtained (Fig. 10) (Bailey *et al.*, 2009).

The enzyme consists of two subunits: a large subunit SorA (~ 40.6 kDa) harboring a single Moco-binding domain (blue) and a small subunit SorB (8.8 kDa), a c_{552}-type heme-binding domain (cyan) (Toghrol & Southerland, 1983, Kappler *et al.*, 2000). In contrast to the chicken liver and plant SO in which the dimerization domain mediates the formation of the dimer, the dimerization domain of SorAB does not mediate the interaction and their function remains unclear. Both subunits (SorA and SorB) form a tight heterodimeric complex with strong electrostatic interactions and the close approach of the redox centres (Mo-Fe distance 16.6 Å (Feng *et al.*, 2003a) allow for rapid electron transfer without the need of conformational changes (Kappler *et al.*, 2000; Bailey *et al.*, 2009).

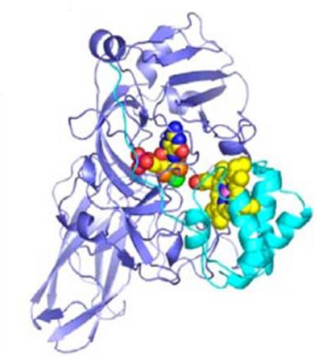


Fig. 10 Ribbon presentation of bacterial sulfite dehydrogenase SorAB from *Starkeya novella*. The SorA and SorB subunits are coloured blue and cyan, and the redox cofactors (molybdopterin cofactor, cytochrome *c*) are coloured yellow with the Mo atom coloured green and the Fe atom coloured violet. Oxygen atoms are coloured with red dots and nitrogen atoms are coloured with blue dots. Adapted from (Bailey *et al.*, 2009).

Since cytochrome *c* is the preferred electron acceptor for SorA these proteins are directly linked to the respiratory chain and thus contribute to energy generation *via* cytochrome *c* oxidase (Yamanaka *et al.*, 1981).

The assembled protein is located in the periplasm, while SorA is exported *via* the *Tat* system, SorB is targeted for *Sec*-dependent transport (Kappler *et al.*, 2000).

SorAB is involved in lithotrophic growth of *Starkeya novella* in which the enzyme is induced by the presence of thiosulfate and can amount to about 1.6% of the total cell protein (Kappler *et al.*, 2000). SDHs are essential not just for lithotrophic but also for organotrophic growth. SorA (B) homologues were detected during growth with taurine in the β-Proteobacterium *Cupriavidus necator* H16. Although it was shown that *sorA* and *sorB* genes are located in the same operon and that they are co-transcribed, it remains unclear whether SorA and SorB form a complex, or even if SorB is part of the enzyme (Denger *et al.*, 2008). Another SorAB homolog was detected in the case of sulfite respiration by the chemoheterotrophic human pathogen *Campylobacter jejuni*. This bacterium is the leading cause of acute bacterial gastroenteritis, both in industrialised and developing countries (Friedman *et al.*, 2000). With SorAB as detoxifying enzyme and sulfite respiration, the bacterium is able to survive in foodstuffs that have been treated with sulfite for preservation reasons, or in the human host where the system serves as protection against immune defence by neutrophils (chapter 1.1.2.2) (Myers & Kelly, 2005; Tareen *et al.*, 2011).

In 1999, Reichenbecher *et al*. reported purification of an 'unusual sulfite dehydrogenase' induced by taurine or isethionate in *Delftia acidovorans* strains P53 and strain 39. In contrast to SorA(B), the enzyme did not contain native cytochrome *c* or reduced added cytochrome *c* instead the enzyme activity was specific *in vitro* for ferricyanide (Reichenbecher *et al.*, 1999). In addition, the native enzyme was much more heat-stable and had a molecular weight of

74.4 kDa indicating the enzyme is a homodimer. The tolerance for sulfate and chloride was much higher compared to SorAB. In another strain of *Delftia acidovorans* (strain SPH-1) this 'atypical sulfite dehydrogenase', named SorA, was also purified and the results of Reichenbecher and colleagues were confirmed (Denger *et al.*, 2008). Ten years after the first purification attempt, the enzyme was renamed as **SorT** since it shares only 32% sequence identity to SorA and has clearly a different structure (Wilson & Kappler, 2009). The enzyme, characterized in the plant symbiotic soil bacterium *Sinorhizobium meliloti*, was induced by taurine and thiosulfate and is a heme-free, periplasmic homodimer (78 kDa) whose apparent $K_m^{sulfite}$ and k_{cat} values are pH-independent and which was found to be insensitive to inhibition, e. g. by changes in ionic strength, in contrast to SorAB. *SorT* is part of an operon containing genes for a cytochrome *c* and pseudoazurin for which it was shown that they are co-transcribed with *sorT*. However, neither cytochrome *c* nor pseudoazurin appear to be part of SorT. *S. meliloti* cells show respiratory activity with sulfite and the results obtained with recombinantly expressed *sorT*, *cyt c* and *pseudoazurin* led to the assumption of the following electron transfer pathway: SorT might use the cytochrome *c* as its *in vivo* electron acceptor which are most likely transferred to cytochome oxidase (Low *et al.*, 2011). However, at present the role of the pseudoazurin in SorT electron transfer is unclear, even though the X-ray crystal structure for this protein has been solved (Laming *et al.*, 2012). A fairly similar electron transfer pathway was described for the SorT homologue in the thermophilic bacterium *Thermus thermophilus*. The heat-stable SorT homologue was first described by Di Salle and collegues (Di Salle *et al.*, 2006). The corresponding gene operon contains a gene encoding a putative novel c_{550}-type cytochrome which could be the electron acceptor for SorT. Cytochrome c_{550} funnels the electrons to another c_{552}-type cytochrome which was recognized as the physiological substrate of the ba_3 oxidase / caa_3 cytochrome *c* oxidase. Therefore cytochrome c_{550} couples sulfite oxidation to cell respiration (Robin *et al.*, 2011). Although all SorT-like proteins contain a dimerization domain, some of them occur as monomers while others are dimeric in structure or can even occur in both forms such as the SorT homologue, termed *draSO*, in the polyextremophilic bacterium *Deinococcus radiodurans*. Here, no cytochrome *c* or pseudoazurin-encoding genes were found (D'Errico *et al.*, 2006).

Interestingly, although the two bacterial sulfite-oxidizing enzymes (SorAB, SorT) are structurally diverse and play a role to a different metabolic context, the operons encoding

these enzymes look strikingly similar in some organisms (*Delftia acidovorans*, *Sinorhizobium meliloti*, and *Starkeya novella*). The operons contain genes encoding an extracytoplasmic function (ECF)-type sigma factor, belonging to the large subgroup ECF-26, and a putative anti-sigma factor (Kappler *et al.*, 2001; Wilson & Kappler, 2009; Kappler, 2011a; Kappler *et al.*, 2012). These ECF sigma-factors are responsible for signal transduction from the bacterial cell envelope to the cytoplasm, and are often found upstream of the genes they regulate and known functions include the detection of envelope stress, such as unfolded proteins, or osmotic stress (Huang *et al.*, 1999b; Staroń *et al.*, 2009).

Recent research has shown that sulfite-oxidizing enzymes are induced in response to exposure to sulfite and that this process is independent of the presence of reduced sulfur substrates that can also potentially be used for energy generation. This indicates that bacterial sulfite oxidation is a stress response, probably due to potentially harmful levels of sulfite or one of its reaction products (Kappler *et al.*, 2012). Since sulfite oxidation in eukaryotic cells is also regarded as a detoxification reaction, both processes can be put into the same category of metabolic response.

However, details of the exact metabolic function and regulation of these periplasmic bacterial enzymes remain unclear at present. For some organisms it has been shown to be regulated by ECF type 26 sigma factors but it is not clear yet how widespread this system is. Among the six SDHs that have been characterized at protein level, only three seem to be regulated by the ECF-26 group while the others are encoded by gene regions devoid of ECF sigma factor genes. However, it has been proposed that the other SDH genes are part of regulons responding to an ECF sigma factor encoded elsewhere in the genome (Kappler *et al.*, 2012).

1.4. Aims of this study

Bacterial sulfite dehydrogenases are poorly known enzymes which are involved in organotrophic growth of aerobic bacteria. They are highly diverse in terms of their overall quaternary structures and reactions compared to eukaryotic sulfite oxidases.

The aim of this study was to contribute to filling the gap of knowledge about this enzyme class, or more generally, about bacterial sulfite oxidation. Therefore, the chief objective was to identify new genes and to characterize their encoding proteins for sulfite-oxidizing activities. Furthermore, understanding the regulation of these genes (or gene clusters) and the composition of the encoded proteins or protein complexes were a major aim. This study also aimed to elucidate which metals or cofactors, respectively, are bound in the enzyme(s). Finally, understanding the nature of the electron transport associated with these intracellular enzymes funnelling electrons from sulfite to a yet unknown transporter chain was an important aim.

To answer those questions, proteomic approaches, such as orbitrap and 2-D gel electrophoresis, were used, intending to identify candidate genes by peptide fingerprinting-mass spectrometry (PF-MS). This was complemented by bioinformatics providing information about the presence and distribution of genes encoding novel sulfite-oxidizing enzymes within genome-sequenced bacteria. Transcriptional analyses were employed to confirm the regulation of those genes which further should allow the assessment of putative regulator sequences. Alternatively and complementing this, *lacZ*-fusion plasmids were used for regulator studies. Generating insertional knockout mutants of putative sulfite oxidation genes enabled confirming the involvement in sulfite oxidation and further in desulfonation. Some organisms have been reported to have no measurable sulfite dehydrogenase, however this may be artefactual due to inadequate assay conditions. This study also aimed to establish new assay conditions or alternative methods to measure the specific enzyme activity. It appeared plausible that with an appropriate enzyme assay, the purification, optionally of heterologously overexpressed proteins, or separation of relevant enzymes would become more easily feasible. Conversely, sufficient amounts of purified enzyme would enable investigating the structural composition of such enzymes, also in terms of their putative cofactor or the associated electron transport chain. To this end, different methods, such as MALDI-TOF-MS analysis, inductively coupled plasma-mass spectroscopy (ICP-MS),

dynamic light scattering (DLS), or Clark-type oxgen electrode experiments were used in this study.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals, kits, and equipment

Unless otherwise specified, standard chemicals were of the highest purity available and purchased from Sigma-Aldrich (Hamburg, Germany, and Gillingham, UK), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Biomol (Hamburg, Germany), Fluka (Buchs, Switzerland), Fermentas (St. Leon-Rot, Germany), BioRad (Munich, Germany) or Biomol (Hamburg, Germany). Sulfoacetaldehyde, as the bisulfite addition complex, was synthesized and characterized previously (Denger *et al.*, 2001). RNAlater™ solution was from Ambion (Frankfurt, Germany), blotting nylon membrane (hybrid-N^+) from Amersham Bioscience (Glattbrugg, Switzerland), and the colour solution (BCIP/NBT premixed solution) for Southern blotting from Sigma-Aldrich (Hamburg, Germany, and Gillingham, UK). RNA was isolated using the E.Z.N.A. bacterial RNA Kit from Omega Bio-Tek (Norcross GA, United States). DNA was isolated by bacteria genomic Prep Mini Spin Kit™ from GE Healthcare (Munich, Germany) or a DNA purification kit from Promega (Mannheim, Germany). Hybridization and labelling of the corresponding probes for Southern blot analysis were done by using the Roche DIG High prime™ DNA labelling detection starter kit and by using the Roche DIG EASY Hib™ solution (Penzberg, Germany). PCR experiments were performed using the Taq PCR Master Mix™, ready-to-use (Qiagen, UK). Proteins for 2-D gel electrophoresis were loaded on ReadyStrip™ immobilized pH gradient (IPG) strips from BIO-RAD (Munich, Germany). Vivaspin™ concentrators with a polyethersulfone membrane were purchased from Sartorius (Göttingen, Germany), and gel filtration columns (Superose 12 HR 10/30) from Pharmacia (Munich, Germany). All buffer exchanges were done with PD-10 columns (Sephadex G-25) purchased from Pharmacia. Nucleic acids were fixed on a nylon membrane *via* UV cross linking (Stratlinker™, Strategene). Quantification and documentation of protein and nucleic acid bands were done using the Gel Doc XR™ gel documentation system (Bio-Rad) and NanoDrop ND-1000 spectrophotometer from Thermo Fisher Scientific (Schwerte, Germany). Isoelectric focussing was done in a Bio-RAD PROTEAN™ IEF cell. The

anion exchange column used for protein purification (MonoQ, HR 10/10) was from Pharmacia. Enzyme assays were carried out using a Kontron instrument Uvikon 922 spectrometer. Dynamic light scattering (DLS) was done by using 802 DLS photometer from Viscotek, Dual Attenuation Technology (Herrenberg, Germany).

2.1.2. Organisms, and growth media

The organisms and the cultivation conditions used are described in Table 2. The marine bacteria *Ruegeria* (formerly *Silicibacter*) *pomeroyi* DSS-3 and *Roseovarius nubinhibens* ISM were grown in a PIPES-buffered *Silicibacter* basal medium (SBM), which is a modified salt medium described by Denger *et al.* (Denger *et al.*, 2006). The growth medium for strain ISM was supplemented with 0.05% yeast extract (YE) (Denger *et al.*, 2009). The complex medium used for *Ruegeria pomeroyi* DSS-3 was 0.5-fold YTSS medium (2 g yeast extract, 1.25 g tryptone, 20 g sea salts in 1 l distilled water). *Roseovarius* sp. strain 217 was grown in a modified Tris-buffered seawater medium (BSWM) supplemented with vitamins (Pfennig, 1978) described by Krejčík *et al.* (Krejčík *et al.*, 2008). The β-Proteobacteria *Cupriavidus necator* (*Ralstonia eutropha*) H16 and α-Proteobacteria *Sinorhizobium meliloti* 1021 were grown in a potassium phosphate-buffered (KP) mineral salt medium described by (Thurnheer *et al.*, 1986). As sole carbon and energy source, the organosulfonates isethionate (2-hydroxyethanesulfonate), taurine, *N*-acteyltaurine, *N*-methyltaurine, homotaurine (APS), each in a concentration of 10 mM, or cysteate, or 2,3-dihydroxypropane-1-sulfonate (Dhps), each in a concentration of 6.7 mM were used. Ammonium chloride (10 mM) served as the nitrogen source. In negative controls the carbon source was omitted (no growth), while in positive controls (no sulfite stress) the organosulfonate was replaced by either 10 mM acetate, malate or succinate, in the appropriate media.

To transfer cultures of *Ruegeria pomeroyi* DSS-3 pre-grown in complex medium to basal medium, cells from 1 ml of outgrown culture were collected by centrifugation (15,000 *g* for 5 min), washed three times with sterile basal medium, centrifuged again, and resuspended in an equal volume of basal medium. The cell suspension was then diluted 20-fold in basal medium containing the appropriate carbon source.

The *E. coli* strains used were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), supplemented with the appropriate antibiotics [μg ml^{-1}]: Kanamycin (Kan) [20], spectinomycin (Spec) [200], rifampicin (Rif) [20], ampicillin (Amp) [100], tetracyclin (Tet) [5].

E. coli BL21DE3 and *E. coli* TP1000 were used for heterologous overexpression of SPO3559, *E. coli* JM101 was chosen as host for the pk19 plasmid, and *E. coli* 803 as host for the pMP220 and pET16 plasmids, respectively.

Table 2 Organisms used in this study and the corresponding growth media and conditions. Abbreviations and compositions of the media are described in detail in the main text.

Organisms	**Media**	**Growth conditions, antibiotic resistence**	**Source**
Ruegeria pomeroyi DSS-3	½ YTSS, SBM	aerobic, 30 °C, Rif	González *et al.*, 2003
Roseovarious nubinhibens ISM	SBM+ 0.05 % YE	aerobic, 30 °C	González *et al.*, 2003
Roseovarius sp. strain 217	BSWM	aerobic, 30 °C	Schäfer *et al.*, 2005
Cupriavidus necator H16 (DSM 428)	KP, pH 7.2	aerobic, 30 °C	Pohlmann *et al.*, 2006
Sinorhizobium meliloti 1021	KP, pH 7.2+ 0.05 % YE	aerobic, 30 °C	Finan *et al.*, 2001b
Escherichia coli BL21DE3	LB	aerobic, 37 °C	Miroux & Walker, 1996
Escherichia coli TP1000	LB	aerobic, 37 °C, Kan	Palmer *et al.*, 1996
Escherichia coli JM101	LB	aerobic, 37 °C	Messing, 1979
Escherichia coli 803	LB	aerobic, 37 °C	Wood, 1966

2.1.3. Plasmids and oligonucleotides

The plasmids used in this study and their features, applications, and sources are listed in Table 3. For antibiotic resistance cassettes (^{R}) the following abbreviations were chosen: Amp, Ampicillin; Kan, Kanamycin; Spc, Spectomycin; Tet, Tetracyclin.

Table 3 Plasmids used in this study. Abbreviations are described in the main text.

Plasmids	Characteristics	Application	Source
pET-16b	Wide host-range expression vector, Amp^R	Heterologous overexpression of SPO3559	*Novagen*
pK19mob, Spc^R	Spc^R cassette cloned into suicide insertion plasmid pK19 mob, Kan^R	Insertional knockout of SPO3559	Todd *et al.*, 2011
pMP220	Wide host-range promotorless-*lacZ* probe vector, Tet^R	Promotor studies, *lacZ*-fusions	Spaink *et al.*, 1987
pBluescript M13	M13 phagemid, T_7 T_3 *lacI lacZ*, Amp^R, high copy plasmid	Subcloning of *lacZ*-SPO3559 and *lacZ*-SPO3562	Short *et al.*, 1988
pRK2013	Kan^R	Mobilising plasmid in *tri*-parental crosses	Figurski & Helinski, 1979

The oligonucleotides used in this study are listed in Table 4 and were synthesized by Microsynth (Balgach, Switzerland) or Eurofins (Ebersberg, Germany).

Table 4 Oligonucleotides used in this study. Recognition sites for restriction enzymes are bold and underlined. Fwd, forward primer, Rev, reverse primer.

Primer		Sequence (5' → 3')	Application
soeA knock-out	Fwd	GC**GGATCC**CGGCGGCGCCGATCATGGCC	Internal mutation of *soeA*, hybridisation probe for Southern blot analysis
	Rev	GC**GAATTC**GATATAGCGATCGAGCTGATCG	
soeA overexpression	Fwd	GGGAAACC**CTCGAG**AGCCACCACCAGCC	Heterologous overexpression of *soeA*
	Rev	GC**GAATTC**ATTTCCCCACCTTCCATGTCAGC	
soeA-lacZ	Fwd	GGCTGAGC**TCTAGA**TCGCCATGCTGTCC	Promotor studies of SPO3562→SPO3557
	Rev	CCATGTTGACC**CTGCAG**AAGCCGCCATGGG	
tauR-lacZ	Fwd	CCGCAAACAG**TCTAGA**GGGCGCCGTGCCC	
	Rev	GGGATCGGCCTGG**CTGCAG**ATGAACGG	
soeA full-length gene	Fwd	GGGAAACCCGAATGAGCCACCACCAGCC	Amplifying and sequencing of *soeA*
	Rev	TCATTTCCCCACCTTCCATGTCAGC	
	Fwd, internal	TTGACGGCCCGCATCTGG	Sequencing of *soeA* revertant
	Rev, internal	CGCCGCACCGCTTTCATC	
soeA, internal fragment	Fwd	TTGACGGCCCGCATCTGG	Transcription studies of *soeABC*
	Rev	CGCCGCACCGCTTTCATC	
soeB, internal fragment	Fwd	GCAGGGGCGCCAGAATGTC	
	Rev	GGCCGAAGGCGTGATGAAGA	
soeC, internal fragment	Fwd	CAGCGCGGCCGAAACAAG	
	Rev	TTGAGCCGCGAGGGATGTG	
soeA → *soeC*	Fwd	GGACCGGGTGCGGATGGT	Co-transcription studies of *soeABC*
	Rev	ACCCAAGCCCGAGAGCGTGGTGAA	
soeA → *soeB*	Fwd	GGACCGGGTGCGGATGGT	
	Rev	GGCCGAAGGCGTGATGAAGA	
pta → *soeA*	Fwd	GCGGGCAACATCGGCTACA	
	Rev	GCCATCCGGGCCATCCTT	
xsc → *soeA*	Fwd	AGTGGCCGGCGGTTACCC	
	Rev	GCCATCCGGGCCATCCTT	

(continued)

Table 4 (continued)

Primer		Sequence (5' → 3')	Application
SPO3123	Fwd	AACCGCCGCCCTGTTCG	Trancription studies of SPO3124 gene cluster
	Rev	GCAGCCGCGTGCCAGAA	
SPO3124	Fwd	GCGCCGCGATCCAGACC	
	Rev	GACGCCGTCACCGGAGATGT	
SPO3125	Fwd	TCCCGCAGGCCAGGACAG	
	Rev	GCGCCCGGTCATGGTTCTC	
SPO3126	Fwd	TGACCGCGCACAGGACATTT	
	Rev	CTGCGGCGCCACATAGAGG	
SPO3127	Fwd	ACATGCGGGCCGAGACGAT	
	Rev	CGCCGCAATGAGGACAGGTT	
SPO3124-*lacZ*	Fwd	GCAGCGCGCT**CTAGA**CATTGAAGGCG	Promotor studies of SPO3124
	Rev	GCAATCGTCA**CTGCAG**TTCGACACG	
SPO0719	Fwd	CGCCGATGGCTACGAGCATA	Transcription of genes which could be involved in sulfite oxidation in *Ruegeria pomeroyi* DSS-3
	Rev	GTCGCCGACCGAGATGGTTT	
SPO0720	Fwd	AACAGCCGGGCATCGTTCTG	
	Rev	GTAATCGGCAGCGGTCTTTTCATC	
SPO2340	Fwd	GCGCCGAGACGCTGGAATA	
	Rev	AGCTCGCCCGGCATCTTG	
SPOA0168	Fwd	CCCGCGGCGGTCCTTATCA	
	Rev	TACCCGCGCTCGGCTTCA	
SPOA0169	Fwd	CGGCGTTCGGGGAAAACA	
	Rev	TGGCGCTCAATAAGGCTAATCTGA	
ROS217_11926	Fwd	GCCGGCCCGCATAGACAA	Transcription of *soeA* in *Roseovarious* sp. 217
	Rev	CAGATCGGCATAGGCAACCATTT	
16S rRNA	Fwd	CAGAGTTTGATCCTGGCTCAG	Positive control for RNA isolation described in (Weisburg *et al.*, 1991)
	Rev	TTACCGCGGCTGCTGGCAC	

2.2. Cultivation, harvesting and preparation of cell extracts

Precultures at the scale of 3 ml were grown in 30 ml screw-cap tubes in a roller. For growth experiments, RNA and DNA isolation and enzyme assays, or for protein purification, respectively, cultures were grown at the 50 ml scale in 300 ml Erlenmeyer flasks, or at the 1 l scale in 5 l Erlenmeyer flasks, respectively, on a shaker (160 rpm, 30 °C). Samples were taken at intervals to monitor optical density ($OD_{580\ nm}$), to assay protein (chapter 2.5), and to determine the concentrations of substrates and products (chapter 2.5). Cultures were harvested in the mid-exponential growth phase for enzyme assays and protein purification by centrifugation (20,000 *g*, 20 min, 4 °C), with the exception of cultures for RNA and DNA isolation, which were harvested in the early exponential phase by centrifugation (16,000 *g*, 10 min, 4 °C). Cells for protein purification were washed twice by centrifugation using the starting buffer of the first purification step (20 mM Tris/H_2SO_4 buffer, pH 8.5, 5 mM $MgCl_2$), and the cell pellet was stored frozen. Cells for preparation of the total RNA were used either immediately, or treated with RNAlater™ (3 ml, 1 h, 4 °C) according to the manufacturers' protocol (Ambion, Frankfurt/Germany) and stored frozen (-20 °C).

Cell-free extracts free of nucleic acids (DNase-I-treated, 50 µg ml^{-1}) were generated by four passages through a chilled French pressure cell at 140 MPa (Junker *et al.*, 1994) followed by centrifugation (17,000 *g*, 5 min, 4 °C). The supernatant was designated 'crude extract' and was ultracentrifuged (170,000 *g*, 20 min, 4 °C) for preparation of 'membrane fraction' (pellet) and 'soluble fraction' (supernatant). The membrane fraction was washed twice by centrifugation and resuspended in the appropriate buffer.

Glycerol stocks were prepared by diluting cultures with glycerol (50% v/v final concentration) and stored frozen at -70 °C, whereas *Ruegeria pomeroy*i DSS-3 and *E. coli* cultures were diluted with glycerol to 25% v/v final concentration, and further supplemented with DMSO (15% v/v final concentration for *Ruegeria pomeroyi* DSS-3, and 25% v/v final concentration for *E. coli*.).

2.3. Molecular methods

2.3.1. *In vitro* and *in vivo* genetic manipulation

For construction of *lacZ* fusions of genes SPO3124, SPO3559, SPO3562 (see Results and Discussion), chromosomal DNA of *Ruegeria pomeroyi* DSS-3 was used as template to amplify the respective genes by PCR using oligonucleotides (Table 4) that introduced restriction sites for *Xba*I and *Pst*I. The PCR products were digested, purified and ligated into plasmid pMP220 (Table 3), and transformed into *E. coli* 803 (Table 2) *via* heat shock (Cohen *et al.*, 1972); correct insertion was confirmed by sequencing. Then, the plasmids were transferred into *Ruegeria pomeroyi* DSS-3 by conjugation *via tri*-parental mating using helper plasmid pRK2013 (Table 3) (Figurski & Helinski, 1979). If transformation rate was insufficient, the constructs were subcloned into high-copy plasmid pBluescript™, which allowed blue-white screening.

For heterologous overexpression of His-tagged SPO3559, the gene was amplified by PCR using oligonucleotides (Table 4) that introduced restriction sites for *Eco*RI and *Xho*I, and the product was digested, purified, ligated into pET16 (Table 3), and transformed into *E. coli* 803 *via* heat shock (Table 2); correct insertion was confirmed by sequencing. The pET16-SPO3559 construct was then transformed into overexpression vectors *E. coli* BL21DE3 and *E. coli* TP1000 *via* heat shock.

For the insertional mutation of SPO3559 in *Ruegeria pomeroyi* DSS-3, an internal fragment (1300 bp) of the gene was amplified by PCR using oligonucleotides (Table 4) that introduced restriction sites for *Bam*HI and *Eco*RI, respectively, followed by cloning into pK19mob, Spc^R in *E. coli* JM101 (Table 3). Plasmids were transferred into *Ruegeria pomeroyi* DSS-3 by *tri*-parental conjugational mating using helper plasmid pRK2013. Transconjugants, in which the plasmid had integrated into SPO3559 were verified by PCR. Furthermore, the mutation was confirmed by Southern blotting of genomic DNA obtained from the mutant compared to the wild type strain.

2.3.2. Southern blotting

Insertional mutations of SPO3559 in *Ruegeria pomeroyi* DSS-3 were confirmed by Southern blot analysis (Southern, 1975). Therefore, 200 ng of genomic DNA of wild type and SPO3559-mutant strains were digested overnight with *Bam*HI or *Eco*RI, and the DNA fragments were separated by gel electrophoresis (1% agarose, 70 V). The blotting procedure was according to the manufacturers' protocol (Roche, Penzberg/Germany).
For hybridization, an internal PCR-amplified fragment of SPO3559 (Table 4) was fluorescence-labelled. Briefly, 16 µl of the purified PCR product was denaturated and 4 µl of the fluorescence-dye was added. The membrane was stored in distilled water for analysis by gel documentation.

2.3.3. Transcriptional analysis

Total RNA preparations were tested for residual DNA by PCR before the reverse transcriptions. Reverse transcription (RT) was done as described previously (Krejčík *et al.*, 2008) using the gene-specific primers listed in Table 4; a 16S rRNA primer was used to generate a positive control of complementary DNA (cDNA). The cDNA was used as template for RT-PCR with the cycling conditions shown in Table 5; chromosomal DNA was used to generate positive controls. The PCR products were visualized on agarose gels (0.7% -1.5%).
Colony PCR experiments were carried out by picking a single colony, which was resuspended in 10 µl distilled water, which in turn was boiled for 10 min. Subsequently, 1 µl of this resuspension was used for PCR.

Table 5 Cycling conditions for PCR experiments. *, conditions were depending on the primer.

Step	Temp. [°C]	Time	Comments	
Prewarming	95	Pause		
Initial denaturation	95	5 min		
Denaturation	95	30 sec		30-35 cycles
Annealing	*	30 sec	Calculated as lowest melting temp. (T_m)- 5 °C	
Elongation	72	*	Calculated as 1 min per 1 kbp of PCR fragment	
Final extending	72	10 min		

2.3.4. Protein expression

Denaturated proteins were separated by one-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 13% or 16% gels) and stained with Coomassie brilliant blue R250 (Laemmli, 1970). For the separation of proteins smaller than 30 kDa (e. g. SPO3124, 11 kDa), Tricine-SDS-PAGE gels were used (Schägger, 2006). To compare protein patterns in crude extracts and membrane fractions of different bacteria, gradient gels were used anticipating sharper protein bands and better separation. Therefore, denaturated protein (250 µg total protein) was loaded onto 1-D SDS-PAGE (5.0-19% acrylamide).

2-D gel electrophoresis was used for high-resolution protein separation. Therefore, samples (500 µg total protein) were precipitated with four volumes of ice-cold acetone and incubated at -20 °C overnight. The precipitated proteins were collected (17,000 *g*, 10 min, 4 °C), dried, and resuspended in 400 µl rehydratization buffer according to the manufacturers' protocol (Bio-Rad, Munich/Germany). ReadyStrip™ IPG strips (pH range 3-10, 4-7, 5-8) were loaded with the sample and incubated according to the manufacturers' protocol (Bio-Rad, Munich/Germany). The focussing conditions were 0-250 V within 15 min, 250-10,000 V within 3 h and 10,000 V constant until a total of 60,000 Vh had been reached.

2.4. Biochemical methods

2.4.1. Solubilization of membrane-bound sulfite-oxidizing activity

Detergents (0.5% (w/v)) were mixed with membrane fraction (1.9 mg protein ml^{-1}), which was resuspended in 50 mM potassium phosphate buffer, pH 7.2, supplemented with 5 mM $MgCl_2$. When optimizing the solubilization procedure, the following detergents were tested: non-denaturating, ionic sodium cholate-hydrate, non-denaturating, non-ionic *n*-dodecyl-β-D-maltoside, zwitterionic CHAPS, non-ionic Triton X-100, non-ionic *n*-octyl-β-D-glucopyranoside and non-ionic *n*-dodecyl-β-D-glucoside. The protein-detergent mixture was incubated on ice for 60 min while vortexing every 10 min. Subsequently, the mixture was ultracentrifuged (170,000 *g*, 90 min, 4 °C) and the supernatant was tested for enzyme activity.

2.4.2. Enzyme purification

In order to purify SPO3124 (11 kDa protein), soluble fractions of cysteate-grown *Ruegeria pomeroyi* DSS-3 were subjected to anion exchange chromatography. The column was equilibrated with 20 mM Tris/H_2SO_4 buffer, pH 8.5 at a flow rate of 1.0 ml min^{-1}. A gradient of sodium sulfate (0–75 mM over 5 min followed by 75–200 mM over 50 min and then 200–500 mM over 10 min) in equilibration buffer was applied and all fractions (3 ml) were tested for sulfite dehydrogenase activity. The enzyme did not bind to the column and eluted immediately, but most other proteins did bind. Active fractions were pooled, concentrated using Vivaspin™ concentrators (10 kDa cut-off), transferred to 50 mM Tris/H_2SO_4 buffer, pH 9.5, and loaded onto the same anion-exchange chromatography column using the same gradient as in the first purification step. The enzyme bound at this pH and eluted at about 120 mM sodium sulfate. Concentrated active fractions were subjected to gel filtration in 50 mM Tris/H_2SO_4, pH 9.0 including 150 mM sodium sulfate at a flow rate of 0.4 ml min^{-1}. Standard high molecular mass proteins (aprotinin, RNase A, carbonic anhydrase, ovalbumin, conalbumin and ferritin) served to calibrate the column. The molecular mass of native SPO3124 was estimated by interpolation.

For purification attempts of SPO3559 (SoeA), soluble fractions were loaded onto the anion exchange column under different purification conditions (between pH 7 and pH 9). To

exclude the possibility that sulfate inhibits sulfite-oxidizing enzyme (SOE), H_2SO_4 was replaced with HCl.

2.4.3. Enzyme assay

Sulfite-oxidizing enzyme (SOE) activity was determined photometrically as described in the literature (Reichenbecher *et al.*, 1999; Denger *et al.*, 2008; Wilson & Kappler, 2009). The standard reaction mixture contained 1 µmol potassium ferricyanide as the electron acceptor and 2 µmol sodium sulfite as substrate in a final volume of 1 ml 50 mM Tris/HCl buffer, pH 8.0. Native cytochrome *c* (bovine, horse, and *Ruegeria pomeroyi* DSS-3) as well as menaquinone, dichlorophenol indophenol (DCPIP), FAD^+, NAD^+ and $NADP^+$ in different concentrations (up to 4 mM) were tested as alternative electron acceptors. It should be noted that sulfite inhibits the enzyme activity due to substrate inhibition. Therefore, the sufite concentration was reduced to 0.4 mM (Denger *et al.*, 2008). The protein concentration in the assay was in the range of 3 (purified enzyme) to 100 µg ml^{-1} (crude cell extracts). All activity determinations were corrected for non-enzymatic sulfite oxidation.

Alternatively, sulfite and sulfate concentrations were quantified discontinuously (chapter 2.5). Therefore, the total volume of the reaction mixture, as described previously, was increased up to 5 ml and samples were taken over a time period of up to 10 min.

The oxygen consumption rate of intact cells was measured in a Clark-type oxygen electrode at 30 °C. The reaction volume was 500 µl (50 mM potassium phosphate buffer, pH 8.0 supplemented with 5 mM $MgCl_2$) with 0.4 mM sulfite as substrate and 0.1 to 1.5 mg protein ml^{-1}.

Kinetic constants for SPO3124 (11 kDa protein) were determined with reaction mixtures containing various sulfite concentrations (up to 30 mM). The pH optimum (between pH 4 and pH 11) for this enzyme was determined in a buffer system with constant ionic strength, composed of *N*-(2-acetamido)-2-aminoethanesulfonate, Tris and ethanolamine (Ellis & Morrison, 1982). To test the influence of iron chelators, purified SPO3124 (3 µg ml^{-1}) was incubated with EDTA (up to 100 mM) or 2,2-bipyridyl (up to 5 mM) at room temperature for 15 min, before the sulfite-oxidizing activity was measured.

In order to determine *β*-galactosidase activity, *Ruegeria pomeroyi* DSS-3 (5 ml) cells

containing pMP220-*lacZ* fusions were grown in basal medium with different carbon sources (succinate, succinate supplemented with sulfite (1 mM), taurine, isethionate, cysteate, sulfoacetaldehyde, each at a concentration of 10 mM) for four days. After harvesting, the cells were permeabilized with chloroform and SDS. *β*-galactosidase was assayed by measuring hydrolysis of the chromogenic substrate *o*-nitrophenyl-ß-D-galactoside (ONPG), according to the procedure of Miller (Miller, 1972). All assays were performed in triplicates.

2.5. Analytical methods

Growth was followed as turbidity ($OD_{580\ nm}$), or quantified as protein in a Lowry-type reaction (Cook & Hütter, 1981) without the initial addition of acid as described in the original Lowry protocol, in order to avoid precipitation of salts from seawater medium. Soluble protein was assayed by protein-dye binding (Bradford, 1976).

Taurine and cysteate were derivatized with dinitrofluorobenzene (DNFB) and quantified by reverse-phase HPLC-UV (Denger *et al.*, 1997; Laue *et al.*, 1997).

Sulfite was quantified as the fuchsin derivative (Denger & Cook, 2001).

Sulfate was determined turbidimetrically as a suspension of $BaSO_4$ (Sörbo, 1987), for which a 1:10 pre-dilution of the sample was necessary to avoid precipitation of other components of the seawater medium. As alternative method for sulfite/sulfate quantification, ion chromatography (IC) with suppression as described elsewhere (Denger *et al.*, 2004) was tested. However, this method was not suitable due to fast sulfite autoxidation under the alkaline conditions of the ion chromatography (5 mM Na_2CO_3, pH 11).

Peptide fingerprinting-mass spectrometry (PF-MS) and Orbitrap™ analyses were done at the Proteomics Facility of the University of Konstanz. PF-MS was used to identify the corresponding genes of individual protein bands (or spots on 2-D gels) and Orbitrap™ analysis was used for a proteomic approach. For Orbitrap™ analysis, 1 mg denaturated protein was separated by SDS-PAGE. After the protein bands were stained (Coomassie-brillant blue R250), they were cut from the gel. The identity of a purified fraction of SPO3124 (m/z = 11245) was also confirmed by MALDI-TOF MS analyses in the negative ion mode (Tholey *et al.*, 2002).

DNA sequencing was conducted at GATC (Konstanz, Germany) using appropriate primers listed in Table 4. The sequences were analyzed using the Lasergene™ package (DNASTAR).

Metal analyses were done by inductively coupled plasma mass spectroscopy (ICP-MS) under contract by the Spurenanalytisches Laboratorium Dr. Baumann (Pirkensee, Maxhütte-Haidhof, Germany). The molecular masses of native, purified proteins were determined by dynamic light scattering (DLS) and calculated using OmniSIZE 3.0 software. DNA was quantified by using NanoDrop technology and analyzed using the ImageJ™ software (http://rsb.info.nih.gov/ij/).

2.6. Bioinformatics

Analyses of the genomes of *Ruegeria pomeroyi* DSS-3 (formerly *Silicibacter pomeroyi* DSS-3) [gene bank accession number CP000031.1 (chromosome); CP000032.1 (megaplasmid)] (Moran *et al.*, 2004), *Roseovarius nubinhibens* ISM [AALY00000000] (González *et al.*, 2003), *Roseovarius* sp. strain 217 [AAMV00000000] (Schäfer *et al.*, 2005), *Cupriavidus necator* H16 (formerly: *Ralstonia eutropha* H16) [NC_008313.1 (chromosome 1, (Pohlmann *et al.*, 2006)), NC_008314.1 (chromosome 2, (Pohlmann *et al.*, 2006)), NC_005241.1 (megaplasmid pHG1, (Schwartz *et al.*, 2003))] CP000031.1 (chromosome); CP000032.1 (megaplasmid)] and of *Sinorhizobium meliloti* 1021 [AL591688.1 (chromosome, (Capela *et al.*, 2001)), AE006469.1 (plasmid pSymA, (Barnett *et al.*, 2001)), AL591985.1 (plasmid pSymB, (Finan *et al.*, 2001a))], were done using the BLAST algorithm (Altschul *et al.*, 1990) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Subroutines from the LASERGENE™ program package (DNASTAR, Madison WI) and the annotation tool Artemis™ (release 11, http://www.sanger.ac.uk/resources/software/artemis/) (Rutherford *et al.*, 2000) were used for handling sequence data (before October 2012) and design of oligonucleotides. Alignments were made using ClustalX™, and phylogenetic trees plotted in NJplot™ (http://pbil.univ-lyon1.fr/software/njplot.html) (Thompson *et al.*, 1997) and edited in Dendroscope™ (http://ab.inf.uni-tuebingen.de/software/dendroscope/) (version 2.7.4) (Huson *et al.*, 2007). Orthologue neighborhood regions were compared with the IMG system of the DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

The following prediction tools were used:

cleavage sites for signal peptides: http://www.cbs.dtu.dk/services/SignalP/,

cysteine disulfide bonds: http://disulfind.dsi.unifi.it/monitor.php?query=otWeRL.

transmembrane helices: http://www.ch.embnet.org/cgi-bin/TMPRED_form_parser,

protein-protein interactions : http://string-db.org/,

ECF-Sigma-factor identification: http://ecf.g2l.bio.uni-goettingen.de:8080/ECFfinder/, and

metal binding sites: http://jing.cz3.nus.edu.sg/cgi-bin/svmprot.cgi.

3. Results and Discussion

3.1. A sulfite-stress inducible 11 kDa protein in *R. pomeroyi* DSS-3

3.1.1. Physiology of growth of *R. pomeroyi* DSS-3 with taurine and cysteate

Ruegeria pomeroyi DSS-3 grew exponentially with taurine as the sole source of carbon and energy with a specific growth rate (μ) of 0.12 h^{-1}. The utilization of taurine was both concomitant with growth and quantitative, as confirmed by the molar growth yield determined, 6.0 g protein (mol C)$^{-1}$ (Cook, 1987). The sulfonate-sulfur was released into the growth medium as sulfate, concomitant with growth and quantitatively, whereas no sulfite was detectable (Fig. 11 A). The specific rate for taurine degradation was calculated at 5.5 mkat (kg protein)$^{-1}$.

Growth with cysteate as sole carbon and energy source was neither exponential nor quantitative. The highest specific growth rate (μ) was 0.036 h^{-1} in an early, rapid growth phase, but this growth rate fell to zero before the limiting substrate cysteate was consumed: utilization of cysteate ceased when about 30% of the substrate was still present, and the molar growth yield for cysteate was 5.0 g protein (mol C)$^{-1}$ at that time (Fig. 11 B). The highest specific degradation rate was 1.9 mkat (kg protein)$^{-1}$ during the rapid growth phase (below 30 μg protein ml^{-1}), while about 80% of the sulfonate-sulfur was excreted as sulfite, and only 20% was oxidized to sulfate, most likely from the autoxidation of sulfite.

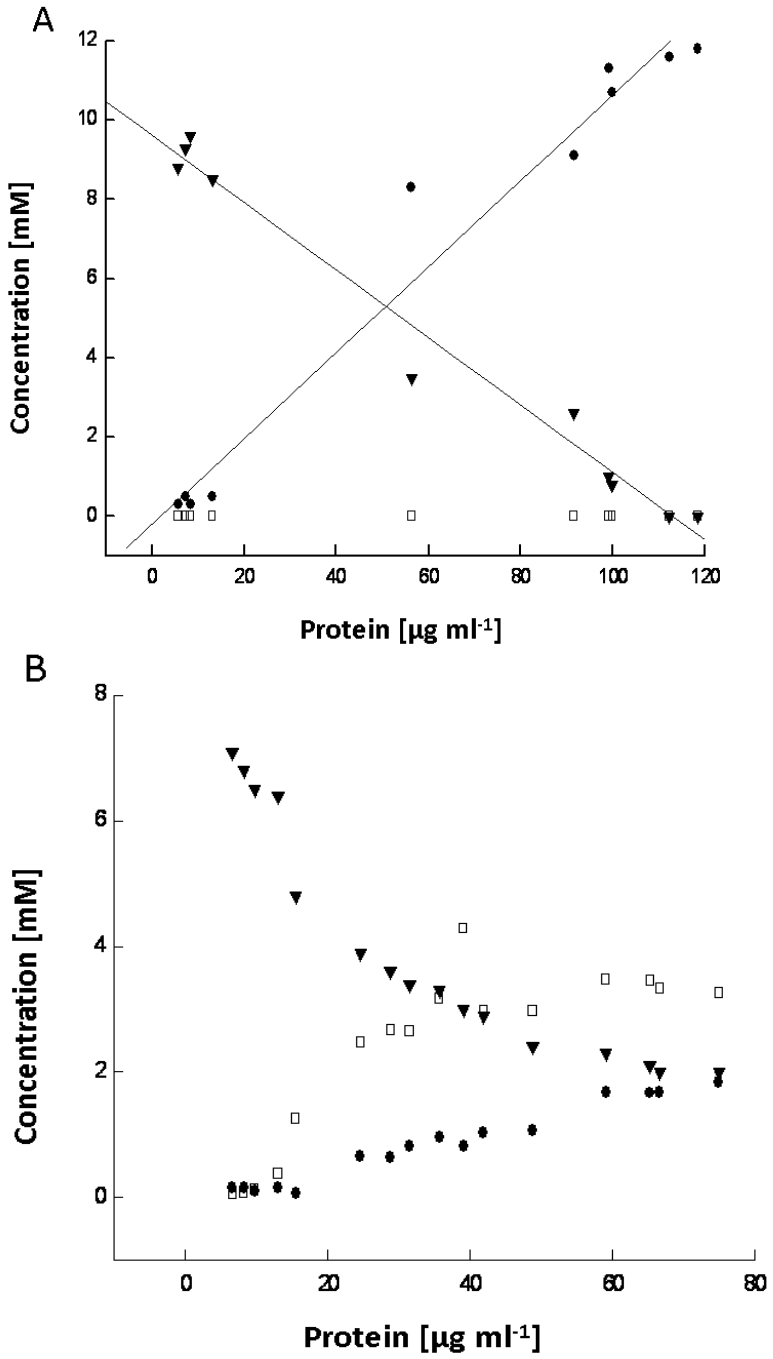


Fig. 11 Concentrations of substrates and products plotted as a function of protein concentration during growth of *R. pomeroyi* with 10 mM taurine (**A**) or 6.7 mM cysteate (**B**). Legend: ▼, taurine / cysteate; •, sulfate; ▫, sulfite.

Hence, it appeared that taurine-growing *R. pomeroyi* DSS-3 cells obviously were able to oxidize sulfite to sulfate, most likely by an active sulfite dehydrogenase, whereas cysteate-growing *R. pomeroyi* DSS-3 cells exhibited no detoxification of sulfite to sulfate, and presumably suffered from sulfite intoxication (chapter 1.1.2.2).

3.1.2. Sulfite dehydrogenase (SDH) activities in *R. pomeroyi* DSS-3

Crude extracts, soluble fractions, and membrane fractions of *R. pomeroyi* DSS-3 grown with taurine and cysteate, and also of cells grown with isethionate (metabolised *via* sulfoacetaldehyde, like taurine, chapter 1.1.2.1, Fig. 2 and Fig. 3) and 2,3-dihydroxypropane-1-sulfonate (Dhps) (metabolised *via* cysteate, chapter 1.1.2.1 Fig. 2 and Fig. 3), were assayed for apparent sulfite dehydrogenase (SDH) activities. Extracts of acetate-grown cells were used as a presumed negative control, since these cells express no taurine-degrading enzymes (Gorzynska *et al.*, 2006). SDH was assayed as sulfite:ferricyanide oxidoreductase activity (Reichenbecher *et al.*, 1999) and the obtained results are summarized in Fig. 12.

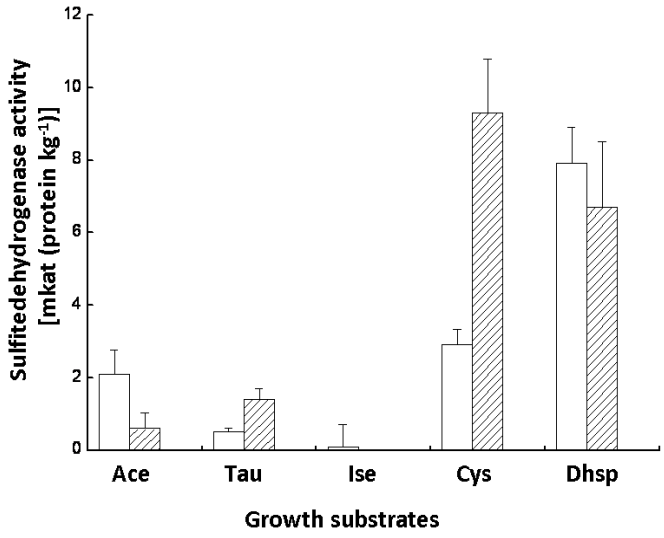


Fig. 12 Specific activities of apparent sulfite dehydrogenase when assayed as sulfite: ferricyanide oxidoreductase activity in cell-free extracts of *R. pomeroyi* DSS-3 grown with different substrates. Legend: Ace, acetate; Tau, taurine; Ise, isethionate; Cys, cysteate; Dhps, 2,3-dihydroxypropane-1-sulfonate; clear box, crude extract; stripped box, soluble fraction

No significant activity was detectable in the membrane fractions (not shown) and no significant differences were observed in extracts of acetate- , taurine-, or isethionate-grown cells: the observed sulfite dehydrogenase activity, if present, was constitutive and, at about 1 mkat (kg protein)$^{-1}$, too low to account for sulfite oxidation in taurine-grown cells *in vivo* (5.5 mkat (kg protein)$^{-1}$) (Fig. 11 A).

Interestingly, high sulfite dehydrogenase activity was detectable (up to 6 mkat (kg protein)$^{-1}$) in soluble fractions of cysteate- or Dhps-grown cells, although such cells obviously were unable to oxidize sulfite *in vivo* (Fig. 11 B).

Therefore, the apparent SDH activity in cysteate-grown cells was investigated in more detail.

3.1.3. Purification and identification of an 11 kDa protein with SDH activity in cysteate-grown cells

Sulfite deydrogenase (SDH) was enriched from cysteate-grown cells which exhibited the highest enzyme activity (Fig. 12). The rise in specific activity on removal of the particulate (membrane) material was confirmed on the larger scale (Table 6). The major steps in the purification were a first anion-exchange column; further purification was achieved by a second passage through an anion exchanger, and by gel filtration.

Table 6. Purification of the 11 kDa protein from *Ruegeria pomeroyi* DSS-3.

Purification step	Total protein [mg]	Total activity [nkat]	Recovery [%]	Specific activity [mkat (protein kg^{-1})]	Purification [-fold]
Crude extract	70.0	163.0	100	2.3	1.0
Soluble fraction	37.9	137.0	84	3.6	1.6
Anion exchanger 1	0.8	68.0	42	86.7	37.7
Anion exchanger 2	0.4	42.0	26	114.2	49.7
Gel filtration	0.3	5.6	3	179.6	78.1

After separation by SDS-PAGE (Fig. 13 A) and Tris-SDS-PAGE (Fig. 13 B) the purified enzyme had an apparent molecular mass of 11 kDa, which was confirmed by MALDI-TOF-MS (Matrix-Assisted-Laser-Desorption/Ionization-Time-Of-Flight-Mass-Spectrometry) analysis.

The native enzyme eluting from the calibrated gel filtration column had a molecular mass of approximately 32 kDa; the value determined with dynamic light scattering (DLS) was 33 kDa. These data suggest that the enzyme was a homotrimer.

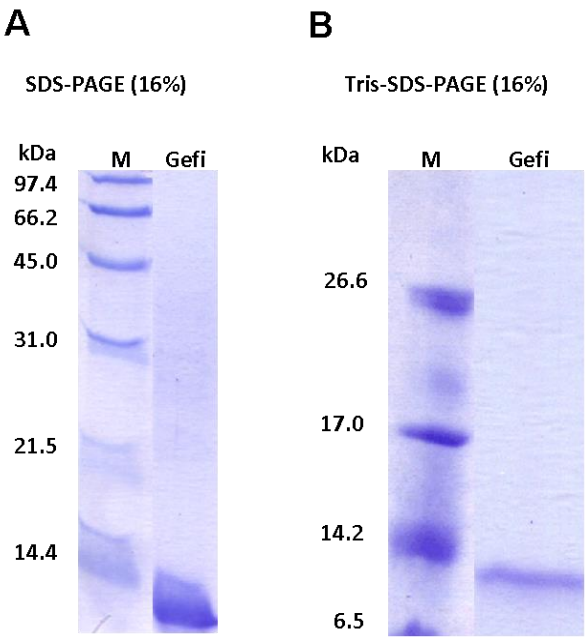


Fig. 13 SDS-PAGE (**A**) and Tris-SDS-PAGE (**B**) analysis of the fraction with the highest specific SDH activity in *R. pomeroyi* DSS-3. The single protein band observed at approximately 11 kDa was cut out of the gel and submitted to peptide fingerprinting-mass spectrometry. Legend: M, molecular mass marker; Gefi, fraction from gel filtration.

Peptide fingerprinting-mass spectrometry (PF-MS) of the purified enzyme identified locus tag (gene) SPO3124 (score, 500; sequence coverage, 35%) in the genome of *R. pomeroyi* DSS-3, which was annotated as 'hypothetical protein' (see below), and whose product had a predicted molecular mass of 14.9 kDa. Prediction software (SignalP) indicated a cleavage site for a leader peptide between residues 37 and 38. Hence, the mature monomer would most likely be a soluble protein with a molecular mass of 11 kDa that is located in the periplasm. These predictions are consistent with the experimental data.

3.1.4. Biochemical characterization of the 11 kDa protein

The highest specific sulfite dehydrogenase (SDH) activity was obtained in cysteate-grown *Ruegeria pomeroyi* DSS-3 cells, although these cells presumably suffered from sulfite intoxication. However it turned out that the responsible protein is an 11 kDa protein. To investigate this enzyme activity in more detail, sulfite and sulfate concentrations were discontinuously determined (Fig. 14). Sulfite disappearance concomitant with sulfate formation was detectable after the addition of a purified fraction of the 11 kDa protein (black triangle); whereas no sulfite oxidation was detectable when a boiled (2 h, 99 °C) fraction of the 11 kDa protein (inactive enzyme, round circle) was tested, or in the absence of any electron acceptor (rectangle), assuming that dioxygen was not used as final electron acceptor.

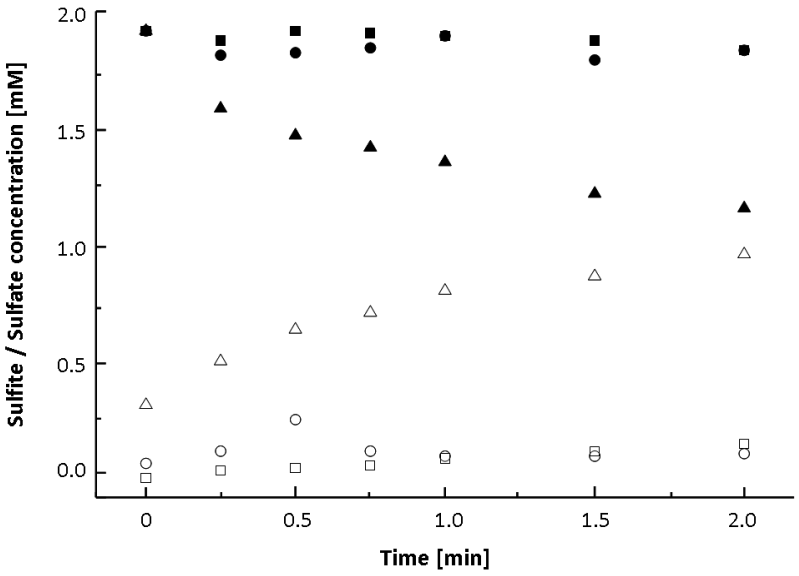


Fig. 14. Sulfite (black symbols) and sulfate (white symbols) determination of a reaction mixture containing 2 mM sulfite and 1 mM ferricyanide in an appropriate volume. Samples were taken at intervals within two minutes.
Legend: ▵, purified 11 kDa protein; ▫, inactive 11 kDa protein; ○, no additional electron acceptor.

Low sulfite-oxidizing activity (0.4% of the activity with ferricyanide) was obtained when bovine cytochrome *c* (cyt. *c*) was tested as electron acceptor; a native cyt. *c* was co-purified and also tested as electron acceptor for the 11 kDa protein, but no reaction was observed (data not shown). Also a substitution of ferricyanide with a membrane fraction of *R. pomeroyi* DSS-3 showed no sulfite oxidation.

The purified colourless 11 kDa protein had an apparent $K_M^{sulfite}$ value of 9.7±1.8 mM. Further, the protein was analyzed for bound metals by inductively-coupled-plasma mass-spectrometry (ICP-MS), which indicated 0.3 mol iron per mol of protein (error probability: ± 25%); a prediction software for metal-binding in the protein sequence confirmed these findings (76% probability for iron-binding site). However, the addition of different iron-chelators (EDTA, 2,2-bipyridyl) did not affect the activity.

The pH and temperature optima for the 11 kDa protein were determined at pH 8.0 and at 35 °C. The buffer composition (KP or Tris buffer) did not affect the enzyme activity. Furthermore, several substrate analogues for sulfite (nitrite and phosphite: 0.02-20 mM, thiosulfate: 0.02-2 mM) were tested, but none of these gave a reaction. Thus, the 11 kDa protein is insensitive against buffer compositions and ionic strength, and specific for sulfite oxidation.

3.1.5. Characterization of the 11 kDa protein (SPO3124) gene cluster

The corresponding gene (SPO3124) of the 11 kDa protein is located in a presumed three-gene operon with genes annotated to encode a RNA polymerase σ-factor of the ECF-family (SPO3125) and an anti σ-factor (SPO3126) (Fig. 15).

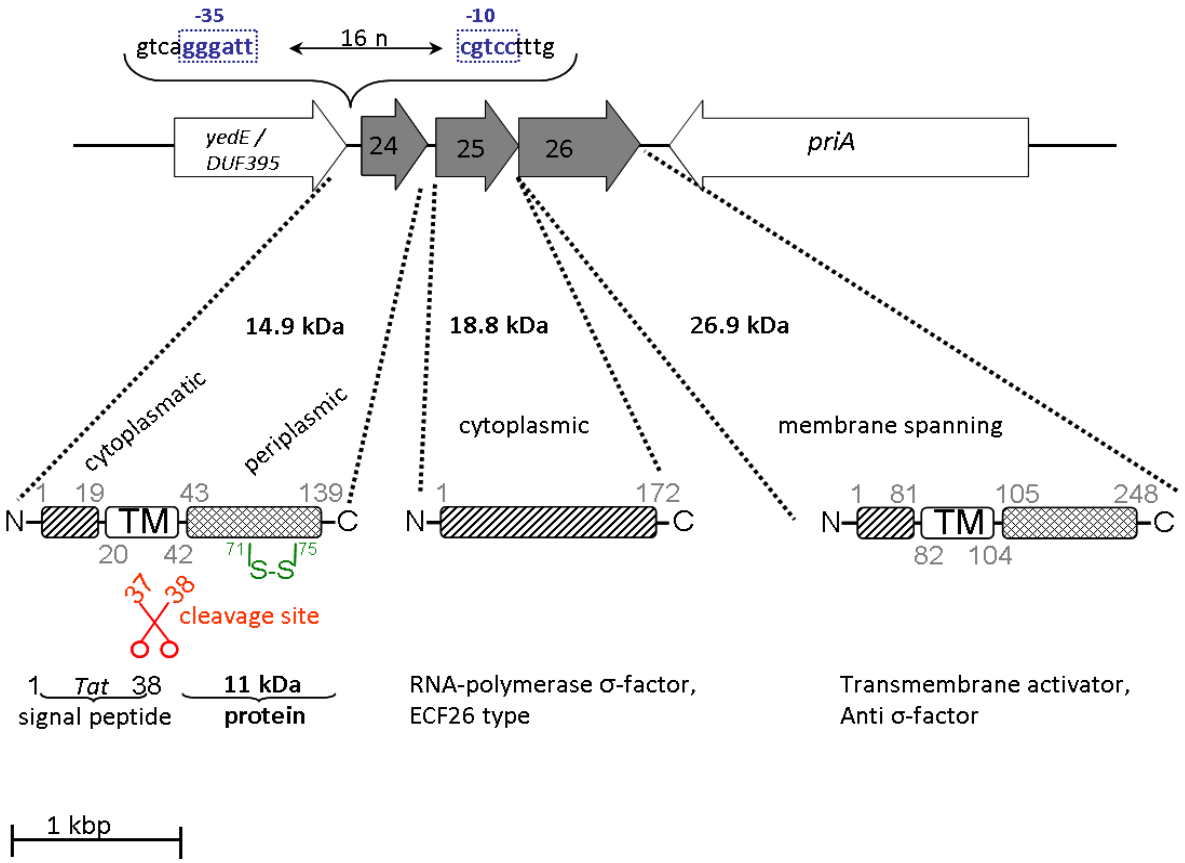


Fig. 15 Three-gene cluster encoding the 11 kDa protein (SPO3124). TM, transmembrane helix; n, nucleotides. Legend: white arrows, surrounding neighbourhood genes; grey arrows, three-gene cluster: SPO3124-SPO3126 abbreviated as 24-26; striped box, cytoplasmatic protein domain; chequered box, periplasmic protein domain.

The SPO3124 protein sequence exhibited a 'secreted repeat of unknown function' domain (pfam03640, lipoprotein-15), which is widespread in bacterial genomes (717 sequences, mainly *Actinomycetales* and Proteobacteria) as either single gene or as part of a three-gene cluster. The sequence exhibits a *TAT* signal peptide for secretion of the folded protein across the cytoplasmatic membrane, and an N-terminal transmembrane helix, each with a predicted cleavage site between amino acid residues 37 and 38, which would result in a

periplasmic 11 kDa protein. Notably, a prediction tool for cysteine-disulfide bonds revealed a disulfide bond between amino acid residues 71 and 75.

The cytoplasmatic RNA polymerase σ-factor (SPO3125) is presumed to belong to ECF σ-factor subgroup 26. The stopcodon of this σ-factor overlaps with the start codon of SPO3126 for PrtR-type σ-factor regulator. This latter gene encodes a single transmembrane spanning protein (between amino acid residues 82 and 104) with an N-terminal cytoplasmatic and a C-terminal periplasmic region. Furthermore, a conserved sequence motif of the -35 and –10 region, which has previously been attributed as potential regulator sequence for ECF σ-factors in other bacteria (Staroń *et al.*, 2009), was also found for the SPO3124 gene cluster.

3.1.6. Regulation of the SPO3124 gene cluster

Reverse transcription-PCR (RT-PCR) was used to test the transcription of the three-gene cluster SPO3124-26 in cysteate-, taurine-, and acetate-grown cells (Fig. 16). The results indicated a basal transcription of the SPO3124 gene for taurine- and acetate-grown cells, and a high transcription in cysteate-grown cells. The transcription of the regulator segments (SPO3125, SPO3126) appeared to be induced in cysteate-grown cells (Fig. 16 A). No transcription was detectable for two neighbouring genes, *yedE* and *priA* (Fig. 15), under any growth condition tested (data not shown).

Furthermore, the protein expression pattern in cysteate- and taurine-grown cells was compared by 2-D PAGE (Fig. 16 B), which indicated an inducible 11 kDa protein in cysteate-grown cells. The protein spot was cut out and confirmed by PF-MS to represent SPO3124 (11 kDa protein).

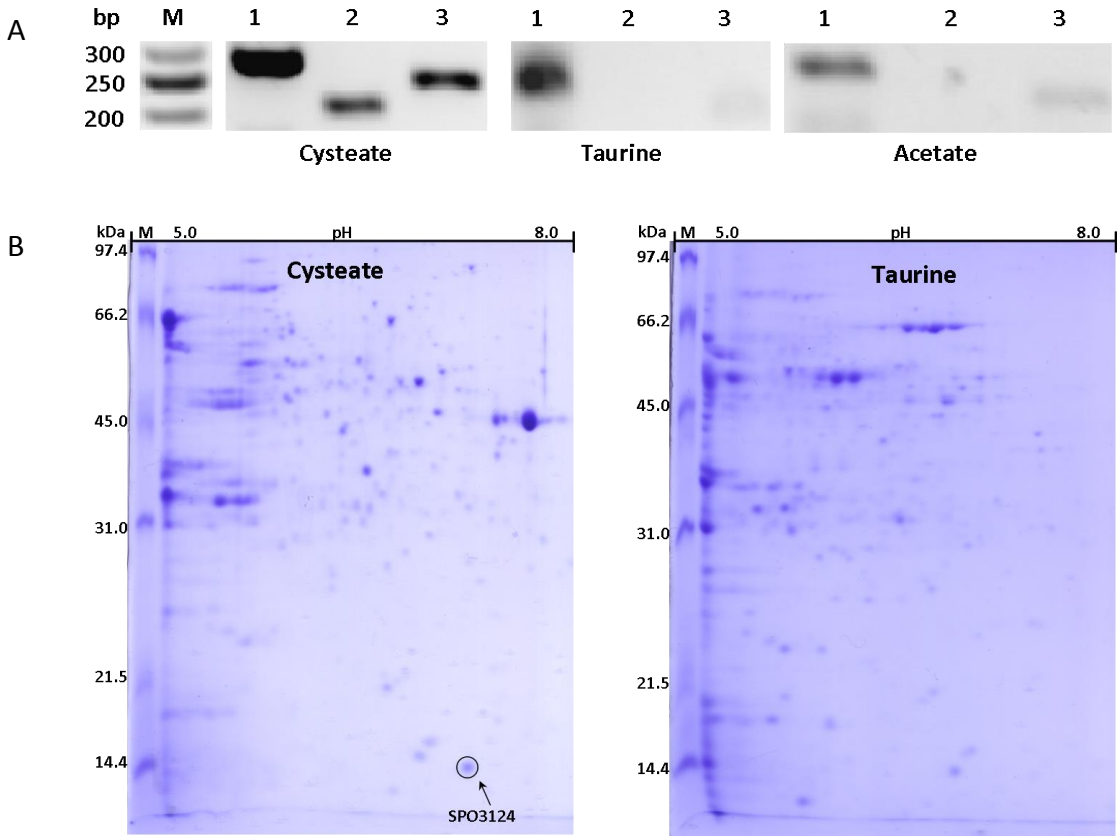


Fig. 16 Reverse transcription (RT) PCR experiments for genes SPO3124 (lane 1, 284 bp), SPO3125 (lane 2, 208 bp), and SPO3126 (lane 3, 245 bp) when testing cysteate-, taurine- and acetate-grown cells (**A**), and 2-D PAGE gels of soluble fractions of taurine- and cysteate-grown cells (**B**). PF-MS identified SPO3124 (marked with ○). M, molecular size/mass markers.

Hence, the results indicated that the three-gene SPO3124-26 cluster is inducibly transcribed in cysteate-grown cells. This observation led to the hypothesis that the transcription in cysteate-grown cells is upregulated in response to sulfite stress in *R. pomeroyi* DSS-3. When RT-PCR was done before and after the addition of 0.5 mM sulfite to acetate-grown cells, the transcription pattern observed indicated a strong induction of all three genes comparable to cysteate-grown cells (Fig. 17).

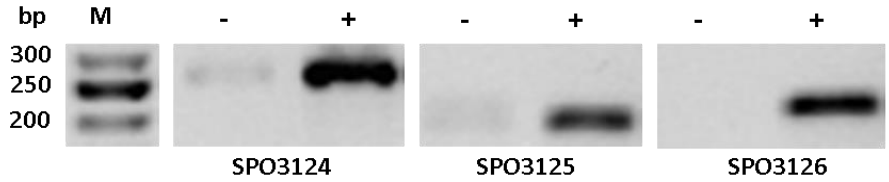


Fig. 17. Effect of sulfite (0.5 mM) on the transcription of the SPO3124-26 gene cluster in *R. pomeroyi* DSS-3 cells. Legend: -, acetate as growth substrate; +, acetate and sulfite treated cells. M, 1 kbp marker.

In order to confirm that sulfite acts as an inducer of the SPO3124-26 gene cluster, a transcriptional *SPO3124-lacZ* fusion plasmid was created and transferred into *R. pomeroyi* DSS-3. The highest β-galactosidase activity was observed in sulfite-treated succinate-grown cells (21.5-fold induction) and in cysteate-grown cells (11-fold induction) compared to only basal activities detectable for taurine- and succinate-grown cells (Fig. 18).

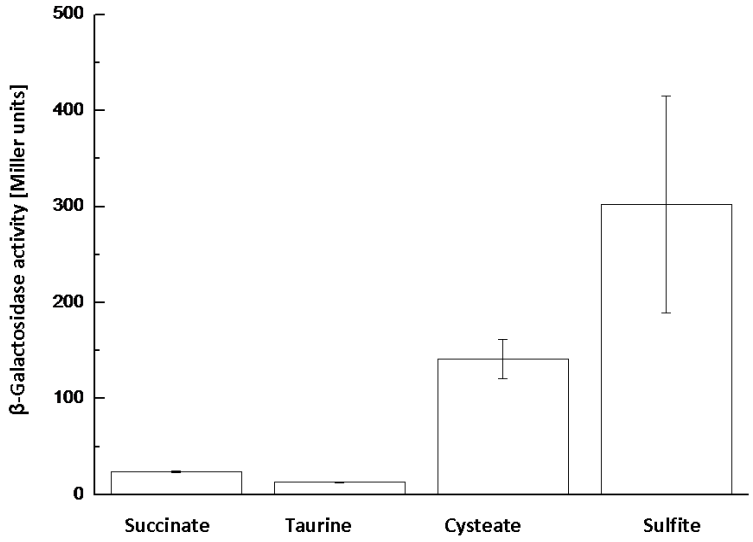


Fig. 18 Regulator studies for the three-gene operon (SPO3124-26) in *R. pomeroyi* DSS-3 harbouring a *lacZ*-fusion plasmid with a potential regulator sequence for SPO3124-26. Cells were grown with succinate, taurine, cysteate or succinate/sulfite.

3.1.7. The effect of sulfite on growth of *Ruegeria pomeroyi* DSS-3

It has been known for a long time that sulfite derivatives (e.g. bisulfite, hydrosulfite) can act as bacteriostatic compounds (chapter 1.1.2.2; e. g. Fischer *et al.*, 1946). This gave rise to the assumption that also the limited growth of *Ruegeria pomeroyi* DSS-3 with cysteate is due to the sulfite excretion (Fig. 11). Following this, the influence of sulfite on the growth of *R. pomeroyi* DSS-3 with taurine was tested. Fig. 19 shows that the growth was normal up to a concentration of approximately 3.5 mM sulfite, but that the presence of more than 4.0 mM sulfite completely prevented growth entirely.

Fig. 19. Effect of increasing concentrations of sodium-sulfite (0-10 mM) on the growth of *R. pomeroyi* DSS-3 with 10 mM taurine.

These results were confirmed when different cysteate concentrations (up to 10 mM) were used as growth substrate for *R. pomeroyi* DSS-3 (Fig. 20). Up to a concentration of 4 mM cysteate a quantitative consumption was observed, since the sulfonate moiety is excreted as sulfite into the growth medium.

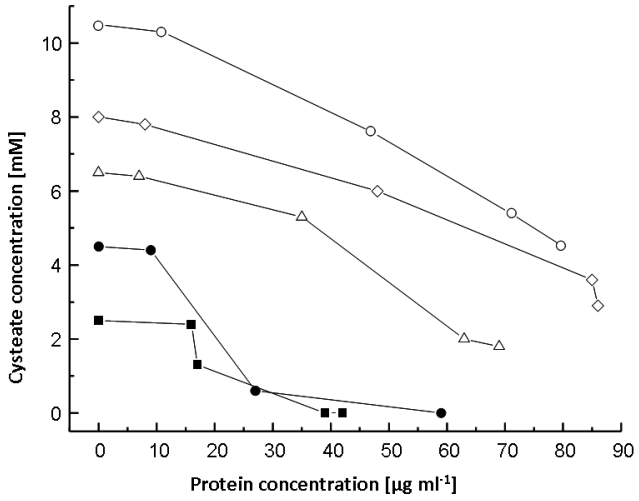


Fig. 20 Growth of *R. pomeroyi* DSS-3 with different cysteate concentrations as growth substrate. Growth was quantified as protein concentration. Legend: ○, 10 mM; ◊, 8 mM; Δ, 6 mM, ●, 4mM; ▪, 2 mM cysteate.

3.1.8. Discussion

Taurine-grown cells obviously were able to oxidize sulfite to sulfate *in vivo* (Fig. 11), but this sulfite dehydrogenase (SDH) activity was negligible *in vitro* when the 'traditional' enzyme assay (measured as sulfite:ferricyanide oxidoreductase) for known SDHs, like SorA and SorT, was used (Fig. 12) (Reichenbecher *et al.*, 1999, Wilson & Kappler, 2009). Therefore, it is reasonable to assume that a yet unknown, third type of sulfite dehydrogenase exists in *Ruegeria pomeroyi* DSS-3, which obviously cannot be measured under the established assay conditions, presumably due to different cofactors or co-substrate requirements (e. g. a yet undefined electron acceptor). In addition, it is conceivable that the enzyme was completely inactivated upon preparation of cell extracts: on the one hand it would be possible that the enzyme is oxygen-labile, but also that the enzyme consists of different subunits like SorAB (Kappler *et al.*, 2000), or even that it is part of a whole enzyme complex like the TOMES (thiosulfate oxidizing multi-enzyme system) complex of *Paracoccus* species (Friedrich *et al.*, 2008).

In contrast, cysteate- and Dhps-grown cells exhibited high apparent SDH activities when measured as a sulfite:ferricyanide oxidoreductase *in vitro* (Fig. 12), but these cells excreted sulfite and not sulfate into the medium (Fig. 11). That the Dhps-grown cells showed similarly high SDH activities as cysteate-grown cells was not surprising, since Dhps is very likely degraded *via* sulfolactate in *R. pomeroyi* DSS-3 (Mayer et al., 2010), and the sulfolactate degradation pathway proceeds *via* cysteate (Denger *et al.*, 2009).

The sulfite:ferricyanide oxidoreductase activity detectable in cysteate-grown cells was purified to homogeneity and identified by PF-MS (Fig. 13). The identified gene, SPO3124, is part of a three-gene cluster comprising ECF σ-factor gene (SPO3125) and its regulator gene (SPO3126) (Fig. 15). RT-PCR experiments and SPO3124-*lacZ*-fusions have shown that this gene cluster is strongly induced in cysteate-grown *R. pomeroyi* DSS-3 cells which is a response of sulfite (Fig. 17 and Fig. 18).

ECF σ-factors play an essential role in signal transduction from the bacterial cell envelope to the cytoplasm, while other known functions of ECF σ-factors are the detection of envelope stress (e. g. unfolded proteins, thio/disulfide stress) (Missiakas *et al.*, 1997; Huang *et al.*, 1999a; Thompson *et al.*, 2007) as well as of other adverse environmental conditions (e. g. high temperatures) (Wösten, 1998). Importantly, a classification tool for ECF-σ-factors attributed SPO3125 as a member of the ECF subfamily 26, a subclass which has previously

been described to contain also the ECF-σ-factors that regulate the transcription of SorAB in *Starkeya novella* and SorT in *Sinorhizobium meliloti* (Kappler *et al.*, 2000; Staroń *et al.*, 2009; Kappler *et al.*, 2012). SPO3126, is a PrtR-type anti-σ-factor. Anti-σ-factors of these types are transmembrane activators, rather than anti-σ-factors (Burger *et al.*, 2000).

The gene product of SPO3124 is a soluble, homotrimer (three monomers of 11 kDa) located in the periplasm. This protein was responsible for high specific apparent sulfite dehydrogenase activities which were obtained by the enzyme assay of Reichenbecher and colleagues (1999). The purified enzyme clearly catalyzed sulfite oxidation, which was confirmed by an increasing concentration of sulfate with concomitant decrease in sulfite concentration (Fig. 14). However, the apparent $k_M^{sulfite}$ value for the 11 kDa protein was in the millimolar range (9.7 mM), which is up to 1000-fold higher compared to known sulfite dehydrogenases, whose $k_M^{sulfite}$ values are between 16 and 100 µM (Kappler *et al.*, 2000; Cook *et al.*, 2008; Wilson & Kappler, 2009). This might suggest that sulfite is not the natural substrate for the enzyme, or that ferricyanide substituted only poorly for the natural electron acceptor.

A protein-protein interaction database search indicated an interaction, in terms of co-occurrence, score: 0.426, of SPO3124 with a protein (SPO2536) annotated to encode a transcriptional response regulator containing a CheY-like receiver domain and a C-terminal effector domain acting as a two-component response regulator. Therefore, the question arises if the 11 kDa protein also plays a role in bacterial signal transduction? A phylogenetic tree was generated, whereas the 11 kDa protein sequence (SPO3124) from *R. pomeroyi* DSS-3 and other homologues mainly originating from Proteobacteria and Actinobacteria were aligned with sequences of representative sensor proteins acting in the one- and two-component regulatory systems (Fig. 21). The 11 kDa sequence appeared as a member of a deep-branching clade of sequences within the class of sensor proteins of one- and two-component regulatory systems.

Hence, it is tempting to speculate whether the 11 kDa protein SPO3124, which is not effective as sulfite dehydrogenase *in vivo*, acts as a periplasmic sensory protein for an ECF-type signal transduction through the cytoplasmatic membrane, e. g. for sensing of sulfite stress or of other environmental stress, and that the apparent SDH activity measurable for this protein is a reflection of its ‘sensory domain’.

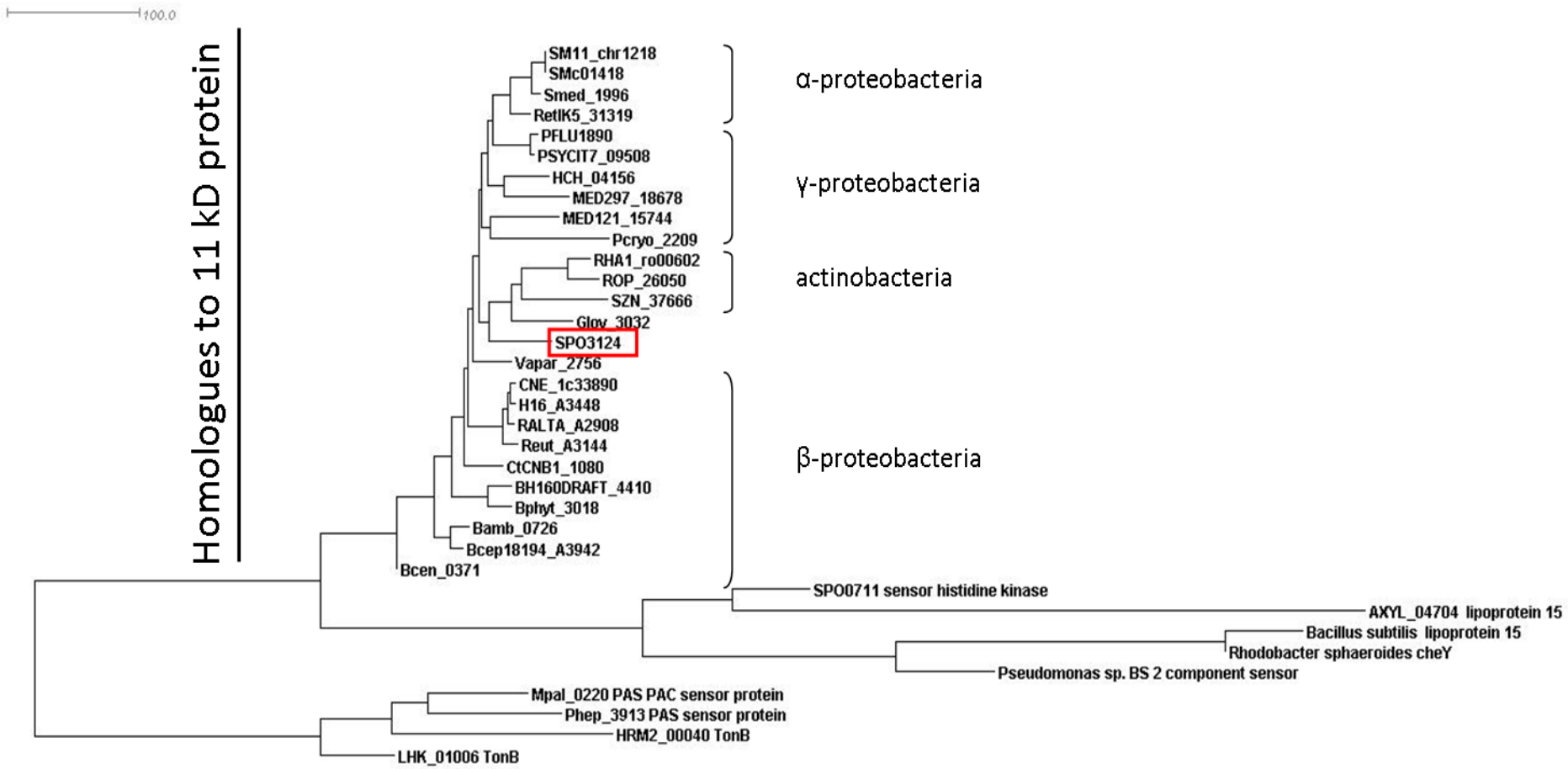


Fig. 21 Phylogenetic analysis of SPO3124 from *Ruegeria pomeroyi* DSS-3, its homologues in other Proteobacteria and Actinobacteria, and of other known sensor proteins of the one- and two-component regulatory systems. Red rectangle shows the investigated 11 kDa protein in *R. pomeroyi* DSS-3.

3.2. The missing majority of sulfite-oxidizing enzymes (SOEs) in sulfonate-degrading bacteria

As mentioned in chapter 1.3.3.2, two bacterial types of sulfite dehydrogenases (SDHs) have been characterized to date: SorA and SorT. Among all genome-sequenced bacteria which are known to utilize and desulfonate organosulfonates for growth, only about 50% encode candidate gene(s) for *sorA* and/or *sorT* (Table 9). Interestingly, some desulfonating bacteria which do not possess *sorA* or *sorT* do in fact excrete sulfate into the medium, as it was shown in *Ruegeria pomeroyi* DSS-3 during growth with taurine (chapter 3.1.1). Therefore, it can be inferred that a yet completely unknown type of SOE must be present in those bacteria.

3.2.1. Genomic and proteomic approaches to identify new genes and proteins involved in sulfite oxidation

Previous studies have shown that genes involved in organosulfonate degradation are inducibly transcribed in bacteria (e. g. González *et al.*, 2003; Gorzynska *et al.*, 2006; Baldock *et al.*, 2007). Therefore, for the following experiments extracts of taurine-grown cells were compared with extracts of acetate-grown cells – i. e., comparing the effect of a non-sulfonated *vs*. a sulfonated substrate.

As described in chapter 1.2.3, one possibility to oxidize sulfite other than by using SOEs would be the indirect sulfite oxidation pathway *via* adenosine-5'-phospho-sulfate (APS'). In order to exclude that the APS' pathway is involved in taurine-degradation in *R. pomeroyi* DSS-3 cells, up to 1 mM AMP were added to the traditional standard enzyme assay for sulfite dehydrogenases (Reichenbecher *et al.*, 1999). However, the addition of AMP did not stimulate the enzyme reaction and it is unlikely that this pathway is involved in sulfite detoxification in *Ruegeria* pomeroyi DSS-3 during growth with taurine. These findings were confirmed by transcriptional analyses (RT-PCR experiments) with *R. pomeroyi* DSS-3 and *Roseovarius* sp. strain 217, in which the PAPS reductase gene was not induced under any growth conditions (data not shown).

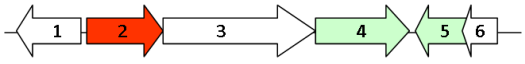
Furthermore, differential 2-D PAGE was conducted (one set of gels is shown in Fig. 16 B), and proteins which seemed to be induced in the soluble fraction (SF) of taurine-grown (compared to SF of cysteate- and acetate-grown) *Ruegeria* pomeroyi DSS-3 cells were excised from the gels and identified by PF-MS. Genes which had been singled out as potentially being involved in sulfite oxidation due to their sequence homologies were tested by RT-PCR. The results are summarized in Table 7.

Table 7 Genes (locus tags) of *R. pomeroyi* DSS-3, found to be taurine-induced in 2-D PAGE and tested in RT (reverse transcription)-PCR experiments. n. d., not detected; tau, taurine.

Locus Tag (SPO)	Annotation	Induction	
		2-D PAGE	RT-PCR
0719, 0720	EtfA/B	tau	tau
2340	SodFe	tau	n. d.
A0168	FAD-binding oxidoreductase	See text & Fig. 22	n. d.
A0169	MOSC domain containing protein	See text & Fig. 22	n. d.

The electron transfer flavoproteins A/B (EtfA/B) were detected at both gene and protein level in taurine-grown cells. No transcription was observed for *sodFe*, although it was detected by 2-D gel electrophoresis.

The genes (locus tags) SPOA0168 and SPOA0169 are located on the megaplasmid of *R. pomeroyi* DSS-3. Their protein products were not directly detected by 2-D PAGE, but gene SPOA0166 (taurine-induced). The corresponding gene cluster is shown in Fig. 22. From this it appears that SPOA0166 (red arrow) is located in the same gene cluster together with an FAD-binding oxidoreductase (SPOA0168, green arrow) and an MOSC (Moco sulfurase C-terminal domain) domain-containing protein (SPOA0169, green arrow). However, none of those genes were inducible transcribed.



No.	Locus Tag (SPOA)	Annotation
1	0165	Transcriptional regulator, LysR-type
2	0166	TRAP transporter soluble receptor, TAXI family
3	0167	TRAP transporter, fusion protein
4	0168	Oxidoreductase, FAD-binding
5	0169	MOSC domain protein
6	0170	Transcriptional regulator, MarR-type

Fig. 22 Potential gene cluster of SPOA0166, which was found inducibly translated in taurine-grown *R. pomeroyi* DSS-3 cells *via* 2-D gel electrophoresis. Genes were tested for induction by RT-PCR.

Orbitrap analyses of total proteins of crude extracts of taurine- and acetate- (as negative control) grown *Ruegeria pomeroyi* DSS-3, *Roseovarius* sp. strain 217 and *Roseovarius nubinhibens* ISM cells were conducted in order to identify further, potentially inducibly translated proteins. However, no evidence for a taurine-inducible SOE were obtained, with only one exception: SoeA. This protein is presumably part of an enzyme complex: SoeABC (**s**ulfite-**o**xidizing **e**nzyme complex ABC) which might represent a novel, third type of SOE. SoeA, a molybdenum-binding oxidoreductase, was found to be inducibly expressed in all three bacteria during growth with taurine. This protein and the corresponding gene cluster (*soeABC*) were investigated in more detail and the obtained results are described in chapter 3.3. The following sub-chapters describe attempts to detect and measure this novel enzyme activity in various bacteria.

3.2.2. Attempts to measure SOE activities in three different bacteria

In order to explore this unknown type of SOE, three different bacterial strains were used as model bacteria: *Ruegeria pomeroyi* DSS-3, *Roseovarius* sp. strain 217, and *Roseovarius nubinhibens* ISM.

Neither *R*. *pomeroyi* DSS-3 nor *Roseovarius* sp. strain 217 exhibit any sequence homologies to *sorA* or *sorT*, instead, the enzymes encoded by both bacteria show sequence homologies to *soeABC* and utilize various organosulfonates *via* known degradation pathways while excreting the sulfonate-moiety as sulfate (with the exception cysteate degradation in *R*. *pomeroyi* DSS-3, chapter 3.1.1). Notably, the genome sequence of *R. pomeroyi* DSS-3 has been published (Moran *et al.*, 2004) and the genome sequence of *Roseovarius* sp. strain 217 is available in the draft version (González *et al.*, 2003; Schäfer *et al.*, 2005).

In contrast, *Roseovarius nubinhibens* ISM exhibit sequence homologies to *sorT* which was found to be inducibly expressed in the soluble fraction of homotaurine-grown cells using 2-D gels (Mayer *et al.*, submission pending). The highly interesting but also confusing point is that two homotaurine-induced SorTs were found, however no enzyme activity could be measured using the traditional enzyme assay with any sulfonate (Denger *et al.*, 2009). The potential novel SDH, SoeABC, was also found in the genome sequence of this bacterium, which is available in the draft version (González *et al.*, 2003).

3.2.2.1. *Roseovarius* sp. strain 217

The marine α-Proteobacterium *Roseovarius* sp. strain 217 is able to utilize *N*-acetyltaurine, *N*-methyltaurine, taurine, isethionate, cysteate and homotaurine as sole carbon and energy source, with the sulfonate-moiety of each of these being quantitatively excreted in the form of sulfate (example for degradation pathway: Baldock *et al.*, 2007) (data not shown). The apparent specific SDH activities measured in cell extracts prepared of cells grown with taurine and cysteate are shown in Table 8, whereas cell extracts of malate-grown cells were used as negative control. The enzyme activity was determined using the traditional enzyme assay described by Reichenbecher and colleagues (in 1999, chapter 2.4.3).

Table 8 Specific sulfite dehydrogenase (SDH) activities measured in different fractions of *Roseovarius* sp. strain 217 cells in dependence of the growth substrate. b. d. l., below detection limit.

	Specific SDH activity [mkat (kg protein^{-1})]		
	Substrate		
Fraction	**Taurine**	**Cysteate**	**Malate**
Crude extract	1.9	0.5	0.5
Soluble fraction	0.1	b. d. l.	b. d. l.
Membrane fraction	4.4	b. d. l.	b. d. l.

The highest SDH activity (3.8-fold higher compared to cysteate- or malate-grown cells) was found in the membrane fraction of taurine-grown cells. Interestingly, no enzyme activity was measured in cysteate-grown cells, although these cells were obviously capable of sulfite oxidation since the sulfonate moiety is excreted as sulfate. Thus, the SDH appeared to be associated to the membrane. However, in all attempts to solubilize the enzyme from the membrane fraction, e. g. by addition of various solubilizing agents (chapter 2.4.1) or by up to 8 passages through a chilled French pressure cell, the activity was lost completely (data not shown).

3.2.2.2. *Roseovarius* nubinhibens ISM

Roseovarius nubinhibens ISM is able to quantitatively degrade sulfolactate, cysteate, taurine, isethionate, sulfoacetate, homotaurine and *N*-methyltaurine, with each of these substrates resulting in the excretion of sulfate into the medium (example for degradation pathways: (Denger *et al.*, 2009; Mayer *et al.*, submission pending) (data not shown). As already mentioned above, it was shown that the specific SDH activities detectable in crude extracts are either below the detection limit (homotaurine) or below 0.5 mkat (kg protein^{-1}) (sulfolactate, 0.4 mkat (kg protein^{-1}); taurine, 0.4 mkat (kg protein^{-1}); cysteate, 0.3 mkat (kg protein^{-1})). The value for the presumed negative control (acetate-grown cells) was in a similar range: 0.2 mkat (kg protein^{-1}) indicating that the SDH cannot be measured with the traditional enzyme assay (Denger *et al.*, 2009). However, two inducible SorT-type SDHs were found in the soluble fractions of homotaurine-grown cells *via* 2-D gel electrophoresis (Mayer *et al.*, submission pending). Additionally, *R. nubinhibens* ISM show sequence homologies to SoeABC.

Since the highest SDH activity was found in taurine-grown cells, these were used to enrich the activity. However, 57% of the specific activity was lost after fractionation of crude extract into a soluble fraction and no activity was found after the first purification step of soluble fraction through anion exchange chromatography (data not shown).

3.2.2.3. *Ruegeria pomeroyi* DSS-3

The main work of this thesis was done with the marine α-Proteobacterium *Ruegeria pomeroyi* DSS-3 which utilizes taurine and cysteate (growth curves in chapter 3.1.1), isethionate, 2,3-dihydroxypropane-1-sulfonate (Dhps), sulfolactate, *N*-methyltaurine, hypotaurine and tauropine. Depending on the sulfonate utilized, the bacterium excretes either sulfite (with Dhps or cysteate as growth substrate) or sulfate (with any other sulfonate) into the medium. The apparent SDH activities detectable are described in chapter 3.1.2, with the highest enzyme activities observed in the soluble fractions of cysteate- and Dhps-grown cells. Under these growth conditions an 11 kDa protein was purified to homogeneity that apparently is induced in response to sulfite stress (see chapter 3.1.6). However, taurine-grown cells excreted sulfate into the medium, presumably due to SOE activity. The specific enzyme activity was only about 1 mkat (kg protein^{-1}), therefore, an

attempt to enrich this activity was made. However, the specific enzyme activity disappeared completely after the first purification step (anion exchange column) indicating that the enzyme is either unstable (e. g. sensitive against atmospheric oxygen), that ferricyanide ($K_3Fe[CN]_6$) is not suitable to replace the natural electron acceptor, or that the enzyme is part of an enzyme complex, as it would be in the case of SoeABC which was destroyed during the purification process.

3.2.3. Methods to measure specific sulfite dehydrogenase activity

3.2.3.1. Traditional enzyme assay for classical sulfite dehydrogenases

Based on the enzyme assay described by Reichenbecher and colleagues in 1999, SDH activity can be followed using ferricyanide as artifical electron acceptor (Fig. 23), thus as sulfite:ferricyanide oxidoreductase activitiy. With this approach, the two known and well-characterized bacterial sulfite dehydrogenases, SorA and SorT, could be detected and characterized (e. g. Kappler *et al.*, 2000; Wilson & Kappler, 2009).

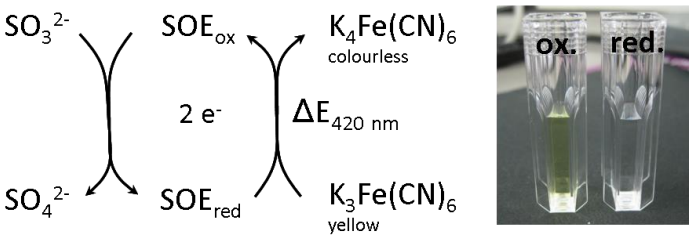


Fig. 23 The traditional enzyme assay for classical SDH (SorA, SorT) described by Reichenbecher *et al.* in 1999. Reaction scheme (left) and electron transfer to the artificial electron acceptor ferricyanide, which is yellow in its oxidized state and gets colourless when it is reduced. The change in colour can be detected photometrically and can be used to determine enzymatic activity as sulfite:ferricyanide oxidoreductase.

This colorimetric enzyme assay is based upon the reduction of ferricyanide (yellow) to ferrocyanide (colourless), however, this artificial electron acceptor can also interact with other enzymes (e. g. with the 11 kDa protein, chapter 3.1.2) and also result in the artefactual detection of unspecific reactions. Subsequent experiments with other electron acceptors were tested for this enzyme assay, but the addition of cytochrome *c* (bovine, horse or native

cyt *c* of *Ruegeria pomeroyi* DSS-3), atmospheric oxygen, menaquinone, DCPIP, FAD^+, NAD^+, or $NADP^+$ did not yield any enzyme activity at all.

3.2.3.2. Clark-type oxygen electrode experiments with intact cells

In order to find an alternative method to assay sulfite-oxidizing activity, Clark-type oxygen electrode measurements were carried out using intact cells of various bacteria, before and after the addition of sulfite (Fig. 24). The rationale for this experimental setup was that electrons derived from sulfite might be transferred to the membrane and *via* the electron transport chain subsequently to the terminal electron acceptor, e. g. molecular oxygen. Therefore, the electrons would be funnelled from an SOE through to a cytochrome *c* oxidase (complex IV), and thus, sulfite-oxidizing activities might be measurable indirectly as an increased oxygen uptake of intact cells after the addition of sulfite. As a representative control for a SorA-mediated electron transfer from sulfite through to oxygen, *Cupriavidus necator* H16 cells were used (Denger *et al.*, 2008). Conversely, for testing for a SorT-mediated electron transfer, *Sinorhizobium meliloti* 1021 cells were used (Wilson & Kappler, 2009). In both cases, the bacteria had been pre-grown with taurine. Acetate-grown cells were used as negative control. *R. pomeroyi* DSS-3 and *Roseovarius* sp. strain 217 were used as representatives for SoeABC. *R*. *nubinhibens* ISM exhibit sequence homologies to both SorT and SorABC.

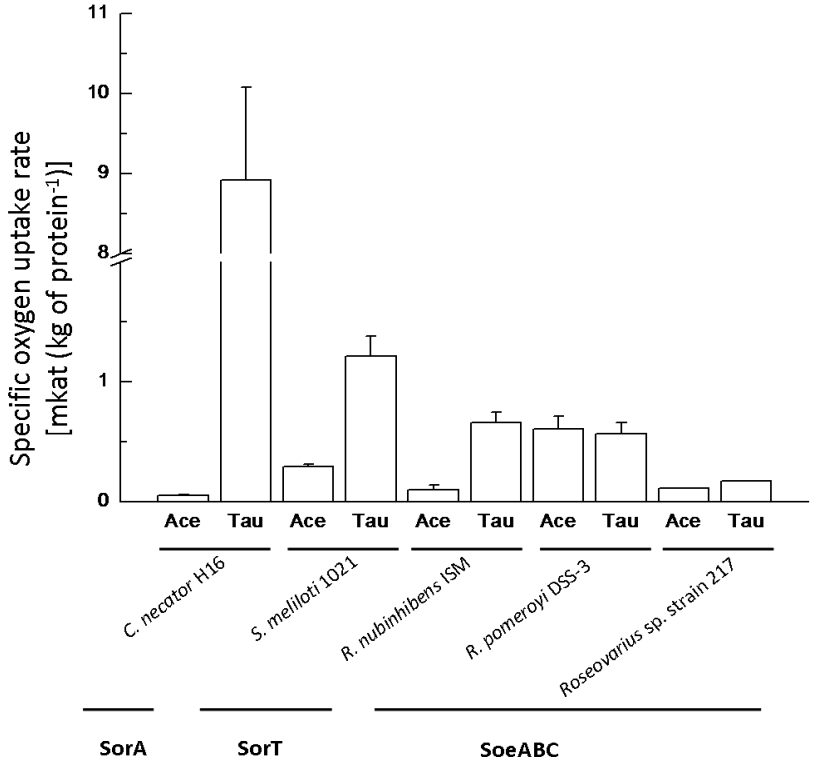


Fig. 24 Specific oxygen uptake rates of intact cells of various bacteria with known and unknown SOEs. Ace, acetate-grown cells served as negative control; Tau, taurine-grown cells; SoeABC, sulfite-oxidizing enzyme complex ABC.

The highest sulfite-dependent oxygen uptake was observed with taurine-grown *C. necator* H16 cells, which was approximately 178-fold higher than with acetate-grown cells. In taurine-grown *S. meliloti* 1021 cells, a 4.2-fold higher oxygen uptake was detectable compared to acetate-grown cells. A similar value, a 6.6-fold higher oxygen uptake, could be detected in taurine-grown *R. nubinhibens* ISM cells compared to acetate-grown cells. However, the absolute activities, which were measured in the SorT-representatives, were much lower compared to the activities detected in SorA-exhibiting *C. necator* H16. However, no significant differences in the oxygen consumption rate comparing acetate- and taurine-grown cells were found in *R. pomeroyi* DSS-3 and only a slightly higher oxygen consumption rate was measured in *Roseovarius* sp. strain 217. Particularly with the latter strain, the absolute oxygen consumption rate was very low.

3.2.3.3. Sulfite and sulfate determination

The results described in chapter 3.2.3.2 indicated that the yet unknown SOE in *R. pomeroyi* DSS-3 and *Roseovarius* sp. strain 217 cannot be assayed indirectly *via* oxygen uptake of intact cells after sulfite addition.

As a further method to measure specific sulfite dehydrogenase activity, sulfite disappearance and sulfate formation were continuously determined over time (35 min) after sulfite (2 mM) had been added to the soluble and the membrane fractions of cysteate-, taurine- and acetate-grown (negative control) *R. pomeroyi* DSS-3 cells. As already shown (chapter 3.1.2), there were various side reactions with ferricyanide as electron acceptor. Therefore, the experiment was carried out with (striped bars) and without (white bars) the addition of any electron acceptor, respectively (Fig. 25).

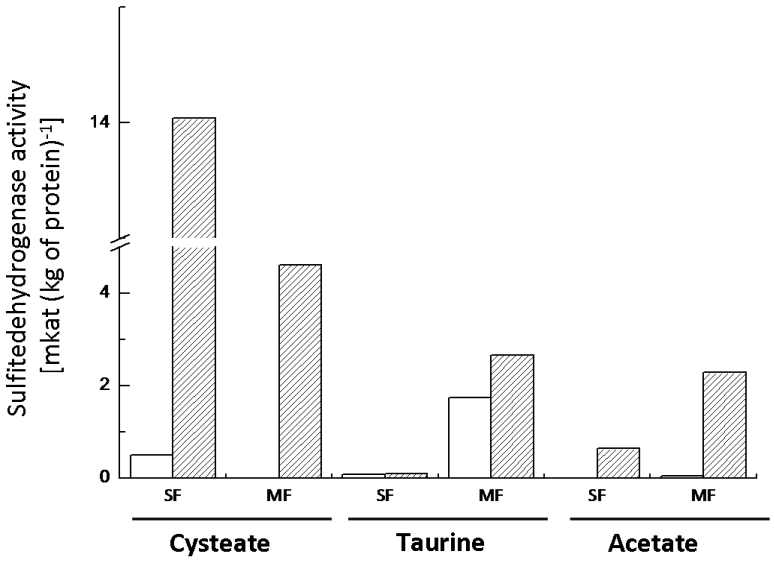


Fig. 25 Apparent specific SDH activities measured continuously by sulfite and sulfate determination (within 35 min) in *R. pomeroyi* DSS-3 grown with different carbon sources. Illustrated is the localization of the enzyme activity and the effect of ferricyanide on the specific sulfite dehydrogenase activity. Legend: white bars: without ferricyanide; striped bars: with ferricyanid; SF, soluble fraction; MF, membrane

If ferricyanide was present in the reaction mixture (striped bars), the highest SDH activity was found in the soluble fraction of cysteate-grown cells, for which it has been shown that these cells suffer and therefore express an 11 kDa protein due to sulfite stress (chapter 3.1.6). No enzyme activity was measured in the soluble fraction of taurine- and acetate-grown cells, whereas negligible enzyme activity was measured in the membrane fraction of these extracts. However, it is reasonable to assume that this activity is due to side reactions of ferricyanide with components of the membrane fraction.

If the enzyme assay was carried out without ferricyanide or any other electron acceptor (white bars), the highest sulfite dehydrogenase activity was observed under taurine-grown conditions in the membrane fraction of *R. pomeroyi* DSS-3. Negligible enzyme activity was measured in the soluble fraction of cysteate-grown cells, and no SDH activity was measured

in acetate-grown cells. The obtained results are consistent with the physiological data shown in chapter 3.1.1.

Since the well-characterized sulfite oxidases are soluble proteins located in the periplasm, (chapter 1.3.3) the latter findings were surprising. In order to get a better understanding of the localization of the sulfite-oxidizing activity in the three model bacteria, the same experiment was carried out using soluble and membrane fractions of taurine- and acetate-grown cells without any electron acceptor (Fig. 26).

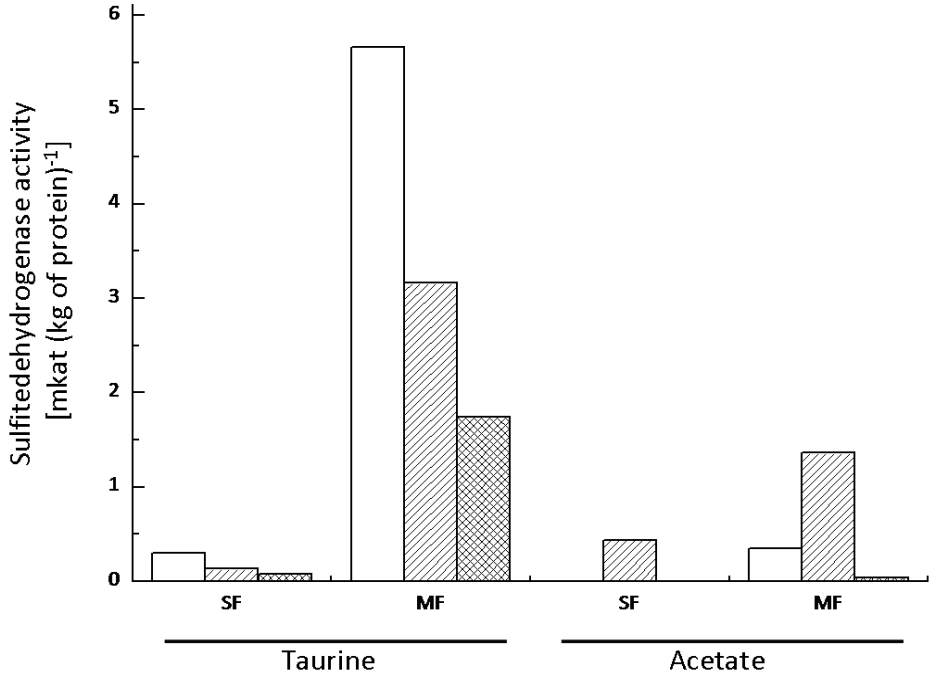


Fig. 26 Localization of SDH activity in taurine- or acetate-grown cells of *R. nubinhibens* ISM (white bars), *Roseovarius* sp. strain 217 (stripped bars), *R*. *pomeroy*i DSS-3 (chequered bars). The specific apparent enzyme activity was measured continuously by determination of sulfite concentration. To avoid side reactions caused by ferricyanide, the experiment was carried out without any additional electron acceptor. SF, soluble fraction; MF, membrane fraction.

The obtained results clearly indicated a sulfite-oxidizing activity in the membrane fraction of all three bacteria grown with taurine as growth substrate, whereas the highest SDH activity was found in *R. nubinhibens* ISM. In contrast, in acetate-grown cells or the soluble fractions (SF) almost no sulfite-oxidizing activity was found.

The same results were obtained when Clark-type oxygen electrode experiments were carried out with soluble and membrane fractions of these bacteria. Again, the highest oxygen consumption rate (and therefore the highest enzyme activity) was found in the membrane fractions all three bacteria, with *Roseovarius* sp. strain 217 showing the highest oxygen consumption rate with a determined specific SDH activity of 7.8 mkat (kg protein)$^{-1}$, *R. nubinhibens* ISM had a determined specific SDH activity of 1.8 mkat (kg protein)$^{-1}$ and *R. pomeroyi* DSS-3 a specific SDH activity of 0.5 mkat (kg protein)$^{-1}$ (data not shown).

3.2.4. Discussion

Only about 50% of known desulfonating bacteria have genes with sequence homologies to the known bacterial sulfite dehydrogenases SorA and SorT. However, these bacteria are known to degrade organosulfonates while excreting the sulfonate-moiety as sulfate or to some extent as sulfite *via* sulfite exporter. It is therefore reasonable to assume that at least a third type of SOE exist in these bacteria.

To exclude the possibility that the indirect pathway *via* APS reductase is involved in sulfite oxidation during sulfonate degradation, reverse transcription (RT) PCR experiments were carried out and the traditional enzyme assay was supplemented with AMP'. However, the obtained results clearly indicate that this alternative pathway of sulfite oxidation can be ruled out. These results were not surprising, since it is presumed that with regard to sulfite detoxification, the responsible enzymes are sulfite dehydrogenases, whereas in respect to energy generating processes, an APS' reductase is involved (Wilson & Kappler, 2009).

A proteomic approach in which the soluble fractions of taurine-, cysteate- and acetate-grown *R. pomeroyi* DSS-3 cells were compared *via* 2-D gel electrophoresis identified some proteins/genes which could be involved in sulfite detoxification. These proteins were investigated in more detail *via* RT-PCR experiments. Firstly, under taurine- and cysteate-grown conditions, increased amounts of EtfAB proteins were found. These proteins are known electron carriers that shuttle electrons/hydrogen atoms from dehydrogenase reactions to the membrane. Taurine and cysteate are degraded to metabolites such as acetyl-CoA and pyruvate, which are further metabolized in the Krebs cycle, generating reducing equivalents such as NADH. The generated hydrogen atoms could be transferred to EtfA/B. Thus, it is not surprising to find these proteins in higher concentrations under sulfonate-grown conditions. Secondly, a recently published study reported that sulfite

oxidation can also be mediated by a copper/zinc-containing superoxide dismutase (Ranguelova *et al.*, 2010). A candidate gene for such an enzyme (SPO2340, SodFe: iron-containing superoxide dismutase) was also found to be inducibly expressed in taurine-grown *R*. *pomeroyi* DSS-3 cells. However, RT-PCR experiments did not support this hypothesis. A possible explanation for this inconsistency could be the harvesting time: the cells for RNA preparation were harvested in the mid-exponential phase, whereas the cells for 2-D gel electrophoresis were harvested at the end of the exponential phase, which requires a different set of proteins, as it is described for *E. coli* in (Jishage & Ishihama, 1998). However, the detection of SodFe in taurine-grown cells could be due to oxidative stress in the cytoplasm mediated by transiently formed sulfite, whereas in cysteate-grown cell, sulfite is effectively exported *via* CuyZ.

In a further attempt to find proteins/genes involved in sulfite oxidation, orbitrap analyses were carried out, in which crude extracts of taurine- and as control acetate-grown *R. pomeroyi* DSS-3, *R. nubinhibens* ISM and *Roseovarius* sp. strain 217 cells were compared. In all three bacteria grown with taurine as substrate a protein was found, which is annotated as a molybdenum-containing oxidoreductase and which is termed SoeA in this study.

Three bacteria were chosen for attempts to measure this potential novel sulfite-oxidizing enzyme, SoeABC,: *R. pomeroyi* DSS-3, *Roseovarius* sp. strain 217 and *R. nubinhibens* ISM. The first two bacteria do not share any sequence homologies to SorA or SorT, while for the latter bacterium it was shown that two homotaurine-inducible SorTs were expressed (Mayer *et al.*, submission pending). However, no enzyme activities could be detected under any growth conditions (Denger *et al.*, 2009), which is surprising since the traditional enzyme assay was reported to be suitable to detect SorT-type sulfite dehydrogenases (e. g. Reichenbecher *et al.*, 1999; Denger *et al.*, 2008; Wilson & Kappler, 2009). All attempts to enrich the sulfite-oxidizing activity failed, either due to loss of activity during enrichment, or because the activity was found in the membrane fraction (*Roseovarius* sp. strain 217) which was destroyed during solubilization trials.

One major challenge of thesis was that a suitable enzyme assay for sulfite dehydrogenases other than SorA or SorT was missing. Most notably, the artificial electron acceptor ferricyanide which is used in the traditional enzyme assay interacts with different cellular components and leads to several side reactions (such as with the 11 kDa protein, chapter 3.1.2) as illustrated in (Fig. 25). Therefore, other electron acceptors were tested, but no

sulfite oxidation could be observed, even though the tested electron acceptors would be able to oxidize sulfite to sulfate due to a relatively low redox potential of the sulfite/sulfate redox couple (-524 mV; (Thauer *et al.*, 1977).

In order to find an alternative method to detect this novel enzyme activity, Clark-type oxygen electrode experiments were carried out with intact cells of *R*. *pomeroyi* DSS-3, *Roseovarius* sp. strain 217 and *R*. *nubinhibens* ISM (Fig. 24). The advantage of this experimental setup was that it would potentially be capable of detecting the activity of a multi-component enzyme complex (such as SoeABC) in intact cells. Furthermore, there is no need for artificial electron acceptors. A 4.2-fold higher specific oxygen consumption rate was observed in taurine- compared to acetate-grown *S. meliloti* 1021 cells. Similar values were reported in a previous work, in which 3.9- up to 6.3-fold higher specific enzyme activities during growth with taurine and thiosulfate were found (Wilson & Kappler, 2009). Taurine-grown *C. necator* H16 cells showed a 178-fold higher oxygen consumption rate compared to acetate-grown cells. Taurine-inducible sulfite dehydrogenase activities were previously reported in *C. necator* H16 (Denger *et al.*, 2008). Therefore, it can be assumed that the positive controls for this experiment were reliable. Using *R*. *nubinhibens* ISM cells, a 6.6-fold higher oxygen consumption rate in taurine-grown compared to acetate-grown cells was measured, a value which is in good accordance to the observations made for SorT in *S. meliloti* 1021. Interestingly, almost no inducible, sulfite-dependent oxygen consumption was detected in *Roseovarius* sp. strain 217 and in *R*. *pomeroyi* DSS-3 cells.

The determination of sulfite and sulfate concentrations was used as a third method to detect sulfite-oxidizing activity (Fig. 25, Fig. 26). The obtained results clearly indicate that the enzyme activity is located in the membrane fraction of all three bacteria and that it is induced in taurine-grown cells. These observations are in good agreement with the physiological data obtained in chapter 3.1.1 and were confirmed by Clark-type oxygen electrode experiments. If SoeABC was indeed the novel type of SOE, this would be the first hint that this enzyme complex is located or somehow connected to the membrane.

3.3. SoeABC, a valid candidate for a novel type of sulfite dehydrogenase?

3.3.1. A highly conserved gene cluster: *soeABC*

Many organisms that are known to degrade organosulfonates lack valid candidate genes for one of the known types of sulfite-oxidizing enzymes (such as SorA and SorT).

In order to provide quantitative support for this statement, a table (Table 9) was generated showing all organisms that possess at least one of the different desulfonating enzymes known (Xsc (Cook & Denger, 2002), CuyA (Cook *et al.*, 2006), and/or SuyAB (Rein *et al.*, 2005), which was compared to the co-occurrence of SorA and/or SorT homologous in those organisms.

This table clearly illustrates that about 50% of the desulfonating organisms lack candidate genes for SorA and/or SorT homologues. However, about two thirds possess a three-gene cluster (*soeABC*) that is highly conserved and widespread in Prokaryotes, particularly within the Proteobacteria, Firmicutes, Chlorobi (personal communication from Marc Mussmann, MPI Bremen), but also in Archaea. No sequence homologies were obtained in eukaryotes.

Table 9. Organisms with genes for desulfonating enzymes (*xsc*, *cuyA*, or *suyA/B*) and the candidate loci to encode proteins with a molybdopterin binding domain, such as sorA, sorT, or soeABC.

Organism[a]	Locus tag prefix	Genes encoding desulfonative enzymes			Genes encoding molybdopterin oxidoreductases		
		xsc	***cuyA***	***suyA/B***	***sorA***	***sorT***	***soe ABC***
α-proteobacterium BAL199	BAL199_	-	-	08538/33	-	-	05294
Candidatus Puniceispirillum marinum IMCC1322	SAR116_	-	2240	-	-	-	2105
Dinoroseobacter shibae DFL 12	Dshi_	1828, 2045	-	2037/38	-	2981	1238
Fulvimarina pelagi HTCC2506	FP2506_	-	-	14329/24	-	-	-
Hoeflea phototrophica DFL-43	HPDFL43_	18962	-	17101/96 21052/57	-	-	19312
Jannaschia sp. CCS1	Jann_	2846	-	-	-	-	1443
Labrenzia alexandrii DFL-11	SADFL11_	4535	-	5174/ 1935	-	-	893
Methylobacterium chloromethanicum CM4	Mchl_	4214	-	-	-	4218, 5388, 3664	-
Methylobacterium nodulans ORS 2060	Mnod_	-	-	3705/06	8664	2704, 8369	-
Methylobacterium radiotolerans JCM 2831	Mrad2831_	-	-	5980/79	5267	-	-
Oceanibulbus indolifex HEL-45	OIHEL45_	04865	13550	-	-	-	04875
Ochrobactrum anthropi ATCC 49188	Oant_	3502	-	-	-	-	-
Octadecabacter antarcticus 307	OA307_	4001	-	-	-	-	3246
Oligotropha carboxidovorans OM5	OCAR_	6744	-	7558/57	-	c17450	-

(continued)

Table 9 (continued)

Organism[a]	Locus tag prefix	Genes encoding desulfonative enzymes			Genes encoding molybdopterin oxidoreductases		
		xsc	*cuyA*	*suyA/B*	*sorA*	*sorT*	*soe ABC*
Paracoccus denitrificans PD1222	Pden_	1010, 1642, 4277	-	-	-	-	-
Pelagibacter ubique HTCC1002	PU1002_	02176	-	02181/86	-	-	-
Phaeobacter gallaeciensis 2.10	RG210_	10327	-	-	-	-	10302
Pseudovibrio sp. JE062	PJE062_	2664	-	-	-	-	-
Rhodobacter sphaeroides 2.4.1	RSP_	4011	-	-	-	-	-
Rhodobacter sphaeroides ATCC 17025	Rsph 17025_	0760	-	-	-	-	0767
Rhodobacteraceae bacterium KLH11	RKLH11_	2875	-	-	-	715	1213
Rhodobacterales bacterium HTCC2150	RB2150_	15441	17689	-	-	-	15426
Rhodococcus jostii CP000431	RHA1_ro	03582	-	08848/47	-	01563	-
Rhodopseudomonas palustris BisA53	RPE_	3201	-	-	-	-	-
Roseobacter sp. GAI01	RGAI101_	2868	-	-	-	-	2827
Roseobacter denitrificans OCh 114	RD1_	0826, 0977	0819, 1838	-	-	-	0975
Roseobacter litoralis Och 149	RLO149_c	001860 007850	007450	-	-	-	001840
Roseobacter sp. str. CCS2	RCCS2_	04724	13064	-	-	-	04749
Roseobacter sp. str. MED193	MED193_	12208	-	-	-	-	12228
Roseovarius nubinhibens ISM	ISM_	10690	09626	-	-	01020, 10401	10700
Roseovarius sp. str. 217	ROS217_	11936	09350 07370	-	-	-	11926
Roseovarius sp. str. HTCC2601	R2601_	20194	-	-	-	-	14290

(continued)

Table 9 (continued)

Organism[a]	Locus tag prefix	Genes encoding desulfonative enzymes			Genes encoding molybdopterin oxidoreductases		
		xsc	*cuyA*	*suyA/B*	*sorA*	*sorT*	*soe ABC*
Ruegeria pomeroyi DSS-3	SPO	3561	0158	-	-	-	3559
Ruegeria sp. R11	RR11_	1775	-	-	-	-	2124
Sagittula stellata E-37	SSE37_	18050	12951	-	-	-	18060
Silicibacter lacuscaerulensis ITI-1157	SL1157_	1492	-	-	-	-	1490
Silicibacter sp. str. TM1040	TM1040_	0155	-	-	-	-	-
Sinorhizobium meliloti 1021	SM	b21530	-	-	-	c04049	-
Bordetella petrii DSM 12804	Bpet	-	-	0415/14	-	3232	-
Burkholderia xenovorans LB 400	Bxe_	B0691	-	-	C0151	-	B1743
Comamonas testosteroni KF-1	CtesDRAFT_ PD	0776	-	-	-	5052	-
Cupriavidus necator H16	H16_	B1870	-	A1258	B0860	-	-
Delftia acidovorans SPH-1	Daci_	1992	-	-	-	0055	-
Lutiella nitroferrum 2002	Fura DRAFT_	1865	-	-	-	3795	-
Herminiimonas arsenicoxydans	HEAR	-	-	3336/37	2349	-	3342
Ramlibacter tataouinensis TTB310	Rta_	-	27290	-	-	19240, 14930	-
Variovorax paradoxus S110	Vapar_	3825	-	3818/19	-	-	3815
Verminephrobacter eiseniae EF01-2	Veis_	3999	-	0893/92	-	1599	3996
Chromohalobacter salexigens DSM 3043	Csal_	-	-	1765/66	-	-	-
Photobacterium profundum 3TCK	P3TCK_	09338	-	-	-	05186	-
Vibrio shilonii AK1	VSAK1_	20144	-	-	-	-	-
Vibrio splendidus 12B01	V12B01_	16621	-	-	-	04568	-
Vibrio sp. str. MED222	MED222_	08988	-	-	-	-	-

(continued)

Table 9 (continued)

Organism[a]	Locus tag prefix	Genes encoding desulfonative enzymes			Genes encoding molybdopterin oxidoreductases		
		xsc	*cuyA*	*suyA/B*	*sorA*	*sorT*	*soe ABC*
Vibrionales bacterium SWAT-3	VSWAT3_	12662	-	-	-	-	-
Desulfatibacillum alkenivorans AK-01	Dalk_	1400	-	-	-	-	-
Desulfotalea psychrophila LSv54	DP[b]	0872	-	-	-	-	-
Desulfovibrio desulfuricans subsp. *desulfuricans* str. ATCC 27774	Ddes_	0220	-	2374/75	-	-	-
Candidatus Solibacter usitatus Ellin6076*	Acid_	1084	-	-	7248	-	-
Rubrobacter xylanophilus str. DSM 9941**	Rxyl_	-	-	2591/90	-	-	-
Dictyoglomus turgidum DSM 6724***	Dtur_	-	-	0482/81	-	-	-
Desulfitobacterium hafniense DCB-2****	Dhaf_[b]	4634	-	3274/75	-	-	-
*Thermotoga neapolitana******	CTN_	-	-	1545/46	-	-	-

The phylogenetic affiliation is indicated by a colour code: grey, α-Proteobacteria; green, β-Proteobacteria; yellow, γ-Proteobacteria; purple, δ-Proteobacteria, or by asterisks: *, Acidobacteria; **, Actinobacteria; ***, Dictyoglomia; ****, Clostridia; *****, Termotogae.

[a] Only one representative strain of several genome-sequenced strains is listed.

[b] Strictly anaerobic bacteria.

Gene maps of *soeABC* were created in order to investigate the *soeABC* gene clusters particularly in terms of its gene neighbourhood in those bacteria (Fig. 27, Fig. 28). These gene maps show two striking features. Firstly, *soeABC* is usually encoded in close neighbourhood with genes involved in organosulfonate degradation. Secondly, *soeABC* is presumably encoded in one operon.

A prominent example is the β-Proteobacterium *Variovorax paradoxus* S110 (Fig. 27), which possesses the *soeABC* genes (*soeA* green-coloured, *soeB* yellow-coloured and *soeC* is purple-coloured) clustered between various genes encoding desulfonating enzymes: *xsc*, *ack* and *pta* genes which are required for taurine degradation ({Baldock *et al.*, 2007}), *suyAB*, *comC* and *slcC* for sulfolactate degradation (e. g. Graupner *et al.*, 2000; Irimia *et al.*, 2004; Denger & Cook, 2010), *iseJ* for isethionate degradation (Weinitschke *et al.*, 2010), and *sauS/T* for sulfoacetate degradation (Weinitschke *et al.*, 2010).

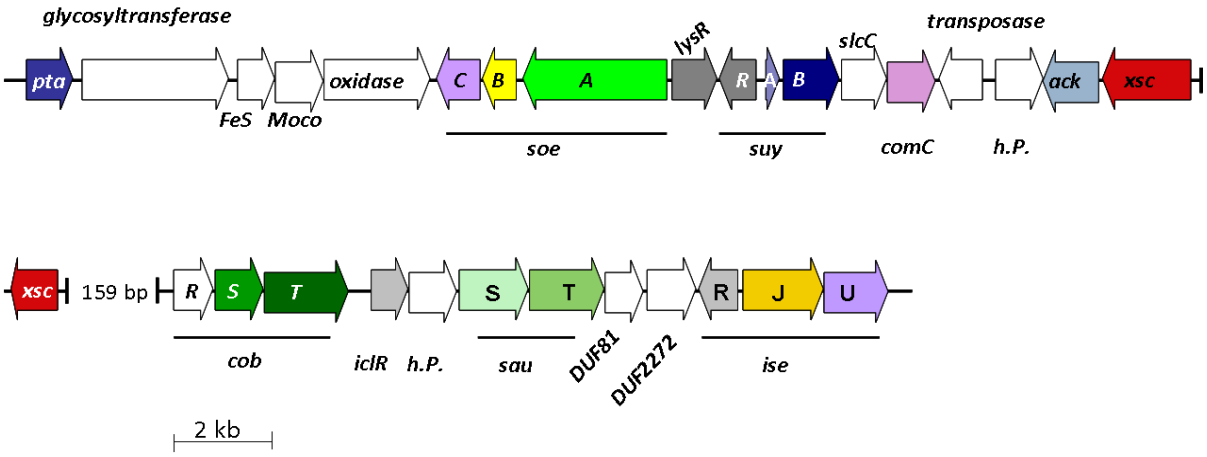


Fig. 27 Detailed gene map of *soeABC* and surrounding genes in *Variovorax paradoxus* S110. The function of the colour-coded genes is described in the main text. Genes that are white-coloured encode enzymes which are not involved in desulfonation. h. P.= hypothetical protein.

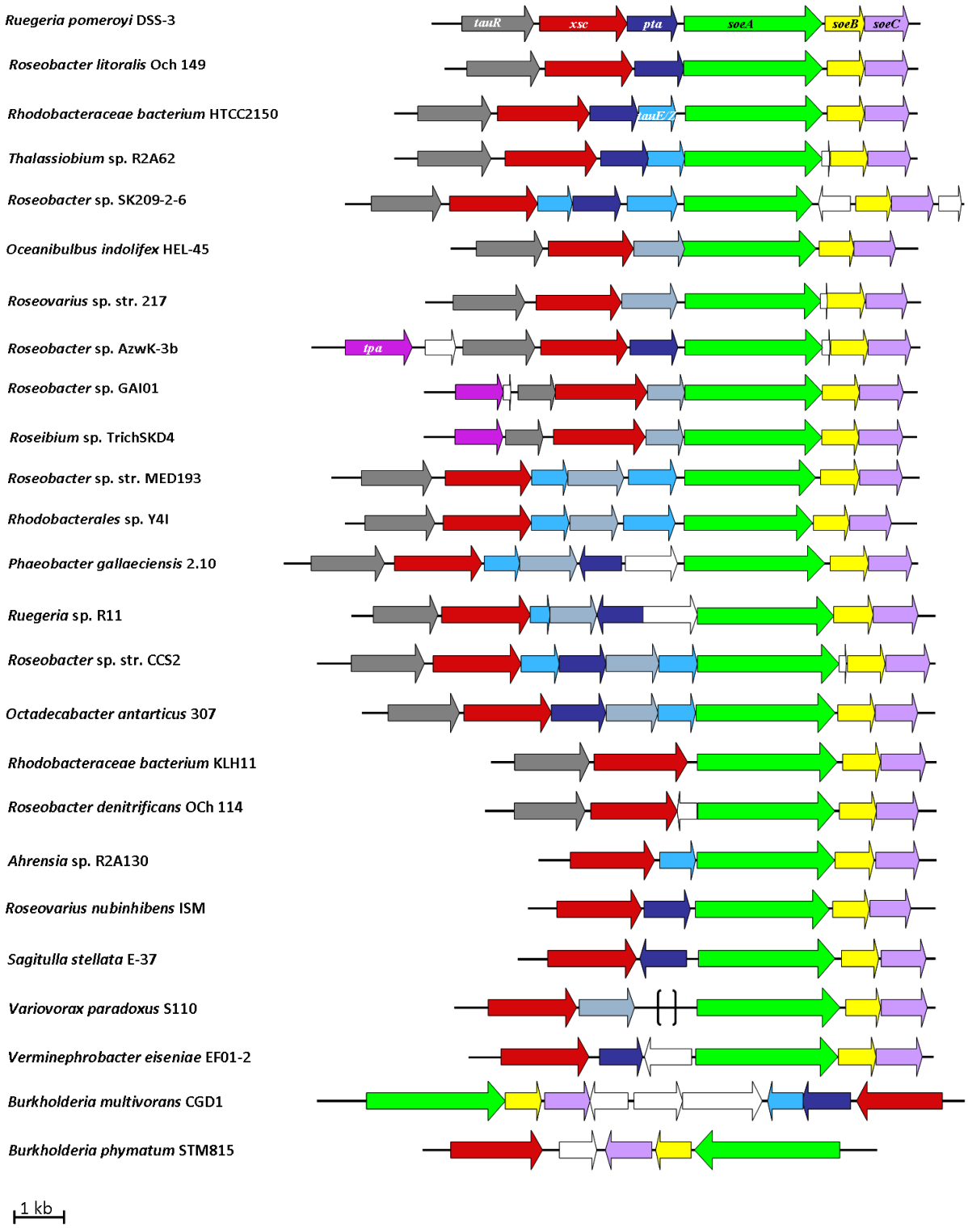


Fig. 28. Gene maps of *soeABC* and surrounding genes. White encodes genes for enzymes which are not involved in desulfonation. Brackets indicate a gene complex of known enzymes catalyzing desulfonation reactions (for more details see Fig. 27). The synthenic gene clusters of the following bacteria are shown: *Ruegeria pomeroyi* DSS-3 = *Silicibacter lacuscaerulensis* ITI-1157, *Phaeobacter gallaeciensis* 2.10 = *Phaeobacter gallaeciensis* BS107, *Roseovarius* sp. str. 217 = *Roseovarius* sp. TM1035, *Octadecabacter antarcticus* 307 = *Octadecabacter antarcticus* 238, *Rhodobacteracea bacterium* HTCC2150 = *Rhodobacteraceae bacterium* HTCC2083, *Burkholderia multivorans* CGD1 = *Burkholderia multivorans* CGD2M, *Burkholderia multivorans* ATCC17616.

Based on these bioinformatic data and prediction tools, a working hypothesis was developed (Fig. 29). SoeABC could be a three-component sulfite-oxidizing enzyme complex consisting of SoeA, SoeB and SoeC which are located in the cytoplasm. SoeA is annotated as a selenocysteine-containing molybdopterin (dinucleotide)-binding oxidoreductase. This subunit could bind sulfite and catalyze sulfite oxidation, with both electrons being transferred to the molybdopterin-cofactor (Moco), the redox centre of SoeA. The reaction mechanism of Mocos is illustrated in Fig. 7. Subsequently, the electrons could be successively transferred to SoeB, which is predicted to bind [4Fe-4S] cluster, as second redox centre. Finally, the electrons could be shuttled to SoeC, a predicted transmembrane protein which itself transfers the electrons to electron acceptors of the respiratory chain in the cytoplasmic membrane (e. g. menaquinone). Another conceivable possibility for SoeC would be to function as sulfate exporter (dashed line). The aim of the following chapters was to find evidence testing this hypothesis.

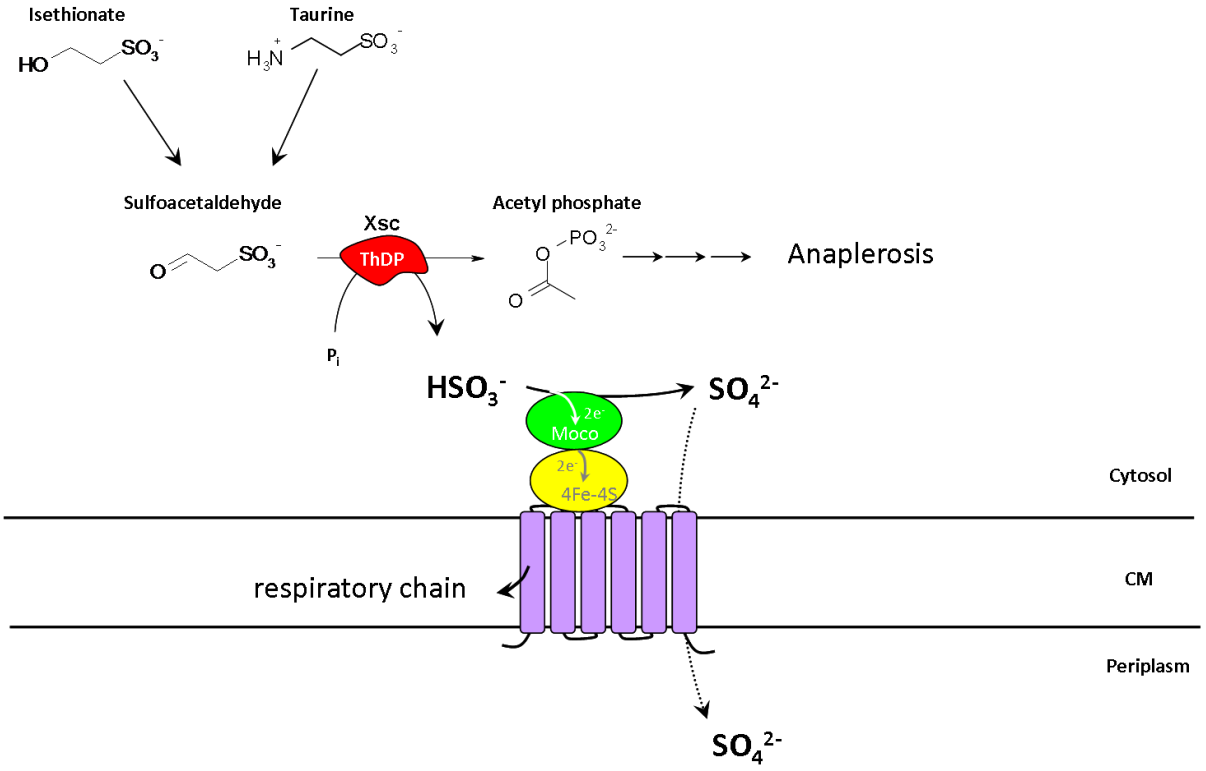


Fig. 29 Illustration of the predicted function and location of SoeABC for taurine and isethionate degradation at the example of *Ruegeria pomeroyi* DSS-3. Green colour indicates SoeA, yellow SoeB and purple SoeC. The dashed line indicates a further conceivable function for SoeC as a sulfate transporter. CM, cytoplasmic membrane. For further abbreviations and explanations see main text and addendum.

3.3.2. Preliminary evidence for the involvement of SoeA in sulfonate degradation

In order to provide evidence for the hypothesis that SoeABC is a three-component sulfite-oxidizing enzyme complex (Fig. 29), transcriptional analyses for *soeA* (reverse transcription (RT) - PCR experiments) and proteome profiles (SDS-PAGE gradient gels, 4.9-19.0% polyacrylamid) of *Ruegeria pomeroyi* DSS-3 cells and *Roseovarius* sp. strain 217 cells were established for different growth conditions (taurine, cysteate, or acetate) (Fig. 30). The inducible transcription of the genes for taurine degradation in *R. pomeroyi* DSS-3 has been shown previously (Gorzynska *et al.*, 2006). Therefore, it was assumed that *soeA* should also be induced during growth with taurine.

The results of the RT-PCR experiment are shown in Fig. 30 A. RNA preparations were tested for DNA contamination, but no DNA was detecable (data not shown). Positive controls with 16S rRNA primers confirmed that same amounts of total RNA were used (Fig. 30 A, lower panel). A strong transcription of *soeA* (expected size, 320 bp) was detected for taurine-grown cells, even though there was also a basal transcription of *soeA* detectable for cysteate- and acetate-grown cells (Fig. 30 A, upper panel).

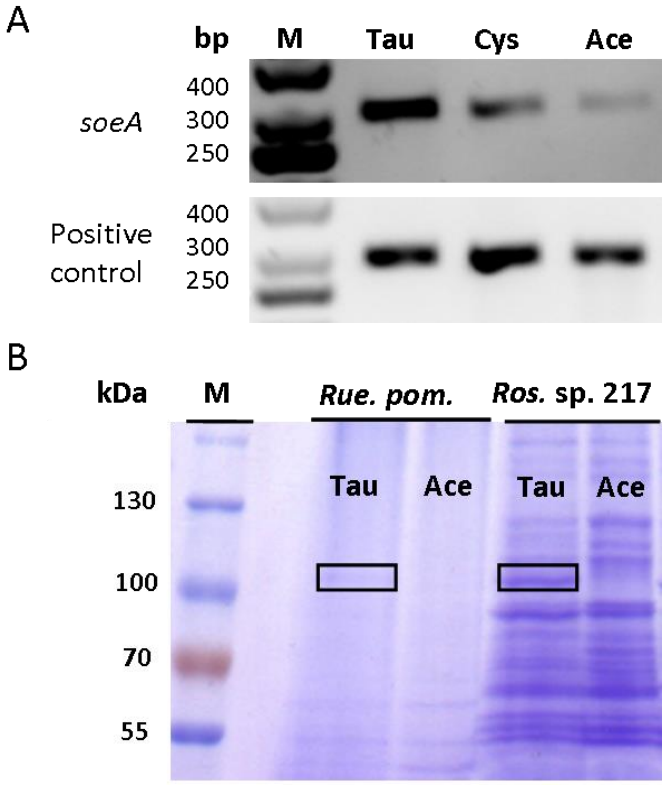


Fig. 30 Agarose gels showing transcripts from RT (reverse transcription) – PCR for *soeA* under different growth conditions (Tau, taurine; Cys, cysteate; Ace, acetate) in *R. pomeroyi* DSS-3 (**upper panel**). 16S rRNA primers were used as positive controls (**lower panel**). M, lenght marker (**A**). Gradient SDS-PAGE gel (4.9-19.0% polyacrylamid) of crude extract of *R. pomeroyi* DSS-3 and of membrane fraction of *Roseovarius* sp. strain 217. Cells were grown with taurine (Tau) or acetate (Ace). Black rectangles indicate taurine induced protein bands which were excised and identified by PF-MS to represent SoeA. M, molecular mass marker (**B**).

As shown in Fig. 26, sulfite-oxidizing activity was found in the membrane fraction of *Roseovarius* sp. strain 217 and of *R. pomeroyi* DSS-3. Therefore, the membrane fraction of *Roseovarius* sp. strain 217 and crude extract of *R. pomeroyi* DSS-3 were analyzed by gradient gel electrophoresis (4.9-19.0% polyacrylamid), which is shown in Fig. 30 B.
A taurine-inducible protein band with a molecular mass of approximately 110 kDa was observed in extracts of both bacteria. These findings are in good accordance with the expected molecular mass of SoeA, which is 105 kDa. Therefore, both proteins bands were excised and submitted to peptide fingerprinting-mass spectrometry (PF-MS), which identified each to be SoeA.

The finding that SoeA is inducibly expressed in taurine-grown cells was supported by orbitrap analyses of crude extracts derived from taurine- or acetate-grown *Roseovarius* sp. strain 217, *R. pomeroyi* DSS-3 and *Roseovarius nubinhibens* ISM cells (see also chapter 3.2.1). The results obtained with regard to SoeA are shown in Table 10 for the taurine-induced data set. However, SoeB or SoeC were not detected in any of the extracts analysed. In acetate-grown cells, the orbitrap analyses did not reveal any matches for all three components of SoeABC.

Table 10 Orbitrap analyses of crude extracts from three different bacteria grown with taurine. The sequence coverages [%] and PSM (peptide spectrum matches) for SoeA are given as quality indicators.

Organism	**SoeA**	
	Coverage [%]	**PSMs**
R. pomeroyi DSS-3	25.68	30
R. nubinhibens ISM	34.54	44
Roseovarius sp. strain 217	28.28	21

In conclusion, the results from the transcriptional and proteomic analyses strongly supported the notion that SoeA is inducibly expressed during growth with the organosulfonate taurine in *R. pomeroyi* DSS-3, *Roseovarius* sp. strain 217, and in *Roseovarius nubinhibens* ISM, and might be involved in detoxification of sulfite in those bacteria. However, as shown in chapter 2, the enzyme activity was hard to measure and also enrichment attempts failed. Therefore, genetic work on SoeABC in *R. pomeroyi* DSS-3 was carried out and the obtained results are shown in the following chapters.

3.3.3. Heterologous overexpression of SoeA

In order to examine the role of SoeA in organosulfonate degradation, a heterologous overexpression of SoeA was attempted and the cloning strategy used is depicted in (Fig. 31). Therefore, *soeA* (SPO3559) was PCR-amplified with a primer pair introducing restriction sites for *Eco*RI and *Xho*I (step 1), the PCR product cloned into vector pET16, which provided an N-terminal His_6-tag (step 2), and the construct transformed into the overexpression strains *E. coli* BL21DE3 and *E. coli* TP1000. While the former strain is suitable for overexpression due to the T7 RNA polymerase and included *lacI* genes, the latter strain was constructed specifically for the overexpression of molybdopterin-containing enzymes (Palmer *et al.*, 1996).

Before transferring the plasmids into the overexpression strains, the latter were tested for their sensitivity against sulfite (up to 1 M). However, no effect could be observed indicating that no side reactions with those strains had to be expected (data not shown).

After IPTG induction (0.2 M, 2 h), the cells were lyzed and denaturated proteins were analyzed by SDS-PAGE in comparison to non-induced cells. The gels are represented in Fig. 31, which indicated a successful overexpression of a protein with an approximate size of 110 kDa in *E. coli* BL21DE3; no over-expression was observed for *E. coli* TP1000 (data not shown). In an attempt to optimize the overexpression conditions, different IPTG concentrations (0.05, 0.1 and 0.2 M), incubation temperatures (22, 28 and 37 °C), and incubation times (2, 4 and 24 h), were tested. However, no increase in protein expression was observed for *E. coli* BL21DE3, and no overexpression at all was obtained for *E. coli* TP1000 (data not shown).

Finally, the *E. coli* BL21DE3 cells were disrupted (sonified) and the sulfite-oxidizing activity was determined in crude extracts using ferricyanide as electron acceptor. However, no enzyme activity could be measured using the traditional enzyme assay (chapter 2.4.3).

Due to unsatisfying overexpression results, the next attempt to discover the function of SoeA was the knockout of this gene in *R. pomeroyi* DSS-3 by insertional mutation. The results are shown and described in the following chapters.

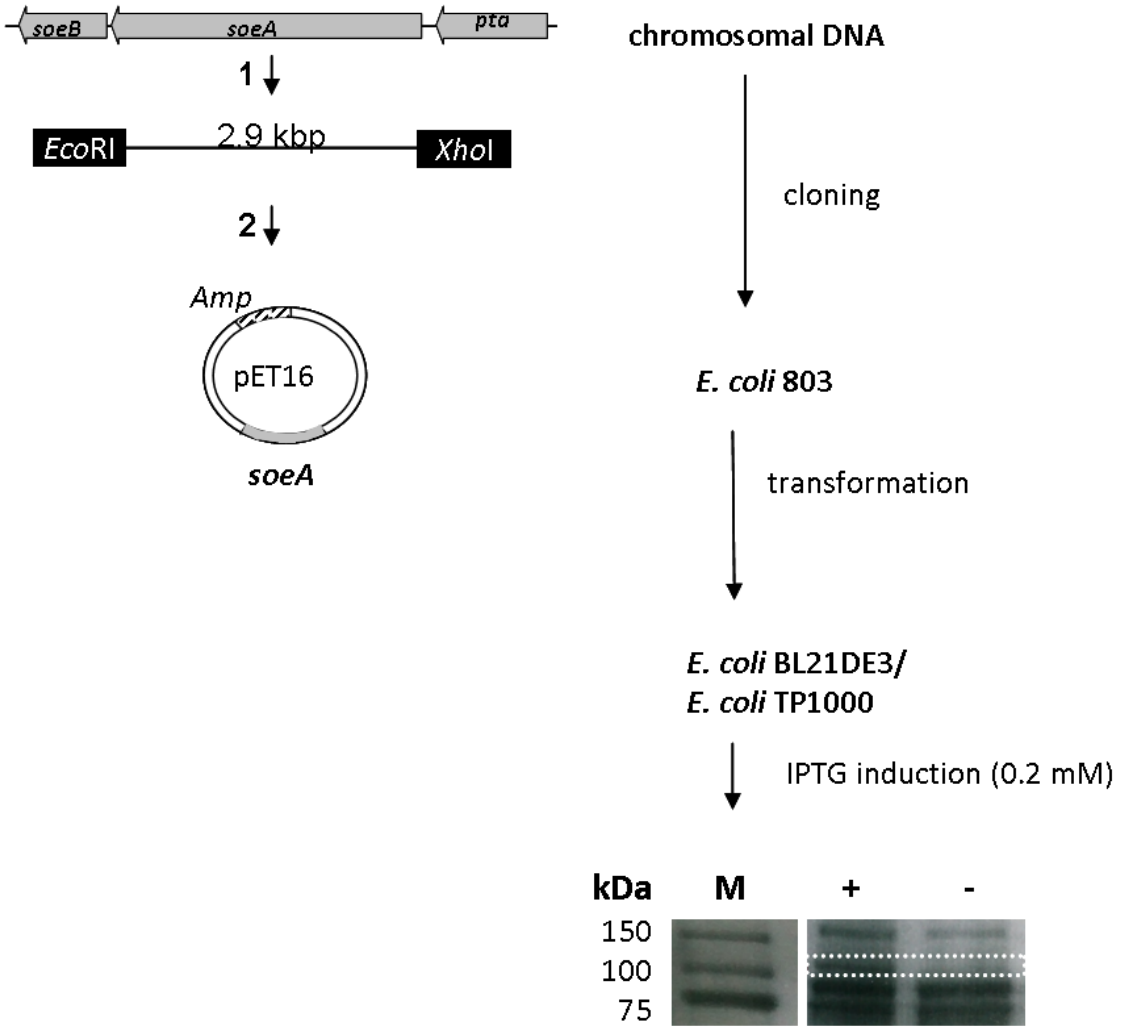


Fig. 31 Cloning strategy for heterologous overexpression of *soeA* (SPO3559) in two different *E. coli* strains (BL21DE3 and TP1000), using the pET vector overexpression system (**upper panel**). For *E. coli* BL21DE3 and after IPTG induction, the overexpression was analyzed *via* SDS PAGE (15%). The white rectangle indicates an IPTG (+) induced protein band with a molecular mass of approximately 110 kDa. Uninduced cells (-) were chosen as negative control (**lower panel**).

3.3.4. Insertional mutation of *soeA* (SPO3559) in *R. pomeroyi* DSS-3

3.3.4.1. Construction and verification of the *soeA*-deficient mutant

A *soeA* knockout mutant of *R. pomeroyi* DSS-3 was generated by insertional mutation as illustrated in Fig. 32. Therefore, a 1.3 kbp fragment of *soeA* was PCR-amplified introducing restriction sites for *Eco*RI and *Bam*HI (step 1). The PCR product was ligated into the suicide vector pK19, which posseses two antibiotic resistant cassettes for kanamycin (kan) and spectinomycin (spec) enabling selection for mutants. Subsequently, the construct was transformed into *E. coli* JM101 (step 2). By *tri*-parental mating with helper strain *E. coli* pRK2013, the vector was conjugated into wild type strain *R. pomeroyi* DSS-3 (step 3) in which the original *soeA* gene was interrupted by homologous recombination.

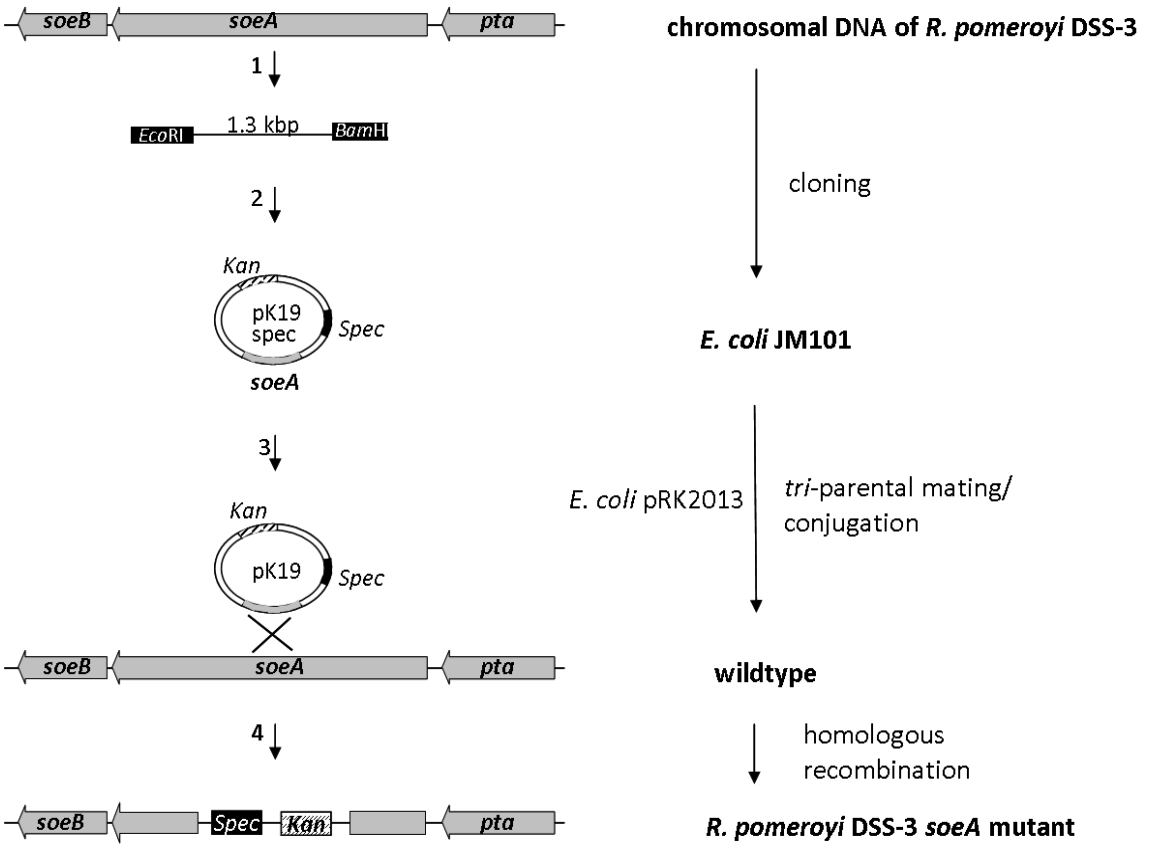


Fig. 32 Strategy to generate the *soeA* deficient *Ruegeria pomeroyi* DSS-3 mutant. Kan, kanamycin; Spec, spectinomycin.

For verification of the *soeA*-deficient *R. pomeroyi* DSS-3 mutants, a Southern blot was carried out (Fig. 33). To this end, genomic DNA of the wild type and three mutants were digested with restriction enzymes *Eco*RI and *Bam*HI. The restriction digest was separated on an agarose gel (1%), blotted onto a nylon membrane, hybridised using a labelled *soeA*-probe (1.3 kbp PCR fragment of *soeA*, orange colour), and the hybridized probe(s)/fragment(s) detected by fluorescence-labelled antibodies. The restriction pattern is shown in the left lower panel of Fig. 33 which is consistent with the expected restriction pattern. Furthermore, a primer pair that covered the full length of *soeA* was used to amplify the *soeA* gene in the wild type and in the mutant. As expected, a PCR product (2.9 kbp) was obtained for the wild type, but not for the mutant under the same PCR conditions (Fig. 33, right panel on the bottom) confirming a successful insertional disruption of the *soeA* gene in *R. pomeroyi* DSS-3.

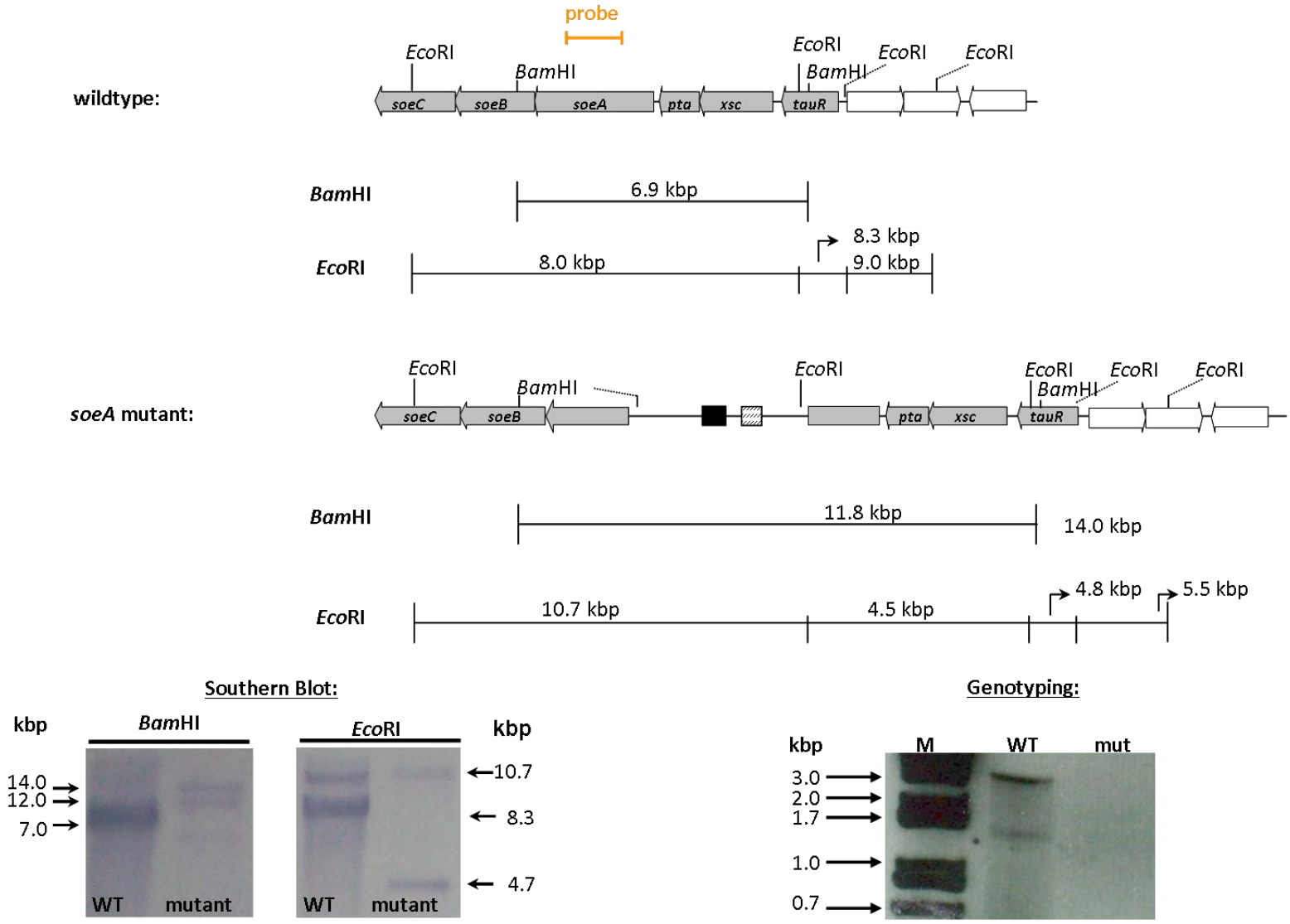


Fig. 33 Verification of *soeA* deficient *Ruegeria pomeroyi* DSS-3 mutant by Southern blotting (**left panel**) and genotyping *via* PCR (**right panel**). WT, wild type.

3.3.4.2. Characterization of the *soeA* mutant

As described in chapter 3.3.4.1 and illustrated in Fig. 33, the *soeA*-deficient mutant was constructed *via tri*-parental mating using *E. coli* pRK2013 and *E. coli* JM101. To exclude the possibility that the mutated *R. pomeroyi* DSS-3 is contaminated with *E. coli* bacteria influencing the subsequent physiological experiments, PCR experiments were carried out with primers for housekeeping genes for *R. pomeroyi* DSS-3 (SPO2157, citrate synthase). Both the wild type and the mutant *R. pomeroyi* DSS-3 strain exhibited a transcript with the expected fragment size (300 bp) for SPO2157 (data not shown).

As a further proof of contamination, the wild type and the mutant was tested for DMS production (*via* gas chromatography) since *R. pomeroyi* DSS-3 is known to cleave DMSP into DMS and acrylate (González *et al.*, 2003). Both the wild type and the mutant excreted DMS while growing with DMSP (1 mM) and succinate (10 mM). Therefore, it can be assumed that the mutant strain is *R. pomeroyi* DSS-3.

To compare the physiology of the wild type and the mutant strain, various growth substrates were chosen: succinate or acetate served as negative controls, taurine, isethionate or cysteate served as organosulfonates. The growth of mutant and wild type strain was monitored measuring the turbidity but also by determination of the sulfite and sulfate concentration and measuring of substrates and products. The results are summarized in Fig. 34.

The wild type and the mutant exhibited normal growth with succincate, acetate or cysteate as C-source (data not shown).

Interestingly, the mutant had lost the ability to grow efficiently with taurine as sole carbon and energy source (within the first 6 days of incubation). In those cultures, no sulfate release was detectable, as opposed to the wild type, but the release of sulfite (0.08 mM). The same phenotype was observed when isethionate was tested as sole carbon and energy source: negligible growth of the mutant with sulfite excretion while the wild type metabolized isethionate completely with concomitant excretion of sulfate (data not shown).

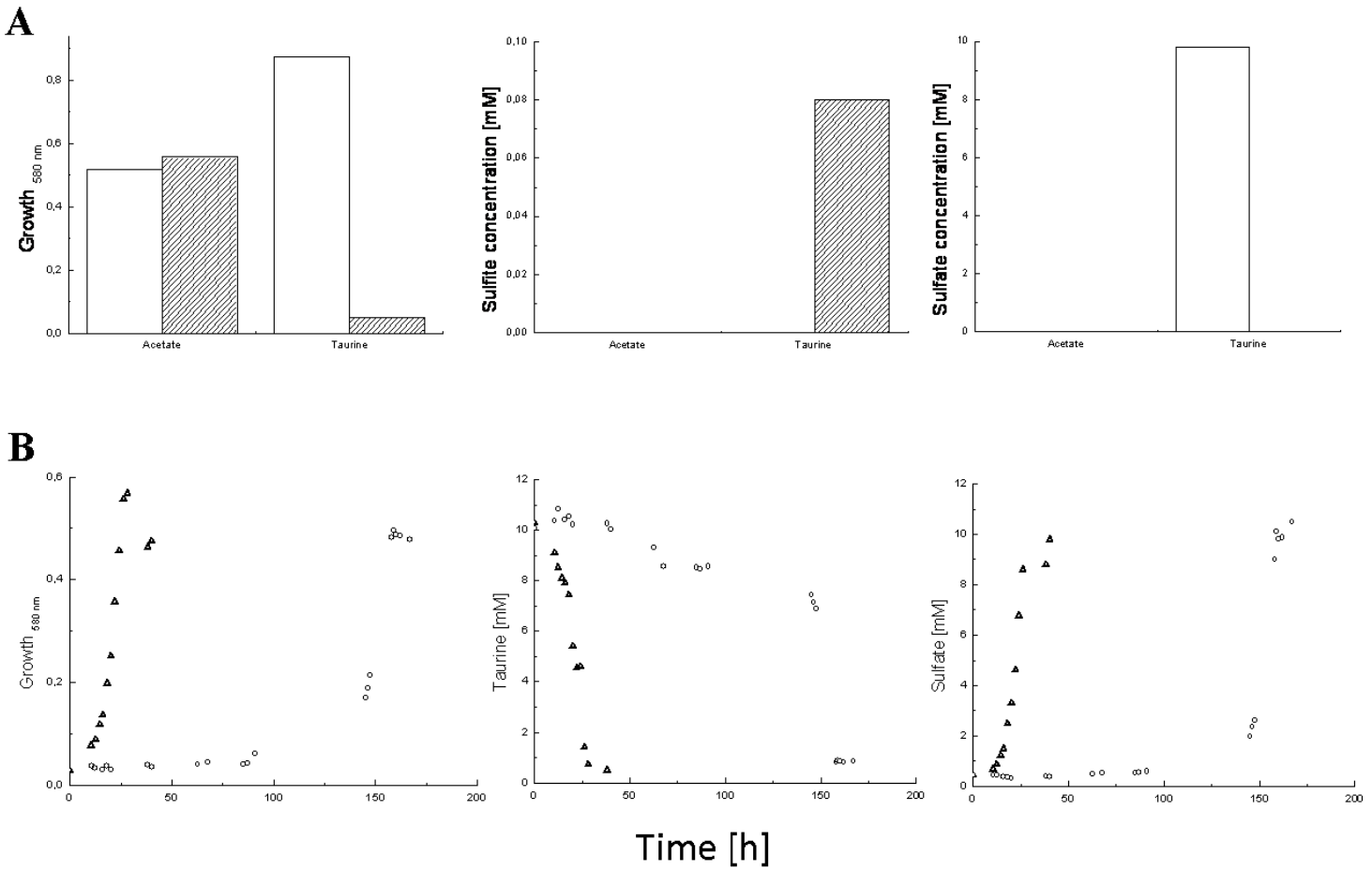


Fig. 34 Growth and sulfite/sulfate release of wild type (white bars) and of *soeA* deficient mutant of *R. pomeroyi* DSS-3 (dashed bars) growing with acetate (negative control) and taurine as sole carbon and energy source. Determination of turbidity, sulfite- and sulfate concentration within two days of growth (**A**). Six-days time course of wild type (black Δ) and mutant (◯) growth with taurine as carbon source, and of sulfite- and sulfate release. Growth was monitored as turbidity (**B**).

Interestingly, after six days of incubation, the mutant culture started taurine-degradation until taurine was completely consumed concomitant with sulfate excretion, whereas no sulfite release was detectable (Fig. 34 B). The same observation was made for the mutant culture with isethionate (data not shown).

These results were interpreted to represent a reversion of the *soeA*-deficient *R. pomeroyi* DSS-3 mutant after prolongued incubation by the excision of the inserted plasmid leading to a fully functional *soeA* gene. In order to confirm this, chromosomal DNA of the mutant and wildype was isolated from taurine- or acetate-grown cultures. Subsequently, *soeA* gene was amplified with primers covering the full-length gene and the amplicons were analyzed on an agarose gel (1%) (Fig. 35). The obtained amplicon from the wild type was a single DNA fragment of the expected size (approximately 2841 bp). The same amplicon was obtained from the mutant culture that had grown with taurine after seven days of incubation. Subsequent sequencing of these fragments confirmed the identity of the *soeA* gene (SPO3559).

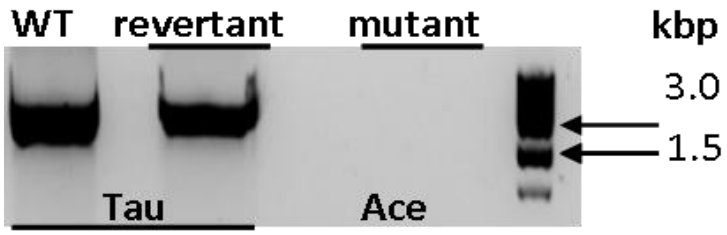


Fig. 35 Agarose gel (1%) showing amplicons of *soeA* with primers covering the full-length of *soeA* (appr. 2.9 kbp). Wild type (WT) and mutant were grown on taurine (Tau) or acetate (Ace).

Therefore, it can be assumed that the mutant reverted the insertional mutation in *soeA* gene within the first six days of growth. In contrast, the mutant culture growing with acetate did not revert the mutation, as indicated by the absence of an amplicon under the same PCR conditions, which can be explained by the lack of selective pressure for functional SOE.

These results strongly supported the notion that *soeA* is involved in taurine- and isethionate-degradation in *R. pomeroyi* DSS-3- most likely, in the detoxification of sulfite to sulfate.

As described previously, SoeA is annotated as a molybdopterin-binding protein. For biosynthesis of the Moco, Mo is required in the growth medium. To investigate growth of *R. pomeroyi* DSS-3 in the absence of Mo, the growth medium was modified: molybdenum was

replaced with tungsten. The effect was interesting and strengthens the hypothesis: *Ruegeria pomeroyi* DSS-3 did not grow with taurine or isethionate, respectively, but with cysteate or acetate (data not shown).

3.3.5. Regulation of *soeABC*

In Fig. 30 A, it was shown that the highest transcription of *soeA* was detectable during growth with taurine in *Ruegeria pomeroyi* DSS-3. In order to investigate and compare the transcriptional pattern of *soeA*, *soeB* and *soeC* in *R. pomeroyi* DSS-3 wild type and in the *soeA*-deficient mutant, RT (reverse transcription) -PCR experiments were carried out (Fig. 36 A). Furthermore, it was tested if the predicted three-gene cluster *soeABC* (operon) is co-transcribed, and if there is also a co-transcription of the *xsc*-gene cluster (*xsc*, *pta*) (Fig. 36 B). A transcript showing the expected fragment sizes (*soeA*, 539 bp; *soeB*, 281 bp; *soeC*, 288 bp) for all genes tested could be detected for the wild type (lane 1), as well as for the mutant (lane 2) under taurine-grown conditions.

The co-transcription experiments yielded a co-transcription of *soeA*/*soeB* and *soeA*/*soeC*, and interestingly, a co-transcription of *pta*/*soeA* and *xsc*/*soeA*.

These findings led to the assumption that most likely *soeABC* is also regulated by *tauR*, as is the *xsc*-gene cluster. This would also explain that the *soeA* mutant is unable to grow with isethionate, since isethionate is metablized *via* sulfoacetaldehyde (SAA) as the substrate for *xsc*.

The *pta* and *soeA* genes are separated by 127 bp, which could encode for an independent promoter. However, bioinformatic analyses for promotor regions revealed no evidence for an independent promoter region upstream of *soeA*.

Furthermore, two *lacZ*-fusions were created with *soeA* (Fig. 36 B, black Δ, 2) and *tauR* (Fig. 36 b, black Δ, 1). Although construction of the *lacZ*-fusions was successful, no clones were obtained carrying the *lacZ* constructs, and therefore, no conclusion can be drawn from these experiments (data not shown).

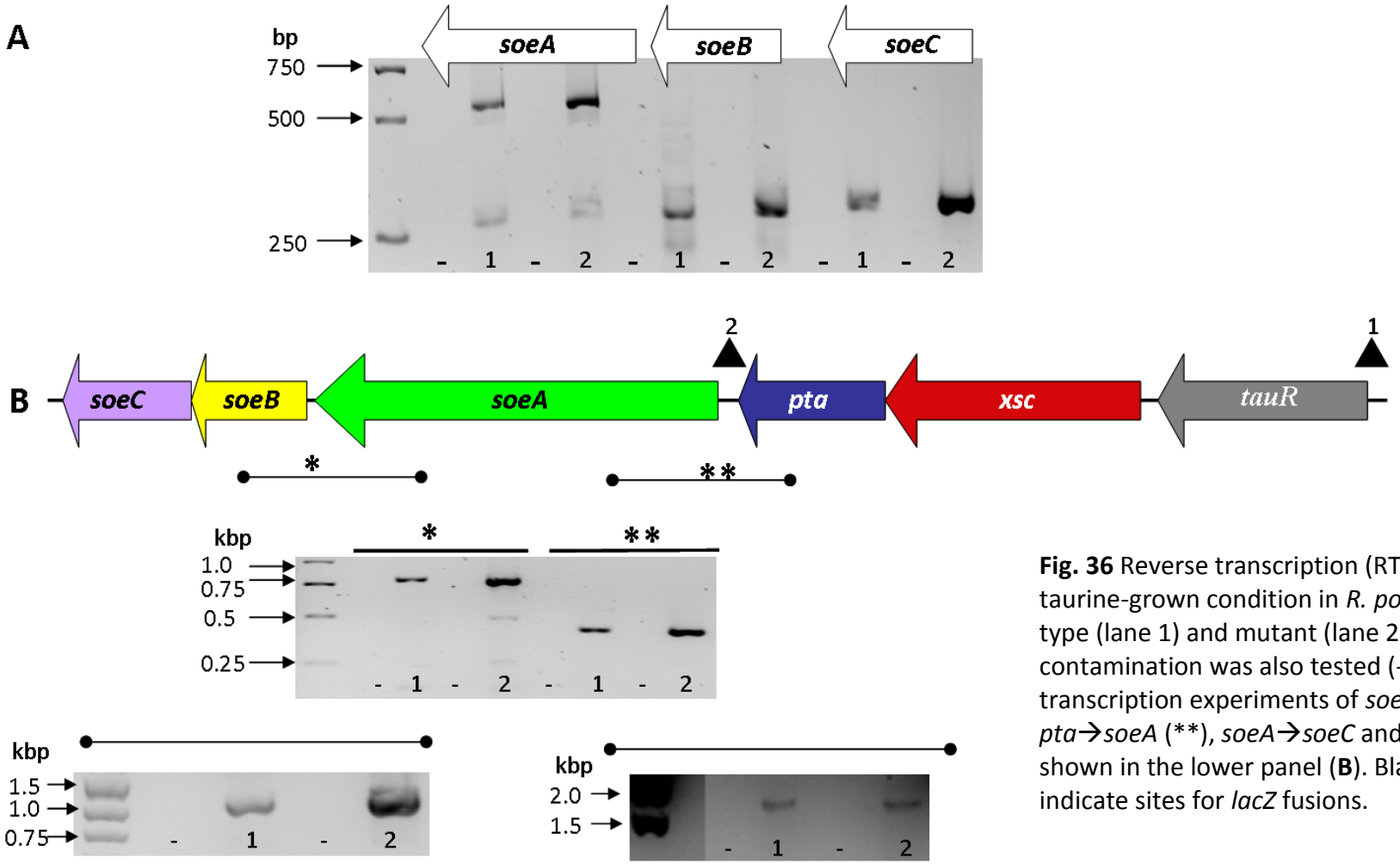


Fig. 36 Reverse transcription (RT)-PCR data under taurine-grown condition in *R. pomeroyi* DSS-3 wild type (lane 1) and mutant (lane 2) strain. DNA contamination was also tested (-) (**A**). Co-transcription experiments of *soeA*→*soeB* (*), *pta*→*soeA* (**), *soeA*→*soeC* and *xsc*→ *soeA* are shown in the lower panel (**B**). Black triangles indicate sites for *lacZ* fusions.

In order to further investigate whether SAA is the inductor for *tauR*, cysteate (10 mM)-grown *R. pomeroyi* DSS-3 cultures were supplemented with different amounts of SAA (0, 1, 5 and 10 mM). As controls, taurine (10 mM)-grown cultures as well as cultures without any carbon source, were also supplemented with SAA (5 and 10 mM). Interestingly, no effect of SAA was observed: cysteate-grown cultures excreted sulfite as shown in Fig. 11, taurine-grown cultures grew as described in chapter 3.1.1, and SAA-inoculated cultures did not grow at all. Since SAA is not available commercially, it was synthesized as described by Denger *et al.*. The synthetic material (bisulphite addition complex of 2-sulfoacetaldehyde dihydrate) was 45 % pure according to elemental analysis and was directly used in the experiments. It therefore might be possible that the impurities in the synthetic SAA preparation had negative effects on the growth of *Ruegeria pomeroyi* DSS-3.

In order to supply SAA intracellularily as inducer to the cells, a mixture of taurine (1 mM), which is metabolised to SAA, and cysteate (10 mM) was added to *R. pomeroyi* DSS-3, and the growth was monitored (Fig. 37 A). After taurine had been completely utilized, a rapid consumption of cysteate (about 4.7 mM cysteate within approximatly 20.5 h) was detectable while the sulfonate moiety was excreted mainly in the form of sulfate (75%). Interestingly, the 10 mM cysteate were completely utilized although it had appeared in previous experiments that more than 4 mM cysteate cannot be metabolised (chapter 3.1.7). These observations imply that SoeABC is indeed induced by SAA, and that enzymatic sulfite oxidation occured. An astonishing observation was that cysteate degradation ceased (for approximately 1.5 h) and continued decelerated in time with a slower degradation rate (about 5.5 mM cysteate within approximately 49 h), while sulfite was temporarily excreted. The additional sulfate detectable in the medium presumably resulted from autoxidation of sulfite.

These results led to the assumption that the induction of the *soeABC* genes is to short: only 1 mM taurine was used. To answer the question whether the induction of *soeABC* is longer with a higher concentration of taurine and therefore, cysteate consumption is also rapid while the sulfonate moiety is excreted as sulfate, different taurine:cysteate ratios were taken. Therefore, *R. pomeroyi* DSS-3 cells were inoculated with 10 mM cysteate supplemented with up to 10 mM taurine. The results revealed that with a ratio higher than 1:3 taurine:cysteate, the sulfonate group was excreted only as sulfate, whereas no sulfite was detectable (data not shown).

A representative growth curve of *Ruegeria pomeroyi* DSS-3 with 7.5 mM taurine and 5.1 mM cysteate is shown in Fig. 36 B. As expected, taurine was completely utilized in a first phase of growth, and subsequently, cysteate degradation followed. While the cleaved sulfonate moiety is excreted as sulfate, only traces of sulfite were measured.

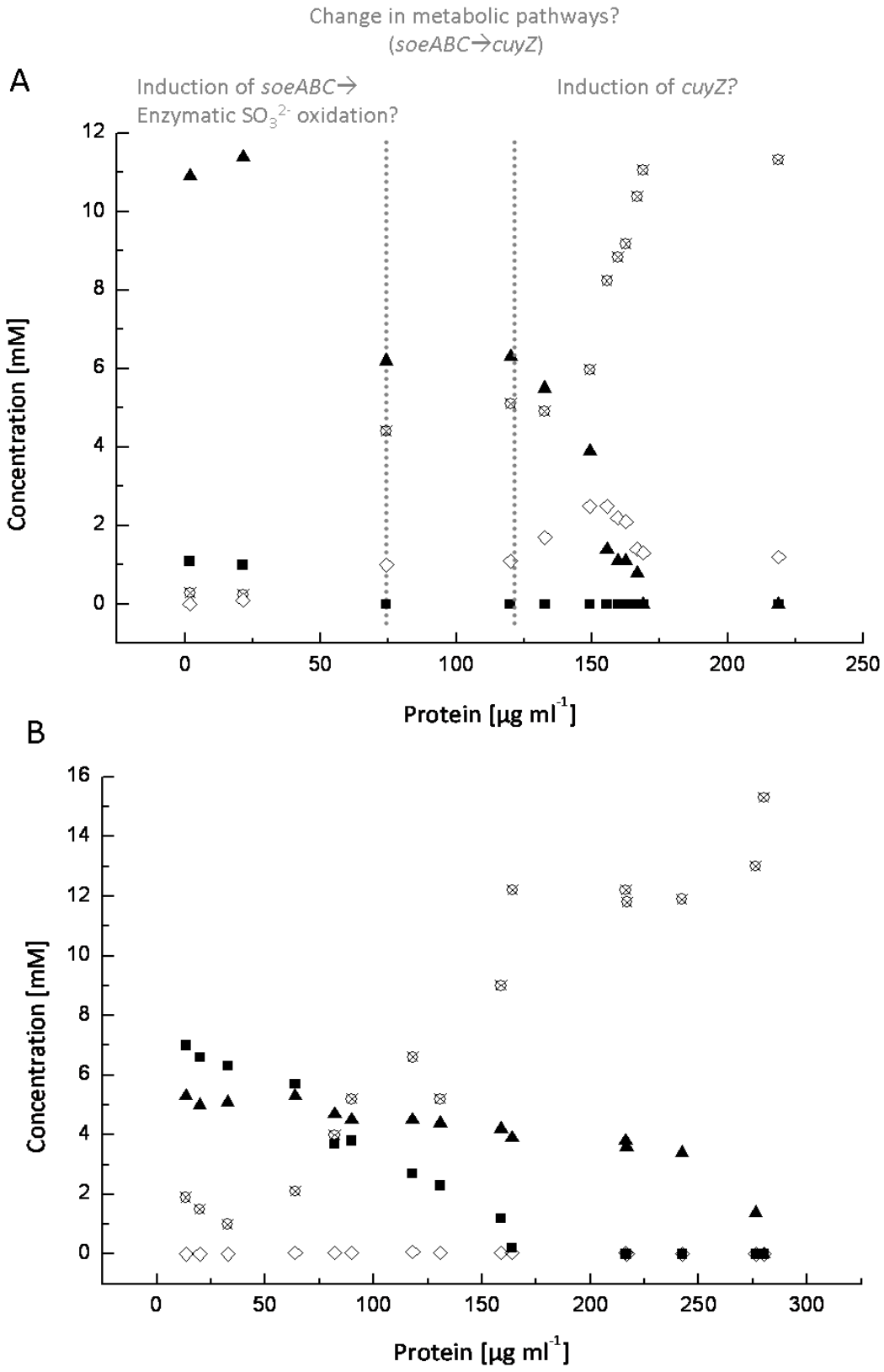


Fig. 37 Cysteate-grown *R. pomeroyi* DSS-3 cells were supplemented with taurine. Growth curve for cysteate:taurine 10 mM:1 mM (**A**). Growth curve for cysteate:taurine 5.1 mM:7.5 mM (**B**). Legend: ▲, cysteate; ■, taurine; ◊, sulfite; ⊠, sulfate.

3.3.6. Phylogenetic analysis

SoeA is predicted to belong to the enzyme class of anaerobic dimethylsulfoxide (DMSO) reductases [EC 1.8.99.-]. To obtain a better understanding of the relationship of SoeA (SPO3559) and of other members of the DMSO reductase family, a phylogenetic analysis was carried out. Therefore, homologous protein sequences were retrieved from the NCBI database using the BLAST algorithm (Altschul *et al.*, 1997). In order to simplify further analyses by reducing the size of the dataset, only one representative per bacetrial species was used. The sequences were aligned using ClustalX. The sequences of sulfite dehydrogenases SorA from *Starkeya novella* DSM 506 and *Cupriavidus necator* H16, and of SorT from *Sinorhizobium meliloti* 1021 and *Delftia acidovorans* SPH-1 were used as outgroup. The phylogenetic trees generated from the alignment of SoeA within the DMSO reductase family enzymes are shown in Fig. 38, as well as in the supplementary data (Fig. S1 - Fig. S9).

3.3.6.1. SoeA, PrsA and Tsr

Interestingly, a tight monophyletic clade, which is clustered in one branch, is comprised by SoeA (sulfite-oxidizing enzyme) and PsrA (poly-sulfide reductase) (Fig. 38). PsrA enzymes are required for the activity of sulfide oxidation (Krafft *et al.*, 1995). A comparison of SoeA (*Ruegeria pomeroyi* DSS-3) and PsrA (*Allochromatium vinosum* DSM 180) revealed 50% sequence identity (query coverage: 97%). The close relationship of SoeA and PsrA is also indicated by the fact that the blast results of SoeA and PsrA revealed the same protein in *Ectothiorhodospira* sp. PHS-1 (blue label in Fig. 38). SoeA and PsrA sequences were identified in α-, β- and γ-Proteobacteria, whereas many species containing enzymes homologous to SoeA belong to the α-Proteobacteria, especially to the order *Rhodobacterales* (more than 65%) and to *Rhizobiales*. PsrA sequences were identified especially in the order *Burkholderia* and in the order *Chromatiales* (Fig. S1).

Based on this relatively narrow taxonomic distribution, it is tempting to speculate if the entire group of SoeA and PsrA enzymes arose at a late stage on the micobial evolutionary timescale.

Tsr (tetrathionate reductase) is another protein belonging to the DMSO reductase family (see Fig. S2). These proteins catalyze the reduction of tetrathionate to thiosulfate, which is reduced to sulfide and sulfite by thiosulfate reductase (Hensel *et al.*, 1999). More than 55%

of the Tsr sequences were found in γ-Proteobacteria, but also in α-, β- and ε-Proteobacteria as well as in *Aquificales*, Firmicutes and *Chrysiogenetes*. Based on the taxonomic range, it is likely that the Tsr enzymes architecture arose early in evolution.

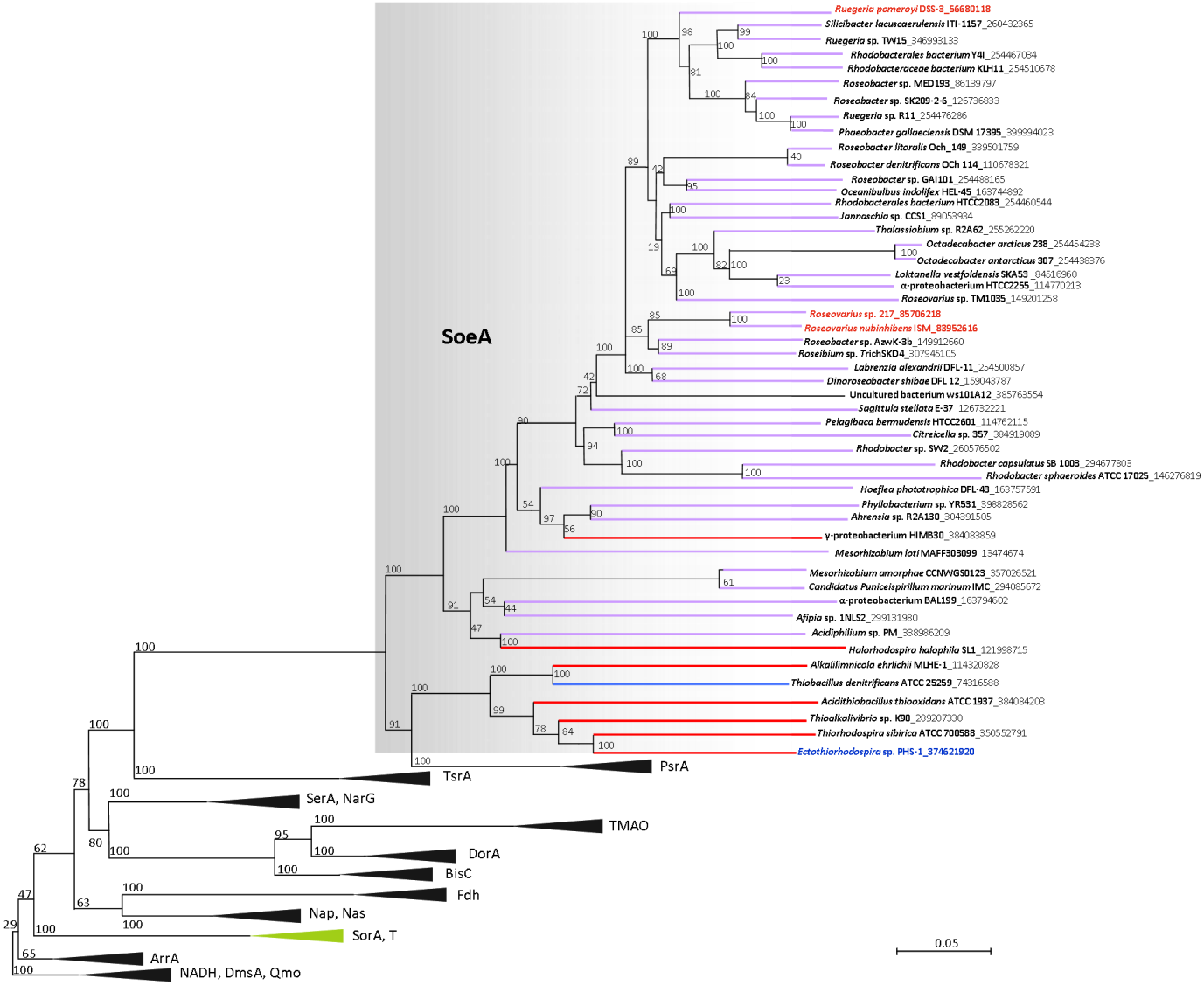


Fig. 38 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. The bar represents 5% sequence divergence. Bootstrap values were calculated from 100 trials. For sequence identification GI numbers (NCBI) are given. The bacteria used in this study are written in red. Purple lines indicate α-, blue lines β-and red lines indicate γ-Proteobacteria. Abbreviations and further explanations: see main text and addendum.

3.3.6.2. SerA, NarG, TMAO, DorA and BisC

Another clade within the DMSO reductase family comprises selenate reductase (SerA) and the membrane-bound respiratory nitrate reductase (NarG) (Fig S3). Unlike SerA, NarG lacks an N-terminal *TAT*-leader peptide, indicating that SerA is exported through the cytoplasmic membrane. Overall, the SerA and NarG group have a high phylogenetic diversity, as they occur in a wide range of organisms of the bacterial and archeal domains of life. The broad distribution of this group is likely a reflection of the early evolutionary emergence of nitrate and selenate respiration.

Another tight monophyletic clade is shown in Fig. S4 and Fig. **S5**, which comprises the respiratory periplasmic DMSO reductase, trimethylamine-N-oxide-reductase (DorA and TMAO), and the cytoplasmic biotin-sulfoxide reductase (BisC). These enzymes are monomeric proteins containing only the catalytic MPT subunit. These sequences are exclusively represented in members of the Proteobacteria, with the exception of the TMAO family occuring in *Deinoccus* species. Based on this relatively narrow taxonomic distribution, it is tempting to speculate whether the entire group of DorA/TorA/BisC enzymes arose at a late stage on the microbial evolutionary timescale and within the Proteobacteria.

3.3.6.3. Fdh, NasA and NapA

The formate dehydrogenases (Fdh), assimilatory cytoplasmic nitrate reductase (NasA), and periplasmic nitrate reductase (NapA), comprise a monophyletic clade (Fig. S6 andFig. S7) in this tree, indicating a common ancestor to this much broader group of multimeric proteins. Within this clade, the NasA and NapA proteins are also monophyletic (Fig. S7). Most of the sequences of the Fdh family were found in Proteobacteria, but also in Firmicutes, *Aquificales* and in *Deferribacter* species. Members of the nitrate reductase family were also identified predominantly within the Proteobacteria, and in some Actinobacteria. In contrast to NapA, the cytoplasmic nitrate reductases are monomeric proteins as described for TMAO, DorA and BisC.

3.3.6.4. Outgroups: SorA, SorT, ArrA, NADH, DmsA, and Qmo

As mentioned before, SorA and SorT were chosen as outgroup since these enzymes also belong to the Moco-containing superfamily of enzymes - but to the sulfite oxidase subclass. As expected, these proteins form a single clade while they are more closely related to the enzymes described in previous chapters (chapter 3.3.6.1-3.3.6.3).

All sequences identified as ArrA occurred in Proteobacteria (Fig. S8). Proteins which are identified as NADH, DmsA and Qmo comprise a monophyletic clade, whereas Qmo is forming a single clade. It is noticable that DmsA proteins only occur in γ-Proteobacteria, sequences identified as Qmo occur in many Firmicutes, Chlorobi and Nitrospira.

3.3.7. Discussion

Many genome-sequenced bacteria which are known to be able to degrade organosulfonates do not exhibit any homologues for *sorA* or *sorT* sulfite dehydrogenase genes, but instead harbour a three-gene cluster (*soeABC*) which could be involved in the detoxification of sulfite to sulfate. The hypothesis about the function and location of SoeABC in a bacterial cell is depicted in Fig. 29.

First evidence supporting an involvement of SoeA in taurine degradation was obtained by transcriptional analyses (Fig. 30 A) and proteomic analyses (chapter 3.2.1, Table 10), which both indicated that SoeA is specifically expressed during growth with taurine in *R. pomeroyi* DSS-3, *Roseovarius* sp. strain 217 and *R. nubinhibens* ISM. It remains unclear why there was a basal transcription in cysteate- and acetate-grown cells since the operon can be regarded to be tightly regulated by TauR, a GntR-like regulator. An involvement of SoeB or SoeC in sulfite detoxification could not be confirmed by Orbitrap analyses, although it could be shown that these genes are co-transcribed under taurine-grown conditions with *soeA* (Fig. 36).

For the heterologous overexpression of SorA, two strains were used for expression: *E. coli* BL21 DE3 and *E. coli* TP1000. Although different overexpression conditions have been tried, no significant sulfite dehydrogenase activity could be measured or enriched by activity-guided fractionation. Although the *E. coli* TP1000 strain has been optimized for the overexpression of eukaryotic molybdoenzymes (Palmer *et al.*, 1996), the strain is apparently not able to overexpress the guanine-dinucleotide form of the cofactor since a kanamycin-resistance cassette has replaced the *mobA* and *mobB* genes which are required for the

synthesis of the molybdopterin guanine-dinucleotide (MGD) (Johnson *et al.*, 1991). It is assumed that SoeA exhibits a MGD-type cofactor-since SoeA belongs to the DMSO reductase family, it would not be surprising that no sulfite dehydrogenase activity could be found. Another possible explanation may be the oxygen-sensitive nature of the Moco and the [4Fe-4S] cluster in SoeA and SoeB (Nason *et al.*, 1971; Hänzelmann *et al.*, 2004). Further, it would be possible that SoeB and SoeC have been missing, since only SoeA was overexpressed.

The missing enzyme assay to detect SOE activity led to the construction of an insertional mutant which was deficient in a functional *soeA* gene. The characterization of the mutant confirmed a loss-of-growth for taurine and isethionate, indicating that *soeA* is essential for growth with these organosulfonates, most likely through loss-of-function to detoxify sulfite: isethionate and taurine are metabolized to sulfoacetaldeyhde (Gorzynska *et al.*, 2006b; Weinitschke *et al.*, 2010) which is further dissimilated to acetyl phosphate and intracellular sulfite (Fig. 3). In contrast, cysteate degradation was not affected in the mutant strain, which was not surprising since cysteate is degraded *via* an independent pathway involving different set of proteins (Fig. 3).

The physiological data led to the assumption that TauR (SPO3562) is a sulfoacetaldehyde (SAA)- rather than a taurine-induced regulator. An attempt to create a *lacZ-tauR* and *lacZ-soeA* fusion failed. Instead, co-transcription experiments were carried out using inter-genic RT-PCR. Indeed, not only the *soeABC* gene cluster is co-transcribed on a polycystronic mRNA, but also *pta-soeA* and *xsc-soeA*. These results strongly indicate that *tauR* acts also as regulator for *soeABC*.

Further proof that SAA is the inducer of the *xsc-pta-soeABC* operon was obtained when cysteate-growing cells were co-incubated with SAA. Under these conditions, it was expected that *soeABC* is induced and that sulfite generated *via* cysteate degradation is also oxidized to sulfate and excreted into the medium. However, no effect was observed when exogenously supplied SAA was used maybe due to the impurity of SAA (contaminated with sodium sulfate). Therefore, endogenously supplied SAA (*via* the addition of 1 mM taurine) was added to cysteate (10 mM)-grown *R. pomeroyi* DSS-3 cells. After taurine was completely ultilized, cysteate degradation followed while the sulfonate moitety was excreted as sulfate indicating enzymatic sulfite oxidation, most likely by SoeABC. However, after approximately 4.7 mM cysteate were utilized growth and consumption stopped. It would be reasonable that a change in the metabolic pathways of *R. pomeroyi* DSS-3 occurred (visible in the stop of

growth and cysteate degradation): for example induction of the *cuyAZ* genes which are located on the megaplasmid. After 1.5 h cysteate degradation started again while transiently formed sulfite could be detected in the growth medium. During this phase it would be conceivable that *soeABC* genes are not induced any longer and therefore CuyZ exported sulfite (Fig. 37).

The sequence alignments and phylogenetic analyses further strengthened the hypothesis that SoeABC is a novel type of bacterial sulfite dehydrogenase. The phylogenetic trees clearly indicated that SoeA sequences clustered in the same clade as the polysulfide reductase (PsrA) sequences. The latter enzyme is not soluble but part of a membrane-bound complex (PsrABC)-just as SoeA. In contrast to SoeA, PsrA proteins exhibit a *TAT*-leader peptide for active transport of the folded protein to a periplasmic localization, a distinctive feature within the DMSO reductase family which determines the direction of electron transfer. In a recent publication (Frigaard & Bryant, 2008; Gregersen *et al.*, 2011) about the oxidative sulfur metabolism in green sulfur bacteria, the authors describe a protein complex: PsrLC (polysulfide reductase-like complex). The genes for PsrLC are located in a cluster with genes that encode for the formation of sulfite, such as the *dsr* genes for dissimilatory sulfite reductase complex. Therefore, the authors assumed that this protein complex could be a putative sulfite dehydrogenase in some green sulfur bacteria. The protein sequence identities for PsrLC to PsrA (from *Wolinella succinogenes*) and to SoeA (from *R. pomeroyi* DSS-3) are approximately 23% (query coverage: 46%) and 50% (query coverage: 50%), respectively. Due to the tight phylogenetic identity of SoeABC and PsrLC one might could conclude that subunit SoeC acts rather as electron mediator for the respiratory chain than as sulfate transporter as it would be for PsrLC. Further, these findings strengthen the hypothesis that SoeA acts as sulfite dehydrogenase especially in the order *Rhodobacterales*.

The findings described above support the hypothesis that SoeA, a molybdopterin-binding protein, is involved in taurine- and isethionate-degradation in *R. pomeroyi* DSS-3, and that it presumably acts as sulfite dehydrogenase, which is induced by SAA.

4. Conclusion and Outlook

Organosulfonates are widespread and ubiquitous compounds of natural and anthropogenic origin which are classified as *N*-, *O*- and *C*-organosulfonates. The first two mentioned classes of organosulfonates can be easily hydrolyzed, whereas the latter one is highly stable, and only bacteria and fungi are able to cleave the *C*-sulfonate bond in a so-called desulfonation reaction. The product of desulfonation is sulfite, a strong nucleophile and reducing agent, which can cause various reactions within the cell, such as sulfitolysis of proteins or modification of DNA. Therefore, sulfite has to be detoxified by oxidation catalyzed by sulfite dehydrogenases (SDHs). Up to date, only two bacterial SDHs are known and have been partially characterized: SorA and SorT. However, about 50% of the organisms which are known to degrade organosulfonates do not share any sequence homologies to SorA and SorT, leading to the assumption that there are more yet unknown SDHs in desulfonating organisms.

Three different bacterial strains were used as model organisms to identify and characterize novel SDHs: *Ruegeria pomeroyi* DSS-3, *Roseovarius* sp. strain 217, and *Roseovarius nubinhibens* ISM. All three strains belong to the *Roseobacter clade* which comprises a group of marine α-Proteobacteria within the order *Rhodobacterales*. All of them are capable of degrading various organosulfonates *via* well-characterized pathways. The sulfonate moiety is excreted as sulfate during desulfonation, with the only exception being cysteate degradation in *R*. *pomeroyi* DSS-3.

Roseovarius sp. strain 217 does not share any sequence homologies to SorA or SorT, although in taurine-grown cells an inducible SDH activity was measured. The activity was located in the membrane and seems to be tightly bound to it. Enzyme activity was determined *via* the direct quantification of sulfite and sulfate concentration and spectrometrically using ferricyanide as electron acceptor (Reichenbecher *et al.,* 1999). The obtained values were in the same range (spectrometric enzyme assay: 5.7 mkat (kg of protein)$^{-1}$, SO_3^{2-}/SO_4^{2-} determination: 4.4 mkat (kg of protein)$^{-1}$). Therefore, it can be concluded that both methods are suitable and reliable for SDH activity measurements. Interestingly, no significant SDH activities were determined in *R. nubinhibens* ISM and *R. pomeroyi* DSS-3 under any growth conditions using the enzyme assay described by

Reichenbecher and colleagues. However, significant SDH activities were obtained by determination of sulfite and sulfate concentration leading to the assumption that the enzyme is also located in the membrane. Moreover, Clark-type oxygen electrode experiments revealed significant SDH activities in *R. nubinhibens* ISM in taurine-grown cells. A similar, specific activity using this method was obtained for *Sinorhizobium meliloti* cells, the representative for SorT. *R. nubinhibens* ISM shares sequence identities to the soluble and periplasmic SorT and earlier work provided circumstantial evidence that growth on homotaurine will lead to inducible expression of two SorT-type SDH isoenzymes (Mayer *et al.*, submission pending) even though no SDH activity was detectable (Denger *et al.,* 2009) which was in accordance with the observations in this study.

R. pomeroyi DSS-3 does not share any sequence homologies with SorA or SorT. Instead, an 11 kDa protein was inducibly expressed in cysteate-grown cells. It reduces ferricyanide at high SDH activities in the described enzyme assay by Reichenbecher and colleagues. It turned out that the 11 kDa protein is expressed in response to sulfite, which was generated during cysteate consumption.

The common feature of all three bacteria is that all of them share sequence homologies with a three-gene cluster which is located in close neighbourhood of genes encoding enzymes involved in desulfonation. Indeed, two thirds of the known organisms which are capable of degrading organosulfonates but which are lacking SorA and SorT possess these genes.

The obtained results relating to the 11 kDa protein as well as evidence for an involvement of SoeABC in desulfonation are summarized in the following two subchapters and are illustrated in Fig. 39, whereas new findings are highlighted by red arrows.

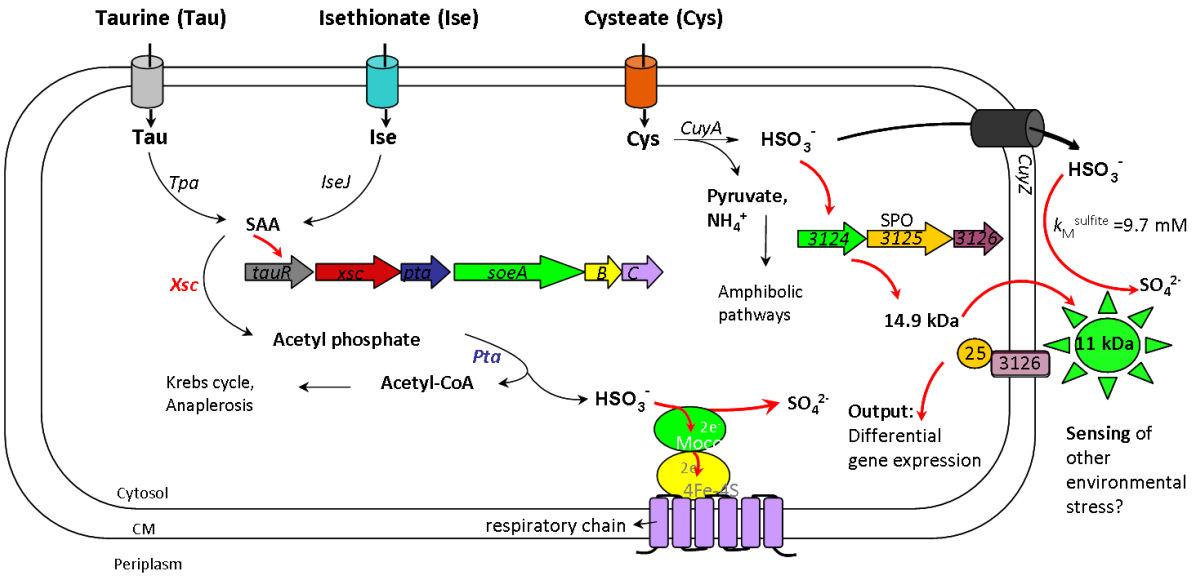


Fig. 39 Overview about the degradation pathways of taurine, isethionate and cysteate in *Ruegeria pomeroyi* DSS-3. Depicted is the import of the organosulfonates into the cytoplasm. The corresponding enzymes are written in italics, intermediates in bold. The *soeABC* gene clusters as well as the upstream genes are also shown since it is assumed that all genes form one operon which is regulated by TauR which itself is induced by sulfoacetaldehyde (SAA). Red arrows indicate new findings in this work. More details are written in the main text of the sub-chapters. CM, cytoplasmic membrane; SAA, sulfoacetaldehyde. Abbreviations of enzymes are written in the addendum.

A sulfite stress-inducible 11 kDa protein in *Ruegeria pomeroyi* DSS-3

The utilization of cysteate was incomplete because the sulfonate moiety was excreted as sulfite, which has bacteriostatic effects on growth. Paradoxically, the highest SDH enzyme activities were measured in those cell extracts. The apparent $K_M^{sulfite}$ value of this activity was determined to be 9.7±1.8 mM, one order of magnitude higher compared to other SDHs. The enrichment and purification yielded an 11 kDa protein (SPO3124) which is a soluble homotrimer (33 kDa) located in the periplasm. It could be shown that sulfite is the inducer for the corresponding gene cluster which comprises three genes: SPO3124 encoding the 11 kDa protein, SPO3125 encoding a cytoplasmic RNA polymerase σ-factor, and SPO3126 encoding a membrane-spanning anti σ-factor. Based on the obtained results and phylogenetic analyses, it is tempting to speculate that the 11 kDa protein acts as a sensor protein in the periplasm. Therefore, it might be possible that the sensory domain of the 11 kDa protein could be a periplasmic sensor, not just for sulfite but also for other forms of environmental stress. The transmitter domain could then interact with the receiver domain

of SPO3126, which is reported to act rather as transmembrane activator than as an anti σ-factor. The receiver domain of this transmembrane spanning protein could transmit the signal into the cytoplasm to the regulator domain of SPO3125, which could finally lead to a differential gene expression of the organism. However, much more evidence is required to confirm this function of a novel type of sensor protein acting in the two-component-signal transduction pathway.

A novel sulfite dehydrogenase in the order *Rhodobacterales*: SoeABC

Two thirds of desulfonating organisms which do not possess sequence homologues to SorA or SorT exhibit a three-gene cluster which was termed SoeABC and which is thought to encode a novel type of SDH, especially in the order *Rhodobacterales*. This gene cluster is in close neighbourhood, if not even in one operon, to genes which encode desulfonating enzymes. The gene cluster encodes proteins which are annotated as molybdopterin containing oxidoreductase (SoeA), as a [4Fe-4S]-cluster binding protein (SoeB), and as the C-subunit of the DMSO reductase (SoeC) which is predicted to exhibit six transmembrane helixes. Based on these bioinformatic data it was assumed that this gene cluster could be a novel type of bacterial SDH.

Several lines of evidence support the notion that SoeABC plays an important role in desulfonation reactions in the order of *Rhodobacterales*, most likely as a novel type of SDH.

The first hint of an involvement of SoeA in sulfite oxidation was that SoeA was found to be inducibly expressed in taurine-grown *R. pomeroyi* DSS-3 cells. However, an appropriate enzyme assay was missing, which would have allowed to measure and enrich the enzyme activity. Therefore, genetic techniques were used to confirm the predicted functions of *soeABC*. It was surprising that the *soeA*-deficient *R. pomeroyi* DSS-3 mutant was defective in growth with taurine, since the presence of functional *xsc* and *pta* should generate the necessary acetyl-CoA. Moreover, the mutant was also unable to grow with isethionate although the corresponding genes are located somewhere else in the genome but not close to *soeABC*. This led to the hypothesis that sulfoacetaldehyde (SAA), which is an intermediate in the degradation of both of these organosulfonates could be an inducer for the TauR regulator which regulates the transcription of the *xsc*, *pta* and *soeABC* gene clusters. To confirm this, cysteate-grown cells were supplemented with traces of taurine leading to the formation of SAA in the cytoplasm. The results were rather astonishing because cysteate-

grown cells were now able to metabolize cysteate quantitatively while excreting the sulfonate moiety as sulfate, only traces of sulfite were detected. Transcriptional analyses confirmed this hypothesis since not just *soeABC* but also *xsc* and *pta* are co-transcribed under taurine-grown conditions.

The obtained results strongly suggest that SoeABC is a novel type of SDH, which would be the fourth type of bacterial SDH, with extraordinary features compared to known sulfite-oxidizing enzymes (SorA, SorT, and sulfite oxidases) such as the localization in the cytoplasm, the attachment or connection to the membrane, or the composition as a three-subunit enzyme complex. SoeABC plays an important role as detoxification enzyme in organosulfonate degradation, especially in the order of *Rhodobacterales*, but also in the oxidation of inorganic and organic sulfur compounds in the *Roseobacter* clade (Lenk *et al.,* 2012). A phylogenetic tree (Fig. 39) was generated with all known bacterial SDH in order to give a rough overview about the current knowledge of this structural highly diverse enzyme class. However, further research is necessary to investigate and characterize this novel type of SDH in the order *Rhodobacterales*. Especially functional evidence such as *lacZ* fusions (upstream of *tauR* and in the *pta-soeA* gene gap) is missing. Furthermore, it would be useful to obtain a *tauR* chromosomal mutant which might provide evidence about the regulation system of *xsc-pta-soeaABC*. RACE (5'-rapid amplification of cDNA ends) technique could be used in order to map the transcription start sites of *xsc/pta* and *soeABC.* For further investigation of the novel SDH (e. g. binding metals, electron transport chain), it would be useful to heterologously overexpress SoeABC or to purify the active enzyme. However, it must be considered that SoeA presumably interacts with SoeB and SoeC, and that it is just functional in the whole complex. To this end, all three genes would need to be overexpressed. Also importantly, suitable overexpression strains which are able to synthesize the Moco as a dinucleotide are necessary. Furthermore, it would be interesting to investigate the role of CuyZ, the sulfite exporter in *R. pomeroyi* DSS-3. If it was possible to clone *cuyZ* downstream of *xsc* using the *soeA*-deficient mutant, it would be conceivable that this mutant is able to grow with taurine or isethionate, contrasting with the *soeA* mutant, while excreting sulfite into the medium.

There is strong evidence that SoeABC is a novel bacterial SDH. However, not all organisms which lack sequence homologies to SorA and SorT share sequence identities to SoeABC. Therefore, one might conclude that there are more unknown SDHs in prokaryotes.

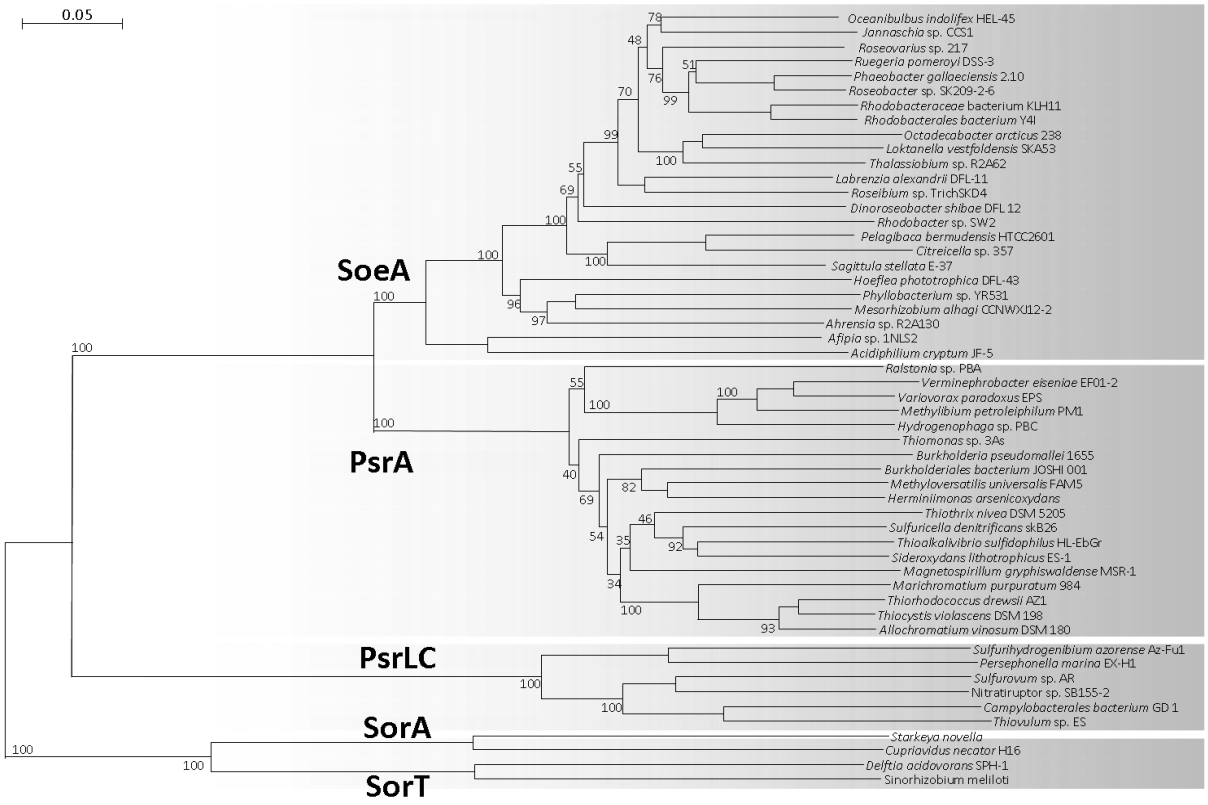


Fig. 39 Phylogenetic tree of bacterial sulfite dehydrogenases (SDHs). SorA and SorT are the best characterized SDHs to date. PsrLC is assumed to act as SDH in some green sulfur bacteria. This work is the first study yielding evidence for a novel SDH, SoeABC, in the order Rhodobacterales.

5. Appendix

5.1. Broader aspects of the overall projects

Further research, which is not included in this thesis:

Denger, K., S. Lehmann and A. M. Cook (2011). Molecular genetics and biochemistry of *N*-acetyltaurine degradation by *Cupriavidus necator* H16. *Microbiology* **157**: 2983-2991.

Lehmann, S., M. Serif and F. C. Küpper (2012). Sulfoglycolysis in marine algae.
Submission pending.

5.2. Abbreviations

ABC transporter	ATP-binding cassette transporter
Ack	acetate kinase
APAT	adenylylsulfate: phosphate adenylyltransferase
APS	homotaurine
APS’	adenosine 5’-phosphosulfate
ArrA	arsenite oxidase
ATP	adenosine 5’-triphosphate
BisC	biotin sulfoxide reductase
cDNA	complementary DNA
CHAPS	3 [(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CobST	cobaltochelatase
ComC	(*R*)-sulfolactate dehydrogenase
CuyA	(*R*)-cysteate sulfolyase
DCPIP	2,6-dichlorophenolindophenol
Dhps	2,3-dihydroxypropane-1-sulfonate
DLS	dynamic light scattering
DMS	dimethylsulfide
DMSP	dimethylsulfoniopropionate
DorA	dimethylsulfoxide reductase
Dsr	dissimilatory sulfite reductase
Duf	domain of unknown function
ECF	extracytoplasmic function
EDTA	ethylenediaminetetraacetic acid
Etf	electron transferring flavoprotein
FAD^+	flavin adenine dinucleotide
Fdh	formate dehydrogenase
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma-mass spectroscopy
IET	intramolecular electron transfer

IPTG	isopropyl-β-D-1-thiogalactopyranoside
IseJ	isethionate dehydrogenase
Kan	kanamycin
KP buffer	potassium phosphate buffer
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MGD	molybdopterin guanine dinucleotide
Moco	molybdopterin cofactor
MOSC	Moco sulfurase C-terminal domain
MPT	molybdopterin
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NADH	NADH quinone oxidoreductase
NapA	periplasmic nitrate reductase
NarG	membrane bound respiratory nitrate reductase
NasA	assimilatory nitrate reductase
OD	optical density
ONPG	*o*-nitrophenyl-ß-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate reductase
PCR	polymerase chain reaction
PIPES	1,4-Piperazinediethanesulfonic acid
PLP	pyridoxal phosphate
PF-MS	peptide fingerprinting-mass spectrometry
PrtR	protease temperature regulation
PSM	peptide spectrum matches
Psr	polysulfide reductase
Pta	phosphate acetyltransferase
Qmo	quinone reductase
RT-PCR	reverse-transcriptase-PCR
SAA	aulfoacetaldehyde
SauST	sulfoacetate utilization

SDH	sulfite dehydrogenase
SDS	sodium dodecylsulfate
SerA	selenate reductase
Sir	sulfite reductase
SlcC	(*S*)-sulfolactate dehydrogenase
SO	sulfite oxidase
SodFe	superoxide dismutase, Fe-binding
SOE	sulfite-oxidizing enzyme
SorA/SorT	sulfite acceptor oxidoreductase
Spec	spectinomycin
SuyAB	(*R*)-sulfolactate sulfo-lyase
Tat	twin-arginine targeting
ThDP	thiamine diphosphate
TMAO	trimethylamine-N-oxide
TPA	taurine:pyruvate aminotransferase
TRAP	tripartite ATP-independent periplasmic transporter
Tris	tris(hydroxymethyl)aminomethane
Tsr	tetrathionate reductase
Xsc	sulfoacetaldehyde acetyltransferase

5.3. Supplementary data

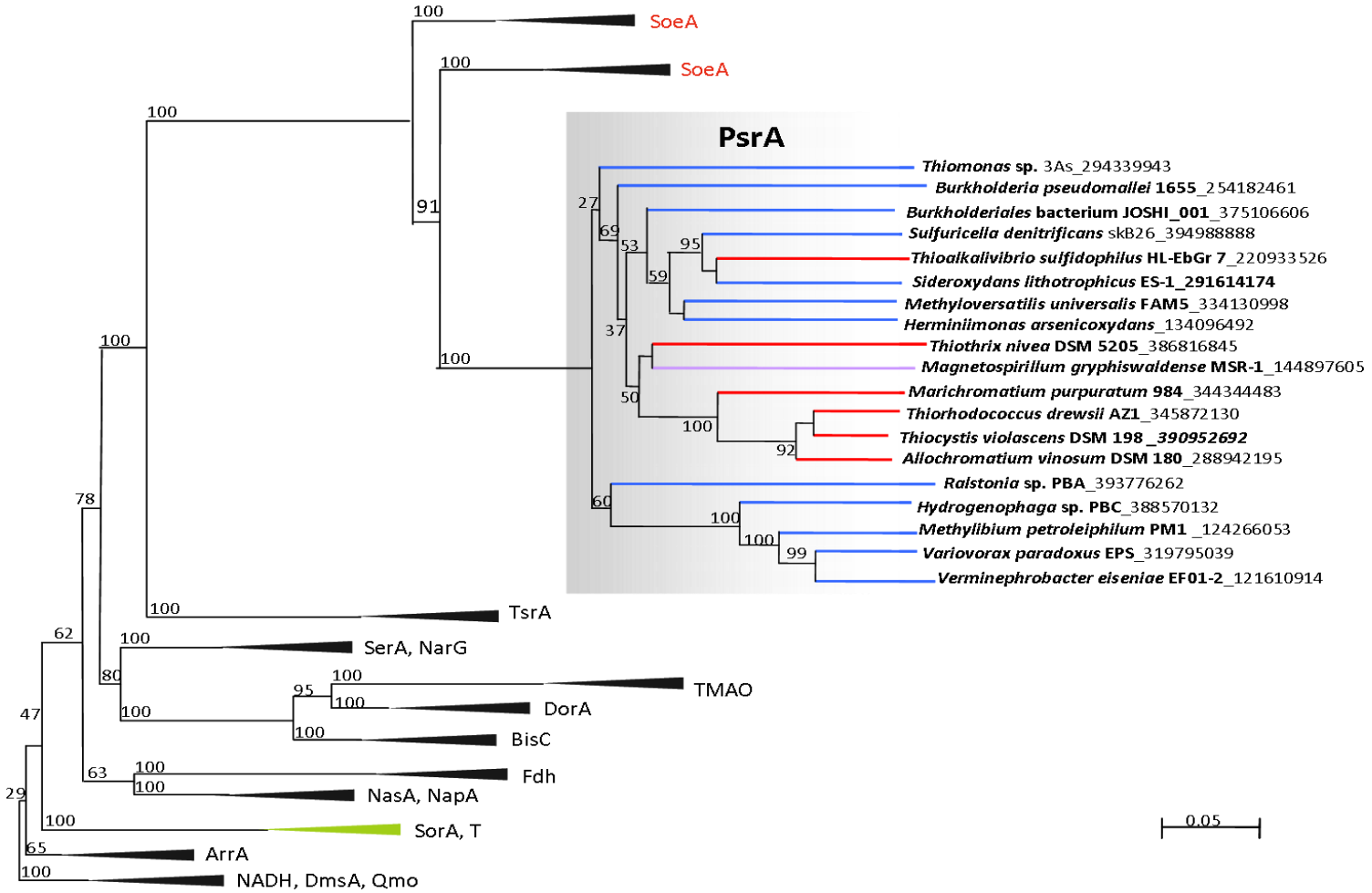


Fig. S1 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA & T and enzymes in the DMSO reductase family. Psr (polysulfide reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β- and red lines γ- Proteobacteria.

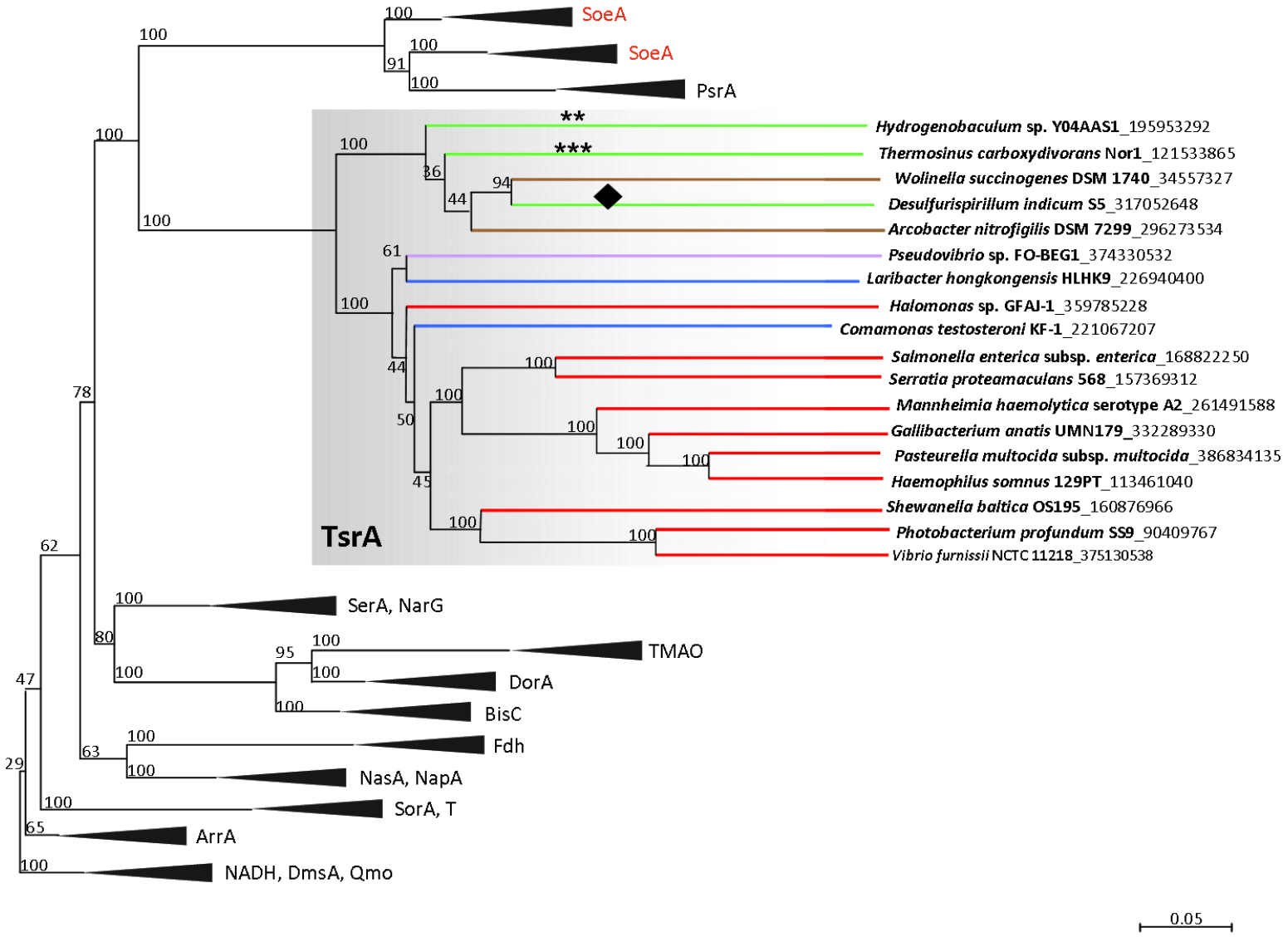


Fig. S2 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase reductase family. Tsr (tetrathionate reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β-, red lines γ- and brown lines ε-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols :

** = *Aquificales*

*** = Firmicutes

◆ = Chrysiogenetes

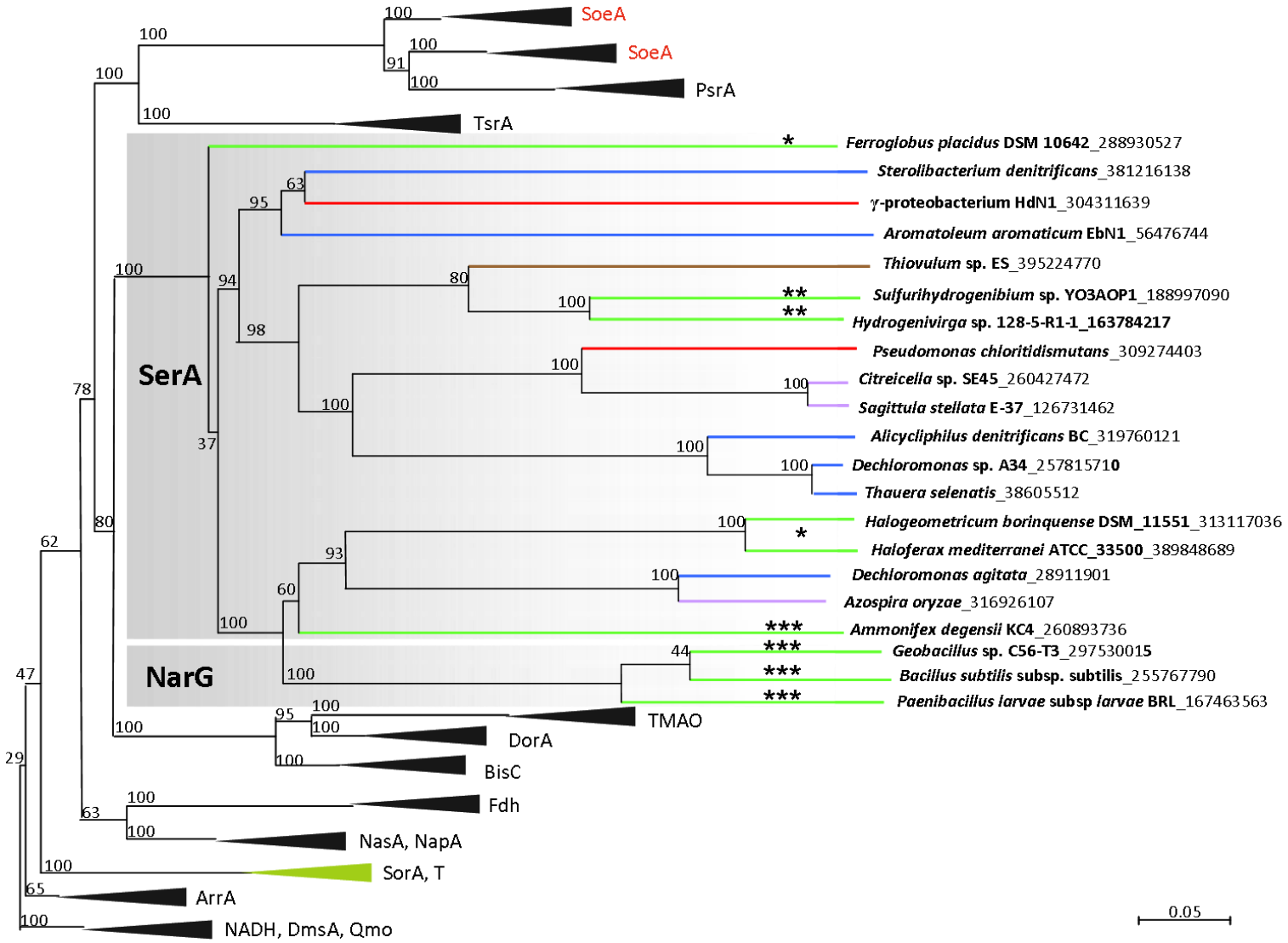


Fig S3 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. SerA (selenate reductase) and NarG (membrane bound respiratory nitrate reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β-, red lines γ- and brown lines ε-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols indicating the following taxonomie:
* = Archaea
** = *Aquificales*
*** = Firmicutes

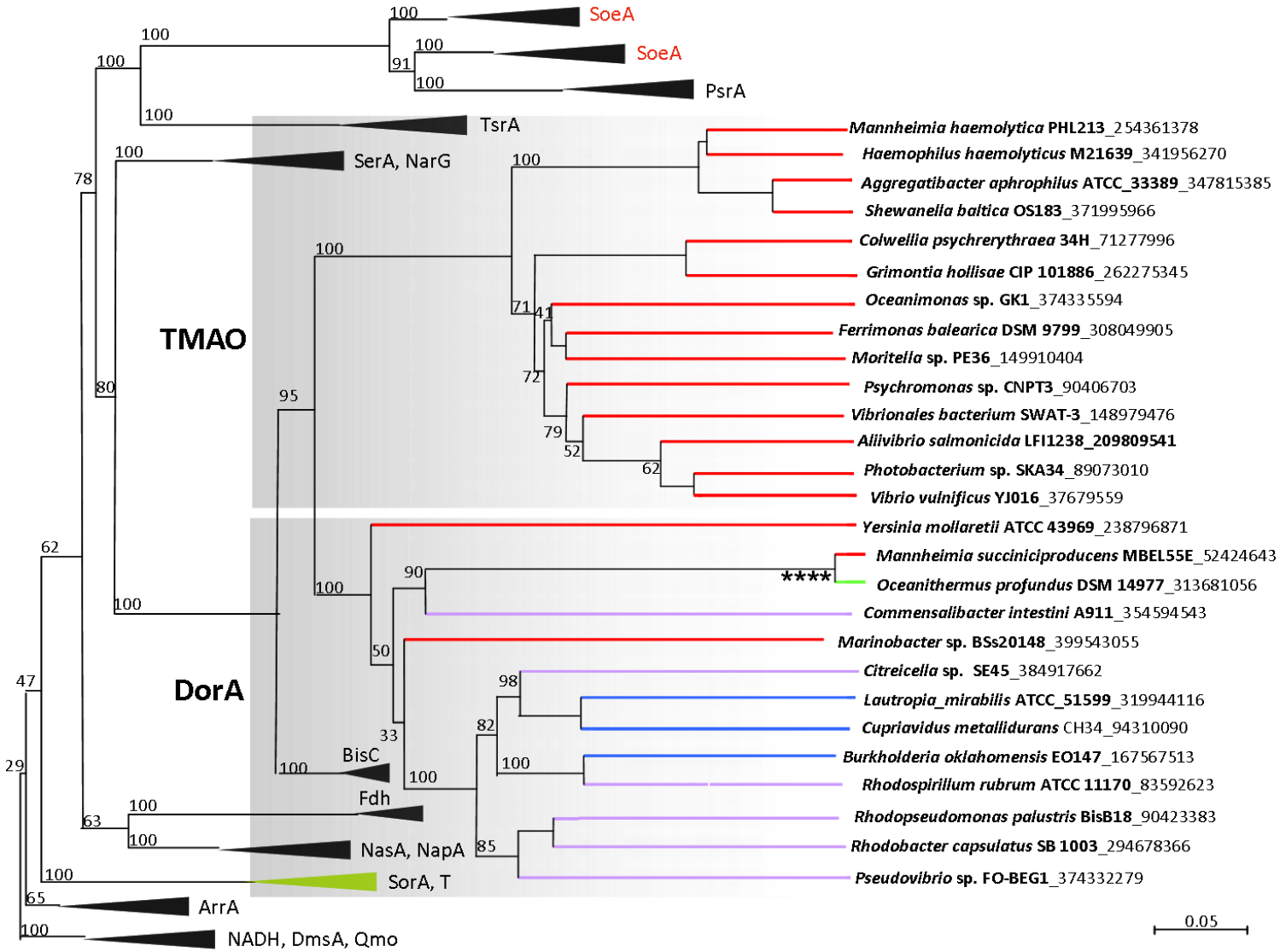


Fig. S4 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA & T and enzymes in the DMSO reductase family. TMAO (trimethylamine-N-oxide) and DorA (dimethylsulfoxide reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β- and red lines indicate γ-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols indicating the following taxonomie:
**** = *Deinoccocus*

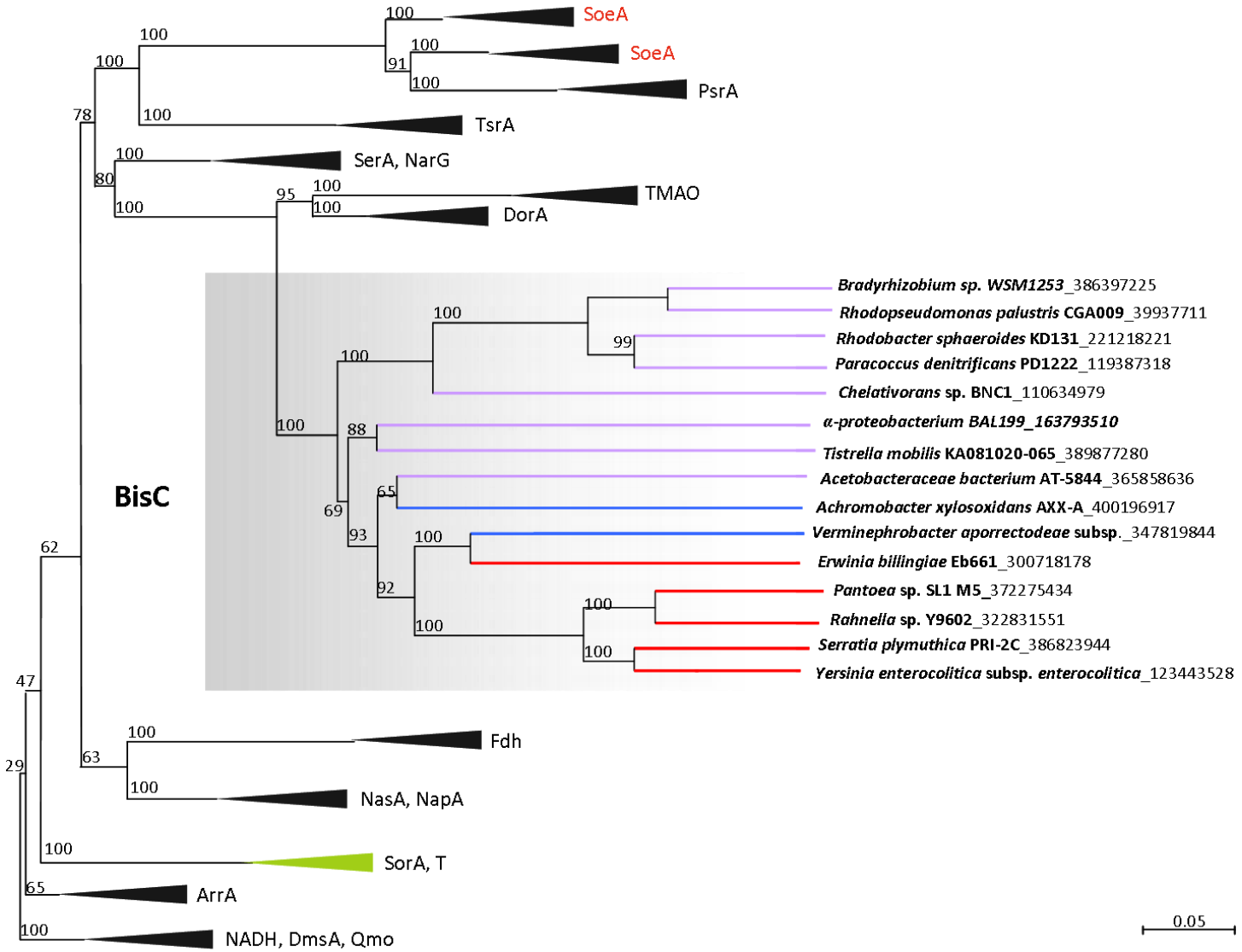


Fig. S5 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. BisC (biotin sulfoxide reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β- and red lines indicate γ-Proteobacteria.

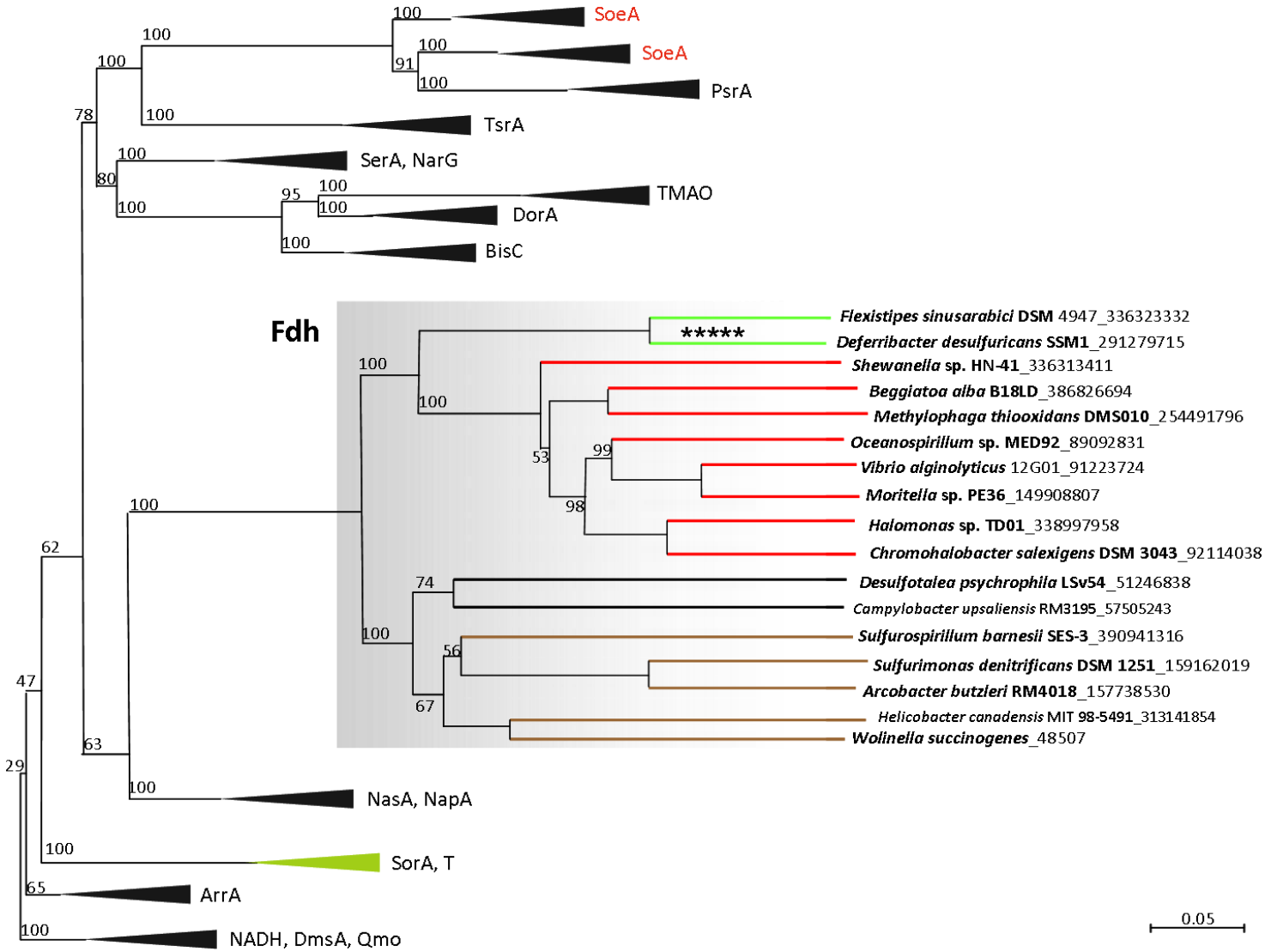


Fig. S6 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. Fdh (formate dehydrogenase) orthologues are shown in more detail. Red lines indicate γ- , black lines δ- and brown lines ε-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols indicating the following taxonomie: ***** = *Deferribacter*

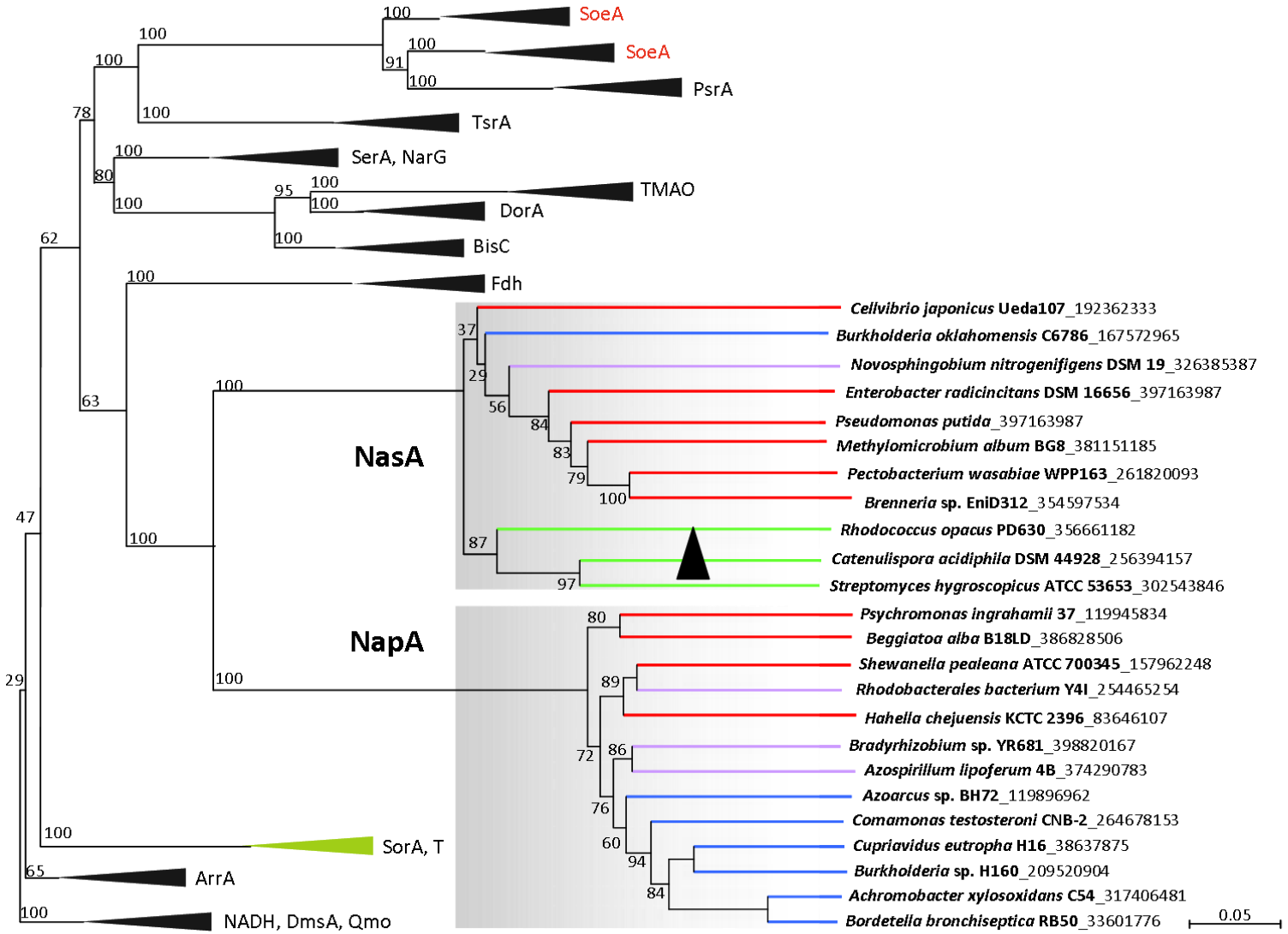


Fig. S7 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. NasA (assimilatory nitrate reductase) and NapA (periplasmic nitrate reductase) orthologues are shown in more detail. Purple lines indicate α-, blue lines β- and red lines γ-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols indicating the following taxonomie: ▲= Actinobacteria

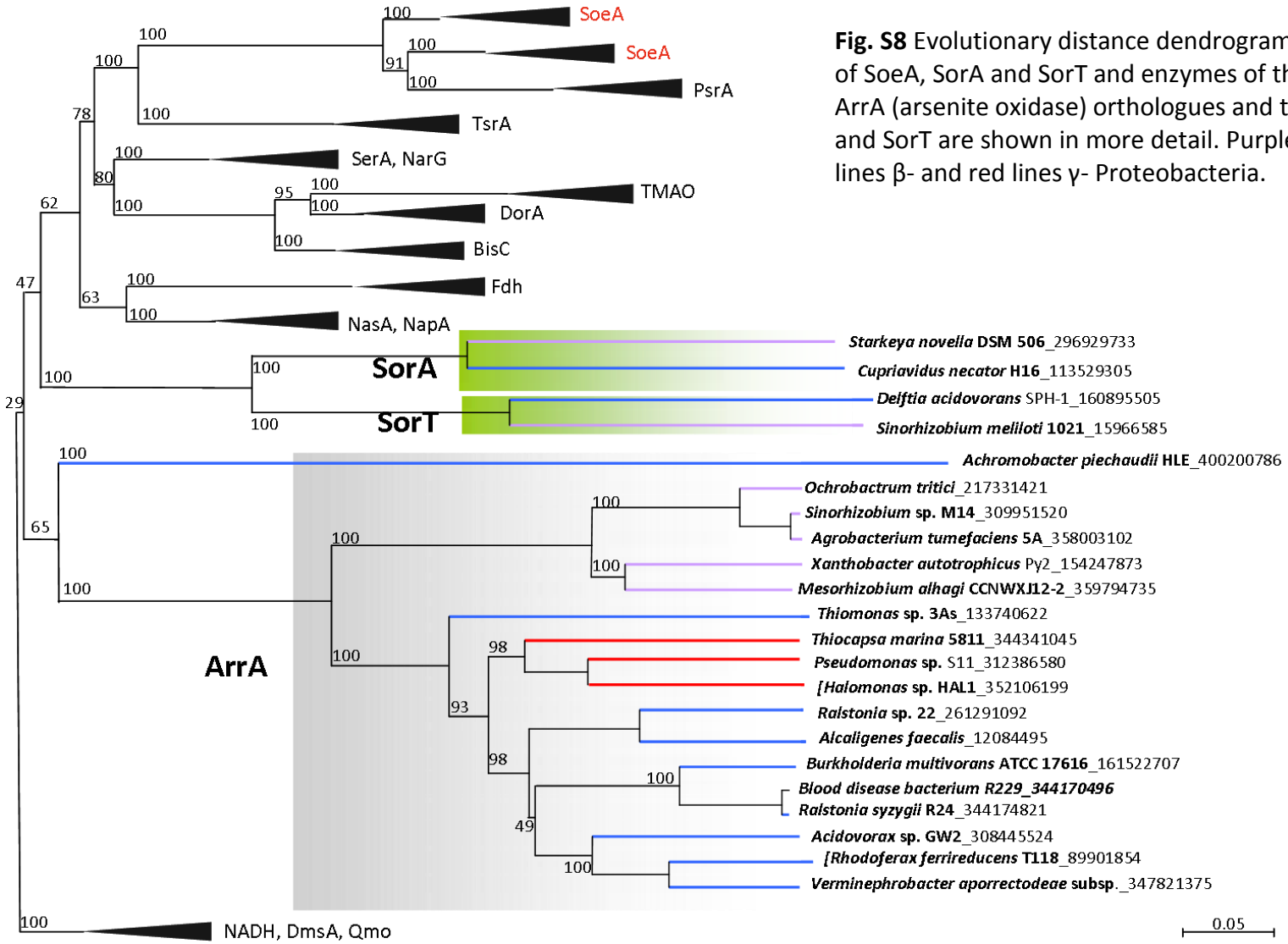


Fig. S8 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. ArrA (arsenite oxidase) orthologues and two representatives of SorA and SorT are shown in more detail. Purple lines indicate α-, blue lines β- and red lines γ- Proteobacteria.

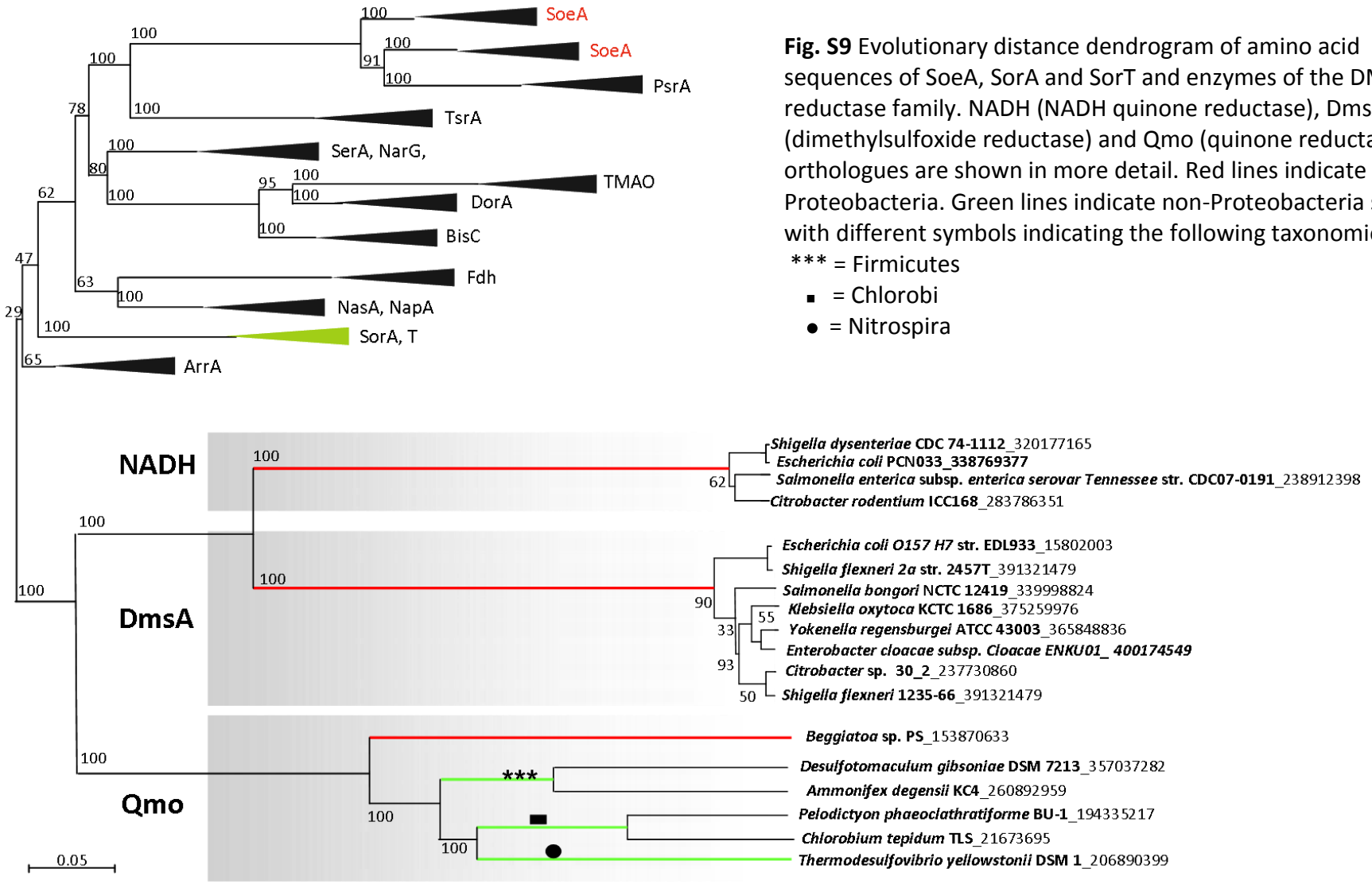


Fig. S9 Evolutionary distance dendrogram of amino acid sequences of SoeA, SorA and SorT and enzymes of the DMSO reductase family. NADH (NADH quinone reductase), DmsA (dimethylsulfoxide reductase) and Qmo (quinone reductase) orthologues are shown in more detail. Red lines indicate γ-Proteobacteria. Green lines indicate non-Proteobacteria signed with different symbols indicating the following taxonomie:

*** = Firmicutes

■ = Chlorobi

● = Nitrospira

5.4. Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.
Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Weitere Personen, insbesondere Promotionsberater, waren an der inhaltlich materiallen Erstellung dieser Arbeit nicht beteiligt. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Konstanz, im Februar 2013

(Sabine Lehmann)

5.5. Lebenslauf

Seit Februar 2009	Promotion in der Arbeitsgruppe von Prof. Dr. Alasdair M. Cook und Prof. Dr. Bernhard Schink, Universität Konstanz, über das Thema „Sulfite dehydrogenases in organothropic bacteria: enzymes, genes and regulation“
Okt. 2006 – Okt. 2008	Masterstudium im Fach Life Science an der Universität Konstanz, Abschluss: Master of Science Schwerpunkte: *In vitro*-Methoden zum Tierversuchsersatz, Molekulare Toxikologie, Mikrobielle Ökologie, Chemische Biologie von Kohlenhydraten, Chemie der Aromaten und Heterocyclen Masterarbeit in der Arbeitsgruppe von Prof. Dr. Alexander Bürkle, Universität Konstanz, über das Thema: „Konstruktion von chimären Maus und humanen PARP-1 Versionen und Optimierung ihrer rekombinanten Expression”
Mai 2006 – Okt. 2006	Praktikum am Klinikum Konstanz in der onkologischen Abteilung
Sept. 2005 - Apr. 2006	Praktikum bei ALTANA Pharma, Konstanz
Apr. 2003 – Okt. 2005	Bachelorstudium im Fach Life Science an der Universität Konstanz, Abschluss: Bachelor of Science Bachelorarbeit in der Arbeitsgruppe von Prof. Dr. Andreas Marx, Universität Konstanz, über das Thema: „Allelspezifische Primerverlängerungsstudien und Polymerasekettenreaktionen auf Glasoberflächen“
Okt. 2001 - März 2003	Chemiestudium an der Universität Konstanz
Sept. 1998 - Juni 2001	Allgemeine Hochschulreife am Ernährungswissenschaftlichen Gymnasium Balingen

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