Analysis of cytoplasmic sulfur trafficking during sulfur globule oxidation in *Allochromatium vinosum*

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Contents

Abbreviations

1,5-IAEDANS	*N*-(iodoacetyl)-*N*'-(5-sulfo-1-naphtyl)-ethylenediamine
aa	Amino acid(s)
Ap	Ampicillin
APS	Ammonium persulfate
bp	Base pair(s)
CIAP	Calf intestine alkaline phosphatase
CV	Column volume
Da	Dalton
ddH_20	Demineralized deionized water
dH_20	Demineralized water
dig	Digoxigenin
DMSO	DMSO dimethylsulfoxide
dNTP	d-nucleosidtriphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g	Gravitational constant
Hdr	Heterodisulfide reductase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-β-D-thiogalactoside
ISC	Iron sulfur cluster
kb	10^3 bases
Km	Kanamycin
Ni-NTA	Nickel-nitrilotriacetic acid
MWCO	Molecular weight cut off
OD	Optical density
p. a.	per analysis
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Rif	Rifampicin
rpm	Rounds per minute
Sm	Streptomycin
SDS	Dodium dodecyl sulfate
SRP	Surface Plasmon Resonance
SUF	Sulfur mobilization
qRT-PCR	Quantitative reverse transcriptase PCR
TCA	Trichloracetic acid
TCEP	Tris (2-carboxyethyl) phosphine
TEMED	N,N,N',N'-tetramethylendiamine
T_a	Annealing temperature
Tris	Trishydroxymethylaminomethane

Introduction

1. The biological sulfur cycle

Sulfur is universally abundant and essential for life. According to the iron–sulfur world theory sulfur was crucial for the origin of life itself (Wächtershäuser, 1988, 1992). The non-metallic element entered the earth's litho- , hydro- and biosphere in gaseous form by volcanic emission and as metal sulfides released by hydrothermal vents. Sulfur is highly versatile; in fact, sulfur is the element with the highest number of allotrops (Beinert, 2000; Steudel, 2000). As such, sulfur occurs in numerous oxidation states ranging from -2 (sulfide) to +6 (sulfate) and undergoes a constant cycle of oxidative and reductive processes. Sulfur is introduced into the biomass by assimilatory sulfate reduction, an energy consuming mechanism that is restricted to prokaryotes, fungi and plants. Sulfate is reduced to the state of sulfide, which is then incorporated into a stable organic molecule: L-cysteine. This amino acid is the central precursor for other sulfur containing biomolecules in the cell, i.e. proteins, cofactors, carbohydrates, sulfolipids, vitamins and antibiotics (Brüser *et al*., 2000; Dahl *et al*., 2002; Kessler, 2006). The use of inorganic sulfur compounds as electron donors or terminal electron acceptors for the purpose of energy conservation is referred to as dissimilatory sulfur metabolism and is restricted to prokaryotes. Sulfate reducing microorganisms are widely spread in anoxic habitats and contribute massively to the global sulfur and carbon cycles by coupling the degradation of organic matter with the reduction of sulfate in marine sediments. Sulfur oxidizing microorganisms use reduced inorganic sulfur compounds like sulfide and elemental sulfur as electron donors for energy conservation and autotrophic CO_2 fixation. The issued electrons are either fed into the respiratory chain by chemolithoautotrophic bacteria and archaea or can be used by a number of bacteria for anoxygenic photosynthesis. The dissimilatory sulfur oxidation in anoxygenic photosynthesis is best studied within the green sulfur bacteria (*Chlorobiaceae*) and the purple sulfur bacteria (*Chromatiaceae* and *Ectothiorhodospiraceae*) with the respective model organisms *Chlorobaculum tepidum* and *Allochromatium vinosum*. As an obligate intermediate of the oxidation of reduced sulfur compounds both groups form sulfur globules that are stored either extra- or intracellular. Intermediary stored sulfur is further oxidized to the final product sulfate. Sulfate is released from the cells and can serve as electron acceptor for the sulfate reducer.

2. Sulfurtransferases

The relevance of sulfur in biomolecules has long been acknowledged. In proteins sulfur is present in the side chains of the amino acids cysteine and methionine. The thiol group (-SH) in cysteine stabilizes proteins by the formation of an intra- or intermolecular disulfide bond with the thiol group of a second cysteine. It also acts as acid or base, as hydrogen donor or acceptor, metal ligand and catalytic nucleophile (Kessler, 2006).

The biosynthesis of L-cysteine is one of the very few reactions for which free sulfide is used as sulfur donor. The incorporation of sulfide into O-acetlyserine is catalyzed by O-acetylserine(thiol)-lyase under the release of acetate and results in the formation of L-cysteine. Though sulfide is a suitable donor for sulfur containing molecules *in vitro*, its toxicity and the requirement for a greater degree of regulation demand the delivery of sulfur in form of a "safer" sulfur species (Mueller, 2006). Accordingly, an activated form of sulfur, namely sulfane or persulfide sulfur (RS-SH / RS-S$^-$) is used for the biosynthesis of most sulfur containing compounds and originally derives from cysteine (Kessler, 2006; Mueller, 2006). Sulfane sulfur has a formal oxidation state of zero (S^0) and is defined as a divalent sulfur atom bonded only to other sulfur atoms, with exception of an ionisable hydrogen depending on the pH value. Apart from the outer sulfur atoms of persulfides and thiosulfate the pool of sulfane sulfur includes inner chain atoms of polysulfides, polythionates and all atoms of elemental sulfur (Westley & Westley, 1991). Low molecular weight persulfides are labile and quickly decompose into the corresponding thiol and elemental sulfur. The protected environment of an active site in enzymes can overcome this destabilization (Kessler, 2006). Proteins that catalyse the formation, conversion and reactions of compounds containing sulfane sulfur atoms are called sulfurtransferases (Westley *et al*., 1983). Sulfurtransferases contain redox-active cysteine residues with a low pK_a value that accepts and donates electrons easily. The neighbouring of positively charged amino acids, electrostatic interactions and a stabilized proton-dissociated state of these cysteine residues are considered to maintain the decreased pK_a value (Nagahara, 2010).The Enzyme Commission number EC 2.8.1 combines acknowledged sulfurtransferases and is to date divided into 12 classes. Enzyme classes relevant to this thesis are briefly introduced.

L-cysteine desulfurase

L-cysteine desulfurases (EC 2.8.1.7) initiate sulfur trafficking for the biosynthesis of numerous sulfur compounds by abstracting sulfane sulfur from L-cysteine. The sulfane sulfur is transiently bound to pyridoxal-5'-phosphate, the prosthetic group of cysteine desulfurases, before it is nucleophilically attacked by the conserved cysteine residue in the active site. Subsequently, sulfane sulfur is transferred to acceptor proteins for further transportation to the biosynthesis site (Zheng *et al*., 1994; Mihara & Esaki, 2002; Fontecave *et al*., 2008). NifS from nitrogen-fixing *Azotobacter vinelandii* was the first characterized cysteine desulfurase and is required for nitrogenase maturation (Zheng *et al*., 1993). The descriptions of IscS, SufS and CsdA from *E. coli* followed. IscS is the cysteine desulfurase for the housekeeping FeS cluster biosynthesis system (ISC), while the SUF system is active under stress conditions, e.g. heavy metal stress or iron limiting conditons (Zheng *et al*., 1998). IscS is also the sulfur-providing enzyme for the biosynthesis of thiamine and the thionucleosides 4-thiouridine and 5-methylaminomethyl-2-thiouridine (Lauhon & Kambampati, 2000; Lauhon, 2002; Takahshi & Tokumoto, 2002; Cupp-Vickery *et al*., 2003; Lee *et al*., 2004; Outten *et al*., 2004; Jang & Imlay., 2010) The function of CsdA, the third cysteine desulfurase in *E. coli*, is unclear (Loiseau *et al*., 2005; Trotter *et al*., 2009).

Thiosulfate:cyanide sulfurtransferase

The common term for thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1) is rhodanese. These enzymes are conserved in all domains of life and their originally assigned *in vivo* role was the detoxification of cyanide (Lang, 1933; Ray *et al*., 2000). Since then several other functions have been proposed; this includes a role in assimilatory sulfur metabolism, mobilization of sulfur for FeS cluster biosynthesis, biosynthesis of molypdopterin and glutathione regeneration (Pagani *et al*., 1984; Donadio *et al*., 1990; Dahl, J. U. *et al*., 2011; Remelli *et al*., 2012). The best studied rhodaneses are the bovine rhodanese (Rhobov) and RhdA from *Azotobacter vinelandii*. Both structures have been solved (Ploegman *et al*., 1978; Bordo *et al*., 2000). Rhobov and RhdA both exhibit a tandem domain architecture and are assembled of two rhodanese domains. Nevertheless, only the carboxy-terminal domain contains the catalytic cysteine residue (Bordo & Bork, 2002). PspE and GlpE from *E. coli* are examples for single-domain rhodaneses (Ray *et al*., 2000; Cheng *et al*, 2008). Like cysteine desulfurases this class of sulfurtransferases generates protein-bound persulfides. The sulfur transfer conducted by rhodaneses is a double displacement or "ping-pong" mechanism (Westley & Westley, 1983). After thiosulfate enters the active site the sulfur-sulfur bond of the substrate is polarized by the cationic environment. This eases the split of the bond by a nucleophilic attack of the active site cysteine. While sulfite is released sulfane sulfur is covalently bound to the sulfurtransferase. In the next step, the bound sulfur atom readily reacts with cyanide or another thiophilic acceptor (Westley & Westley, 1983).

Molybdopterin synthase

Molybdopterin (MPT) synthase (EC 2.8.1.12) catalyses the second step of the molybdenum cofactor biosynthesis, namely the conversion of cPMP to MPT by introduction of two sulfur atoms at the C1' and C2' positions in cPMP. In *E. coli* the enzyme consists of two MoaD and two MoaE subunits (Pitterle & Rajagopalan, 1993; Rudolph *et al*., 2001). Unlike cysteine desulfurases and rhodaneses a glycine residue in the carboxy-terminus of MoaD was identified as the sulfur binding site and MPT synthase depends on IscS for the delivery of sulfur. Before the active MPT synthase is assembled, MoaD forms a complex with MoeB. MoeB originally was supposed to act as molybdopterin synthase sulfurtransferase (EC 2.8.1.11) (Rajagopalan, 1997). However, neither thioester nor disulfide linkage between MoeB and MoaD was found. Instead, the contribution of MoeB is thought to acitvate MoaD under the consumption of ATP (Lake *et al*., 2001; Leimkühler *et al*., 2001). IscS is the primary *in vivo* sulfur donor for MoaD and the persulfide of IscS was suggested to nucleophilically attack the activated glycine residue which leads to the formation of an acyl-disulfide bond between MoaD and IscS (Zhang *et al*., 2010). After the release of AMP MoeB is supposed to catalyse the reduction of thiocarboxylated MoaD and IscS (Leimkühler *et al*., 2011). Subsequently, the catalytically active heterotetramer is formed and performs the thiolation reaction that transforms cPMP into MPT. The rhodanese YnjE and the sulfurtransferase TusA have recently been shown to be involved in the formation of MPT; however neither is essential (Dahl, J. U. *et al*., 2011, 2013).

tRNA sulfurtransferase

tRNA sulfurtransferases (EC 2.8.1.4) perform thiol-modifications on nucleosides in tRNA. Generally, modified nucleosides influence the translation fidelity and improve the efficiency of tRNA in translation. Each type of thiolation is performed by different enzymes (Ajitkumar & Cherayil, 1988; Hagervall *et al*., 1990; Perrson *et al*., 1994; Li *et al*., 1997). In *E. coli* sulfur for the biosynthesis of 4-thiouridine and 5-methylaminomethyl-2-thiouridine is provided by IscS. In case of 4-thiouridine sulfur is transferred to the tRNA sulfurtransferase ThiI where it is bound to Cys456 in the rhodanese domain prior to modification of uridine (Mueller *et al*., 1998; Kambampati & Lauhon, 1999, 2000). ThiI is a bifunctionary enzyme and also engages in the biosynthesis of thiamine (Palenchar *et al*., 2000). The enzyme responsible for 5-methylaminomethyl-2-thiouridine, thiolation of uridine in the wobble position of tRNAs for glutamic acid, glutamine and lysine, is MnmA. The earliest report states that while IscS and MnmA alone were able to reconstitute the modification *in vitro*, the product yield and specific activities were low (Kambampati & Lauhon, 2003). Later, three additional proteins were identified that are necessary for the production of 2-thiouridine *in vivo*: TusA, TusBCD and TusE (Ikeuchi *et al*., 2006). Each of the proteins contains a conserved cysteine residue which enables the polypeptide to bind sulfane sulfur. In total, sulfur for the biosynthesis of 2-thiouridine is delivered by a cascade of sulfur transfer reactions that includes five different enzymes. In the first step sulfur is mobilized from IscS and transferred to TusA. The next acceptor is TusBCD from where it further transferred to TusE. The thiolation reaction itself is then catalysed by MnmA (Figure I.1) (Ikeuchi *et al*., 2006).

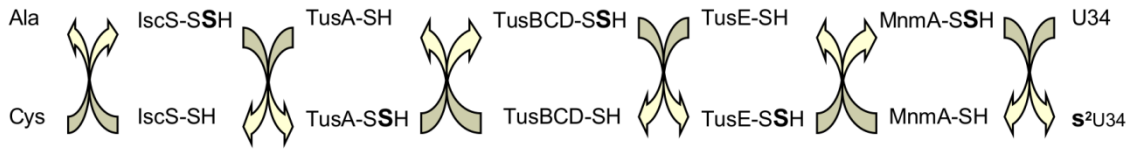

Figure I.1. Schematic overview of the sulfur relay system during the biosynthesis of 5-methylaminomethyl-2-thiouridine at the wobble position of tRNAs in *E. coli* (after Numata *et al*., 2006). Sulfane sulfur is abstracted from L-cysteine by L-cysteine desulfurase IscS and transferred to TusA. From TusA sulfur is further transferred to TusBCD and from there to TusE. In the final step MnmA uses this sulfur atom to modify uridine.

The other sulfurtransferase classes comprehend 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.3), thiosulfate—thiol sulfurtransferase (EC 2.8.1.4), thiosulfate—dithiol sulfurtransferase (EC 2.8.1.5), biotin synthase (EC 2.8.1.6), lipoyl synthase (EC 2.8.1.8), molybdenum cofactor sulfurtransferase (EC 2.8.1.9) and thiazole synthase (EC 2.8.1.10).

In summary, sulfurtransferases are a versatile group that involves enzymes which catalyse one step in a single pathway as well as enzymes which act at the basis of cellular sulfur trafficking and provide

sulfur for various pathways. The number of sulfurtransferases that exist in a single cell and the solved mechanisms for the biosynthesis of sulfur containing compounds imply that sulfur is generally handled in a very regulated and controlled way; most likely to avoid the toxicity of free sulfide. Given that various sulfur compounds are present in high concentrations and processed simultaneously during dissimilatory sulfate reduction or sulfur oxidation it would be feasible that these sulfur species are also subjected to cautious handling via numerous sulfurtransferases. Rhodanese activity has been reported in a number of bacteria using reduced sulfur compounds for energy conservation including *Acidithiobacillus ferrooxidans (*Tabita *et al*., 1969) and the anoxygenic phototrophic sulfur bacteria (Brune, 1989). However, only for a limited number of sulfurtransferaese the activity could actually be related to energy metabolism. For the periplasmic Sud protein from the ε-Proteobacterium *Wolinella succinogenes* the significance for polysulfide respiration is firmly established. Up to ten sulfur atoms were found bound to the active site cysteine Cys109. In form of a polysulfide chain bound to Sud sulfur is supposed to be transferred to the active site of the membrane associated polysulfide reductase (Klimmek *et al*., 1998, 1999). SbdP (Aq-477) from *Aquifex aeolicus* is a more recent example. Like Sud this protein binds a short polysulfide chain and it is supposed to act as sulfur mediator between sulfur globules and the sulfur oxygenase reductase and the sulfur reductase, respectively (Aussignargues *et al*., 2012).

As obvious from transcriptomic studies of *Atb. ferrooxidans* and *Allochromatium vinosum* organisms with dissimilatory sulfur metabolism provide excellent opportunities to identify new sulfurtransferases (Quatrini *et al*., 2009; Weissgerber *et al*, 2013). Both studies identified putative sulfurtransferase genes that responded rates to sulfur oxidizing conditions with increased gene expression.

3. *Allochromatium vinosum*

Allochromatium vinosum is a gram-negative, rod-shaped γ-Proteobacterium and belongs to the *Chromatiaceae*. This purple sulfur bacterium contains a vesicular photosynthetic membrane system and thrives in aquatic habitats with stagnant, sulfide-containing freshwater (Pfennig & Trüper, 1989; Imhoff *et al*., 1998).Under micro- to semiaerobic conditions the bacterium can grow chemotroph in the dark with oxygen as terminal electron acceptor (Kämpf & Pfennig, 1980; Dincturk *et al*., 2011), but typically gains energy by anoxygenic photosynthesis. It can grow photolithoautotrophically using reduced sulfur species as electron source (sulfide, polysulfide, thiosulfate, sulfite or elemental sulfur) or photoorganoheterotrophically with various organic substrates, e.g. formate, acetate, propionate, fumarate, succinate and malate (Brune, 1989; Pfennig & Trüper, 1989; Weissgerber *et al*., 2011). CO_2 fixation is achieved via the reductive pentose phosphate pathway (Brune, 1989; Weissgerber *et al*., 2011). Together with an adaptable metabolism, the genetic accessibility, the sequenced genome (NC_013851) and studies regarding the transcriptome, proteome and metabolome make *Alc. vinosum* a model organism for dissimilatory sulfur metabolism in phototrophic purple sulfur bacteria

(Pattaragulwanit & Dahl, 1995; Weissgerber *et al*., 2011; Weissgerber *et al*., 2013; Weissgerber *et al*., 2014 submitted, 2014 revision submitted).

4. The dissimilatory sulfur metabolism in *Alc. vinosum*

The oxidative sulfur metabolism in *Alc. vinosum* includes three major steps. At first, sulfur globules are accumulated in the periplasm as an obligate intermediate during the oxidation of reduced sulfur compounds. The stored sulfur is then further oxidized to sulfite in the cytoplasm. In the final step sulfite is converted to sulfate, which is excreted as endproduct (Trüper & Fischer, 1982; Dahl & Trüper, 1994). An overview of the sulfur-converting enzymes in *Alc. vinosum* is given in Figure I.2.

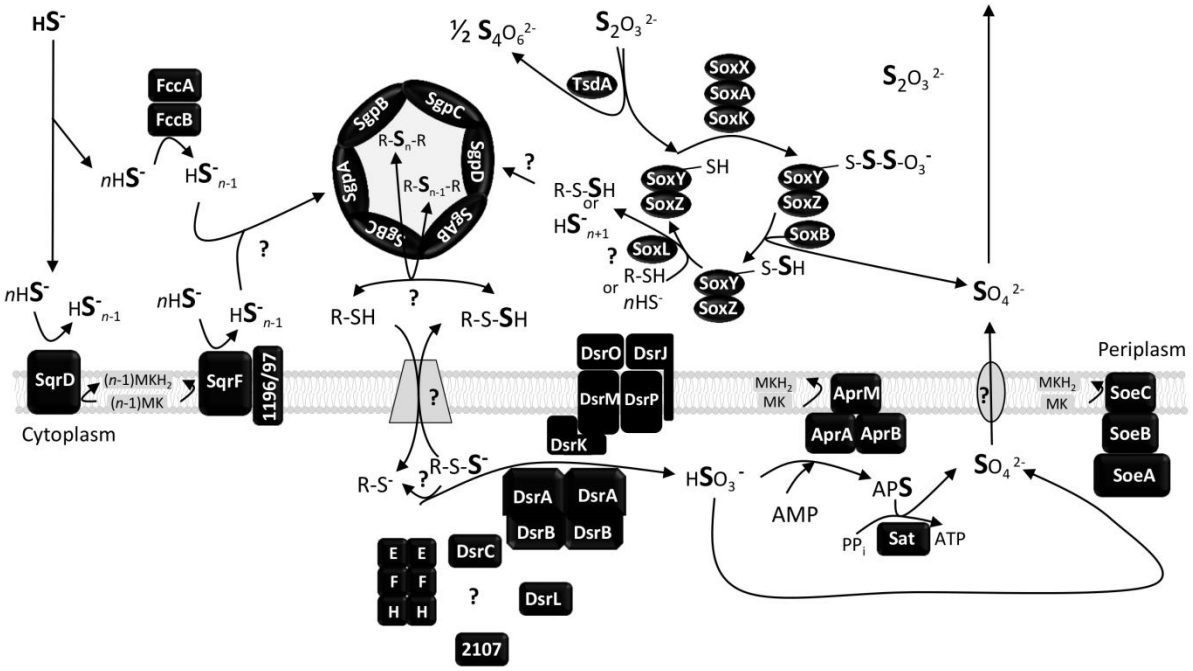

Figure I.2. Currently proposed model of dissimilatory sulfur metabolism in *Alc. vinosum* (after Weissgerber *et al*., 2014, revision submitted). Ratio of sulfur globule proteins SgpA, SgpB, SgpC and SgpD is not stoichiometric. E: DsrE. F: DsrF. DsrH: DsrH. 1196/97: Alvin_1996 and Alvin_1197. 2107: Alvin_2107. MK: menaquinone.

The enzyme which is primarily responsible for the oxidation of sulfide in *Alc. vinosum* is still under debate. *Alc.vinosum* contains at least three different enyzmes that might be involved in this step, soluble FccAB (Alvin_1192/93) and the membrane-bound sulfide:quinone oxidoreductases (SQR) SqrD (Alvin_2145) and SqrF (Alvin_1195). FccAB is not essential for sulfide oxidation; the deletion of *fccAB* yielded similar sulfide oxidation rates as *Alc. vinosum* wild type (Reinartz *et al*., 1998). SqrD and SqrF are both predicted to be oriented towards the periplasm, but have not been isolated yet. However, *Alc. vinosum* exhibits SQR activity and the deletion of *sqrF* affected the formation of sulfur globules during growth on high sulfide concentrations (Dahl, 2008; Weissgerber *et al*., 2013; Zigann & Dahl, unpublished data). The inactivation of Alvin_1196/97 yielded a similar phenotype and it was

proposed that these membrane proteins conduct the electron transfer from SqrF to the quinone pool since the predictions for transmembrane helices in SqrF are unclear (Weissgerber *et al*., 2013). *In vitro* the primary product of *Rhodobacter capsulatus* SQR are polysulfides, which were also found in *Alc. vinosum* during sulfide oxidation (Griesbeck *et al*., 2002; Franz *et al*., 2009).

Thiosulfate is either oxidized to tetrathionate or it is converted to sulfate (Hensen *et al*., 2006). Under acidic conditions the thiosulfate dehydrogenase TsdA (Alvin_1091), a c-type cytochrome, oxidizes two thiosulfate anions to tetrathionate, which is not further processed (Hensen *et al*., 2006; Denkmann *et al*., 2012). For the oxidation of thiosulfate to sulfate the periplasmic proteins SoxXAK (Alvin_2168-70), SoxYZ (Alvin_2111/12) and SoxB (Alvin_2167) are essential (Hensen *et al*., 2006; Lehmann, 2010). Thiosulfate is oxidatively coupled to the substrate-binding molecule SoxYZ by the c-type cytochrome SoxXA. SoxK acts as binding protein for SoxXA (Lehmann, 2010). The hydrolytic cleavage of the bound thiosulfate and release of the more oxidized sulfone sulfur as sulfate is carried out by the manganese cluster containg SoxB, for which a function as sulfate thiohydrolase was proposed (Epel *et al*., 2005; Friedrich *et al*., 2005). In *Paracoccus pantotrophus* the sulfur dehydrogenase SoxCD, composed of the molybdoprotein SoxC and the hybrid diheme c-type cytochrome SoxD, catalyses the oxidation of the remaining sulfane sulfur, which is still bound to SoxYZ. After release of the second sulfate molecule by SoxB SoxYZ can engage in a new cycle (Friedrich *et al*., 2001; Zander *et al*., 2010). However, SoxCD is not present in sulfur oxidizers that contain Dsr proteins like *Alc. vinosum* (Gregersen *et al*., 2011). Instead, the sulfane sulfur is fed into the sulfur globules. The exact mechanism that regenerates SoxYZ is still unsolved, but the rhodanese-like protein SoxL (Alvin_2171) is possible candidate (Welte *et al*., 2009).

Periplasmically stored sulfur globules contain chains of polysulfane sulfur terminated by an organic residue at one or both ends ($R\text{-}S_n\text{-}R$; with $n > 2$) at which the organic residue is believed to be glutathione or its amidated derivate, glutathione amide (Prange *et al*., 1999, 2002). They are coated by a monolayer of hydrophobic sulfur globule proteins SgpABCD (Alvin_1905, Alvin_0358, Alvin_1325 and Alvin_2515, respectively). SgpABC have long been known to envelop the transiently stored sulfur and they have been assigned a structural function as they carry no cysteine residue to which sulfur might bind. SgpD on the other side has just recenctly been idenitified during the analysis of the sulfur globule proteome; a strictly structural function for SgpD is assumed as well, although this protein contains a cysteine residue (Schmidt *et al*., 1971; Brune, 1995; Pattaragulwanit *et al*., 1998; Weissgerber *et al*., 2014, revision submitted).

The oxidation of the stored sulfur to sulfite is catalysed by the cytoplasmic dissimilatory sulfite reductase DsrAB (Alvin_1251/52) (Pott & Dahl, 1998; Dahl *et al*., 2005). Sulfur in the globules must be activated and transported from the periplasm across the cytoplasmic membrane into the cytoplasm. The mechanism by which the transport is accomplished belongs to the least understood topics in dissimilatory sulfur metabolism. The transfer of sulfur in its persulfidic state, bound to an organic

carrier molecule has been suggested, though (Dahl *et al*., 2008). DsrAB from *Alc. vinosum* is homologous to the dissimilatory sulfite reductase in sulfate reducing bacteria, which reduces sulfite to sulfide (Hipp *et al*., 1997). In sulfur oxidizing prokaryotes the enzyme is supposed to operate in reverse (Schedel *et al*., 1979). *dsrAB* forms a transcriptional unit with the 13 genes that are encoded directly downstream of *dsrB* (Alvin_1253-65). Yet, another protein has been detected that probably participates in the degradation of sulfur globules. The absence of Alvin_2107 led to an impairment of the oxidation of intermediately stored sulfur. The exact function of the protein is yet unknown. Homologues of Alvin_2107 are conserved within the *Chromatiaceae* and other sulfur oxidizing γ-Proteobacteria, that also encode the *dsr* operon (Weissgerber *et al*., 2014, revision submitted).

In the final step sulfite is converted to sulfate. The membrane-bound polysulfide reductase-like iron–sulfur molybdoprotein SoeABC (Alvin_2489-91) has recently been identified as the prime enzyme to catalyse this reaction in *Alc. vinosum* (Dahl *et al*., 2013). The enzyme is anchored to the cytoplasmic membrane via SoeC. The iron-sulfur protein SoeB and the molybdoprotein SoeA, which carries an amino-terminal FeS cluster binding site, are located in the cytoplasm. The complex is present not only in phototrophic but also chemotrophic sulfur oxidizers. Alternatively, sulfite is indirectly oxidized by APS reductase (Alvin_1119-21) and ATP sulfurylase (Alvin_1118). First, APS is formed from sulfite and AMP by the APS reductase. Subsequently, the AMP moiety of APS is transferred to pyrophosphate by ATP sulfurylase and sulfate is released (Dahl, 2008). However, gene inactivation showed this pathway is non-essential in *Alc. vinosum* (Dahl, 1996).

5. Oxidation of sulfur globules and a putative involvement of sulfurtransferases

The degradation of sulfur globules in *Alc. vinosum* strictly depends on proteins encoded in the *dsr* operon (Alvin_1251-1265) (Figure I.3) (Dahl *et al*., 2005; Lübbe *et al*., 2006; Sander *et al*., 2006, Dahl *et al*., 2008; Grimm, 2004; Grimm *et al*., 2010a, b; Grimm, 2011). The gene expression of the *dsr* operon is induced under photolithoautotrophic conditions. Thus, contradicting the findings for *Thiobacillus denitrificans* where the *dsr* genes are thought to be highly and probably constitutively expressed (Pott & Dahl, 1998; Beller *et al*., 2006; Grimm *et al*., 2010b; Weissgerber *et al*., 2013). Apart from a sulfide inducible promoter upstream of *dsrA*, secondary promoters for *dsrC* and *dsrS* are present (Dahl *et al*, 2005; Grimm *et al*., 2010b).

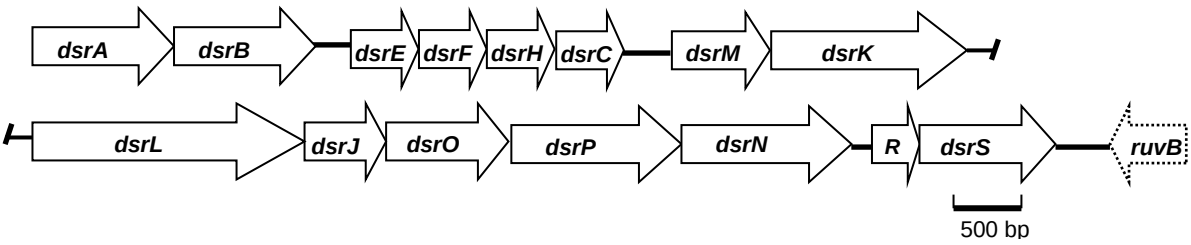

Figure I.3. The *dsr* operon in *Alc. vinosum*. R: DsrR.

The **d**issimilatory **s**ulfite **r**eductase DsrAB is the key enzyme and thought to oxidize transiently stored sulfur to sulfite (Schedel *et al*., 1979; Hipp *et al*., 1997). DsrAB is a cytoplasmic, soluble and $\alpha_2\beta_2$-structured protein with siroamide-[Fe4S4] as prosthetic group (Lübbe *et al*., 2006). Siroamide is the amidated form of classical siroheme; the glutamine-dependent derivatisation reaction is thought to be catalysed by DsrN, a homologue of the cobyrinic acid a,c-diamide synthase (Dahl *et al*., 2005; Lübbe *et al*., 2006). The structure of DsrAB from several sulfate reducers have been solved and show that not all cofactors are catalytically active (Oliveira *et al*., 2008; Schiffer *et al*., 2008; Hsieh *et al*., 2010; Oliveira *et al*., 2011).

The transmembrane complex DsrMKJOP is essential for the oxidation of intermediary sulfur globules and is homologous to DsrMKJOP in sulfate reducing bacteria and archaea, where it transfers electrons to DsrAB (Sander *et al*., 2006; Pires *et al*., 2006). Since the sulfur metabolism of *Alc. vinosum* is reverse to that of sulfate reducers, DsrMKJOP was originally believed to be the exit point for electrons issued by DsrAB out of the cytoplasm. However, analysis of the complex refuted this assumption; analogous to DsrMKJOP from sulfate reducers the complex in *Alc. vinosum* provides an electron flow from the periplasm into the cytoplasm (Grein *et al*., 2010a).
The triheme cytochrome c DsrJ is thought the have a function in catalytic sulfur chemistry and oxidize a yet unknown periplasmic substrate. The electrons are supposed to be transferred across the membrane via other components of the complex. DsrJ is attached to the membrane via its signal peptide that is not cleaved off (Grein *et al*., 2010b). The periplasmic, FeS cluster containing DsrO would be the next electron accepting protein. The integral membrane proteins DsrM and DsrP both contain b-type cytochromes and might interact with the quinone pool in the membrane as quinol oxidase, supplying DsrK with electrons, and as quinone reductase, respectively (Grein *et al*., 2010a). DsrK is a cytoplasmic iron-sulfur protein, which is monotopically anchored in the cytoplasmic membrane. Native DsrK was co-purified with DsrMJOP, DsrEFH, DsrC and DsrAB. Based on the similarity to the catalytic subunit of heterodisulfide reductases in methanogenic archaea DsrK was proposed to be the catalytic subunit of the DsrMKJOP complex (Dahl *et al*., 2005; Grein *et al*., 2010a).

The function of the soluble, cytoplasmic proteins DsrL, DsrR and DsrS is unclear. For DsrR and DsrS a function in the post-translational control of the *dsr* operon was proposed. DsrR is similar to A-type scaffolds that participate in the maturation of protein-bound FeS clusters, whereas no conserved motifs were detected in DsrS (Grimm *et al*., 2010a; Grimm, 2011). The homodimeric iron-sulfur flavoprotein DsrL is critical for the degradation of sulfur globules and exhibits NADH:acceptor oxidoreductase reductase activity *in vitro*. The protein carries the thioredoxin motif Cys-X-X-Cys that is preceded by the FeS cluster binding site and is co-purified with DsrAB from the soluble fraction of *Alc. vinosum* (Lübbe, 2005; Lübbe *et al*., 2006). A disulfide reductase activity was proposed for DsrL using NADH as electron donor. As potential electron acceptor DsrC in its oxidized state and the persulfidic carrier

molecule for sulfur from the periplasm to the cytoplasm were suggested. The latter reaction would yield in the release of sulfide, a possible substrate for DsrAB (Dahl *et al.*, 2005; Cort *et al*., 2008).

If sulfide was indeed the substrate for DsrAB the degradation of sulfur globules would be accompanied with intracellular sulfide concentrations that easily excel the concentrations that are usually tolerated to ensure the biosynthesis of cysteine without causing damage to the cell. Reported intracellular sulfide concentrations range from 20 to 160 µM (Schmidt & Jäger, 1992; Wang, 2002; Theissen *et al*., 2003). It was therefore hypothesized that the sulfur to be oxidized is presented to DsrAB in form of a persulfide bound to DsrC. In early publications DsrC was described as the γ-subunit of DsrAB (Mander *et al*., 2005). However, based on more recent studies on DsrC from sulfate reducing *Dsv. vulgaris*, *Desulfovibrio gigas* and *Desulfomicrobium norvegicum* DsrC was established as an individual protein interacting with DsrAB rather than a subunit of the latter (Oliveira *et al*., 2008; Hsieh *et al*., 2010; Oliveira *et al*., 2011; Venceslau *et al*., 2013). *dsrC* belongs to the core set of *dsr* genes and was found to be the most abundant genes among communities of sulfur oxidizers and sulfate reducers (Stewart *et al*., 2011; Canfield *et al*., 2010; Grimm *et al*., 2008). Apart from organisms with sulfur driven metabolism homologues of DsrC are also found in bacteria lacking DsrAB (Cort *et al*., 2008). The best studied example is the aforementioned TusE from *E. coli* (Ikeuchi *et al*., 2006). The tertiary structure of *Alc. vinosum* DsrC showed the characteristic fold that is shared by all known DsrC proteins: at the amino-terminal end a two-strand β-hairpin is followed by five helices that form an orthogonal bundle while the highly conserved carboxy-terminus extends from the rest of the globular protein. NMR spectroscopy showed an increased flexibility of the last seven residues [Pro-Lys-Pro-Thr-Gly-Cys-Val] at the carboxy-terminus beginning with Pro106. The point of exit is located in a positively charged region (Cort *et al*., 2008). The only cysteines present in DsrC are located in the carboxy-terminus: while Cys111 in the penultimate position is strictly conserved in all DsrC/TusE homologues, Cys100 is only present in organisms with a sulfur based energy metabolism. Dimerization via an intermolecular disulfide bond between the cysteine residues Cys111 of two DsrC molecules has been detected as well as an intramolecular disulfide bond between Cys100 and Cys111 (Cort *et al*., 2008). Structures of DsrAB from sulfate reducing organisms showed DsrC bound to DsrAB with the flexible carboxy-terminus inserted into the putative substrate channel that is connected to siroheme (Oliveira *et al*., 2008; Hsieh *et al*., 2010; Oliveira *et al*., 2011). Based on these reports it was suggested that DsrC in *Alc. vinosum* acts as substrate donor for DsrAB and leave DsrAB after the oxidation step with a sulfonate group bound to one of the cysteine residues. The final release of sulfite is supposed to be achieved by the formation of a disulfide bond between Cys111 and Cys100 (Cort *et al*., 2008). The electrons for the subsequent reduction of DsrC could be donated by DsrK, which was shown to interact with DsrC (Grein *et al*., 2010a).

It was further proposed that DsrEFH might be the sulfur donor for DsrC. DsrEFH is restricted to sulfur oxidizers and not present in sulfate reducing bacteria; it is therefore fair to assume that this protein

exclusively engages in the direction of sulfur oxidation. The homologous proteins DsrE, DsrF and DsrH form the hexameric $\alpha_2\beta_2\gamma_2$-structured DsrEFH holo-protein that shows characteristics of the YchN-fold from *E. coli* (Dahl *et al*., 2008). Like DsrC the protein binds no prosthetic groups. According to the number and position of strictly conserved cysteines in the putative active sites DsrEFH proteins can be subdivided into different groups. With one conserved cysteine residue in DsrE (Cys78) and one in DsrH (Cys20) *Alc. vinosum* DsrEFH is grouped with proteins from other well established sulfur oxidizers including *Tbc. denitrificans*, the green sulfur bacteria and the magnetotactic bacteria. In all of these organisms the *dsrEFH* genes are clustered with other *dsr* genes (Dahl *et al*., 2008). The cysteine residues DsrH-Cys95 and DsrF-Cys40 are not conserved. The deletion of *dsrEFH* led to a genetically instable mutant. Analysis of the gene sequence revealed that the secondary promoter for constitutively expressed *dsrC* is present in the region of *dsrFH* (Dahl *et al*., 2008, Grimm *et al*., 2010a). However, the single-locus deletion of *dsrE* allowed insights into the physiological relevance of DsrEFH for sulfur oxidation. After deletion of *dsrE Alc. vinosum* was no longer able to degrade sulfur globules while neither the oxidation rate of sulfide nor the formation of sulfur globules was compromised. Complementation with *dsrEFH* led to a fully restored wild type phenotype (Dahl *et al*., 2008, Stockdreher, 2009). Unlike the reintroduction of *dsrEFH* neither the complementation with *dsrE-Cys78Ser-FH* mutant nor with *dsrEFH-Cys20Ser* could reinstall the ability to degrade sulfur globules (Stockdreher *et al*., 2012). Thus, indicating that DsrE-Cys78 and DsrH-Cys20 are crucial for the oxidation of sulfur globules in *Alc. vinosum*.

The proposed role for DsrEFH and DsrC is the transfer of sulfur atoms to DsrAB during the degradation of sulfur globules (Cort *et al*., 2008). This strategy would avoid free sulfide as possible substrate for DsrAB and is in good accordance the observation that thiol-binding reagents as well as Cys-Ser exchanges in DsrE and DsrH inhibit the oxidation of transiently stored sulfur (Hurlbert *et al*., 1968; Stockdreher *et al*., 2012). DsrEFH and DsrC have already been shown to interact *in vitro*, so the transfer of sulfur might be possible. The presence of DsrE-Cys78 and DsrC-Cys111 is vital for the interaction (Cort *et al*., 2008; Dahl *et al*., 2008; Stockdreher, 2009). Furthermore, DsrEFH and DsrC are homologous to the *E. coli* sulfurtransferases TusBCD and TusE, respectively, which participate in in a sulfur relay system (Ikeuchi *et al*., 2006).

So far, there is no indication that would lead to the conclusion that DsrEFH or DsrC might be able to mobilize sulfane sulfur from a potential carrier molecule. This raises the question how sulfur is initially introduced to the Dsr system and enhances the possibility that further sulfurtransferases are involved in a potential cytoplasmic sulfur relay system. Again, a Tus protein might be the answer. In *E. coli* TusA is the sulfur donor for TusBCD. A TusA homologue is also present in *Alc. vinosum*, Alvin_2600 (Dobler, 2008). The protein showed interaction with DsrEFH and the ability to bind sulfur via the only cysteine residue, Cys15 (Stockdreher, 2009). TusA-like proteins are wide-spread; however, the TusA homologues in sulfur oxidizers share a higher similarity with each other than with

TusA from *E.coli* (Dobler, 2008). In *Alc. vinosum tusA* is preceded by a gene for a rhodanese (Alvin_2599) and followed by *dsrE2* (Alvin_2601). The gene product of the latter belongs to the DsrE/DsrF/DsrH family; it is annotated as hypothetical protein of unknown function. The attempt to predict the function of the homotrimer MTH1491, homologous to DsrE2, from *Methanobacterium thermoautotrophicum* by analysis of the protein's structure yielded no conclusive results (Christendat *et al.*, 2002). The rhodanese was previously characterised as monomeric thiosulfate:cyanide sulfurtransferase and will subsequently be referred to as Rhd_2599 (Sturm, 2009)

Besides the interaction of TusA with DsrEFH a further potential link of Rhd_2599, TusA and DsrE2 to sulfur metabolism is given by the enhanced gene expression of *tusA* and *dsrE2* when *Alc. vinosum* is grown photolithoautotrophically (Weissgerber *et al.*, 2013). Furthermore, the same gene cluster has been reported to be present in chemotrophic sulfur oxidizers *Atb ferrooxidans*, *Acidithiobacillus caldus* and *Thioalkalivibrio sp*. K90mix (Quatrini *et al*., 2009; Muyzer *et al*., 2011; Mangold *et al*., 2011; Osorio *et al*., 2013). In *Atb. ferrooxidans tusA* and *dsrE2* were increasingly expressed under sulfur oxidizing and aerobic conditions relative to growth on ferrous iron. The rhodanese attracted attention in an earlier macroarray study on *Atb. ferrooxidans* where higher expression were shown when the organism was grown on sulfur (Acosta *et al*., 2005; here, the rhodanese was designated P11). Downstream of the *rhd-tusA-dsrE2* cluster in *Atb. ferrooxidans* genes for a putative heterodisulfide complex are located, *hdrC1B1A1orf2hdrC2B2*. Like *tusA* and *dsrE2* the expression of the *hdr* genes was upregulated under sulfur oxidizing conditions (Quatrini *et al*., 2009). On this ground Quatrini and co-workers proposed that the Hdr complex in *Atb. ferrooxidans* might use the natural existing proton gradient and oxidize disulfide intermediaries that result from the oxidation of sulfur to sulfite and deliver the electrons to the quinone pool (Quatrini *et al*., 2009). Hence, Hdr in *Atb. ferrooxidans* is supposed to operate in the opposite direction than heterodisulfide reductases in methanogenic archaea where they are responsible for the reduction of the heterodisulfide CoM-S-S-CoB (Hedderich *et al*., 2005; Thauer *et al*., 2008). Quatrini *et al.* refer to the rhodanese, TusA and DsrE2 as accessory sulfurtransferases that conduct the transfer of sulfur to the Hdr complex (Quatrini *et al*., 2009). An alternative role for TusA and DsrE2 in *Atb. ferrooxidans* was offered by Osorio *et al*.; in their model TusA and DsrE2 are only relevant under anaerobic conditions and responsible for the efflux of H_2S that is produced by the membrane-bound sulfur reductase SreABCD (Osorio *et al*., 2013).

6. Objective

Research on the degradation of sulfur globules in *Alc. vinosum* has been focused on the characterization of individual proteins of the Dsr system and their relevance for the process, electron flow during sulfur oxidation and the regulation of the *dsr* operon. The main focus of this thesis lay on the cytoplasmic substrate delivery for DsrAB.

In the first part components of the Dsr system itself, DsrEFH and DsrC, were analysed in regard to a putative sulfurtransferase activity. First, their ability to mobilize and bind sulfur atoms was investigated and the respective sulfur binding sites were identified. Furthermore, the transfer of sulfur between DsrEFH and DsrC was examined. The nature of the interaction between both proteins was characterized in more detail.

A potential involvement of Rhd_2599, TusA and DsrE2 in the oxidation of intermediary stored sulfur was explored in the second part of this thesis. To this end, genome-sequenced sulfur oxidizing prokaryotes were systematically surveyed for the presence of the *rhd-tusA-dsrE2* gene cluster and the expression of the three genes in *Alc. vinosum* during the oxidation of reduced sulfur compounds was analysed at various time points. The physiological relevance of the proteins was approached by the deletion of the gene cluster. Finally, the proteins were characterized individually and possible sulfur transfer reactions among Rhd_2599, TusA and DsrE2 were tested as well as the transfer to the Dsr proteins and *vice versa*.

II Materials and Methods

1 Bacterial strains

Table II.1. Bacterial strains used in this study

Strains	Genotype or phenotype	Reference or source
Escherichia coli		
DH5α	F$^-$ Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44λ*$^-$ *thi-1 gyrA relA1*	Hanahan (1983)
S17-1	294(*recA pro res mod*$^+$) Tp^r Sm^r (pRP4-2-Tc::Mu-Km::Tn7)	Simon *et al*. (1983)
BL21(DE3)	F$^-$ *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm met* (DE3)	Novagen (Madison, USA)
C41(DE3)	derived from BL21(DE3), at least one uncharacterized mutation	Miroux & Walker (1996)
C43(DE3)	derived from C41(DE3), at least two uncharacterized mutations	Miroux & Walker (1996)
K-12	wild type	DSM 498
Allochromatium vinosum		
Rif50	Rif^r; spontaneous rifampicin-resistant mutant of *Alc. vinosum* DSM 180^T	Lübbe *et al*. (2006)
rhd_2599::ΩSm	Rif^r, Sm^r, insertion of ΩSm^r in *rhd_2599* of Rif50	This study
Δ*rtd*	Rif^r; Rif50 with *in frame* deletion of *rhd_2599, tusA* and *dsrE2*	This study
Δ*rtd*::ΩKm	Rif^r Km^r; Rif50 with *in frame* deletion of *rhd_2599, tusA*, *dsrE2* and inserted ΩKm cassette in center of deleted sequence	This study

2 Plasmids

The correct sequences of inserts of plasmids constructed in this study were verified by DNA sequencing carried out by GATC (Konstanz, Germany).

Table II. 2. Plasmids used in this study.

Plasmid	Genotype or phenotype	Reference or source
pHP45Ω-Km	Ap^r, Km^r	Fellay *et al*. (1987)
pK18*mobsacB*	Km^r, *lacZ*', *sacB*, Mob$^+$	Schäfer *et al*. (1994)
pK18*mobsacB*Δ*rtd*	Km^r, 1.21 kb amplicon with deleted *rhd_2599-tusA-dsrE2* cloned into *Bam*HI-restriction site of pK18*mobsacB*	This study
pSUP301	Ap^r, Km^r, RP4 *oriT* p15A *ori* Mob$^+$	Simon *et al*. (1983)
pSUP301Δ*rtd*	Ap^r, 1.21 kb amplicon with deleted *rhd_2599-tusA-dsrE2* cloned into *Hin*dIII-restriction site of pSUP301	This study
pSUP301Δ*rtd*::ΩKm	Ap^r, Km^r, pHP45Ω-Km kanamycin cassette inserted into pSUP301Δ*rtd* using *Eco*RI restriction sites	This study
pET-15b	Ap^r, T7 promoter, His-Tag (N-terminal)	Novagen (Madison, USA)
pETCEX	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsrC* in pET-15b	Cort *et al*. (2008)
pETCEXSer100	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsrC-Cys100Ser* in pET-15b	Cort *et al*. (2008)
pETCEXSer111	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsrC-Cys111Ser* in pET-15b	Cort *et al*. (2008)

Table II. 2. Plasmids used in this study (continued).

Plasmid	Genotype or phenotype	Reference or source
pET15bDsrE2	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsrE2* in pET-15b	This study
pETEFH	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsr*EFH in pET15b	Dahl *et al*. (2007)
pETE$_{78}$FH	Ap^r, in *Nde*I/*Bam*HI *fragment* of amplified *dsrE-Cys78Ser-FH* in pET-15b	Dahl *et al*. (2008)
pETEFH$_{20}$	Ap^r, in *Nde*I/*Bam*HI *fragment* of amplified *dsrEFH-Cys20Ser* in pET-15b	Dahl *et al*. (2008)
pETE$_{78}$FH$_{20}$	Ap^r, in *Nde*I/*Bam*HI *fragment* of amplified *dsrE-Cys78Ser-FH-Cys20Ser* in pET-15	Dahl *et al*. (2008)
pETIscS	Ap^r, *Nde*I/*Xho*I fragment of amplified *E. coli iscS* in pET-15b	Stockdreher (2009)
pET15bRhd	Ap^r, *Nde*I/*Xho*I fragment of amplified *rhd_2599* in pET-15b	Sturm (2009)
pET15bRhd-Cys64Ser	Ap^r, *Nde*I/*Bam*HI fragment of amplified *rhd_2599-Cys64Ser* in pET-15b	This study
pET15bRhd-Cys74Ser	Ap^r, *Nde*I/*Bam*HI fragment of amplified *rhd_2599-Cys74Ser* in pET-15b	This study
pET15bTusA	Ap^r, *Nde*I/*Bam*HI fragment of amplified *tusA* in pET-15b	Stockdreher (2009)
pET15bTusA-Cys15Ser	Ap^r, *Nde*I/*Bam*HI fragment of amplified *tusA-Cys15Ser* in pET-15b	Stockdreher (2009)
pET15bEcTusA	Ap^r, *Nde*I/*Bam*HI fragment of amplified *E. coli tusA* in pET-15b	This study
pET15bEcTusBCD	Ap^r, *Nde*I/*Bam*HI fragment of amplified *E. coli tusBCD* in pET-15b	This study
pET-22b	Ap^r, T7 promoter, His-Tag (C-terminal)	Novagen (Madison, USA)
pET22bDsrE2	Ap^r, *Nde*I/*Bam*HI fragment of amplified *dsrE2* in pET-22b	This study
pASK-IBA5plus	Ap^r, Tet promoter, Strep-Tag (N-terminal)	IBA (Göttingen, Germany)
pIBADsrE2	Ap^r, *Bsa*I fragment of amplified *dsrE2* in pASK-IBA5plus	This study
pIBADsrE2-Cys110Ser	Ap^r, *Bsa*I fragment of amplified *dsrE2-Cys110Ser* in pASK-IBA5plus	This study
pIBADsrE2-Cys120Ser	Ap^r, *Bsa*I fragment of amplified *dsrE2-Cys120Ser* in pASK-IBA5plus	This study
pIBADsrE2-Cys156Ser	Ap^r, *Bsa*I fragment of amplified *dsrE2-Cys156Ser* in pASK-IBA5plus	This study

3 Oligonucleotides

All oligonucleotides were purchased by MWG-Biotech AG (Ebersberg, Germany).

Table II.3 Oligonucleotides used in this study. Restriction sites are underlined and mutated sequences are marked bold.

Purpose	Oligonucleotide	Sequence 5'→3'	Reference
Deletion of *rtd*			
	ΔRTD_F1	CTTTGACAC**GGATCC**GATAACG	This study
	ΔRTD_R1	GAGACTTCAGATGTAGA**CGTTGACCACACTATCG**	This study
	ΔRTD_F2	CGATAGTGTGGTCAACG**TCTACATCTGAAGTCTC**	This study
	ΔRTD_R2	AAGTCGACGGATCCGCGATG	This study
Interposon mutagenesis of *rtd*			
	RTD_Kan_F1	CAGCGCAAGCTTCCGCGTG	This study
	RTD_Kan_R1	CTTCAGATGTAG**GAATTC**ACCACACTATC	This study
	RTD_Kan_F2	GATAGTGTGGT**GAATTC**CTACATCTGAAG	This study
	RTD_Kan_R2	GACGCAAAGCTTGCACATTGG	This study

Table II.3 Oligonucleotides used in this study (continued). Restriction sites are underlined and mutated sequences are marked bold.

Purpose	Oligonucleotide	Sequence 5'→3'	Reference
Cloning of *dsrE2* into pET-15b/pET-22b			
	DsrE2_for	CGCGTGGAGGACCATATGGAACAAAAG	This study
	DsrE2_rev	CGGTTCAGAGCTCGAGGATGTAGAGAC	This study
Cloning of *dsrE2* into pASK-IBA5plus			
	DE2_Strep_for	ATGGTAGGTCTCAGCGCCATGGAACAAAAGAAACT GGCGATC	This study
	DE2_Strep_rev	ATGGTAGGTCTCATATCAGATGTAGAGACAGATGT CGCTCT	This study
Clonining of *E. coli tusA* into pET-15b			
	EcTusA_for	TGA AGCATATGCCCGATCTC	This study
	EcTusA_rev	CATCAGGGATCCTCAACCGCC	This study
Cloning of *tusBCD* into pET-15b			
	TusBCD_for	AGATAACATATGCGTTTTGCCATCG	This study
	TusBCD_rev	ATCGGATCCTCACCAGGCCAT	This study
Cys-Ser exchange			
	DsrE2_C110S_for	CGAGCTCT**CA**CAGGAAGCC	This study
	DsrE2_C110S_rev	GGCTTCCTG**TG**AGAGCTCG	This study
	DsrE2_C120S_for	TGATCGCCT**CA**CAGATGACCG	This study
	DsrE2_C120S_rev	CGGTCATCTG**TG**AGGCGATCA	This study
	DsrE2_C156S_for	AGCGACATCT**CA**CTCTACATC	This study
	DsrE2_C156S_rev	GATGTAGAG**TG**GATGTCGCT	This study
	dsrE78for	CGT GCA GGG CCA GAC TGG	Dahl *et al*. (2008)
	dsrH20rev	GGAAAACGTTCTTCGGGG	Dahl *et al*. (2008)
	IBA5_for	TGAGCTATGAGAAAGCGCC	This study
	IBA5_rev	GGCGACACGGAAATGTTG A	This study
	Rhd_C64S_for	GTGGTCATC**T**ACTCCCGCAGC	This study
	Rhd_C64S_rev	GCTGCGGG**A**GTAGATGACCAC	This study
	Rhd_C74S_for	CAGGCTTC**C**GCCTATCTGATGC	This study
	Rhd_C74S_rev	GCATCAGATAGGC**G**GAAGCCTG	This study
qRT-PCR			
	RT-UROD-for	GTACCGCGCATCGAGGATT	Weissgerber *et al*. (2013)
	RT-UROD-rev	GCATTACCGGCAGCGAGAA	Weissgerber *et al*. (2013)
	RT-Rhd-for	GTGTTGCTGGTGGACATCC	This study
	RT-Rhd-rev	GGCAGTAGATGACCACGTCG	This study
	RT-TusA-for	CGATCAAGAACTCGACGCAAGC	Weissgerber *et al*. (2013)
	RT-TusA-rev	GTTGCCGGTCTGCTTGGC	Weissgerber *et al*. (2013)
	RT-DsrE2-for	CGGTATGCAGGGCATGATGAC	Weissgerber *et al*. (2013)
	RT-DsrE2-rev	TTCGGCATATCGAAGAGGTCG	Weissgerber

et al. (2013)

4 Chemicals and materials

4.1 Chemicals

1,5-IAEDANS	Sigma-Aldrich (Steinheim, Germany)
30% acrylamide/bisacrylamide	Roth (Karlsruhe, Germany)
4-chloro-1-naphthol	Sigma-Aldrich (Steinheim, Germany)
4x Rotiload 1	Roth (Karlsruhe, Germany)
Anhydrotetracycline	IBA (Göttingen, Germany)
Anti-digoxigenin-AP	Roche (Mannheim, Germany)
Blocking reagent	Roche (Mannheim, Germany)
Bradford reagent	Sigma-Aldrich (Steinheim, Germany)
CDP-Star	Roche (Mannheim, Germany)
D-desthiobiotin	IBA (Göttingen, Germany)
dig-dUTP	Roche (Mannheim, Germany)
dNTPs	Fisher Scientific (Schwerte, Germany)
DTT	Roth (Karlsruhe, Germany)
GelRed Nucleic Acid Gel Stain	Biotium (Hayward, USA)
Monobromobimane	Sigma-Aldrich (Steinheim, Germany)
Rifampicin	Sigma-Aldrich (Steinheim, Germany)
SDS	Serva (Heidelberg, Germany)

All other chemicals were obtained from the companies: Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). All chemicals were at least of p. a. quality.

4.2 Enzymes

Alkaline phosphatase (CIAP)	Fisher Scientific (Schwerte, Germany)
Alkaline Phosphatase (FastAP)	Fisher Scientific (Schwerte, Germany)
DNase I, RNase-free	Fisher Scientific (Schwerte, Germany)
Lysozyme	Sigma-Aldrich (Steinheim, Germany)
Pfu DNA polymerase	Fisher Scientific (Schwerte, Germany)
Restriction enzymes	Fisher Scientific (Schwerte, Germany)
Ribonuclease A	Sigma-Aldrich (Steinheim, Germany)
T4 DNA ligase	Fisher Scientific (Schwerte, Germany)
Taq DNA polymerase	Fisher Scientific (Schwerte, Germany)

4.3 Standards for DNA and protein gel electrophoresis

1 kb DNA ladder	Invitrogen (Karlsruhe, Germany)
GeneRuler 1 kb	Fisher Scientific (Schwerte, Germany)
100 bp DNA ladder	Invitrogen (Karlsruhe, Germany)
PageRuler Prestained Protein Ladder	Fisher Scientific (Schwerte, Germany)

4.4 Kits

BCA Protein Assay	Pierce (Rockford, USA)
GC-RICH PCR System	Roche (Mannheim, Germany)
GeneJET Plasmid Miniprep Kit	Fisher Scientific (Schwerte, Germany)
GeneJET Gel Extraction Kit	Fisher Scientific (Schwerte, Germany)
QuantiTect SYBR Green RT-PCR Kit	Qiagen (Hilden, Germany)
RNeasy Mini Kit	Qiagen (Hilden, Germany)
First Strand cDNA Synthesis Kit	Fisher Scientific (Schwerte, Germany)
First-DNA all-tissue Kit	Genial (Troisdorf, Germany)

4.5 Chromatography material

Ni-NTA Agarose	Qiagen (Hilden, Germany)
Strep-Tactin superflow	IBA (Göttingen, Germany)

4.6 Other materials

Anaerocult A + Anaerotest	Merck (Darmstadt, Germany)
Cellulose nitrate filter	Sartorius (Göttingen, Germany)
Centriplus Centrifugal Filter Device	Millipore (Schwalbach, Germany)
ABgene PCR Plates	Fisher Scientific (Schwerte, Germany)
Adhesive PCR Film	Fisher Scientific (Schwerte, Germany)
Developer	Kodak (Rochester, USA)
Dialysis tube	Serva (Heidelberg, Germany)
Fixer	Kodak (Rochester, USA)
Membrane filters	Whatman (Dassel, Germany)
PD Mini–Trap columns	GE Healthcare (Munich, Germany)
Polypropylene columns	Qiagen (Hilden, Germany)
Roth nylon membrane	Roth (Karlsruhe, Germany)
RNase AWAY	Roth (Karlsruhe, Germany)
Sterile syringe filter	Roth (Karlsruhe, Germany)
Vivaspin 500 centrifugal concentrators	Sartorius (Göttingen, Germany)
Whatman 3MM paper	Millipore (Schwalbach, Germany)
X-ray film X-OMAT AR	Kodak (Rochester, USA)

5 Software and online tools

Table II.4. Software and online tools.

Software	Function	Source or reference
Bioedit	Sequence alignment editor	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
BLAST	Comparison of protein sequences with data bank entries	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Bprom	Recognition of bacterial σ^{70} promoter	http://linux1.softberry.com/berry.phtml
Clone Manager 9	Sequence processing	Sci-Ed software (Cary, USA)
ClustalW	Sequence alignments	www.ebi.ac.uk/clustalw/
Codon usage database	Database for codon usage	http://www.kazusa.or.jp/codon/
Delta mass	Analysis of molecular masses	www.abrf.org
Entrez	Sequence database	www.ncbi.nlm.nih.gov/ENTREZ
ExPasy	Database for DNA and protein analysis	www.expasy.ch
Gimp 2.8	Image processing	www.gimp.org
iCycler iQ software	Control software for iCycler iQ Real Time PCR Detection System	Bio-Rad (Munich, Germany)
INTAS GDS	Control and documentation software for INTAS imaging device	INTAS (Göttingen, Germany)
Microsoft Office 2010	Text and data reprocessing	Microsoft (Redmond, USA)
PC1000	HPLC control software	Thermo Electron (Dreieich, Germany)
PRED-TMBB	Prediction of transmembrane helices in proteins	http://bioinformatics.biol.uoa.gr/PRED-TMBB/
SignalP	Prediction of signal peptides	http://www.cbs.dtu.dk/services/SignalP/
TMHMM v. 2.0	Prediction of transmembrane helices in proteins	http://www.cbs.dtu.dk/services/TMHMM/
TransTermHP	Prediction of terminator sites	http://transterm.cbcb.umd.edu/query.php
UV WinLab	Control of the Perkin Elmer Lambda 11	Perkin Elmer Inc. (Waltham, USA)
WinAspect	Control of the analytic Jena Specord 210	Analytic Jena AG (Jena, Germany)

6 Microbiological methods

6.1 Cultivation of *Alc. vinosum*

Alc. vinosum was grown either photoorganoheterotrophically in RCV medium (Weaver *et al*., 1975) or photolithoautotrophically in modified Pfennig's medium referred to as 0-medium at 30°C anaerobically in the light (Trüper & Pfennig, 1992, Hensen *et al*., 2006). Antibiotics were used in the following concentrations: 10 µg ml^{-1} kanamycin, 50 µg ml^{-1} rifampicin, 10 µg ml^{-1} ampicillin and 20 µg ml^{-1} streptomycin.

6.1.1 Photoorganoheterotrophic growth

RCV medium was used for cultivation of *Alc. vinosum* under photoorganoheterotrophic conditions. The medium was supplemented with the trace element solution SL12 (Overmann *et al*., 1992) RCV medium consists of two solutions that were sterilized separately. When needed, solution A was complemented with solution B. Fresh medium was inoculated with 10% culture and stored in glass bottles filled to the brim to keep access of oxygen to a minimum. Cultures were grown at 30°C in an illuminated incubator (Biotron, Hilden, Germany). Strains were regularly tested for contaminations by incubating samples on LB medium under oxic conditions at 37°.

RCV medium

Solution A

RÄH medium	50 ml
Yeast extract	0.5 g
NaOH	1.8 g
dH_2O	ad 950 ml
pH 7.0	

Solution B

KH_2PO_4	180 mM
K_2HPO_4	180 mM
pH 7.0	

RÄH medium

Malate	60 g
NH4Cl	24 g
$MgSO_4 \times 7\ H_2O$	4 g
$CaCl_2 \times 2\ H_2O$	1.4 g
SL12 (10x)	20 ml
dH_2O	ad 1000 ml

Trace element solution SL12 (10x)

EDTA	3 g
$FeSO_4 \times 7\ H_2O$	1.1 g
$ZnCl_2$	42 mg
$MnCl_2 \times 4\ H_2O$	50 mg
H_3BO_3	300 mg
$CoCl_2 \times 6\ H_2O$	190 mg
$CuCl_2 \times 6\ H_2O$	2 mg
$NiCl_2 \times 6\ H_2O$	24 mg
Na_2MoO_4 x 2 H_2O	18 mg
dH_2O	ad 1000 ml

RCV medium was also used to grow *Alc. vinosum* on solid habitat. For conjugation RCV plates were prepared by adding 1.5% (w/v) agar. After conjugation cells were plated on RCV-phytagel plates. For this purpose RCV medium was solidified by addition of 1% (w/v) phytagel, 0.5% (w/v) NaCl to support gelling as well as 0.02 % (w/v) $Na_2S_2O_3 \times 5\ H_2O$, 2 mM sodium acetate and 2.6 mL feeding solution for growth enhancement. Plates were cultivated within an anaerobic jar; the anaerobic atmosphere was generated inside by using the Anaerocult A reagent.

Feeding solution

$HNaS \times H_2O$	3.1 g
$NaHCO_3$	5.0 g
dH_2O	ad 100 mL

The solution was sterilized and stored in the dark at room temperature.

6.1.2 Photolithoautotrophic growth

Photolithoautotrophic growth was carried out to analyse the phenotype of mutants. To this end *Alc. vinosum* was grown in 0-medium (Trüper & Pfennig, 1992, Hensen *et al*., 2006).

0-medium

Solution 1

KCl	3.3 g
$MgCl_2 \times 6H_2O$	3.3 g
$CaCl_2 \times 2\ H_2O$	4.3 g
NH_4Cl	3.3 g
SL12 (10x)	10 ml
dH_2O	ad 8000 ml

Solution 2

KH_2PO_4	3.3 g
dH_2O	ad 1000 ml

Solution 3

$NaHCO_3$	15 g
dH_2O	ad 1000 ml

Solution 1 was prepared in a 10 L container and the solutions were sterilized separately for 60 min at 121°C. After the liquids had cooled down to room temperature solution 2 and 3 were added to solution 1 under nitrogen atmosphere and constant stirring. The pH was adjusted to 6.7 – 6.9 by bubbling CO_2 into the mix, thereby revoking the precipitation of carbonate. The desired volume was then distributed into glass bottles which were sealed tightly. 0-medium was stored at room temperature in the dark. Before using a new charge the medium was stored over night to check for putative precipitation of contents or contaminations. Reduced sulfur compounds were prepared freshly and added with the inoculum.

6.1.2.1 Turnover of reduced sulfur compounds by *Alc. vinosum* wild type and mutants

The turnover of reduced sulfur compounds by *Alc. vinosum* wild type and mutant strains were determined in batch culture under constant illumination at 30°C and anaerobic conditions. Cells were grown photoorganoheterotrophically in 1000 ml RCV for 3 to 4 day until they reached stationary phase. The culture was harvested (14,000 × g; 10 min; room temperature) and the pellet was washed with 0-medium. After another centrifugation step the resuspended cells served as inoculation for 1 litre 0 medium in a thermostatted fermenter. To keep up the anaerobic conditions a balloon filled with nitrogen was attached to the fermenter. The experiment was started by injecting the freshly prepared and sterile filtrated sulfur compound through a septum into the culture. Elemental sulfur was added without sterilization to avoid changes in speciation due heat. The pH was constantly controlled by a pH-electrode (SteamLine SL 80-325pH, Schott, Mainz, Germany) and adjusted to pH 7.0 with 1 M

HCl or 1 M $NaHCO_3$ (both sterilized) if needed. A water bath ((Lauda M3/MT, MWG, Laude-Königshofen, Germany) kept the temperature steady at 30°C throughout the experiment. For illumination 2 spotlight lamps (Osram, 60 Watt) were positioned at each side of the fermenter at approximately 30 cm. The culture was permanently stirred to guarantee a homogeneous mix. During the experiment samples were taken periodically to determine OD_{690} and concentrations of sulfur compounds as well as protein concentrations.

Alternatively, *Alc. vinosum* was cultivated in glass bottles to monitor the oxidation of reduced sulfur compounds. A 250 ml culture was grown until the stationary phase was reached. The cells were pelleted (14,000 × g; 10 min; room temperature) and washed with 0-medium. After another centrifugation step the cells were resuspended in a small volume of 0-medium and used as inoculum for 250 ml fresh 0-medium. The sulfur compound in question was added and the bottles were filled to the brim to reduce air exposure. Again, the culture was permanently stirred and illuminated. The pH was controlled during sampling and roughly adjusted with 1 M HCl or 1 M $NaHCO_3$.

6.1.3 Conservation of *Alc. vinosum* strains

For long-term conservation *Alc. vinosum* strains were stored in liquid nitrogen. Cells of a well-grown photoorganoheterotrophically grown culture were harvested (4,000 × g; 10 min; room temperature) and resuspended in 5 ml fresh RCV medium. 900 µl of the solution was carefully mixed with 10% (v/v) DMSO (sterilized) and transferred into 2 ml cryo caps (CryoTubes, Nunc).

6.2 Cultivation of *E. coli*

E. coli was aerobically cultivated at 37°C. Cultivation in liquid medium was carried out in a shaker incubator at 180 rpm (HT I FORS AI 70, INFORS, Bottmingen, Germany). Antibiotics for screening purposes were applied in following concentrations: ampicillin 100 µg ml^{-1}, kanamycin 50 µg ml^{-1} and 50 µg ml^{-1} streptomycin.

E. coli DH5α cells were employed for molecular cloning and amplification of plasmids, while *E. coli* S17-1 was used for conjugation with *Alc. vinosum*. *E. coli* BL21(DE3) was used for the overproduction of soluble proteins, for membrane bound proteins *E. coli* strains C41 and C43 were employed.

6.2.1 Luria Bertani (LB) medium

E. coli was mainly cultivated in LB medium (Sambrook *et al*., 1989). For cultivation on solid medium the medium was supplemented with 1.5% (w/v) agar before sterilization. Plated cells were incubated overnight at 37°.

LB medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
dH_2O	ad 1000 ml
pH 7.5	

6.2.2 2xYT medium

E. coli cells intended to be made competent were cultivated in 2x YT medium (Sambrook *et al*., 1989).

2xYT medium

Typtone	16 g
Yeast extract	10 g
NaCl	5 g
dH_2O	ad 1000 ml
pH 7.0	

6.2.3 Conservation of *E. coli* strains

For long-term storage of *E. coli* strains of an overnight grown culture was mixed 1:1 with sterile DMSO or glycerine and kept at -70°C. For short-term storage cell were plated on selective LB medium plates and stored at 4°C after growth overnight at 37°C.

6.2.4 Preparation of competent *E. coli* cells

E. coli cells were prepared for transformation of plasmid DNA using the $CaCl_2$ method of Dagert and Ehrlich (Dagert & Ehrlich, 1974).

$CaCl_2/MgSO_4$ solution

$CaCl_2$	70 mM
$MgSO_4$	20 mM

5 ml 2x YT medium was inoculated with a single *E. coli* colony and incubated overnight at 37°C and 180 rpm. The next day 70 ml 2x YT medium was inoculated 1:100 with the preculture and incubated under the same conditions until an OD_{600} of 0.3-0.5 was reached. The cells were harvested in sterile tubes (4,000 × g; 10 min; 4°C). After discard of the supernatant the cells were carefully resuspended in 10.5 ml cooled $CaCl_2/MgSO_4$ solution and stored on ice for 45 minutes. After the second centrifugation step sedimented cells were resuspended in 3.5 ml of cooled $CaCl_2/MgSO_4$ solution. 875 µl sterile glycerine was added and aliquots were stored at -70°C.

6.2.5 Heterologous overproduction of recombinant proteins

E. coli BL21(DE3) was used for the overproduction of soluble proteins, for membrane bound proteins *E. coli* strains C41 and C43 were employed. Derivates of pET-15b, pET-22b (Novagen, Madison, USA) and pASK-IBA5plus (IBA, Göttingen, Germany) served as expression vectors and provided the host cells with vector-encoded ampicillin resistance. The pET system was induced with 0.1 mM IPTG and resulted in amino-terminally His-tagged proteins, while the latter was induced with 50 µg ml^{-1} anhydrotetracycline and yielded proteins amino-terminally fused to a Strep-tag. For the overproduction of recombinant proteins 500 ml LB medium were inoculated with 5% of an overnight grown pre-culture. The cells were sedimented and washed with fresh LB medium (2,500 × g; 10 min; room temperature). After inoculation the culture was incubated at 37°C and constantly agitated to ensure aeration (shaker-incubator HT I FORS AI 70, INFORS, Bottmingen, Germany) untilan OD_{600} of 0.6 to 0.8 was reached. After induction the cells were cultivated for 2 more hours under the same conditions, harvested (25,000 × g; 20 min; 4°C) and the pellet was stored at -20°C until further use.

7 Molecular biological methods

7.1 Preparation of DNA and RNA

7.1.1 Preparation of chromosomal DNA from *Alc. vinosum*

Chromosomal DNA from *Alc. vinosum* was isolated by using sarcosyl lysis (Bazaral & Helsinski, 1968).

TE buffer	10 mM Tris-HCl; 1mM EDTA; pH 8.0
TES buffer	100 mM NaCl; 10 mM TrisHCl; 1mM EDTA; pH 8.0
Sucrose solution	20% (w/v) sucrose in TES
Lysozyme/RNAse solution	20 mg ml^{-1} lysozyme; 1mg ml^{-1} RNAse
Sarcosine solution	10% (w/v) laurylsarcosine; 250 mM EDTA

Cells of a well-grown culture were harvested (10 min; 2, 500 × g), washed with 50 mM Tris-HCl (pH 8.0) and stored at -20°C for further use. 100 mg cell material was resuspended in 2 ml ice-cold TES buffer and pelleted for 10 min at 4°C and 14,000 × g. The supernatant was discarded and the pellet resuspended in 250 µl sucrose-TES buffer. After cooling for 30 minutes on ice 250 µl lysozyme-RNAse solution was added and the mixture was incubated for 60 minutes at 37°C. 100 µl sarcosine solution was added and after inverting the tube DNA was sheared by pressing it through a sterile cannula (1.2 x 40 mm) several times before adding 300 µl sterile dH_2O. To remove proteins one volume of phenol-chloroform-isoamylalcohol (25:24:1) was added to one volume of DNA containing solution and the mix was vortexed thoroughly before centrifugation (14,000 × g; 4°C; 5 min). The aqueous upper phase was transferred carefully to a new tube to repeat this step at least three times. Phenol was removed from the mix by extraction with chloroform-isoamylalcohol (24:1). The DNA solution was dialysed at 4°C in a dialysis tube (MWCO 12,000) first against TE buffer (2h and 16h, respectively) and in a final step against ddH_2O for 2 h. Chromosomal DNA was stored at 4°C.

7.1.2 Preparation of chromosomal DNA from *E. coli* K-12

Genomic DNA from *E. coli* K-12 was isolated with the First-DNA all-tissue Kit (Genial, Troisdorf, Germany) following the manufacturer's instructions. DNA was stored at 4°C.

7.1.3 Preparation of plasmid DNA

Plasmid DNA from *E. coli* was prepared by either taking advantage of alkalic lysis using the GeneJET Plasmid Miniprep Kit (Fisher Scientific, Schwerte, Germany) according to the manufacturer's instructions or by the non-column prep. For the latter 1.5 ml of an overnight grown *E. coli* culture was pelleted and resuspended in 200 µl buffer 1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg ml^{-1} RNase). After adding 200 µl buffer 2 (200 mM NaOH; 1% SDS; pH 12.5) the tube was inverted several times before 200 µl buffer 3 (3 M KOAc, pH5.5) was added. A centrifugation step (3 min; 16,000 × g; room temperature) followed thorough mixing. The supernatant was transferred into a new tube containing 500 µl chloroform, mixed and centrifuged (16,000 × g; 3 min; room temperature). The resulting supernatant was then mixed with 500 µl isopropanol and DNA was sedimented (16,000 × g; 3 min; room temperature). After discarding the supernatant plasmid DNA was dried at 65°C and resuspended in 50 µl ddH_2O.

7.1.4 Purification of DNA

For cloning DNA fragments were first separated in agarose gels and subsequently excised from the gels. For purification the GeneJet Gel Extraction Kit (Fisher Scientific, Schwerte, Germany) was used according to the manufacturer's instructions. The kit was also employed for the purification of DNA after incubation with restriction endonucleases.

7.1.5 Preparation of RNA from *Alc. vinosum*

Total RNA from *Alc. vinosum* Rif50 was isolated from cells either grown photoorganoheterotrophically on malate (22 mM) or photolithoautotrophically with elemental sulfur (50 mM), sulfide (4 mM) or thiosulfate (5 mM) as electron donor based on the procedure for gram-negative bacteria (Ausubel *et al*., 1997). Precultures were cultivated in 1 litre bottles in the photoorganoheterotrophic growth mode. Cells were harvested (14,000 × g; 10 min; room temperature) and washed with RCV or 0-medium prior to a second centrifugation step. The cells were then used as inoculum for 1 litre of RCV or 0-medium. The culture was grown in thermostatted fermenters to ensure controlled conditions. After addition of reduced sulfur compounds the cultures were grown for 24 hours and samples for RNA extraction (2x 35 ml; 14,000 × g; 10 min; 4°C) were taken at several time points to cover dynamic changes of RNA levels during the oxidation of reduced sulfur compounds. The malate grown culture served as control. Pellets were resuspended in 350 µl RLT buffer (Qiagen, Hilden, Germany) containing 10 mM DTT before the cells were disrupted by vortexing (Ivoclar Vivadent Silamat S6, Ivoclar Vivadent AG, Schaan, Liechtenstein) using 0.1 mm Zirconia/Silica beads (Roth, Karlsruhe, Germany). After sedimentation of beads and cell debris

(15,000 × g; 2 min; 4°C) the supernatant was mixed with 500 µl phenol by thorough vortexing for 1 minute. Subsequently, 500 µl chloroform was added and the mix was again vortexed for 1 minute before it was centrifuged (10,000 × g; 10 min; 4°C). RNA in the supernatant was precipitated by adding $^1/_{10}$ volume 3 M sodium acetate (pH 6) and two volumes of ice-cold ethanol. RNA from 70 ml culture was resuspended in 50 to 100 µl RNase free water (Qiagen, Hilden, Germany). DNase I (Fisher Scientific, Schwerte, Germany) was used for removal of residual DNA. RNA was further purified with the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -70°C.

7.1.5.1 cDNA synthesis

The First Strand cDNA Synthesis Kit (Fisher Scientific, Schwerte, Germany) was used according to the manufacturer's instructions to generate cDNA from RNA isolated from *Alc. vinosum* to check for co-transcription of genes.

7.1.5.2 Quantitative real-time PCR

The transcription of *rhd_2599, tusA* and *dsrE2* was studied via qRT-PCR. As template served 100 ng RNA which was isolated from *Alc. vinosum* at different time points during the oxidation of reduced sulfur compounds. RNA from a malate grown culture was used as control. The QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) and the iCycler iQ real-time detection system (Bio-Rad, Munich, Germany were used following the manufacturer's instructions. Every RNA sample was analysed for residual DNA by omitting reverse transcriptase from the reaction mixture. DNA fragments of approximately 150 bp were amplified. As endogenous control Alvin_0486 was amplified. This gene encodes uroporphyrinogen decarboxylase (Uro-D) and showed no transcriptional changes in the *Alc. vinosum* transcriptome (Weissgerber *et al*., 2013). The reactions were carried out in triplicate. PCR conditions were as follows:

1. 50°C	30 min	Reverse transcription
2. 95°C	15 min	Inactivation of reverse transcriptase / initial activation of polymerase
3. 94°C	15 sec	Denaturation
4. T_a*	30 sec	Annealing of oligonucleotides
4. 72°C	30 sec	Elongation
Repeat steps 3 to 5 40x		
5. 40-100°C	0.5°C (30 sec)$^{-1}$	Melting curve analysis

*T_a (*tusA, dsrE2*): 58°C; T_a (*rhd_2599*); 56°C

Analysis of the melting curve and calculation of C_t (Calculated threshold) values were automatically carried out by the iCycler iQ software. Releative expression ratios (R) were calculated after the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) using equation (1) and (2):

$$\Delta\Delta C_t = (C_{t,target} - C_{t,Alvin\_0486})_{photolithoautotroph} - (C_{t,target} - C_{t,Alvin\_0486})_{photoorganoheterotroph} \quad \text{eq (1)}$$

$$R = 2^{-\Delta\Delta Ct} \quad \text{ep (2)}$$

7.2 Determination of concentration and purity of nucleic acids

The purity and concentration of nucleic acids was determined by UV/Vis spectroscopy (Specord 210, Analytik Jena, Jena, Germany). The absorption of diluted samples was measured at 260 nm and 280 nm. The maximum absorption for nucleic acids is found at 260 nm; here, an absorption value of 1.0 corresponds to 50 µg ml^{-1} double-stranded DNA and 40 µg ml^{-1} RNA (Sambrook *et al*., 1989). The maximum absorption of proteins is 280 nm. The ratio of A_{260}/A_{280} was used to ascertain the purity of nucleic acid solutions. Ratio values of 1.8–2 indicate high degrees of purity.

7.3 Transfer of DNA

7.3.1 Transformation of competent *E. coli* cells

Plasmid DNA was transferred into *E. coli* cells by using the transformation technique (Hanahan, 1983). 1µl plasmid DNA or a complete ligation preparation were added to 100 µl competent cells and stored in ice for 30 minutes. After the heat-shock (42°C, 90 sec) and cooling phase of 2 minutes in ice 500 µl LB or 2x YT medium were added. The cells were further incubated for 45 to 60 minutes at 37°C, subsequently plated on selective plates and incubated overnight at 37°C.

7.3.2 Conjugational transfer of plasmid DNA from *E. coli* to *Alc. vinosum*

Alc. vinosum is genetically accessible through conjugation (Pattaragulwanit & Dahl, 1995). *E. coli* S17-1 donor cells were grown overnight on LB medium with the appropriate antibiotic. 3 ml RCV was inocultated with the donor cells until an OD_{600} of 0.4 was reached. An OD_{600} of 0.1 corresponds to 10^8 *E. coli* cells ml^{-1} (Sambrook *et al*., 1989). *Alc. vinosum* Rif50 served as recipient and was grown photoorganoheterotrophically until stationary phase in which an OD_{690} of 1.0 corresponds to 8×10^8 cells ml^{-1} (Pattaragulwanit & Dahl, 1995). Roughly 12×10^8 cells were sedimented (10,000 × g; 5 min; room temperature) and washed twice with fresh RCV medium before they were resuspended in 500 µl RCV medium. Afterwards 500 µl of the *E. coli* solution was added resulting in a 3-times higher *Alc. vinosum* cell count. The cells were pelleted and resuspended in a small volume RCV medium before they were evenly plated on sterile nitrate cellulose filters (pore size 0.45 µm) that were placed on RCV-agar plates. The incubation of the plates took place in an anaerobic jar for two days in the light at 30°C. Subsequently, the cells were carefully washed from the filter with 1 ml RCV medium and plated on RCV-phytagel plate which contained appropriate antibiotics for the screening process.

Derivatives of pK18*mobsacB* were installed to create *in frame* deletion mutants of *Alc. vinosum*. Since this suicide vector is not replicable in *Alc. vinosum*, it has to be integrated into the genome, thereby providing resistance to kanamycin. Single crossover mutants were grown for 3 generations in liquid medium without kanamycin in order to remove the vector backbone after homologous recombination from the genome and then plated on RCV phytagel plates containing 10% sucrose to induce the expression of *sacB*. The exoenzyme levansucrase SacB is lethal. When plasmids of the pSUP series

were used double crossover mutants were identified by their resistance to kanamycin and loss of the vector-encoded ampicillin resistance. Genotypes were verified via colony PCR and Southern blotting.

7.4 Enzymatic DNA modification

7.4.1 Restriction digestion of DNA

DNA was digested by type II restriction endonucleases obtained from Fisher Scientific (Schwerte, Germany) and used according to the manufacturer's instructions. A typical restriction assay contained 0.5 to 10 µg DNA and 1 to 5 U restriction enzyme. The digestion was performed at 37°C or at another manufacturer-recommended temperature for one hour.

7.4.2 *in vitro* amplification of DNA by Polymerase chain reaction (PCR)

The polymerase chain reaction was developed by Mullis and Faloona to amplify specific DNA sequences via a repeating cycle of denaturation of double stranded DNA, annealing of specific oligonucleotides and elongation of the same. For amplification of sequences for molecular cloning and introduction of restriction sites either the *Pfu* polymerase from Pyrococcus furiosus (Fisher Scientific, Schwerte, Germany) or the GC-RICH system (Roche, Mannheim, Germany) were employed. The latter is a mix of *Tgo* polymerase from *Thermococcus gorganarrius*, providing 3'-5' proof reading acitivity and *Taq* polymerase from *Thermococcus aquaticus*. The *Taq* (Fisher Scientific, Schwerte, Germany) polymerase as sole enzyme was used for screening PCR. Oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany). All PCR were performed according to the manufactures' instructions and carried out either in Trio-Block (Biometra, Göttingen, Germany) or in MyCycler (BioRad, Munich, Germany) thermal cycler. For a standard PCR chromosomal or plasmid DNA served as template. Colony PCR was performed in order to screen for positive clones of *Alc. vinosum* mutants. Here, whole cells either from a liquid culture or from a plate were washed with ddH_2O at least twice and added in a small volume to the reaction mixture. To break the cells the mixture was incubated for 10 min at 95°C before *Taq* polymerase was added.

Standard PCR mixture

Polymerase-specific buffer	1x
DNA	0.1 – 0.5 µg
Primer forward	30 pmol
Primer reverse	30 pmol
Nucleotides	0.2 mM
GC mix /*Taq*/*Pfu* polymerase	1 U/2.5 U/1.25 U
ddH_2O	ad 50 µl

For colony PCRs 5% (v/v) DMSO and 25 mM $MgCl_2$ were added.

Standard PCR protocol

1. 95°C	3 min	Initial denaturation step
2. 95°C	30 sec	Denaturation
3. T_A	1 min	Primer annealing
4. 72°C	1-2 min/kb*	Elongation
repeat steps 2-4 for 25 cycles		
5. 72°C	7 min	Final elongation

* The elongation period depends on the product size and the polymerase used: *Taq* polymerase: 1 min/kb; GC mix and *Pfu* polymerase: 2 min/kb.

7.4.3 Site-directed mutagenesis by gene SOEING

The introduction of pinpoint mutations and *in frame* deletion of genes requires the substitution and alteration of specific nucleotides or nucleotide sequences. These were achieved by gene SOEING (gene splicing by overlap extension), a method developed by Horton (1995). Complementary primers carrying the desired mutation were used in two separate PCR reactions. The resulting DNA fragments harboured overlapping ends that were complementary to each other and were combined in the third PCR reaction in which these overlaps served as oligonucleotides for each other. This fusion product was then amplified. For the procedure either *Pfu* polymerase or the GC rich system were applied.

7.4.4 Construction of digoxigen-labelld DNA probes by PCR

For DNA-DNA hybridization in the process of Southern blotting DNA probes were labelled with dig-dUTP (20 µM) during PCR. Therefore, the concentration of dTTP was reduced to 160 µM. Following an otherwise standard PCR the product was analysed by electrophoresis and excised from the agarose gel. For further use the probe was cooked for 20 min at 100°C added to the Southern blott assay for hybridization.

7.4.5 Alkaline phosphatase

For plasmid construction all vector backbones were incubated either with calf intestinal alkaline phosphatase or FastAP (both purchased from Fisher Scientific, Schwerte, Germany) to remove phosphate groups from linearized vector backbones. 1 µl of the enzyme was directly added to the restriction digestion and incubated for 30 min at 37°C.

7.4.6 Ligation

The T4 ligase (Fisher Scientific, Schwerte, Germany) was used to ligate vector backbones with DNA fragments. Ligations were carried out either for 2 h at 37°C or overnight at 16°C.

7.5 DNA sequencing

Successful cloning of DNA sequences into plasmids constructed in this study was verified via DNA sequencing by GATC (Konstanz, Germany).

7.6 DNA-DNA hybridization

Southern hybridization is used to detect specific sequences within genomic DNA and allows the identification of mutated sequences (Southern, 1975; Southern, 1979).

Solutions

Southern blotting

Depurination solution	0.25 M HCl
Transfer solution	0.4 M NaOH
20x SSC	3 M NaCl; 0.3 M sodium citrate; pH 7.0
2x SSC + 1 % SDS	10% (v/v) 20x SSC; 1% (w/v) SDS

Hybridization

Buffer 1	0.1 M maleic acid; 0.15 M NaCl; pH 7.5
Pre-hybridization solution	20% (v/v) buffer 1; 25% (v/v) 20x SSC; 0.1 % N-lauroyl sarcosine; 0.02% (w/v) SDS; 2% (w/v) blocking reagent
0.1x SSC + 1% SDS	0.5% (v/v) 20x SSC; 1% (w/v) SDS

Chemiluminescent detection

Buffer 2	1% blocking reagent in buffer 1 (storage at 4°C)
Buffer 3	0.1 M Tris-HCl; 0.1 M NaCl; pH 9.5 (storage at 4°C)
Washing buffer	0.3% Tween-20 in buffer 1

To this end chromosomal DNA from *Alc. vinosum* Rif50 and the putative mutant strain was digested by restriction endonucleases and separated by agarose gel electrophorese. The DNA fragments were transferred to a Roti-Nylon plus membrane (Roth, Karlsruhe, Germany) via capillary blot after documentation of the gel.

Afterwards the membrane was rinsed twice with 2x SSC and the DNA was covalently linked to the membrane by UV cross-linking (Stratalinker 1800, Stratagene, La Jolla, USA). The membrane was incubated in pre-hybridization solution for 3 to 5 hours at 68°C to prevent unspecific binding in a hybridization oven (Biometra, Göttingen, Germany). For hybridization the dig-labelled DNA probe, containing the desired sequence (I.7.5.4), was incubated at 100°C for 20 minutes before it was poured into the pre-hybridization solution and allowed to bind to the membrane bound DNA overnight for 16 to 18 hours at 68°C.

For the chemiluminescent detection of the DNA probe the same was coupled with digoxigenin-specific antibody coupled to an alkaline phosphatase (antidigoxigenin-AP, Roche, Mannheim, Germany). All steps were carried out at room temperature. The pre-hybridization solution together with excess DNA probe was discarded and the membrane was washed twice with 100 ml 2xSSC +

0.1% SDS (5 minutes each time) and twice with 100 ml 0.1x SSC + 1% SDS (15 minutes each time). Following a washing step (50 mL washing buffer for 5 minutes), unspecific binding sites were saturated during the incubation with 50 mL buffer 2 for 30 minutes. Buffer 2 was renewed (15 ml) and 2 µl digoxignin-antibody was added. After 30 minutes the buffer was discarded and the membrane washed twice with 100 ml washing buffer (15 minutes). The membrane was now equilibrated with 30 ml buffer 3 for 5 minutes before 10 µl CDP-star (the substrate for alkaline phosphatase) was added. The resulting emission of visible light was documented using X-ray film (X-OMAT AR, Kodak, Rochester, USA).

8 Proteinbiochemical methods

8.1 Cell lysis

Cells were disrupted via sonication. Therefore, the cells were resuspended in 3 ml of the appropriate buffer per 1 g wet weight (at least 6 ml). The cells were exposed to sonication for 1.5 min ml^{-1} (Cell Disruptor B15, Branson, Danbury, USA) at 50% intensity and 4°C. The crude extract was centrifuged (25,000 × g; 4°C; 30 min) to sediment unbroken cells and cell debris. Recombinant proteins were isolated from the supernatant.

8.2 Ultracentrifugation

Ultracentrifugation (145,000 x g; 4°C) was used to prepare the membrane fraction from crude extracts. Extracts from *E. coli* were centrifuged for 2 hours, while this period was expanded to 4 hours when *Alc. vinosum* extracts were used. Membranes were found in the pellet while the supernatant contained the soluble fraction.

8.3 Solubilisation of membrane proteins

Solubilisation was an obligatory step prior to the purification of membrane-bound proteins to convey the proteins from the membrane fraction into soluble solution. Therefore, the pellets attained from ultracentrifugation were resuspended in lysis buffer and incubated with 1% of the detergent Triton X-100 overnight on ice. To support the process the mix was gently stirred. Afterwards the protein solution was again applied to ultracentrifugation and the respective protein was purified from the supernatant.

8.4 Purification of recombinant proteins

Recombinant proteins were isolated using affinity-chromatography.

8.4.1 His-tag purification

Lysis buffer

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	10 mM
pH 8.0	

Elution buffer

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	20-250 mM
pH 8.0	

Proteins fused to a His-tag were purified via immobilized-metal affinity chromatography using Ni-NTA agarose. DsrC, DsrEFH, TusA, Rhd_2599, and IscS were purified with this method. Binding of the proteins to the Ni-NTA resin was done in the column mode: The agarose was equilibrated with 6 CV of lysis buffer before the crude extract was applied. Unspecifically bound proteins were washed from the column with 3 CV of lysis buffer containing 10, 20, 50 and 75 mM imidazole. Elution of the protein of interest was performed with 2 CV of lysis buffer containing 250 mM imidazole.

8.4.2 Strep-tag purification

Buffer W

Tris-HCl	100 mM
NaCl	150 mM
Triton-X100	0.1%
pH 8.0	

Buffer E

Buffer W	
Desthiobiotin	2.5 mM
Triton X-100	0.1%

The Strep-tag binds highly selective to Strep-Tactin and allows a highly efficient and homogenous purification of recombinant proteins. Since the Strep-Tag system is compatible with the use of the detergent Triton X-100 in a concentration up to 2% DsrE2 protein variants were isolated with this system. Binding of the proteins to the Strep-Tactin was carried out in the column mode: The agarose was equilibrated with 4 CV of buffer W before the crude extract was applied. Unspecifically bound proteins were washed from the column with 5 CV of buffer W. The proteins were eluted with 6x 0.5 CV buffer E containing 2.5 mM desthiobiotin.

8.5 Desalting of protein solutions

Protein buffers were desalted and exchanged using dialysis or gel filtration.

8.5.1 Dialysis

For dialysis protein solutions were filled into dialysis tubes (MWCO 3,500 Da (TusA) or 6,000 to 8,000 Da (all other proteins), Serva, Heidelberg, Germany) and gently stirred in 2 litres of the appropriate buffer for 16 to 18 hours at 4°C. Tubes were prepared by autoclaving and rinsing in dH_2O.

8.5.2 Gel filtration

Gel filtration of small volumes was carried out using PD MiniTrap G25 columns (GE Healthcare, Munich, Germany). The columns were equilibrated with 3 CV of the desired buffer before a maximum of 500 µl protein solutions was applied. If the volume of the solution fell below 500 µl the protein solution was applied and the volume was adjusted with equilibration buffer after the protein solution had completely entered the Sephadex G25 medium.

8.5.3 Storage buffers

Proteins were stored at -70°C after quick-freezing in liquid nitrogen in the following buffers:

DsrC	50 mM Tris-HCl; 500 mM NaCl; pH 7.5
DsrEFH	10 mM Tris-HCl; pH 7.5
DsrE2	100 mM Tris-HCl; 150 mM NaCl; 0.1% Triton X-100; pH 8.0
IscS	50 mM Tris-HCl; 200 mM NaCl; pH 7.5
Rhd_2599	50 mM Tris-HCl; 100 mM NaCl; pH 8.0
TusA	50 mM Tris-HCl; 10 mM KCl; pH 7.5

Preparations of DsrC and TusA that were intended for analysis with MALDI-TOF mass spectrometry were dialysed after purification in the stated buffer and desalted before storage.

8.6 Protein quantification

8.6.1 Protein quantification after Bradford

The concentration of *Alc. vinosum* cultures was determined with Bradford reagent (Bradford, 1976). To this end 1 ml of the culture was pelleted and frozen if needed. The thawed sample was resuspended in 1 ml 1 M NaOH and incubated at 95°C for 5 min to disrupt the cells. After a centrifugation step (13000 × g; room temperature; 3 minutes) 25 µl of the supernatant were mixed with 750 µl Bradford reagent. The extinction of the reaction mixture was measured at 595 nm after 10 minutes in the dark against a chemical blank. For the standard curve BSA concentrations ranging from 0 to 1.4 mg ml^{-1} were used.

8.6.2 Protein quantification with BCA

The concentration of purified protein solutions was determined using the BCA Protein Assay-Kit (Pierce, Rockford, USA) following the manufacturer's instructions. The calibration curve was generated with BSA ranging from 0-500 µg ml^{-1}.

8.7 Concentration of protein solutions

For concentration of large volumes of protein solutions Centriplus Centrifugal Filter Device (Millipore, Schwalbach, Germany) with an appropriate MWCO were used. The solutions were centrifuged at 3,000 × g at 4°C until the desired volume was reached. For small volumes Vivaspin 500 centrifugal concentrators (Sartorius,Göttingen, Germany) were applied (13,000 × g; 4°C).

8.8 Analysis of protein-protein interaction

Band shift assays

The formation of protein complexes was analysed either with native PAGE or BN-PAGE. For sample preparation proteins were incubated in varying amounts in 5 mM HEPES (pH 7.8), 0.1 M KCl, 0.01% Tween 20 and 25 µM TCEP in a final volume of 60 µl for 30 minutes at 30°C.

Surface Plasmon Resonance

For SRP DsrEFH (in 20 mM sodium acetate, pH 5.0) was covalently immobilized to a CM5 sensor chip (Biacore, Munich, Germany) using the amine coupling protocol. The protein (150 mg ml^{-1}) was injected during 2 minutes at 10 ml min^{-1}, resulting in 600 resonance units (RU) of immobilized protein on the CM5 chip surface. TusA (10 mM) was incubated in running buffer (10 mM HEPES; 150 mM NaCl; 3 mM EDTA; 0.005% Tween 20, pH 7.4) in the presence of 25 mM TCEP for 30 minutes at room temperature. TusA was then diluted and injected at flow rate of 40 ml min^{-1} for three minutes. At the end of sample injection, the running buffer was flowed for 6 minutes over the sensor surface to allow dissociation. For regeneration of the surface 2 M $MgCl_2$ was used. For the experiment a Biacore 2000 instrument (Biacore, GE Healthcare, Munich, Germany) was used at 25°C.

9. Detection of sulfur binding and sulfurtransferase activity

9.1 Detection of sulfur binding and sulfurtransferase activity using MADLI-TOF mass spectrometry

Buffers

Buffer A	Tris-HCl	50 mM (pH 7.5)
	NaCl	100 mM
Buffer B	Tris-HCl	50 mM (pH 7.5)
	NaCl	50 mM
Buffer C	HEPES	10 mM (pH 7.5)

For the sulfur binding experiments 30 µM of recombinant protein was incubated either with 2 mM NaSH or with 2 µM IscS and 2 mM cysteine for one hour at 30 °C in a final volume of 100 µl buffer A. Sulfite (2 mM), thiosulfate (2mM) and GSSH (0.5 mM) were also tested as substrates. GSSH as was formed by incubating 500 µM oxidized glutathione with 450 µM sulfide for 30 minutes at 30°C (Rohwerder & Sand, 2003). For detecting sulfurtransferase activity the putative sulfur-donating protein was incubated with sulfide as described above. Afterwards, sulfide was removed by gel filtration on PD Mini—Trap columns (GE Healthcare, Munich, Germany). The columns were run according to the manufacturer's instructions using a volume of 700 µl for elution. 30 µM of the putative sulfur acceptor protein was added after the sulfide-free donor protein samples had been concentrated to their initial concentration of 30 µM using Vivaspin 500 centrifugal concentrators (5 kDa MWCO, Sartorius, Göttingen, Germany). The samples were again incubated for one hour in a final volume of 100 µl buffer B. After buffer exchange the samples were stored overnight on ice.
For MALDI-TOF mass spectrometry, buffer B was exchanged for buffer C by using PD Mini—Trap columns. Samples were diluted 1:5 with 0.1% trifluoracetic acid in ddH_2Owater and mixed with one volume of matrix: alpha-cyano-4-hydroxycinnamic and sinapic acid were used. Matrices were dissolved in 0.1% trifluoracetic acid in acetonitrile. Diluted samples were mixed with dissolved matrix in the ration 1:1 and 1 µl of this mix was applied to a MPT 384 target plate ground steel TF (BrukerDaltonik GmbH, Leipzig, Germany). Spectra were recorded in the linear positive mode within a range of 2 kDa to 20 kDa using Biflex III (BrukerDaltonik GmbH, Leipzig, Germany).

9.2 Detection of sulfur binding and sulfurtransferase activity using 1,5-IAEDANS

1,5-IAEDANS *(N*-(iodoacetyl)-*N*'-(5-sulfo-1-naphtyl)-ethylenediamine) is a fluorescent reagent that specifically binds to thiol groups by displacement of the iodide group with the sulfur atom of the thiol group (Zheng *et al*., 1994; Thomè *et al*., 2011). For the detection of persulfides the protocol of Thomè was used (Thomè *et al*., 2011). Here, labelled proteins are analysed via SDS-PAGE and fluorescence is detected under UV-light. The derivatisation with 1,5-IAEDANS alone leads to fluorescent bands mirroring the migration patterns of proteins and does not discriminate between proteins in their thiol and persulfide state. To distinguish between these states Thomè and co-workers treated all samples with DTT prior to electrophoresis. While the protein in the thiol state is unchanged by the presence of DTT, the disulfide bond in the persulfidic protein will be reduced and the sulfane sulfur together with coupled 1,5-AEDANS will dissociate from the protein which leads to a significant decrease of the fluorescence.

Solutions

1,5-IAEDANS	0.5 mM (in 100 mM Na_2HPO_4; pH 7.5)
Cysteine	100 mM
DTT	200 mM
Buffer D	50 mM Tris-HCl (pH7.5); 100 mM NaCl (0.1% Triton X-100)

All proteins were first treated with 2 mM DTT for 30 minutes at room temperature to reduce thiol groups and to remove any pre-existing persulfides. Excess DTT was removed by gel filtration on PD Mini-Trap columns (GE Healthcare, Munich, Germany) using buffer D. When DsrE2 was analysed the buffer contained 0.1% Triton X-100 in addition. For testing the principle reactivity of the reduced proteins with 1,5-IAEDANS the proteins were concentrated using Vivaspin 500 centrifugational concentrators (5 kDa MWCO, Sartorius,Göttingen, Germany) to about 800 µg ml^{-1} at this stage. A volume containing 200 pmol protein was then brought to a volume of 20 µl and 1,5 IAEDANS was added. Conditions and further treatment were as described below for the sulfide treated proteins.

For persulfuration of proteins the reduced protein solutions eluted from PD Mini-Trap columns were immediately incubated with 4 mM NaHS or 4 mM sodium thiosulfate for one hour at 30°C followed by dialysis against buffer D to remove any excess sulfur compound. Subsequently as a control, half of the persulfurated protein solution was again reduced with DTT which was then removed by gel filtration. Both, the untreated and the DTT-treated protein solutions were then concentrated to 300-700 µg ml^{-1}. 100 pmol (in case of DsrEFH) or 200 pmol (all other proteins) were incubated with 0.5 nmol of 1,5-IAEDANS in a final volume of 20 µl for one hour in the dark at 4°C. Unbound 1,5-IAEDANS was allowed to react with 100 nmol L-cysteine for 30 minutes at room temperature to prevent reaction of 1,5-IAEDANS in subsequent reaction steps. All reaction mixtures were now treated with DTT for 30 minutes at room temperature to reductively release possible persulfides as 1,5-AEDANS-sulfide conjugates. The samples were then mixed with 1 µl of native loading buffer and the complete reaction mixture was applied to a 15% Laemmli gel that was run in the dark. Note that the pH of the resolving gel was adjusted to pH 9.5 to improve separation. The gels were then analysed under UV light for visualization of 1,5-AEDANS-labelled proteins. The same gels were later stained with Coomassie-Brilliant Blue to allow comparison of protein amounts in each lane. When sulfur transfer from one protein to another was studied, 100 pmol (in case of DsrEFH) or 200 pmol (all other proteins) persulfurated donor protein was combined with the same amount of acceptor protein in a final volume of 20 buffer D for one hour at 30°C followed by addition of 1,5 IAEDANS and further treatment as described above.

9.3 Determination of thiosulfate:Cyanide sulfurtransferase, glutathionepersulfide:cyanide sulfurtransferase activity

Thiosulfate:cyanide sulfurtransferase (rhodanese) activity was measured according to Ray *et al.*(Ray *et al.*, 2000). The assay contained 100 mM glycine (pH 8.9), 50 mM sodium thiosulfate, 50 mM NaCN and enzyme in a final volume of 500 µl. The reaction was started by adding NaCN and the mixture was incubated for 1 minute at 30°C. 250 µl 15% formaldehyde was used to stop the reaction before 750 µl ferric nitrate reagent (25 g of $Fe(NO_3)_3 \times 9\ H_2O$ and 50 ml of 65% HNO_3 per 375 ml) was added. Absorption was measured at 460 nm. Enzyme units are defined as the amount that catalyzed the production of 1 µmol thiocyanate. As an alternative substrate GSSH was tested in a concentration

of 50 mM. GSSH was synthesized by incubating 500 mM oxidized glutathione with 500 mM sulfide for 30 minutes at 30°C (Rohwerder & Sand, 2003).

10 Electrophoretic methods

10.1 Agarose gel electrophoresis of DNA

The separation of DNA fragments was achieved by agarose gel electrophoresis (Sambrock *et al*., 1989).

TAE buffer

Tris	40 mM
Acetic acid	20 mM
EDTA	10 mM
pH 8.0	

10x loading buffer

Bromphenole blue	0.25% (w/v)
Sucrose	40% (w/v)

To this end DNA was mixed with the appropriate volume of 10x loading buffer and placed into the wells of an agarose gels (1-2% agarose in 1x TAE buffer). As standards 5 µl of 1kb or 100 bp DNA ladder were used (Invitrogen, Karlsruhe, Germany and Fisher Scientific, Schwerte, Germany). After covering the gel with 1x TAE buffer they were run at 80 to 100 V. Ethidium bromide or GelRed (Biotium, Hayward, USA) were used for staining (10 minute; gentle shaking in the dark). After rinsing the gels with dH_2O nucleotides were visualized on a UV transilluminator (INTAS, Göttingen, Germany).

10.2 Electrophoretic separation of proteins

10.2.1 SDS PAGE

For protein separation by molecular mass discontinuous SDS-PAGE (Laemmli, 1970) was performed with the Bio-Rad Mini Protean system (Bio-Rad Mini Protean system, Munich, Germany).

Solutions

Buffer A	1.5 M Tris-HCl; pH 8.8; 0.3% (w/v) SDS
Buffer B	0.5 M Tris-HCl; pH 6.8; 0.4% (w/v) SDS
Acrylamide solution	30% Acrlyamide-bisacrylamide (37.5:1)
Sample buffer (reducing)	4x Rotiload
2x sample buffer (non-reducing)	100 mM Tris-HCl; pH 6.8; 4% SDS; 0.001 % bromphenol blue; 20 % (v/v) glycerine
2x sample buffer (native)	2M sucrose; 1% (w/v) bromphenol blue
5x Electrophoresis buffer	15g Tris; 72 g glycine; 5 g SDS ad 1000 ml ddH_2O

Resolving and stacking gels were prepared by mixing the appropriate volumes of ddH_2O, buffers A and B, acrylamide, APS and TEMED to obtain gels of 7.5 to 15%:

	resolving gel 7.5%	resolving gel 12.5%	resolving gel 15%	stacking gel 4.5%
ddH_2O	6 ml	4 ml	3 ml	3 ml
buffer A	3 ml	3 ml	3 ml	3 ml
buffer B				1.25 ml
acrylamide	3 ml	5 ml	6 ml	750 µl
APS (10%)	100 µl	100 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl	5 µl

After the resolving gel was polymerized the stacking gel was poured above. The protein samples were mixed with 4x Rotiload and cooked at 100°C for 5 minutes or mixed with 2x non-reducing buffer if the analysis of putative disulfide bonds was intended. After the samples were placed in the wells the gel chamber was filled with 1x electrophoresis buffer. The gels were run at 60 V until the proteins reached the resolving gel and completed at 100 V. PageRuler Prestained Protein Ladder (Fisher Scientific, Schwerte, Germany) served as standard.

For the detection of protein bound persulfides (I.9.2.) the pH of the resolving gel was adjusted to pH 9.5 for better resolution and protein samples were mixed with native sample buffer.

10.2.2 Native PAGE

The formation of protein complexes was analysed with native PAGE. Here, the Laemmli system (Laemmli, 1970) was used as well, but SDS was omitted from all buffers. All resolving gels had a concentration of 7.5%. The gel was run at 12 mA at 4°C.

10.2.3. BN-PAGE

Electrophoretic separation of native protein complexes according to their molecular weight was carried out using BN-PAGE (Jagov & Schägger, 1994). The complexes were separated in a linear gradient of the resolving gel from 10 to 15% (10 to 15% acrlyamide-bisacrylamide; 50 mM Bis/Tris-HCl; pH 7.0). The stacking gel equalled that used for SDS-PAGE. The gel was run at 6 mA at 4°C

Cathode buffer	50 mM tricine; 15 mM Bis/Tris-HCl (pH 7.0); 0.02% (w/v) Coomassie Brilliant Blue G250
Anode buffer	50 mM Bis/Tris-HCl (pH 7.0)

10.2.4 Visualization of proteins

After electrophoresis the resolving gel was stained in with Coomassie Brilliant Blue. After at least 60 minutes of staining the gels were transferred to destaining solution; the destaining solution was exchanged several times until protein bands were visible. Subsequently, destained gels were dried (Aldo-Xer gel dryer, Schutt, Göttingen, Germany).

Staining solution

Methanol	50 % (v/v)
Acetic acid	10 % (v/v)
Coomassie Brilliant Blue R250	0.25 % (w/v)
dH_2O	40 % (v/v)

Destaining solution

Methanol	20 % (v/v)
Acetic acid	10 % (v/v)
dH_2O	70 % (v/v)

11 Analytical determination of sulfur compounds

11.1 Determination of elemental sulfur

The modified cyanolysis after Bartlett and Skoog (Bartlett & Skoog, 1954) was used to monitor intracellular sulfur concentrations of *Alc. vinosum* during oxidation of reduced sulfur compounds.

Ferric nitrate reagent

$Fe(NO_3)_3 \times 9\ H_2O$	30 g
HNO_3 (55 %)	40 mL
ddH_2O	ad 100 mL

Sodium cyanide solution

NaCN	0.2 M

200 µl culture sample was centrifuged (13,000 ×g; 3 minutes; room temperature). The pellet was frozen until further use; it was then resuspended in 200 µl dH_2O and cooked at 100°C for 10 minutes after addition of 100 µl NaCN (0.2 mM). 650 µl dH_2O and 50 µl ferric nitrate reagent were added after a short cooling period. The mix was again centrifuged (13,000 ×g; 3 minutes; room temperature) and the absorption of the supernatant was measured at 460 nm against chemical blank. Sodium thiocyanate (0 to 300 nmol) was used to create the calibration curve.

11.2 Determination of sulfate

The protocol of Sörbo (Sörbo, 1987) was employed for the determination of sulfate in the supernatant of culture samples.

$BaCl_2$-PEG reagent

$BaCl_2 \times 2\ H_2O$	0.98 g
PEG6000	15 g
Na_2SO_4 (50 mM)	100 µl (added drop by drop while stirring the solution)
dH_2O	ad 100 ml

1 ml of culture sample was centrifuged (13,000 × g; 3 minutes; room temperature) and the supernatant was frozen for subsequent determination. After another centrifugation step (13,000 ×g; 5 min; room temperature) one volume of supernatant was mixed with one volume of 0.5 M TCA with intent to

release residual sulfide and precipitate proteins. After at least 10 minutes the mix was centrifuged (13,000 × g; 5 minutes; room temperature). Afterwards, 600 µl 0.25 M TCA was mixed with 200 µl of the supernatant and 200 µl BaCl2-PEG solution was precipitate SO_4^{2-} anions as BaSO4. The reaction was carried out for 20 minutes at room temperature under constant gentle shaking and thorough vortexing after 10 minutes. The absorption was measured at 450 nm against chemical blank. Na_2SO_4 was used to generate the standard curve (0 to 100 nmol).

11.3 Determination of thiosulfate

The concentration of thiosulfate was determined using the protocol of Urban (Urban, 1961).

Solutions

Ferric nitrate reagent	
$Fe(NO_3)_3 \times 9\ H_2O$	30 g
HNO_3 (55 %)	40 mL
ddH_2O	ad 100 mL
NaCN	0.2 M
$CuCl_2$	40 mM
NaOAc	0.2 M (pH 4.8)

A volume containing a maximum of 250 nmol thiosulfate was taken from the culture sample and was filled up with dH_2O to a total volume of 650 µl and mixed with 200 µl NaOAc, 50 µl NaCN, 50 µl $CuCl_2$. After thorough mixing 50 µl of the ferric nitrate solution was added and the absorption was measured at 460 nm against the chemical blank. The concentration of thiosulfate was determined directly without freezing the sample. The calibration curve was generated with Na_2SO_3 in concentrations ranging from 0 to 250 nmol.

11.4 Determination of tetrathionate

A slightly modified protocol after Kelly (Kelly, 1969) was employed for the determination of tetrathionate.

Solutions

Ferric nitrate reagent	
$Fe(NO_3)_3 \times 9\ H_2O$	30 g
HNO_3 (55 %)	40 mL
ddH_2O	ad 100 mL
NaCN	0.2 M
Tris-acetate	1 M (pH 8.7)

A volume containing a maximum of 150 nmol tetrathionate was taken from the culture sample and was filled up with dH_2O to a total volume of 500 µl and mixed with 50 µl Tris-acetate and 50 µl NaCN. After thorough vortexing the mix was incubated for 5 minutes at room temperature before 50 µl of ferric nitrate solution was added. The absorption was measured at 460 nm against the chemical

blank. The tetrathionate concentration was determined directly without prior freezing of the sample. The calibration curve was generated with $K_2S_4O_6$ in concentrations ranging from 0 to 150 nmol.

11.5 Determination of thiols using HPLC

Thiols such as sulfide, polysulfides, sulfite and thiosulfate were determined using HPLC. Therefore, the thiols were derivatized with fluorescent monobromobimane and detected by their fluorescence at 480 nm (Rethmeier *et al*., 1997).

Solutions

HEPES buffer	HEPES 50 mM
	EDTA 5 mM
	pH 8.0 (adjusted with NaOH)
Monobromobimane:	96 mM in acetonitrile
Methanesulfonic acid:	65 mM
Solvent A:	0.25% acetic acid
	pH 4.0 (adjusted with NaOH)
Solvent B:	methanol (HPLC grade)

The samples were prepared by mixing 50 µl culture sample with 50 HEPES buffer, 55 µl acetonitrile and 5 µl monobromobimane. After 30 minutes in the dark 100 µl methanesulfonic acid was added and the sample was stored at -20°C. For analysis via HPLC the sample was centrifuged (13,000 ×g; 5 minutes; room temperature) to sediment cell material and 10 µl of the supernatant was added to 190 ddH_2O. The diluted sample was poured into HPLC tubes and 50 µl were injected into the sample loop by the autosampler and directed into the column LiChrospher 100 RP-18e (Merck, Darmstadt, Germany) for analysis. The samples eluted from the column in a linear gradient, consisting of solvents A and B, with a flow rate of 1 ml min^{-1}. The column was heated to 35°C in the column oven.

Gradient

Time [min]	Solvent A [%]	Solvent B [%]
0	85	15
5	85	15
50	55	45
55	0	100
58	0	100
61	85	15
76	85	15

Derivatized thiols were detected by a fluorescence detector FL3000 (excitation at 380 nm, emission at 480 nm) (Thermo-Separation Products, Engelbach, Germany). Concentrations for standards ranged from 50 to 1000 µM of the respective sulfur compounds. Standards for polysulfides were not available, therefore their concentration remained relative.

III Results

1. Analysis of DsrEFH and DsrC

Putative sulfurtransferases of the Dsr system

The proposed function for DsrEFH and DsrC as sulfurtransferases originated from their homologies to TusBCD and TusE, respectively. This notion was further supported by the observation that the conserved cysteine residues DsrE-Cys78 and DsrH-Cys20 are crucial for the oxidation of transiently stored sulfur in *Alc. vinosum*, stressing the relevance of both cysteine residues for the function of DsrEFH. The following chapter comprises the experiments that were undertaken to substantiate the hypothesis that DsrEFH and DsrC serve as sulfurtransferases during the degradation of sulfur globules in *Alc. vinosum*. Both proteins were tested in regard to their ability to mobilize sulfane sulfur and form persulfides using different methods for detection. During further procedure the transfer of sulfur atoms between these proteins was investigated. Variants of DsrEFH and DsrC carrying Cys-Ser mutations allowed analysing the impact of each conserved cysteine residue to a putative sulfurtransferase activity. The nature of the interaction between DsrEFH and DsrC is the topic of the closing section of this chapter.

1.1. Analysis of the sulfur binding capabilities of DsrEFH and DsrC *in vitro*

1.1.1 DsrEFH

In the first step to verify a possible sulfurtransferase activity for DsrEFH the protein's ability to form a persulfide was tested. For this purpose DsrEFH was incubated either with the L-cysteine desulfurase IscS from *E. coli* and L-cysteine or with sulfide alone. Afterwards, the protein was analysed via MALDI-TOF mass spectrometry or using the fluorescent reagent 1,5-IAEDANS.

The characteristic feature to look out for in a persulfurated protein that is analysed via MALDI-TOF mass spectrometry is an additional mass of 32 Da compared to the protein's genuine mass. For interpretation of the spectra singly charged molecules as well as doubly charged molecules were used. During mass spectrometry DsrEFH decomposed into its subunits, so that DsrE, DsrF and DsrH could be analysed individually (Figure III.1). For each protein a mass was detected that agreed with the theoretically calculated molecular mass within a tolerable range of only a few Da (theoretically calculated molecular masses: His-tagged DsrE, 16,620 Da; DsrF, 15,642 Da; DsrH, 10,953 Da).

After incubation with either sulfide or IscS and L-cysteine the spectrum of doubly charged DsrE showed two species of this protein; the first species represented the mass of recombinant DsrE. With

an additional mass of 16 Da, as expected for doubly charged molecules, the second species represents the persulfurated version of DsrE. Reduction of DsrEFH with TCEP prior to the incubation with sulfide yielded the same result. In the control sample that contained DsrEFH in its native state the additional mass was not detected. Neither DsrH nor DsrF bound sulfur atoms, though both polypeptides carry cysteine residues.

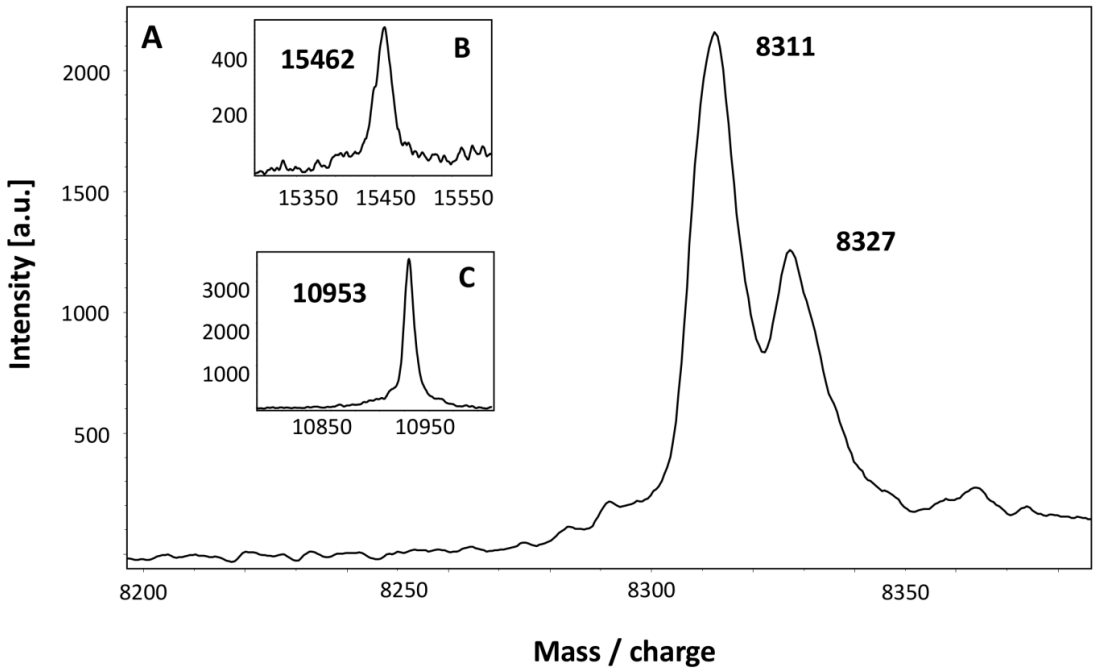

Figure III.1. Sulfur binding of DsrEFH analysed via MALDI-TOF mass spectrometry. 30 µM DsrEFH was incubated with 2 mM sulfide or 2 µM IscS and 2 mM cysteine for 60 minutes at 30°C. **(A)** His-tagged DsrE (theoretically calculated molecular mass: 16,620 Da). **(B)** DsrF (theoretically calculated molecular mass: 15,642 Da). **(C)** DsrH (theoretically calculated molecular mass: 10,953 Da). The formation of a persulfide is documented by a mass increase of 32 Da for singly charged molecules. Note that for DsrE the spectrum for the doubly charged molecules is shown.

Though MALDI-TOF mass spectrometry yields detailed data when ideal conditions for the respective protein are applied, the method is problematic when proteins exceeding a certain molecular mass or membrane proteins are analysed. Thus, a second method was employed to visualize the binding of sulfane sulfur. 1,5-IAEDANS (I.9.2) is a fluorescent reagent that can be coupled to proteins by displacement of its iodide group with the sulfur atom of a thiol group or a persulfide (Thomé *et al*., 2011; Zheng *et al*., 1994).

For this method only sulfide served as substrate. As seen in Figure III.2 DsrEFH migrated mainly in one band around 38 kDa in the gel, which roughly corresponds to the trimeric form, rather than decomposing into its monomers. The small fraction of DsrEFH which ran in the monomeric state decreased after the incubation with sulfide. DsrEFH was successfully labelled with 1,5-IAEDANS and persulfurated DsrEFH was less fluorescent than the native protein. This loss is explained by cleaving off the sulfide-1,5-AEDANS conjugate during the incubation with DTT as the last step before the

protein mix was subjected to electrophoresis (Thomé *et al*., 2011). To demonstrate that the decrease in fluorescence is attributed only to the formation of a persulfide, persulfurated DsrEFH was again reduced with DTT prior to the 1,5-IAEDANS treatment (Figure III.2). That approach led to a level of fluorescence comparable with DsrEFH in its native state; thus, clearly pointing out that the reduced level of fluorescene in persulfidic DsrEFH is attributed to persulfide formation. Note that for better visibility the colours of the gels under UV light were inverted.

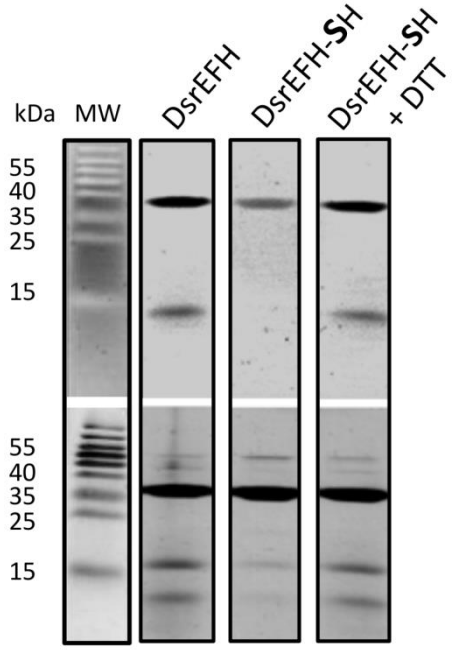

Figure III.2. Sulfur binding by DsrEFH analysed using 1,5-IAEDANS-labelling. DsrEFH was reduced with DTT before incubation with 4 mM sulfide. After dialysis the protein was again treated with DTT before it was subjected to 1,5-IAEDANS treatment. Afterwards all samples were reduced with DTT and 100 pmol of each sample was applied to electrophoresis (15% gel). The upper image shows the gel under UV light, the lower after staining with Coomassie. Molecular weight (MW) of marker proteins is given in kDa.

1.1.2 DsrC

Like DsrEFH DsrC was persulfurated with sulfide or IscS and L-cysteine and first analysed via MALDI-TOF mass spectrometry. The spectrum for doubly charged DsrC is shown in Figure. III.3 The first peak matched the theoretically calculated molecular mass of 14,638 Da for DsrC. The second peak exhibited an additional mass of 16 Da, which is characteristic for the persulfidic state of a protein. Unlike DsrEFH a signal for a second sulfur atom was detected. The same was observed when DsrC was reduced prior to the incubation with sulfide. In untreated control samples of Dsrc the persulfide formation was not observed. Note that successful detection of DsrC with MALDI-TOF mass spectrometry was only possible if the storage buffer did not contain NaCl.

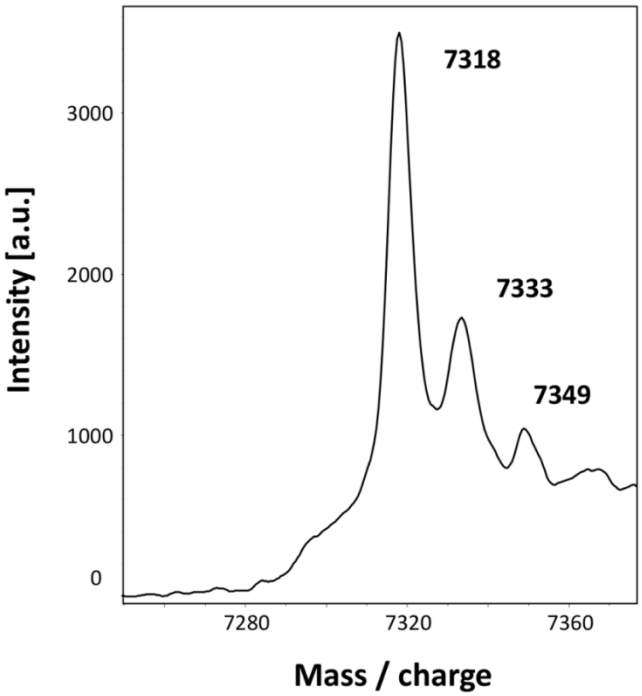

Figure III.3. Sulfur binding of DsrC analysed via MALDI-TOF mass spectrometry. 30 µM DsrC was incubated with 2 mM sulfide or 2 µM IscS and 2 mM cysteine for 60 minutes at 30°C. Theoretically calculated molecular weight for His-tagged DsrC: 14,638 Da. Note that the spectrum for the doubly charged DsrC molecule is shown.

In the next step the formation of a persulfide on DsrC was verified using 1,5-IAEDANS. Here, DsrC was persulfurated with sulfide prior to the 1,5-IAEDANS treatment. As seen in Figure III.4 the reagent bound to DsrC causes the emission of fluorescent light. Upon persulfuration of DsrC the fluorescence decreased. Reduction of persulfurated DsrC with DTT prior to the 1,5-IAEDANS treatment led to fluorescence levels comparable to the protein in its native state.

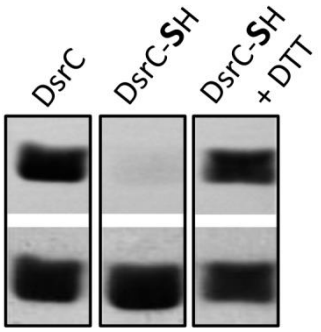

Figure III.4. **Sulfur binding of DsrC analysed with 1,5-IAEDANS.** DsrC was reduced with DTT before incubation with 4 mM sulfide. After dialysis the protein was again treated with DTT before it was subjected to 1,5-IAEDANS treatment. Afterwards the samples were reduced and 200 pmol of each sample was applied to electrophoresis (15% gel). The upper image shows the gel under UV light, the lower after staining with Coomassie.

1.1.3 The significance of conserved cysteine residues for persulfide formation

Generally, sulfurtransferases bind sulfur to strictly conserved cysteines that are redox-active. DsrEFH and DsrC both harbour two conserved cysteine residues which both might be involved in persulfide formation. It was therefore of interest to investigate the impact of the individual cysteines and clarify whether the observed persulfuration reactions were indeed specific. This issue was approached by repeating the experiments that were conducted to establish sulfur binding abilities with variants of DsrEFH and DsrC that carried serine instead of cysteine residues.

First, DsrEFH and the mutated variants, DsrE-Cys78Ser-FH, DsrEFH-Cys20Ser and DsrE-Cys78Ser-FH-Cys20Ser, were treated with 1,5-IAEDANS to check which thiol groups are available for the reagent and putative substrate molecules. As shown in Figure III.5A DsrEFH proteins carrying the single mutations in DsrE and DsrH were labelled with 1,5-IAEDANS. However, the fluorescence was reduced compared to wild-type DsrEFH and the influence of the DsrE-Cys78Ser mutation was clearly higher than that of the DsrH-Cys20Ser. 1,5-IAEDANS did not bind to the protein harbouring the double mutation. Thus, the non-conserved cysteine residues in DsrF and DsrH were not available for labelling.

In the next step these proteins were incubated with sulfide and subsequently analysed via MALDI-TOF mass spectrometry. No additional mass was detected when the mutated proteins carrying DsrE-Ser78 were analysed (Figure III.5B). Since the cysteine residue in DsrH is essential for sulfur oxidation *in vivo* it is noteworthy that the exchange of this residue had no effect on the sulfur binding ability of DsrEFH. The spectrum for DsrEFH-Cys20Ser after incubation with sulfide was identical to the spectrum of unaltered DsrEFH and showed the peak for DsrE and an additional mass of 32 Da (not shown). Analysing the persulfuration reaction of DsrEFH mutants using the 1,5-IAEDANS method yielded the same result (not shown).

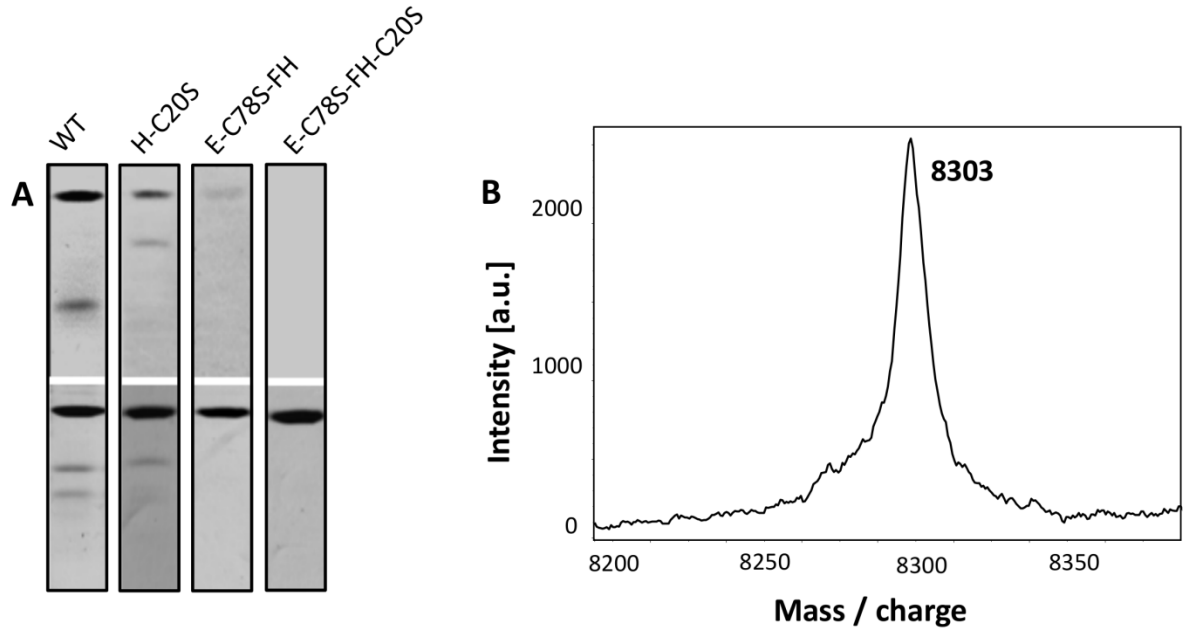

Figure III.5. Labelling (A) and persulfuration (B) of DsrEFH mutant variants. (A) Prior to 1,5-IAEDANS treatment DsrEFH mutant proteins were reduced with DTT. 100 pmol of each protein was analysed via SDS-PAGE (15%). The upper image shows the gel under UV light, the lower after staining with Coomassie. WT, DsrEFH; H-C20S, DsrEFH-Cys20Ser; E-C78S-FH, DsrE-Cys78Ser; E-C78S-FH-C20S, DsrE-Cys78Ser-FH-Cys20Ser. **(B)** 30 µM DsrE-Cys78Ser-FH was incubated with 2 mM sulfide before analysis with MALDI-TOF mass spectrometry. Note that the doubly charged molecule of DsrE is shown and that the exchange of cysteine with serine in the mutated protein causes a molecular weight reduction by 16 Da.

The results for DsrC variants after 1,5-IAEDANS labelling is shown in Figure III.6. Only the protein still containing DsrC-Cys111 was visible under UV-light; DsrC-Cys100Ser was not labelled by the reagent and the mutant protein was therefore not detectable.

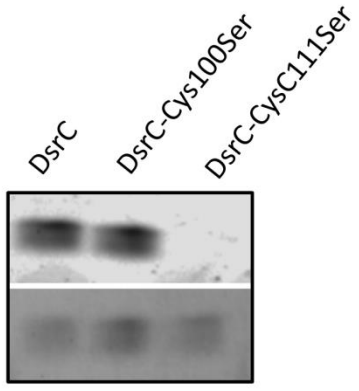

Figure III.6. Labelling of DsrC, DsrC-Cys110Ser and DsrC-Cys111Ser with 1,5-IAEDANS. The DsrC variants were reduced with DTT prior to treatment with 1,5-IAEDANS and 200 pmol of the proteins were subjected to SDS-PAGE (15%). The upper image shows the gel under UV light, the lower after staining with Coomassie.

Mass spectrometry of the mutant proteins after incubation with sulfide showed no additional masses for the protein carrying only DsrC-Cys100 whereas a persulfide was detected for the the Cys111 containing protein (Figure I.7). Implementing the 1,5-IAEDANS method further verified the formation of a persulfide on DsrC-Cys111; the fluorescence of persulfidic DsrC-Cys100Ser was significantly reduced compared to the untreated protein sample (not shown).

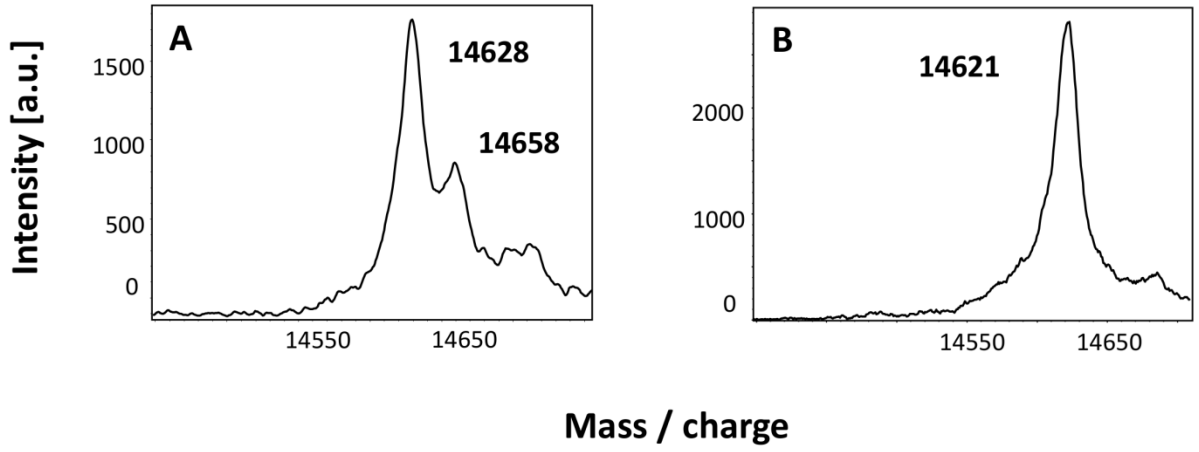

Figure III.7. Persulfuration of DsrC mutant proteins via MALDI-TOF mass spectrometry. 30 µM DsrC-Cys100Ser **(A)** and DsrC-Cys111Ser **(B)** were incubated with 2 mM sulfide for one hour at 30°C and subsequently analysed via MALDI-TOF mass spectrometry.

1.1.4 Sulfur transfer between DsrEFH and DsrC *in vitro*

The next set of experiments was dedicated to a putative transfer of sulfur atoms between DsrEFH and DsrC. For these experiments solely sulfide was used to persulfurate the putative donor protein. Using sulfide as substrate ruled out any involvement of IscS in a possible sulfur transfer between the Dsr proteins and guaranteed that a successful persulfuration of the acceptor protein could be traced back exclusively to the activity of the DsrEFH or DsrC. To further exclude the possibility that the acceptor protein might bind excess sulfide that was not bound in the first reaction step by the donor, the samples were purified via PD Mini-Trap columns. Two methods were employed to confirm the successful removal of excess sulfide from protein samples. First, the elution of sulfide from the gel filtration column was monitored via HPLC. Therefore samples of the first, second and third elution step were analysed. The first elution fraction contained the persulfurated protein that was further used as sulfur donor and contained no sulfide. Unbound sulfide eluted in the second elution step from the column. As a second control, samples for mass spectrometry were prepared by first incubating sulfide in buffer with the omission of proteins. After gel filtration DsrEFH and DsrC were added and to bind any sulfide that was not eliminated by the purification step. However, no mass increases were detected.

In a first attempt, the ability of DsrEFH to serve as sulfur donor for DsrC was tested. DsrEFH was incubated with sulfide and sulfur was successfully bound to DsrE-Cys78. After incubation with DsrC

the mass spectrometric analysis still showed the presence of unaltered DsrC, but in addition three extra signals were observed (Figure III.8). Each of these three extra peaks showed mass increases of 31 or 32 Da compared to the previous peak and therefore each represents a sulfur atom bound to DsrC. Using DsrC-Cys100Ser as acceptor protein also yielded the same result. Based on these findings it can be concluded that DsrEFH does in fact transfer sulfur to DsrC *in vitro* and thereby establishes a short polysulfide chain upon the cysteine DsrC-Cys111.

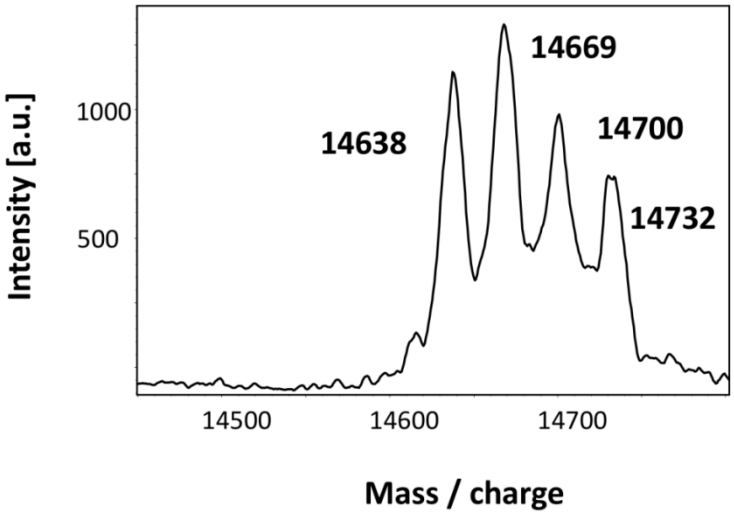

Figure III.8. Transfer of sulfur atoms from persulfidic DsrEFH to DsrC analysed via MALDI-TOF mass spectrometry. 30 µM DsrC was incubated with 30 µM persulfurated DsrEFH for one hour at 30°C. The transfer of up to three sulfur atoms from DsrEFH to DsrC is documented by mass increases in steps of 32 Da. The spectrum for DsrC is shown.

To test whether the 1,5-IAEDANS method would also allow the demonstration of sulfur transfer, the transfer reaction between DsrEFH and DsrC was repeated with this system. Again, DsrEFH was first reduced with DTT and then incubated with sulfide before it was dialysed overnight to remove excess sulfide. The next day DsrEFH was incubated with reduced DsrC for one hour before the mixture was treated as described in II.9.2 The result of the SDS-PAGE is shown in Figure III.9. Compared to untreated DsrC the fluorescence of DsrC was annihilated after incubation with persulfidic DsrEFH. This agrees with the result for persulfurated DsrC in Figure III.4. The opposite was observed for DsrEFH, after incubation with DsrC the thiol specific fluorescence was restored pointing to the loss of the persulfide.

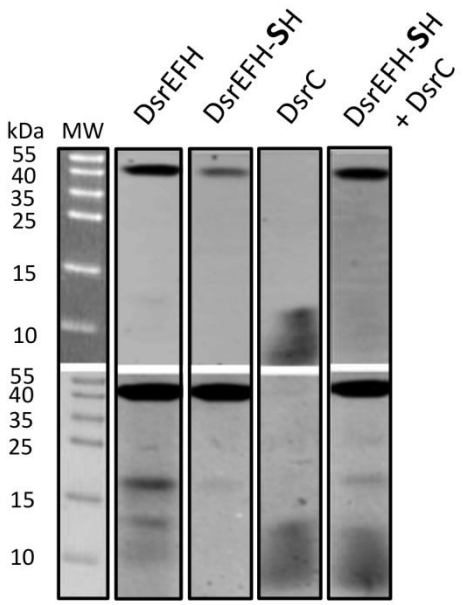

Figure III.9. Transfer of sulfur atoms from persulfidic DsrEFH to DsrC analysed with 1,5-IAEDANS. DsrEFH and DsrC were prepared as described above. For the transfer reaction 100 pmol per-sulfurated DsrEFH was mixed with 200 pmol DsrC and incubated for one hour at 30°C. Afterwards the complete protein mixture was subjected to SDS-PAGE (15%) after 1,5-IAEDANS treatment. The upper image shows the gel under UV light, the lower after staining with Coomassie. Molecular weight (MW) of marker proteins is given in kDa.

The transfer reaction was also tested with DsrEFH-Cys20Ser as sulfur donor. DsrH-Cys20 itself was neither able to bind sulfur nor was it required for the sulfur binding by DsrE. It is however essential for the degradation of sulfur atoms (Stockdreher *et al*., 2012) and could play a role in the transfer reaction from DsrEFH to DsrC. However, the result was the same as shown for wild type DsrEFH. The spectrum retrieved from mass spectrometry showed again three sulfur atoms that were transferred to DsrC and using the 1,5-IAEDANS method yielded in the loss of fluorescence in DsrC (not shown).

The reverse transfer, from persulfidic DsrC to DsrEFH, was neither detected with MALDI-TOF mass spectrometry nor via the 1,5-IAEDANS method.

1.1.5 Binding of sulfite

One of the models proposed for the oxidation of sulfur globules in *Alc. vinosum* depicts DsrC in its persulfidic state as direct substrate for DsrAB (Cort *et al*., 2008). In this scenario the oxidation product, sulfite, would leave DsrAB as sulfonate group bound to DsrC. To test whether DsrC is actually able to bind a sulfonate group, the protein was incubated with 2 mM Na_2SO_3 and analysed via MALDI-TOF mass spectrometry. The result is shown in Figure III.10. Additionally to the peak for unaltered DsrC a second peak with an increased molecular mass of 80 Da occurred in the spectrum. This second peak is consistent with the theoretically calculated mass for a sulfonate group bound to a DsrC molecule. When the experiment was repeated with DsrEFH no additional mass was observed.

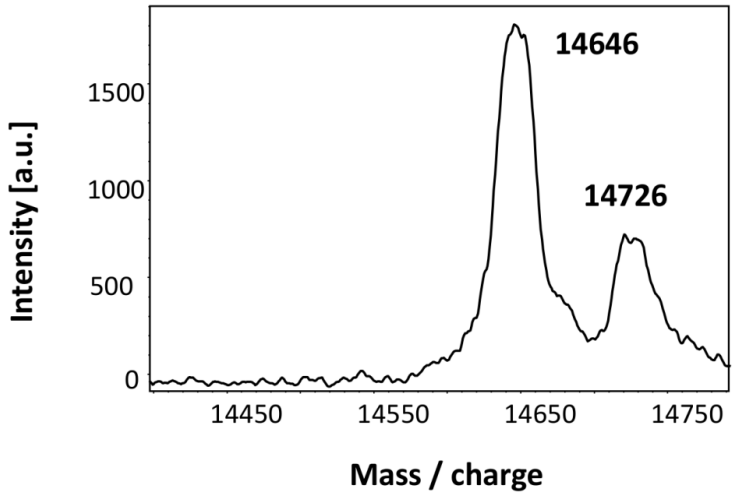

Figure III.10. MALDI-TOF spectrum of DsrC after incubation with Na_2SO_3. 30 µM DsrC was incubated with 2 mM Na_2SO_3 for one hour at 30°C. The binding a sulfonate group is represented by a mass increase of 80 Da.

1.1.6 Mobilization of sulfane sulfur by DsrEFH and DsrC

The transfer of sulfur from periplasmic sulfur globules to the cytoplasmic dissimilatory sulfite reductase DsrAB is a long standing enigma. The introduction of sulfur to the Dsr system is one of the major questions. Is it recruited from the putative carrier molecule GASSH by proteins of the system itself or is an additional protein interconnected that conducts the transfer? To approach this issue DsrEFH and DsrC were tested for their ability to mobilize sulfane sulfur from low molecular weight thiols using the assay for thiosulfate:cyanide sulfurtransfearses as described by Ray (Ray *et al*., 2000). Following the protocol, thiosulfate was first used as substrate. However, neither DsrEFH nor DsrC were able to transfer sulfane sulfur to the acceptor molecule cyanide. Additionally, GSSH was tested as substrate, but again, no sulfur transfer to cyanide was detected. These results were further verified by analysis of DsrEFH and DsrC using MADLI-TOF mass spectrometry after incubation with thiosulfate or GSSH. No additional masses were detected.

GSSH as was formed by incubating 500 µM oxidized glutathione with 450 µM sulfide for 30 minutes at 30°C (Rohwerder & Sand, 2003). Residual sulfide in the mix was ruled out by analysing the solution via HPLC and omitting proteins from the rhodanese assay after Ray (Ray *et al*., 2000). As control sulfide alone was also used for this assay and readily reacted with cyanide to thioccyanate.

1.2 *In vitro* analysis of the interaction between DsrEFH and DsrC

The interaction between DsrEFH and DsrC *in vitro* and its dependency on the residues DsrE-Cys78 and DsrC-Cys111 has first been shown by band shift assays in native polyacrylamide gels (Cort *et al*, 2008; Dahl *et al*., 2008). The migration patterns of DsrC and DsrEFH in native polyacrylamide gels changed after both proteins were incubated together: two additional bands appeared between DsrC and DsrEFH (Figure III.11A). So far, it was not unambiguously shown that these additional bands indeed arose from the formation of complexes between DsrEFH and DsrC. Therefore, these bands were

extracted from the native gel and analysed in the second dimension. The bands were subjecting to SDS-PAGE with the intention to visualize the proteins engaged in the formation of the additional bands. The extracted bands were dehydrated with 50% (v/v) acetonitrile for 20 minutes at room temperature to improve fitting to the wells. As shown in Figure III.11B (lanes 6 and 7) both bands contained DsrEFH as well as DsrC. Notably, the signal for DsrC was significantly stronger in the upper band, indicating that the protein complex in this band (Figure III.11B, lane 7) has a higher DsrC:DsrEFH ratio.

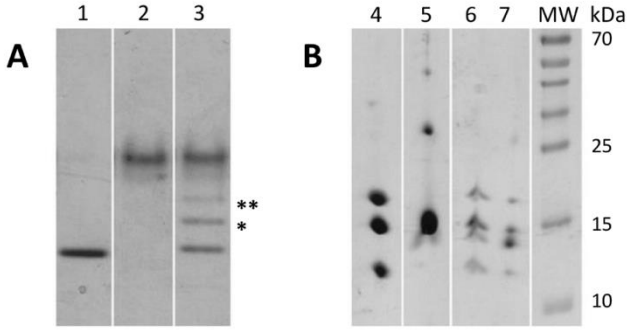

Figure III.11. Formation of stable protein complexes between DsrC and DsrEFH. (A) For the interaction, 200 pmol of DsrEFH and 400 pmol of DsrC were incubated for 30 minutes at 30°C. The protein mixtures were then applied to a native polyacrylamide gel (7.5%). The additional bands that indicate putative DsrEFH/DsrC complexes are marked by * and **. All bands were cut out of the gel and the pieces were applied to SDS-PAGE (15%) after dehydration with 50% (v/v) acetonitrile. Proteins in native gel (lanes 1–3): lane 1 DsrEFH, lane 2 DsrC, lane 3 DsrEFH pre-incubated with DsrC; **(B)** SDS-PAGE (lanes 4–7): lane 4 DsrEFH, lane 5 DsrC, lane 6 DsrEFH and DsrC (lower migrating band), lane 7 DsrEFH and DsrC (upper migrating band). Molecular weight (MW) of marker proteins is given in kDa.

In cooperation with Dr. Sofia S. Venceslau in the laboratory of Prof. Dr. Inês A. C. Pereira at ITQB in Oreias, Portugal, it was possible to elucidate the stoichiometry of the DsrEFHC complex and further characterize the interaction between these proteins. Blue-native PAGE analysis allowed to separate the mix of DsrEFH and DsrC based on their molecular masses (Figure III.12). Hence, the masses of the additional bands could be determined by using a calibration curve based on the molecular masses of the standard. Relative to DsrEFH alone the two additional bands displayed extra masses of 14 kDa and 28 kDa, respectively, corresponding to one and two DsrC molecules. DsrC is not visualized in this gel.

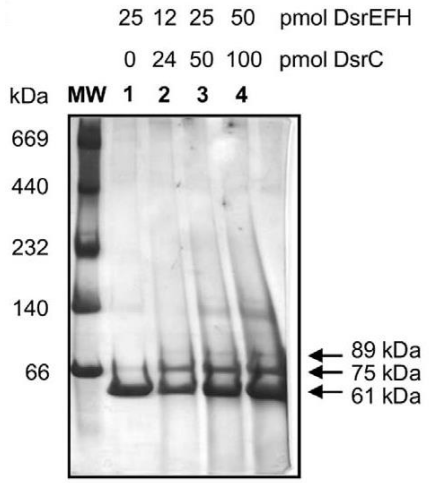

Figure III.12. Analyzing the stoichiometry of the DsrEFHC complex with Blue-native PAGE (10–15%): (1) DsrEFH, (2) to (4) DsrEFH pre-incubated with DsrC in 1:2 ratio but with increasing amounts. Molecular weight (MW) of marker proteins is given in kDa. Performed by S S. Venceslau.

2. Analysis of Rhd_2599, TusA and DsrE2

Proteins involved in cytoplasmic sulfur trafficking?

The second chapter of this work comprehends the strategies that were adopted to elucidate the function of Rhd_2599, TusA and DsrE2 in the cytoplasm of *Alc. vinosum*. A role for the three proteins in the dissimilatory sulfur metabolism has previously been suggested (Dobler, 2008; Stockdreher, 2009; Sturm, 2009), but was not proven so far. TusA was discussed as direct sulfur donor for DsrEFH in analogy to their counterparts in *E. coli;* here, both proteins are part of a sulfur relay system for the biosynthesis of 5-methylaminomethyl-2-thiouridine (Ikeuchi *et al*., 2006). The system involves a third Tus protein, TusE, which is homologous to DsrC. In *Alc. vinosum* the *tusA* gene is flanked by genes encoding the rhodanese Rhd_2599 and DsrE2. The existence of the same gene arrangement has been reported for species of the chemotrophic sulfur oxidizing Acidithiobacilli (Acosta *et al*., 2005; Quatrini *et al*., 2009; Chen *et al*., 2012). Here, the three genes are located directly upstream of another gene cluster that encodes HdrC1B1A1-Orf2-HdrC2B2, a heterodisulfide reductase complex. Quatrini *et al*., proposed that this complex would oxidize disulfidic intermediates resulting from sulfur oxidation to sulfite (Quatrini *et al*., 2009). For the *rhd-tusA-dsrE2* (subsequently referred to as *rtd*) gene products the authors theorized accessory function as sulfur delivery proteins. Following these clues, different lines of evidence were combined to assess the possibility that Rhd_2599, TusA and DsrE2 indeed participate in oxidative sulfur metabolism of *Alc. vinosum*, precisely the degradation of sulfur globules.

2.1 Occurrence of the gene cluster in sulfur oxidizing organisms

The occurrence of a certain gene cluster in a physiologically coherent group of prokaryotes can provide insights into the relevance of the respective genes for metabolic pathways used in these organisms. Therefore, a survey of the currently completely sequenced genomes of sulfur oxidizing prokaryotes was performed in order to assess whether the presence of the *rtd* gene cluster follows a conspicuous pattern. To this end, Blastp analysis was used (Altschul *et al*., 1990) with *Alc. vinosum* TusA as query. The proteins encoded adjacent to the resulting *tusA* homologous gene in the respective genome were then inspected. Table III.1 lists all organisms found that encode TusA in context with either a *rhd* or a *dsrE2* gene. To classify an organism as sulfur oxidizer the genome was subsequently searched for the presence of the *dsr* gene cluster or the genes encoding the Hdr complex.

Table III.1. Occurrence of the *rhd-tusA-dsrE2* gene cluster in genome-sequenced sulfur oxidizing prokaryotes.
[*]The *dsr* gene cluster comprises *dsrABCEFHMKLJOPN* if not stated otherwise, in case of the Chlorobi the cluster consists of *dsrNCABLUEFHTMKJOPVW*.
[**]DsrA-DsrR. [***]DsrA-DsrS. [****]DsrA-DsrP. [*****]DsrEFH is missing, [******]only DsrB.
[†]The *hdr* cluster consists of *hdrC1B1A1-orf2-hdrC2B2*.
[††]*hdrBCAA* are present. [†††]Instead of *hdrC2* this cluster contains a pseudogene. [††††]Only *hdrC1B1A1* are present. [†††††]*hdrB1* is present with two homologues.

	Rhd	TusA	DsrE2	Dsr complex[*]	Hdr complex[†]
Bacteria					
α-Proteobacteria					
Hyphomicrobiaceae					
Hyphomicrobium denitrificans ATCC 51888	no	YP_003754839	YP_003754829	no	YP_003754830-835
Rhodomicrobium vannielii ATCC 17100	no	YP_004010984	YP_004010983	YP_004010978-966	no
Rhodospirillaceae					
Magnetospirillum magneticum AMB-1	no	YP_421179	YP_421178	YP_422730 - 41[****]	no
Magnetococcaceae					
Magnetospirillum gryphiswaldense MSR-1	no	CAM75689	CAM75687	CAM75808-797	no
Magnetococcus marinus MC-1	no	YP_864721	YP_864720	YP_866063-5/5618-24/3983-5	no
β-Proteobacteria					
Burkholderiaceae					
Burkholderiales bacterium JOSHI_001	no	WP_009551576	WP_009551575	WP_009551564-551	no
Gallionellaceae					
Sideroxydans lithotrophicus ES-1	no	YP_003524327	YP_003524326	YP_003524306-292	no
Hydrogenophilaceae					
Sulfuricella denitrificans skB26	WP_009206414	WP_009206413	WP_009206412	WP_009207522-535[**]	YP_008546724-27[††]
Thiobacillus denitrificans ATCC 25259	YP_314331	YP_314332	YP_314333	YP_316243-230[**]	no
Thiobacillus thioparus DSM 505	WP_018508572	WP_018508573	WP_018508574	WP_018507220-207[**]	no

Table III.1. Occurrence of the *rhd-tusA-dsrE2* gene cluster in genome-sequenced sulfur oxidizing prokaryotes (continued)**.**

	Rhd	TusA	DsrE2	Dsr complex*	Hdr complex†
γ-Proteobacteria					
Acidithiobacillaceae					
Acidithiobacillus caldus SM-1	YP_004749705	YP_004749704	YP_004749703	no	YP_004749702-697
Acidithiobacillus ferrivorans SS3	YP_004784899	YP_004784898	YP_004784897	no	YP_004784896-891
Acidithiobacillus ferrooxidans ATCC 23270	YP_002426938	YP_002426937	YP_002426936	no	YP_002426935-930
Acidithiobacillus ferrooxidans ATCC 53993	YP_002220598	YP_002220597	YP_002220596	no	YP_002220595-589†††
Acidithiobacillus thiooxidans ATCC 19377	WP_010637272	WP_010637274	WP_010637276	no	WP_010637278-286
Chromatiaceae					
Allochromatium vinosum DSM 180^T	YP_003444541	YP_003444542	YP_003444543	YP_003443222-236***	no
Lamprocystis purpurea DSM 4197	no	WP_020506580	WP_020506581	WP_020504936-923**	no
Marichromatium purpuratum 984	WP_005225002	WP_005225001	WP_005225000	WP_005223286-308**	no
Thiocapsa marina 5811	WP_007192879	WP_007192878	WP_007192877	WP_007193787-773***	no
Thiocystis violascens DSM 198	YP_006414236	YP_006414235	YP_006414234	YP_006412728-740	no
Thioflavicoccus mobilis 8321	YP_007244989	YP_007244988	YP_007244987	YP_007242648-660	no
Thiorhodococcus drewsii AZ1	WP_007039535	WP_007039534	WP_007039533	WP_007040750-736***	no
Thiorhodovibrio sp. 970	WP_009147444	WP_009147445	WP_009147446	WP_009147786 -773**	no
Ectothiorhodospiraceae					
Alkalilimnicola ehrlichii MLHE-1	no	YP_742508	no	YP_742489-502	no
Ectothiorhodospira sp. PHS-1	no	WP_008932742	WP_008932743	no	WP_008932744-749
Halorhodospira halophila DSM 244	no	YP_001003503	no	YP_001003517-529	no
Thioalkalivibrio nitratireducens DSM 14787	YP_007218369	YP_007218368	no	YP_007216031-018**	YP_007218367-362
Thioalkalivibrio sp. K90mix	YP_003459881	YP_003459882	no	no	YP_003459883-888
Thioalkalivibrio sulfidophilus HL-EbGr7	YP_002514285	YP_002514284	no	YP_002514252-265**	YP_002514283-278

Table III.1. Occurrence of the *rhd-tusA-dsrE2* gene cluster in genome-sequenced sulfur oxidizing prokaryotes (continued)**.**

	Rhd	TusA	DsrE2	Dsr complex*	Hdr complex†
Ectothiorhodospiraceae (cont.)					
Thioalkalivibrio thiocyanodenitrificans ARhD 1	WP_018232042	WP_018232041	no	WP_018232018-031**	no
Thioalkalivibrio thiocyanoxidans ARh 4	WP_006745883	WP_006745882	no	WP_006747914-901**	no
Thiorhodospira sibirica ATCC 700588	no	WP_006787555	WP_006787554	no	WP_006787553-548
Thiotrichaceae					
Thiothrix disciformis DSM 14473	no	WP_020393794	WP_020393578	WP_020397046-032**	no
Endosymbionts					
Bathymodiolus endosymbiont	no	WP_010646158	WP_010646160	WP_010645590-614	no
Candidatus *Ruthia magnifica*	no	YP_903482	YP_903481	YP_904057-045	no
Candidatus *Vesicomyosocius okutanii* HA	no	YP_001219073	YP_001219072	YP_001219625-612**	no
Chlorobi					
Chlorobiaceae					
Chlorobaculum parvum NCIB 8327	YP_001997653	no	no	YP_001997654-667	no
Chlorobaculum tepidum TLS	NP_661737	NP_661741	NP_661755	NP_661745-751	no
Chlorobium chlorochromatii CaD3	YP_380251	YP_380250	YP_380252	YP_380249-233	no
Chlorobium ferrooxidans DSM 13031	no	WP_006367306	no	WP_006367305	no
Chlorobium limicola DSM 245	YP_001942753	YP_001942125	YP_001942126	YP_001942754-771	no
Chlorobium phaeobacteroides BS1	YP_001960259	YP_001960258	YP_001960238	YP_001960257-241	no
Chlorobium phaeobacteroides DSM 266	YP_910618	YP_910619	YP_910617	YP_910620-636	no
Chlorobium phaeovibrioides DSM 265	YP_001129565	YP_001129566	YP_001129564	YP_001129567-583	no
Chloroherpeton thalassium ATCC 35110	no	no	YP_001995564	no	YP_001995056-054††††
Chlorobium luteolum DSM 273	YP_373965	YP_373966	YP_373964	YP_373967-983	no
Pelodictyon phaeoclathratiforme BU-1	YP_002019137	YP_002019136	YP_002019138	YP_002019135-118	no
Prosthecochloris aestuarii DSM 271	YP_002014738	YP_002014739	YP_002014772	YP_002014740-756	no

Table II.1. Occurrence of the *rhd-tusA-dsrE2* gene cluster in genome-sequenced sulfur oxidizing prokaryotes (continued)**.**

	Rhd	TusA	DsrE2	Dsr complex*	Hdr complex†
Aquificae					
Aquificaceae					
Aquifex aeolicus VF5	no	no	NP_213270/271	no	NP_213272-278††††
Hydrogenivirga sp. 128-5-R1-1	no	WP_008286378	WP_008286380/379	no	WP_008286381-86
Hydrogenobacter thermophilus TK-6	no	YP_003433531	YP_003433530/529	no	YP_003433528-23
Hydrogenobaculum sp. HO	no	YP_007500438	YP_007500437/436	no	YP_007500435-30
Hydrogenobaculum sp. Y04AAS1	no	YP_002121749	YP_002121748/747	no	YP_002121746-41
Thermocrinis albus DSM 14484	no	YP_003474109	YP_003474108/107	no	YP_003474106-101
Archaea					
Crenarchaeota					
Sulfolobaceae					
Acidianus hospitalis W1	no	YP_004458872	YP_004458873/874	no	YP_004458871-866
Metallosphaera cuprina Ar-4	no	YP_004409272	YP_004409271/270	no	YP_004409273-278
Metallosphaera sedula DSM 5348	no	YP_001191627	YP_001191628/629	no	YP_001191626-621
Sulfolobus acidocaldarius DSM 639	no	YP_255044	YP_255045/046	no	YP_255043/29-34
Sulfolobus islandicus M.14.25	no	YP_002829162	YP_002829164/163	no	YP_002829161-156
Sulfolobus solfataricus P2	no	NP_342591	NP_342590/589	no	NP_342592-597
Sulfolobus tokodaii str. 7	no	NP_377858	NP_377860/859	no	NP_377857-852

The complete gene cluster is conserved in the phototrophic families *Chromatiaceae and Chlorobiaceae* as well as in the chemotrophic sulfur oxidizing *Acidithiobacillaceae* and *Hydrogenophilaceae.* Within the *Chromatiaceae* the presence of the cluster appears to be restricted to members that also contain DsrAB as neither was found in the *Rheinheimera* and *Nitrosococcus* strains. In *Lamprocystis purpurea* the cluster lacks the rhodanese gene.

The gene order and organisation of *rtd* found in the green sulfur bacteria deviates from all other sulfur oxidizers. The genes were generally found in direct vicinity of the *dsr* gene cluster and genes that encode the cytoplasmically oriented SoeABC-related polysulfide reductase like complex PSRLC3 for which a role in the oxidation of sulfite in the cytoplasm is very probable (Frigaard & Bryant, 2008; Dahl *et al*., 2013). The complete sequence *dsrE2-rhd-tusA* is placed directly upstream of the *dsr* gene cluster in *Chlorobium luteolum*, *Chlorobium chlorochromatii*, *Chlorobium phaeobacteroides* DSM 266, *Chlorobium phaeovibrioides* and *Pelodictyon phaeoclathratiforme*. With exception of *Pld. phaeoclathratiforme* the genes are further preceded by the genes encoding PSRLC3. A different organisation was found in *Chlorobaculum tepidum*, *Chlorobium phaeobacteroides* BS1 and *Prostheocochloris aestuarii*. Here, the *dsrE2-rhd-tusA* sequence is intercepted by the *dsr* genes so that the *dsr* cluster is framed by a combination of *rhd-tusA* upstream of *dsrN* and *dsrE2* was located downstream of *dsrW*. The *Cba. tepidum* genome carries two sets of *dsr* genes, but only the *dsrC1A1B1L1EFH* cluster is situated between *rdh-tusA* and *dsrE2*. *Chlorobium limicola* is the only *Chlorobium* strain in which PSRLC3 is not encoded upstream of the *dsr* genes. Interestingly, the *rtd* genes are split between these two complexes: *rhd* lies upstream of the *dsr* cluster while *tusA* and *dsrE2* are located immediately downstream of *psrLC3*. *Chlorobium* species in which the cluster is absent are *Chlorobium parvum* and *Chlorobium ferrooxidans*. However, in the latter a *tusA* gene was found situated directly adjacent to *dsrB*. *Chloroherpeton thalassium* was the earliest branching green sulfur bacterium that was analysed and this organism is lacking the *rtd* cluster as well as the Dsr protein encoding genes. Only *dsrE2* was detected and the gene is located in a small sulfur island with *fccAB, soxYZ* and *dsrC*. It is worth mentioning that the Dsr system of the Chlorobi varies from the systems found in other sulfur oxidizers that employ the Dsr proteins for the sulfur globule oxidation as it involves DsrV and DsrW (Holkenbrink *et al*., 2011). In the *Chromatiaceae* a homologous protein is encoded immediately upstream of the rhodanese gene in the opposite direction of transcription. In case of *Alc. vinosum* this is Alvin_2598. The gene product is annotated as sirohydrochlorin ferrochelatase and shares 50% identity with the siroheme synthase CysG from *E.coli*. Interestingly, an Alvin_2598 homologue was found directly downstream of a rhodanese gene in *Lpc. purpurea*; the only strain of the *Chromoatiaceae* in which only *tusA* and *dsrE2* were encoded together.

The *Acidithiobacilliaceae* is the only family which harbours the complete *rhd-tusA-dsrE2* sequence, although the Dsr proteins are lacking. Here, homologues of *rhd_2599, tusA* and *dsrE2* are located directly upstream of the genes encoding the Hdr complex. This particular combination appears to follow a general scheme, even in the presence of the *dsr* gene cluster *rhd_2599, tusA and dsrE2* are

located upstream of the *hdr* genes. However, the coexistence of the Dsr and Hdr system seems to be the exception to the rule. Apart from *Thioalkalivibrio sulfidophilus, Thioalkalivibrio nitratereducens* and *Sulfuricella denitrificans* only one of the protein complexes was found in sulfur oxidizing prokaryotes. The Hdr system in *Scl. denitrificans* is truncated (only *hdrBCAA* are present and the *rtd* sequence was found in a different genomic region) and the *Thioalkalivibrio* strains belong to the *Ectothiorhodospiraceae*, the family with the highest flexibility in regard to the analysed genes. In this group the sulfur oxidizing systems appear to be interchangeable and are not restricted to either Hdr or Dsr proteins. The *tusA* gene is conserved either with *rhd* or *dsrE2*, but never with both. Furthermore, the *Ecothiorhodospiraceae* are the only species in which *rhd* alone is conserved with *tusA.* In other groups *dsrE2* is present whereas a rhodanese gene was not found in this particular context. In *Halorhodospira halophila* and *Alkalilimnicola ehrlichii* only *tusA* is conserved and was found together with the *dsr* genes and *soeABC*.

In the thermophilic to hyperthermophilic *Sulfolobaceae* and *Aquificaceae tusA* is conserved together with two homologues of *dsrE2*. The DsrE2 proteins differ in regard to the conserved cysteine residues. In both families the Hdr complex is conserved**.** Note that an individual TusA protein is not present in *Aquifex aeolicus*; merely a TusA domain containing protein is encoded by Aq_1421. However, the *hdr* genes (Aq_391-400) are preceded by the two *dsrE2* homologous genes (Aq_389/390).

The gene cluster was also found in a few Bacillus strains and some members of the Chloroflexi. In these species they are adjacent to genes that encode for proteins which are most likely involved in intracellular sulfur trafficking. In case of the Chloroflexi the genes are located in direct proximity to a sulfate transporter encoding gene and a gene that encodes for a rhodanese/β-lactamase fusion protein. The latter gene is also found next to *rtd* in the Bacillus strains in question. Here, a gene encoding a TusA/Rhd fusion protein and an extra *rhd* gene were also detected. The striking difference to the cluster from sulfur oxidizing microorganisms was found in the reduced number of conserved cysteine residues in DsrE2.

2.2 Transcription of *rhd_2599, tusA* and *dsrE2*

2.2.1 Transcription profiles under sulfur oxidizing conditions

Recently, a profile of the transcriptional response to photolithoautotrophic growth on different sulfur sources relative to -organoheterotrophic growth on malate was established for *Alc. vinosum* (Weissgerber *et al.*, 2013). Here, *tusA* and *dsrE2* showed increased mRNA levels when *Alc. vinosum* was grown on sulfide and thiosulfate whereas the rhodanese gene was completely inconspicuous. The results for *tusA* and *dsrE2* were further verified via qRT-PCR. In the microarray study the time point for cell harvest was chosen to guarantee a maximum oxidation rate for the respective sulfur compound administered to the culture. Since this study represented only a single time point in the complex machinery of sulfur species conversion the mRNA levels for *tusA, dsrE2* and *rhd_2599* were

evaluated again. Now, the mRNA levels were monitored over a course of time that included the maximum oxidation of the sulfur source, accumulation and oxidation of sulfur globules and the excretion of sulfate so that dynamic changes in the relative amount of mRNA over time were covered. Alvin_0486, which encodes an uroporphyrinogen decarboxylase, was used as endogenous reference since this gene showed no changes in the transcriptomic study (Weissgerber *et al*. 2013).

In the phases of maximum oxidation rates, the new results for *tusA* and *dsrE2* matched the old ones; the mRNA levels for both genes were increased when *Alc. vinosum* metabolized sulfide or external sulfur. Contradicting the results retrieved by the microarray analysis the relative transcription of *rhd_2599* was increased when *Alc. vinosum* was grown photolithoautotrophically. One hour after supplementing elemental sulfur to the culture the mRNA levels for *rhd_2599* were 5-fold higher relative to growth on malate and dropped by 60% within 7 hours before the level increased again and almost reached the initial value (Figure III.13). With sulfide as electron source the relative mRNA level increased 6.28-fold and dropped continuously to 2.46-fold over the course of the experiment. The over-all expression patterns for *tusA* and *dsrE2* were remarkably similar and differed only by a few percentage points with sulfide and thiosulfate as substrates. For both genes a ~6.5-fold elevated mRNA level was observed after the first hour with sulfide in the medium which then dropped by 80%. The levels kept stable over the next hours and increased only slightly after 24 hours. In the presence of elemental sulfur the transcription of *tusA* and *dsrE2* also increased at first, but instead of a decrease the levels for both genes first were steady and then increased to 8.3-fold (*tusA*) and 6.3-fold (*dsrE2*) over time. Thiosulfate triggered the lowest transcriptional response for all three genes and the overall fold-changes within 24 hours were less significant than with sulfide and sulfur. This means that for *tusA* and *dsrE2* slightly enhanced mRNA levels (~ 2-fold) continuously dropped over 24 hours until the level of the malate grown cells was reached. The elevated level of *rhd_2599* was only observed within the first hour, after that the mRNA for growth on thiosulfate showed no changes to that on malate and even fell below this level after 24 hours.

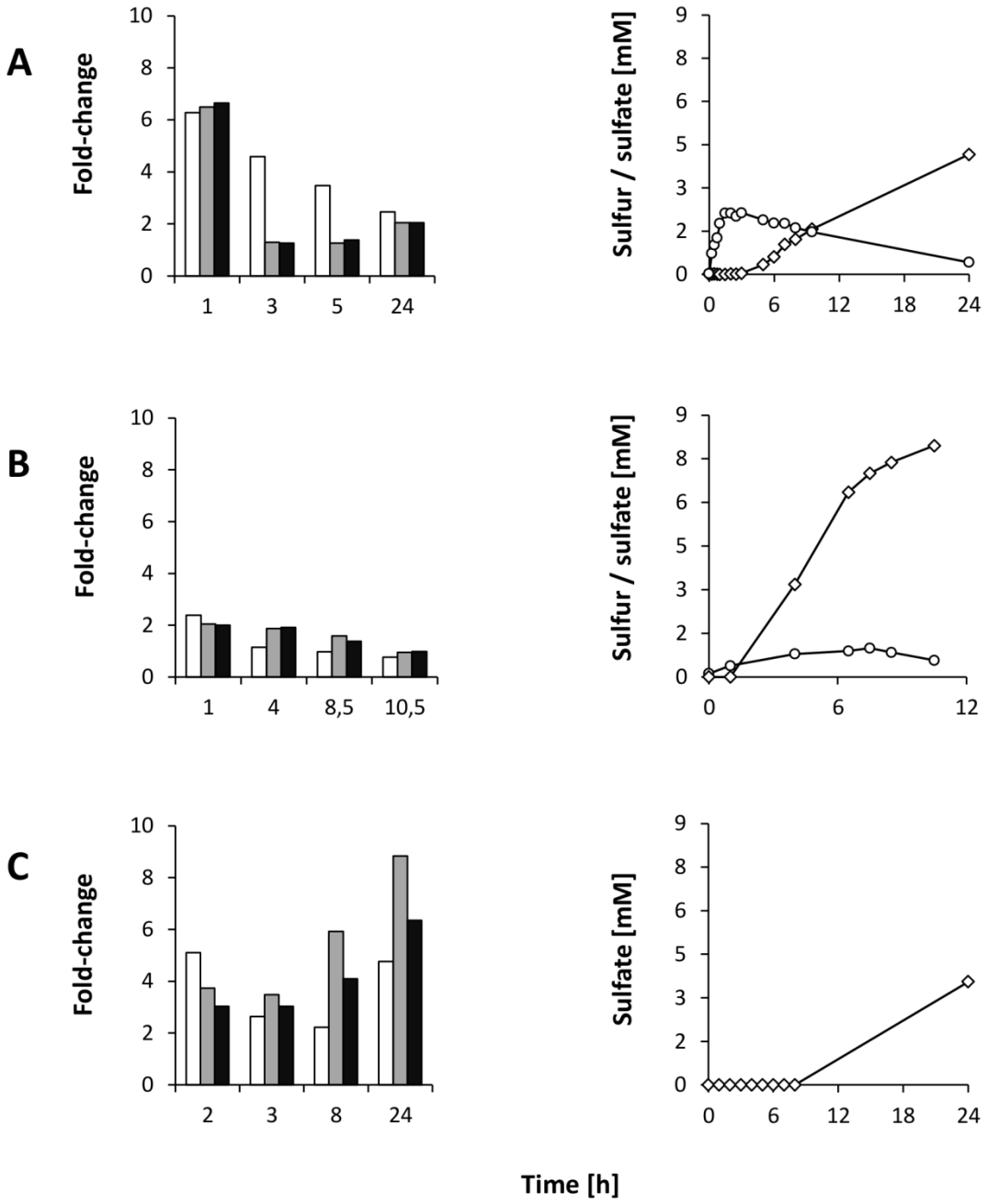

Figure III.13. Relative mRNA levels during growth on 4 mM sulfide (A), 4 mM thiosulfate (B) and 50 mM elemental sulfur (C) compared to growth on malate. Results for *rhd_2599* are shown as white bars, those for *tusA* as grey bars and *dsrE2* as black bars. Fold-changes were calculated using the method of Livak and Schmittgen (2001). Standard deviation of single C_t values from average of triplicates ranged from 0 to 2%. Alvin_0486 served as endogenous reference. Concentrations of internal sulfur (○) and sulfate (◊) are presented in the right panel.

2.2.2 rhd_2599, tusA and *dsrE2* form a transcriptional unit

Overall the transcriptional patterns of *rhd_2599*, *tusA* and *dsrE2* were notably similar. Though the drop in the mRNA level of *rhd_2599* with sulfide as electron source after one hour was not as drastic as for *tusA* and *dsrE2*, the trend was nonetheless the same and thereby pointing to a co-transcription of the genes. To assess this hypothesis, cDNA was synthesized using the DE2_Strep_rev oligonucleotide and RNA that was isolated from sulfide grown cells. Afterwards PCR were performed with oligonucleotide pairs that would result in fragments of the *rhd_2599* – *tusA* sequence and the *tusA* – *dsrE2* sequence in case of co-transcription of the genes. The *tusA* and *dsrE2* genes were amplified to check the success of the cDNA synthesis. Figure III.14 shows that the single genes as well as the *rhd_2599-tusA* and *tusA*- sequences were amplified, indicating that the three genes indeed form a transcriptional unit.

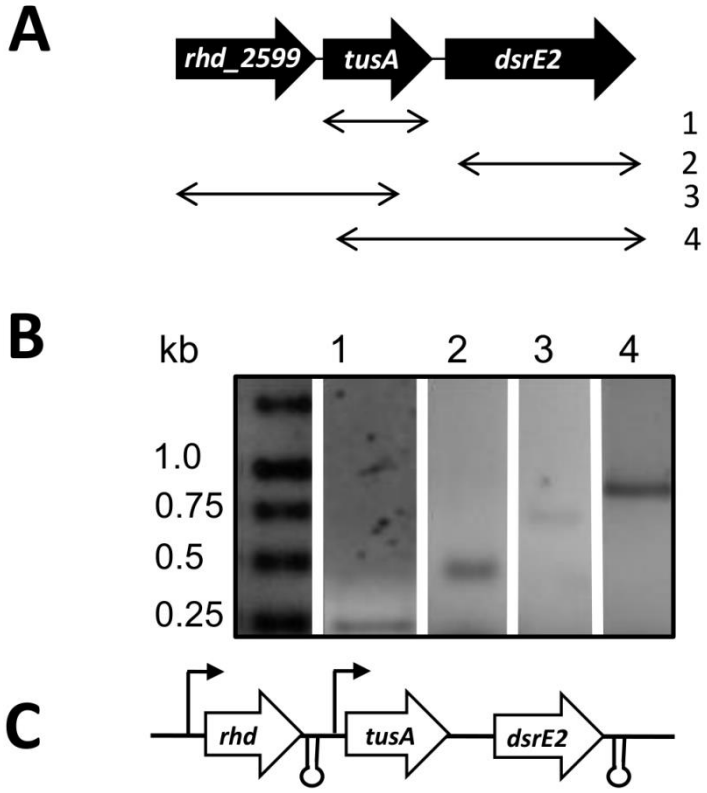

Figure III.14. **Co-transcription of *rhd_2599, tusA* and *dsrE2*. (A)** Genomic organisation of the *rtd* cluster. Arrows indicate amplified sequences in PCR 1 to 4 performed with cDNA that was synthetized with DE2_Strep_rev. **(B)** Agarose gel of amplicons. **(C)** Predicted promoter and terminator sites for *rhd_2599*, *tusA* and *dsrE2*. 1: 235 bp; 2: 495 bp; 3: 700 bp; 4: 860 bp.

The promoter prediction programm BPROM found two promoter sites. The first site is located upstream of *rhd_2599*, the second lies directly upstream of *tusA*. The progam TransTermHP predicted ρ-independent termination sites to be located downstream of *rhd_2599* and *dsrE2*. Thus, indicating a secondary promoter for *tusA* and *dsrE2*.


1 GGGCCGCGCG GCTCCGTTGA GTTGGCCTTG GAAAATCCTT AATATACTTA AATACTTTGT TTTAGGGTAG
71 GACGATAGTG TGGTCAACGA GATCGATTCC GAGTCCCTGA GCCAGCGGCT CGCGGATACT GAAGATGTGT
>>.....rhd_2599.....>
v v n e i d s e s l s q r l a d t e d v
141 TGCTGGTGGA CATCCGCACC CCGGCAGAGA TCGCCCAGGG CATGATCCCC GACGCCCTGC AACTGCCGAT
>.....rhd_2599.....>
l l v d i r t p a e i a q g m i p d a l q l p
211 GCACCTGATC CCGATCCGGA TGAGCGAGAT ACCCAAAGAT CGCGACGTGG TCATCTACTG CCGCAGCGGC
>.....rhd_2599.....>
m h l i p i r m s e i p k d r d v v i y C R S G
281 GCGCGCTCCT ACCAGGCTTG CGCCTATCTG ATGCAGCAGG GTTATGGCCG CGTGCTCAAC CTGCGCGGCG
>.....rhd_2599.....>
A r s y q a c a y l m q q g y g r v l n l r g
351 GCATCATCGC CTGGGCGCGT CACGGCCTGC CGATCGTCGC CCCCGAGGGC TGATCCAAGT ACCCGCCCAA
>.....rhd_2599.....>>
g i i a w a r h g l p i v a p e g -
421 CCGATCCGGC GGCTCCCTTG TTTTTTACGG TGGATTCGCC TATAACCGCG AATTCTTGTT TTTACTGAAA
491 TACTGAAATA AGTAATTGAC GGAGGCTCCG ATGGCTGATT TCGATCAAGA ACTCGACGCA AGCGGCCTGA
>>.....tusA.....>
m a d f d q e l d a s g l
561 ACTGCCCGCT GCCGATCCTG CGCGCCAAGA AGACCCTGAA CGCCATGTCC AGCGGTCAGG TCCTGCACGT
>.....tusA.....>
n C P L P i l r a k k t l n a m s s g q v l h
631 CATCGCCACC GATCCCGGCT CGGTCAAAGA CTTCGACGCC TTCGCCAAGC AGACCGGCAA CGAGCTGATG
>.....tusA.....>
v i a t d p g s v k d f d a f a k q t g n e l m
701 GAGTCCAAGG AAGAAGGCGG CAAGTTCCAC TTCCTGATCA AGAAGTCCTG ATCGATCCGC ACGGCGCGAA
>.....tusA.....>>
e s k e e g g k f h f l i k k s -
771 CCTGAAGCCC CACATCAGGT TCGAACGCCC GCTCCGCACG ACAGACGGCT ATCCAAAACA ACATGATCCG
841 CGAGCCTGAC ACAGGCGCCG CGTGGAGGAC TCGATGGAAC AAAAGAAACT GGCGATCATC GCCACCAAGG
>>.....dsrE2.....>
m e q k k l a i i a t k
911 GTTCGCTCGA CTGGGCCTAC CCGCCCTTCA TCCTCGCCTC GACGGCCGCC GCCCTGGGCT ATGAGGTGCA
>.....dsrE2.....>
g s l d w a y p p f i l a s t a a a l g y e v
981 GGTCTTCTTC ACCTTCTACG GGCTGCAACT CCTGAAGAAG AAGCCGAACC TGGAAGTCAC GCCGCTGGGC
>.....dsrE2.....>
q v f f t f y g l q l l k k k p n l e v t p l g
1051 AACCCCGGCA TGCCGATGCC GATGGGCATG GACAAGTGGT TCCCGGTGCT CGGTCTGGCG CTGCCCGGTA
>.....dsrE2.....>
n p g m p m p m g m d k w f p v l g l a l p g

```
1121  TGCAGGGCAT GATGACCGCC ATGATGAAGC AGAAGATGAA GAGCAAGGGC GTGGCCAGCA TCGAGGAACT
      >.....................................dsrE2.....................................>
       m  q  g   m  m  t  a   m  m  k   q  k  m   k  s  k  g   v  a  s   i  e  e

1191  GCGCGAGCTC TGTCAGGAAG CCGAAGTCAA GATGATCGCC TGTCAGATGA CCGTCGACCT CTTCGATATG
      >.....................................dsrE2.....................................>
      l  r  e  l   c  q  e   a  e  v   k  m  i  a   c  q  m   t  v  d   l  f  d  m

1261  CCGAAAGCCG AGTTCATCGA CGGCGTCGAG TACGCCGGCG CGGCGGCCTT CTTCGAGTTC GCCGGCGAGA
      >.....................................dsrE2.....................................>
        p  k  a   e  f  i   d  g  v  e   y  a  g   a  a  a   f  f  e  f   a  g  e

1331  GCGACATCTG TCTCTACATC TGAAGTCTCT GAACCGCACG TCCGGAGCGA CTTCGGTCGC TCCGTTCCAC
      >.........dsrE2........>>
       s  d  i   c  l  y  i   -

1401  TGATCGAAGC
```

Figure III.15. Predicted promoter and termination sites for the transcription of *rhd_2599, tusA* and *dsrE2*. The derived amino acid sequence is shown below the each triplet in grey. Conserved motifs in Rhd_2599 and TusA are printed in bold and red. The -35 boxes and -10 boxes are framed in dark and light green, respectively. Termination sites are underlined and printed bold.

2.3 Deletion of the *rhd_2599, tusA and dsrE2* sequence

The deletion of a gene and the subsequent analysis of the resulting phenotype of the mutant strain in comparison to the wild type is a common method to approach the *in vivo* function of the encoded protein. To study a potential impact of Rhd_2599, TusA and DsrE2 for the oxidative sulfur metabolism in *Alc. vinosum* the complete *rhd_2599-tusA-dsrE2* should be deleted and the phenotype of the mutant strain during growth on reduced sulfur compounds should be determined.

2.3.1 Construction of *Alc. vinosum* Δ*rtd*

At first *rhd_2599-tusA-dsrE2* should be deleted *in frame*. This technique has been proven to be valid for the creation of stable mutant strains of *Alc. vinosum* (Sander *et al*., 2006; Hensen *et al*., 2006; Dahl *et al*., 2008) and allows the deletion of genes without disrupting the reading frames situated downstream of the deletion. The amplicon containing the deletion of *rhd_2599-tusA-dsrE2* and recognition sites for *Bam*HI was generated via gene SOEing (Horton, 1975) using the oligonucleotides ΔRTD_F1/F2 and ΔRTD_R1/R2 and genomic DNA of *Alc. vinosum*. The final PCR product was digested with *Bam*HI and cloned into the corresponding site in pk18*mobsacB*. The resulting plasmid pk18*mobsacB*Δ*rtd* was transferred to *Alc. vinosum* Rif50 via conjugation as described in section II.7.3.2 with *E. coli* S17-1 hosting the plasmid. Single crossover mutants were verified via colony PCR and positive clones were brought into liquid culture. After three generations in non-selective medium the cells were plated on medium containing 10% (w/v) sucrose to eliminate single crossover mutants. This approach yielded exclusively recombinants to the wild type genotype.

2.3.2 Construction of *Alc. vinosum* Δ*rtd*::ΩKm

In the next step interposon mutagenesis was deployed to create a *rtd* negative mutant strain. This method is advantageous relative to *in frame* deletion since a positive selective marker maintains constant selective pressure. In the past this method was also applicable to *Alc. vinosum* (Pott & Dahl, 1998; Dahl *et al*., 2005). Eventual polar effects, caused by transcription terminator sites at either site of the interposon Ω-cassette, usually have to be taken into consideration. Here, the Ω-Km cassette was inserted into the remains of the *rhd_2599-tusA-dsrE2* gene cluster for which a terminator site in the intergenic region between *dsrE2* (Alvin_2601) and Alvin_2602 was predicted. The predicted promoter for Alvin_2598 is located in the intergenic region between Alvin_2598 and Alvin_2599 and should therefore not be affected by the insertion of the Ω-cassette. For a subsequent introduction of the Ω-Km cassette the *Eco*RI restriction site was introduced into the remains of the *rhd_2599-tusA-dsrE2* locus via gene SOEing (Horton, 1975) using the oligonucleotides RTD_Kan_F1/F2 and RTD_Kan_R1/R2 and pk18*mobsacB*Δ*rtd* as template. The amplicon was cloned into the *Hin*dIII restriction site of pSUP301. Afterwards the Ω-Km cassette was isolated from pHP45Ω-Km using *Eco*RI and cloned into the corresponding site of pSUP301Δ*rtd*. Again, conjugation was used to introduce the protein into *Alc. vinosum* Rif50 cells via *E. coli* S17-1 hosting the plasmid. Two weeks after the cells were plated on the selective medium, which routinely contains 0.8 mM $Na_2S_2O_3$ and 1.4 mM NaHS to support growth, and incubation under anoxic conditions in the light, two colony types were observed that differed with regard to diameter and colour (Figure III.15).

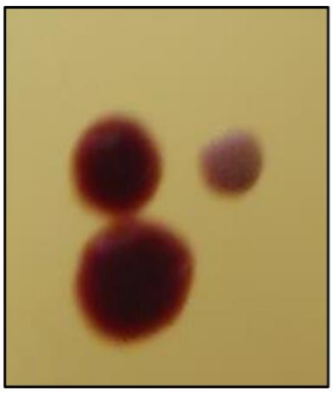

Figure III.15. Different colony types of *Alc. vinosum* Δ*rtd*::ΩKm on selective medium.

While the first kind resembled typical *Alc. vinosum* colonies in radius, dark red colour and a shiny surface, the second kind was significantly smaller, had a non-shiny surface and the colour was a milky pink. Light microscopy showed that the milky appearance of the smaller colonies was due to massive accumulation of intercellular sulfur globules. Apparently, these cells were impaired in their ability to degrade the sulfur globules formed during the oxidation of sulfide and thiosulfate. Cells did not grow on plates without reduced sulfur compounds. A difference in the appearance of individual cells as reported for the *E. coli tusA* deficient mutant (Yamashino *et al.*, 1998) was not observed. Single colonies of both types were transferred to kanamycin/ampicillin and kanamycin only plates to

discriminate between single crossover recombinants, that still contained the ampicillin-resistance conferring shuttle plasmid, and double crossover recombinants, that had lost the vector-encoded ampicillin resistance. All of the regular colonies turned out to contain only single crossover mutants whereas the slowly growing cells of the smaller colonies were solely kanamycin resistant. A considerable number of attempts to restreak these colonies and transfer them into liquid medium with and without reduced sulfur compounds remained unsuccessful. Inevitably, the cultures died after a few generations pointing to a crucial role of Rhd_2599, TusA and DsrE2.

2.4 Insights into the *Alc. vinosum* transcriptome

Genes encoding for proteins of the same functional unit commonly exhibit similar patterns regarding their transcription and the presence of the respective gene products in the cell under specific growth conditions. It was therefore of interest to compare the transcriptional profiles of *rhd_2599, tusA* and *dsrE2* with those of the genes encoding for proteins involved in the biosynthesis of sulfur containing cofactors since TusA reportedly affects the biosynthesis of 5-methylaminomethyl-2-thiouridine, FeS clusters and the molybdenum cofactor (Moco) in *E. coli* (Dahl, J. U. *et al.*, 2013). Besides proteins of central carbon metabolism several important enzymes of the sulfur oxidation pathway in *Alc. vinsoum* contain FeS clusters, including DsrAB, DsrL and the membrane complex DsrMKJOP(Pott & Dahl, 1998; Dahl *et al.*, 2005; Lübbe *et al.*, 2006; Grein *et al.*, 2010a). Another example is the FeS-molybdoenzyme SoeABC, the major player in the oxidation of the DsrAB product sulfite (Dahl, *et al*., 2013). It can therefore not *a priori* be excluded that the close genomic association of the *rtd* genes with sulfur oxidation genes is related with a function of the former in biosynthesis of co-factors of the latter. If the functions of *Alc. vinosum* TusA and possibly also Rhd_2599 and DsrE2 were indeed limited to biosynthesis this should be reflected in congruent transcription patterns of *rhd_2599, tusA and dsrE2* and the genes encoding proteins that are involved in biosynthetic machineries. Data were taken from Weissgerber (Weissgerber *et al.*, 2013) and complemented by the qRT-PCR results described above (Figure III.13). Furthermore, a comparison was drawn between these profiles and that of the *dsr* genes.

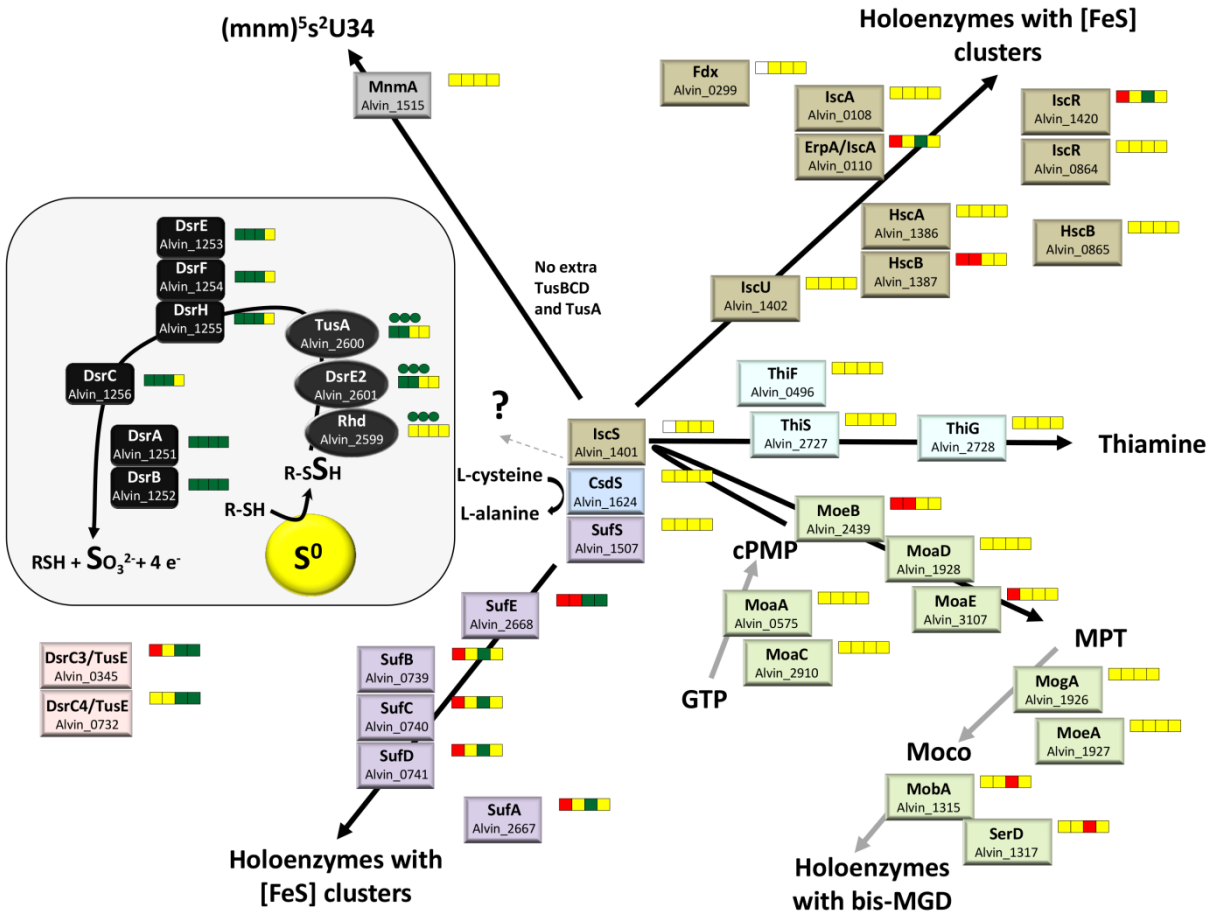

Figure III.16. Analysis of pathways for biosynthesis of FeS cluster, the molybdenum cofactor, thiamine and 5-methylaminomethyl-2-thiouridine in *Alc. vinosum* as predicted from the genome sequence and the transcription patterns of the respective genes. The transcriptomic profiles are depicted next to the respective protein and colour-coded: Relative fold changes in mRNA levels above 2 (green) were considered significantly enhanced. Relative changes smaller than 0.5 (red) were considered as significant decreases in mRNA levels. Relative fold changes between 0.5 and 2 (yellow) indicated indifferent mRNA levels. For *rhd_2599*, *tusA* and *dsrE2* the results obtained via qRT-PCR in this study (values taken from the experiments presented in Figure III.13) are depicted in cycles. Note that thiosulfate grown cells for RNA extraction used for qRT-PCR were harvested after 1 hour, cells for the microarray experiment by Weissgerber *et al.* were harvested after 2 hours. Administered sulfur compounds from left to right: sulfide, thiosulfate, elemental sulfur and sulfite. DsrC/TusE proteins Alvin_0028 and Alvin_1508 are omitted from the figure since they harbour no cysteine residues.

Alc. vinosum encodes the ISC (*iscRSUAhscBAfdx*) and the SUF (*sufABCDSE*) systems for biosynthesis of FeS-clusters. Genes crucial for the biosynthesis of the molybdenum cofactor and thiamine were also found. As depicted in Figure III.16 the transcriptional response of these genes was mostly unchanged. Merely the mRNA levels of the *suf* genes along with Alvin_0110 and Alvin_1420, encoding the Fe-carrier IscA and the regulator IscR, respectively, were enhanced during growth on elemental sulfur. The gene expression of the same genes decreased when sulfide was used as substrate. This observation was also made for the relative mRNA levels of *hscB*, *moeB* and *moaE* of which the gene products of the latter two participate in the conversion of cPMP to molypdopterin, a precursor of

Moco. In *E. coli* TusA is involved in this very step (Dahl, J. U. *et al*., 2013). The relative mRNA levels for other genes for the molybdenum cofactor biosynthesis were mostly unaffected by the change of growth conditions or responded negatively. *Alc. vinosum* is lacking *thiI, fdhD, ynjE, iscX* and *cyaY* which are also associated with the biosynthesis of sulfur containing molecules as they encode further interaction partners for IscS (Tokumoto *et al*., 2002; Adinolfi *et al*., 2009; Thomé *et al*., 2011). It is therefore unclear to which extent the established mechanisms from *E. coli* can be directly adopted for *Alc. vinosum*. This holds true for the biosynthesis of 5-methylaminomethyl-2-thiouridine, too. In *E. coli* a multiprotein sulfur relay system involving TusA, TusBCD, TusE and finally MnmA is responsible for this process. *Alc. vinosum* harbours a *mnmA* homologue, of which the relative mRNA levels did not respond to reduced sulfur compounds. Extra homologues of *tusBCD* apart from *dsrEFH* are not present; *tusA* is also present in only a single copy. The *tusE* homologue *dsrC* on the other hand is present with five genomic homologues, though not all of the proteins carry cysteine residues (Figure III.17).

```
Alvin_1256  KYLYSLFPYGPAKQACRFAGLPKPTGCV
Alvin_0345  KDLYDIFKKGPMKLICKWGGLPKPTGCV
Alvin_0732  KYLYGLFPGGPVAQGCRFAGLTAPSGAADKSFGSVQ
Alvin_0028  RGLHRLFPRGGPQKQGNRLAGLLRTKGEH
Alvin_1508  KDLYLLFPLAPTKQGTKIAGLPAVKRKGGY
```

Figure III.17. Alignment analysis of the carboxy-terminus of the DsrC/TusE proteins present in *Alc. vinosum*. The conserved cysteine residues are shown in red.

Alvin_1256 (encoded in the *dsr* operon) and Alvin_0345 each harbour two cysteine residues: the one characteristic for DsrC/TusE proteins involved in sulfur transfer at the penultimate position of the carboxy-terminus and at a distance of 10 amino acids the cysteine specific for DsrC proteins found in sulfur oxidizers and sulfate reducers (Cort *et al*., 2008). The latter was also found in Alvin_0732 whereas Alvin_0028 and Alvin_1508 do not contain any cysteines. As evident from Figure III.16 the gene regulation of the *tusE/dsrC* homologues differed from the biosynthesis related genes (for reasons of clarity only the cysteine encoding genes are depicted). Alvin_0732 and Alvin_0345 exhibited raised relative mRNA levels when elemental sulfur and sulfite served as substrate while the gene expression of Alvin_1256 was increased when *Alc. vinosum* was grown on sulfide, thiosulfate and elemental sulfur. This is in line with the results for *dsrEFH* and *dsrAB*; *dsrAB* further responded positively to sulfite. For the genes *tusA, rhd_2599* and *dsrE2* the results from the qRT-PCR experiment showed enhanced mRNA levels for all three genes in the presence of sulfide, thiosulfate or elemental sulfur which agrees with the transcriptomic profiles of the *dsr* genes rather than the genes affiliated with the biosynthesis of sulfur containing cofactors.

2.5 Individual characterization of Rhd_2599

2.5.1 Analysis of the primary structure

Containing only one rhodanese domain and 107 amino acids Rhd_2599 (Alvin_2599; theoretically calculated molecular mass of recombinant Rhd_2599: 13,991 Da) belongs into the group of single-domain rhodaneses (pfam00581) together with GlpE and PspE from *E. coli*, with which Rhd_2599 shares 37% and 31% identity, respectively. Unlike the *E. coli* enzymes Rhd_2599 contains a second cysteine residue, Cys74, in close proximity to the active site cysteine, Cys64. Both cysteines are strictly conserved among homologous rhodanese-like proteins of the *Chromatiaceae* and the *Ectothiorhodospiraceae* (Figure III.19).

```
Alv     M~~~~VNEIDSESLSQRLADTEDVLLVDIRTPAEIAQGMIPDALQLPMHLIPIRMSEIPK 56
Mcp     M~~~~INEIDSESLQQRISDGEDQLLVDIRTPAEMAQGMIPDAMQLPMHLIPLRLAELPR 56
Tcm     M~~~~INEIDSESLHGRIASGEDVLLVDIRTPAEVAQGAIPDAMRLPMHLIPIRINELPK 56
T90     MYG~FSEITADELEQWRTEGKAFRLVDVRSPGETSRGVIPGAELVPLTVLPLRKDEFLGG 59
Tan     MFGISISEVTPQTLEEWDAEGRPYRLVDVRSMAETERGVLPGAELVPLHLIPLRKDELSG 60

Alv     DRDVVIYCRSGARSYQACAYLMQQGYGRVLNLRGGIIAWARHGLPIVAPEG 107
Mcp     DRDIVLYCRSGARSYQACAYMQQQGYDRVINLRGGIIAWARHGYPVVAPA  106
Tcm     DRDVVLYCRSGARSYQACAYMMQQGYDRVLNLRGGIIAWARHGYPIA     103
T90     DIPLVLYCQSGARSAQACAFLAQQGLETANSLRGGIVGWAQAGKSVVTPD  109
Tan     DRPVVLYCQTGARSGQACAFLAQQGITNVHNLVGGIAGWARSGKPIVAPD  110
```

Figure III.19. Sequence alignments of rhodaneses from Chromatiales. Conserved cysteine residues are highlighted. *Allochromatium vinosum* (Alv, YP_003444541), *Marichromatium purpuratum* (Mcp, WP_005225002), *Thiocapsa marina* (Tcm, WP_007192879) *Thioalkalivibrio sp*. K90mix (K90, YP_003459881), *Thioalkalivibrio nitratireducens* (Tan, YP_007218369).

2.5.2 *In vivo* analysis of Rhd_2599

In order to elucidate the *in vivo* function of Rhd_2599 the phenotype of the mutant *Alc. vinosum rhd_2599*::ΩSm was determined. The mutant strain *Alc. vinosum rhd_2599*::ΩSm was constructed by inserting the Ω-Sm/Spc cassette into *rhd_2599* and thereby inactivating the gene (Sturm, 2009; here, the strain is named *Alc. vinosum 1106*::Ω-Sm^r according to the DRAFT genome annotation). The turnover of elemental sulfur, sulfide and thiosulfate within 48 hours by this strain was tested as described in section II.6.1.2.1 and the results are shown in Figure III.20. For reasons of clarity only the changes in the concentrations of internally stored sulfur and sulfate are displayed.

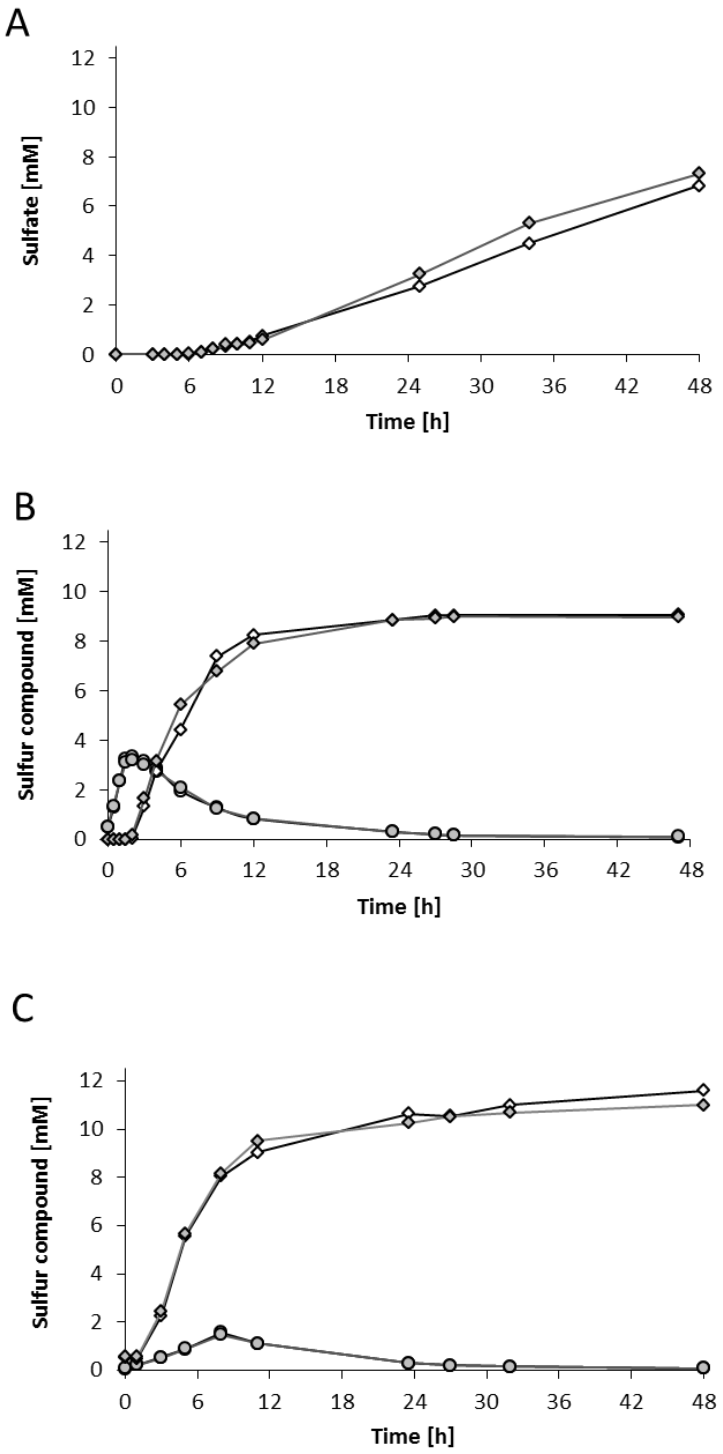

Figure III.20. Turnover of reduced sulfur compounds by *Alc. vinosum* Rif50 and *Alc. vinosum rhd_2599*::ΩSm within 48 hours. (A) Oxidation of 50 mM externally added sulfur. The protein concentration increased over time from 238 to 283 µg ml^{-1} (*Alc. vinosum* Rif50) and 255 to 293 µg ml^{-1} (*Alc. vinosum rhd_2599*::ΩSm). **(B)** Oxidation of 8 mM sulfide. The protein concentration increased over the time of the experiment from 248 to 341 µg ml^{-1} (*Alc. vinosum* Rif50) and 266 to 330 µg ml^{-1} (*Alc. vinosum rhd_2599*::ΩSm). **(C)** Oxidation of 5 mM thiosulfate. The protein concentration of *Alc. vinosum* Rif50 rose from 258 to 352 µg ml^{-1} and for the mutant strain from 264 to 328 µg ml^{-1}. Data points for *Alc. vinosum* wild type are presented in white, data for *Alc. vinosum rhd_2599*ΩSm in grey. ○: concentration of internal sulfur; ◊: concentration of sulfate. Representative experiments are shown.

When elemental sulfur (50 mM) served as electron donor sulfur globules were first observed after three hours in both cultures. The exact concentration, though, was not quantifiable, because it is impossible to separate sulfur storing cells from insoluble sulfur particles in the medium (Franz *et al*., 2009). The final product sulfate was first detected after 8 hours simultaneously in both cultures. The substrate sulfide (8 mM) was rapidly oxidized by both strains and led to the almost immediate formation of sulfur globules; the maximum sulfur concentrations were reached after two hours and further oxidized to sulfate within the monitored period. Feeding thiosulfate (5 mM) to the cultures instantly led to detectable sulfate concentrations. The concentration of intermediately stored sulfur increased progressively in both cultures over the first eight hours when they reached their maxima. In summary, the phenotype of *Alc. vinosum rhd_2599*::ΩSm under photolithoautotrophic conditions did not deviate from the phenotype observed for the wild type. Regarding the concentration of sulfide, thiosulfate and tetrathionate the mutant behaved similar to the wild type as well.

2.5.3 Overall rhodanese activity in *Alc. vinosum* extracts

The disruption of *rhd_2599* appeared to be of no consequence for *Alc. vinosum* under photolithoautotrophic conditions. Therefore the general involvement of rhodaneses in the dissimilatory sulfur metabolism of *Alc. vinosum* should be assessed. To this end *Alc. vinosum* was cultivated photoorganoheterotrophically in 250 ml RCV medium for four days before the cells were pelleted and resuspended in 250 ml 0-medium. 8 mM sulfide was added to induce dissimilatory sulfur oxidation. After eight hours in the light the cells were harvested. As control a photoorganoheterotrophically grown culture was used. Pelleted cells were resuspended in 100 mM Tris-HCl, 50 mM NaCl, pH 7.5 and disrupted by sonication. After separating cell debris, whole cells and sulfur globules from the crude extract (30,000 × g; 30 min; 4°C) the soluble fraction was prepared by two ultracentrifugation steps (each 145,000 × g; 2 h; 4°C). The pellet of the first ultracentrifugation step was used to prepare the membrane fraction. To dissociate peripheral membrane proteins from the membrane the pellet was washed with 1 M NaCl (in 100 mM Tris-HCl, pH 7.5) and again resuspended in 100 mM Tris-HCl, 50 mM NaCl, pH 7.5 after the second ultracentrifugation step. The rhodanese activity was then evaluated after the protocol of Ray (Ray *et al*., 2000).

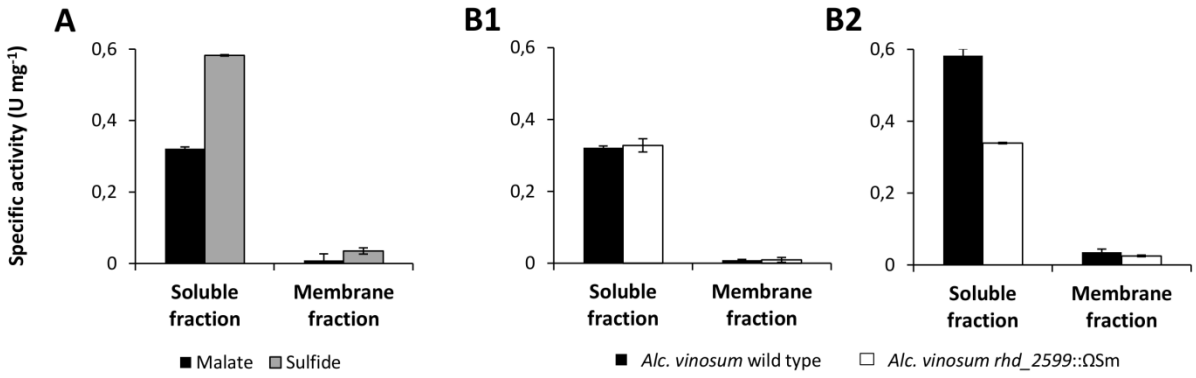

Figure III.21. Rhodanese activity in soluble and membrane fractions of *Alc. vinosum* wild type and *Alc. vinosum rhd_2599*::ΩSm. (A) Comparison of malate and sulfide grown cells of *Alc. vinosum* wild type. **(B)** Comparison of malate **(B1)** and sulfide grown **(B2)** cells of *Alc. vinosum rhd_2599*::ΩSm and the wild type.

As evident from Figure III.21 the rhodanese activity was almost completely limited to the soluble fraction which is due the cellular localisation of the rhodanese domain containing proteins: only Alvin_3028 was predicted to be bound to the membrane (Table III.2). The comparison between malate and sulfide grown cells showed that the rhodanese activity indeed increased under sulfur oxidizing conditions in the soluble and the membrane fraction. In the soluble fraction it was raised by 75% and in the membrane fractions of sulfide grown cells the activity was 6-fold higher relative to malate grown cells.

The experiment was then repeated with *Alc. vinosum rhd_2599*::ΩSm to survey the relevance of Rhd_2599 to the overall rhodanese activity. Under photoorganoheterotrophic conditions no significant difference was observed between the wild type and the *rhd_2599* negative mutant strain (Fig. III.21 B1). However, in the soluble fraction of sulfide grown cells of the mutant the rhodanese activity only reached 58% of the activity determined for the wild type (Fig. III21 B2). Such a reduction was not observed for the membrane fraction. It is noteworthy that the overall increase in enzyme activity in the soluble fraction of *Alc. vinosum rhd_2599*::ΩSm of sulfide grown cells relative to growth on malate was below 10%. In the membrane fraction the activity increased 5.9-fold and is thereby consistent with the development that was observed in the wild type.

Table III.2. Cellular localisation of rhodanese domain containing proteins in *Alc. vinosum*. Note that Alvin_1439 is annotated as FAD-dependent pyridine nucleotide-disulfide oxidoreductase, that contains a rhodanese domain whereas the other proteins listed consist of a single rhodanese domain.

Locus-Tag	Cytoplasm	Periplasm	Membrane
Alvin_0258		•	
Alvin_0866	•		
Alvin_0868	•		
Alvin_1439	•		
Alvin_1587	•		
Alvin_2171		•	
Alvin_2599	•		
Alvin_3028			•

2.5.4 Thiosulfate/GSSH:cyanide sulfurtransferase activity of Rhd_2599

The thiosulfate:cyanide sulfurtransferase activity of Rhd_2599 has been reported before (Sturm, 2009). Here, the experiment was repeated following the protocol of Ray modified by Sturm (Ray *et al*., 2000; Sturm, 2009) and the yielded specific activity of 857 U mg^{-1} matched the 878 U mg^{-1} determined by Sturm. Since thiosulfate is already metabolized in the periplasm by the Sox proteins in *Alc. vinosum* (Hensen *et al*., 2006) it is an unlikely *in vivo* substrate for cytoplasmic Rhd_2599. To date persulfidic glutathione amide (GASSH) and glutathione (GSSH) are discussed as the organic carrier molecules for sulfane sulfur that is transported from the periplasmic sulfur globules to the cytoplasm (Bartsch *et al*., 1996; Prange *et al*., 2002) and might therefore be a substrate for Rhd_2599 *in vivo*. GSSH was used as substrate for Rhd_2599. Indeed, Rhd_2599 showed a specific activity of 25 U mg^{-1}.

2.5.5 Cloning of mutant variants of *rhd_2599*; heterologous overproduction and purification of recombinant Rhd_2599 mutant proteins

Mutant variants of Rhd_2599 were generated to study the relevance of Cys64 and Cys74 the sulfurtransferase activity of Rhd_2599. Mutated amplicons of *rhd_2599* were generated by gene SOEing (Horton, 1975) using plasmid DNA of pET15bRhd and the oligonucleotide pairings Rhd_C64S_for/dsrH20rev, Rhd_C64S_rev/ dsrE78for, Rhd_C74S_for/dsrH20rev and Rhd_C74S_rev/dsrE78for. After digestion with *Nde*I and *Bam*HI each amplicon was separately cloned into the corresponding sites of pET-15b. For the heterologous overproduction the plasmids pET15bRhd_Cys64Ser and pET15bCys74Ser were separately transferred to *E. coli* BL21(DE3); for details see II.6.2.5. After cell disruption via sonication the proteins were isolated from the soluble fraction of the crude extract via the amino-terminal His-tag (Figure III.22).

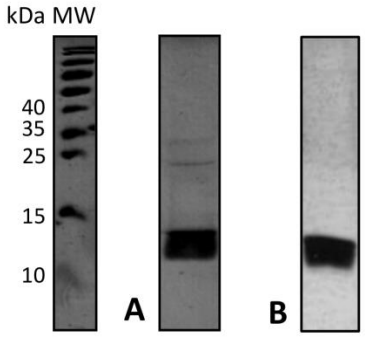

Figure III.22. Identification of Rhd_2599 mutant variants in Coomassie stained SDS-PAGE (15%). (A) Elution fraction of Rhd_2599-Cys64Ser. **(B)** Elution fraction of Rhd_2599-Cys74Ser. Proteins were isolated via the amino-terminal His-tag by elution from a Ni-NTA filled column. Molecular weight (MW) of marker proteins is given in kDa.

2.5.6 Sulfur binding of Rhd_2599

After the thiosulfate/GSSH sulfurtransferase activity was evaluated photometrically, the mobilization and binding of sulfane sulfur from thiosulfate and GSSH to the active site of Rhd_2599 was verified by MADLI-TOF mass spectrometry. Exactly one sulfur atom was bound to the protein after incubation with each of the substrates (Figures III.23A and III.23B). Sturm reported up to three sulfur atoms bound to Rhd_2599 after incubation with sulfide (Sturm, 2009).

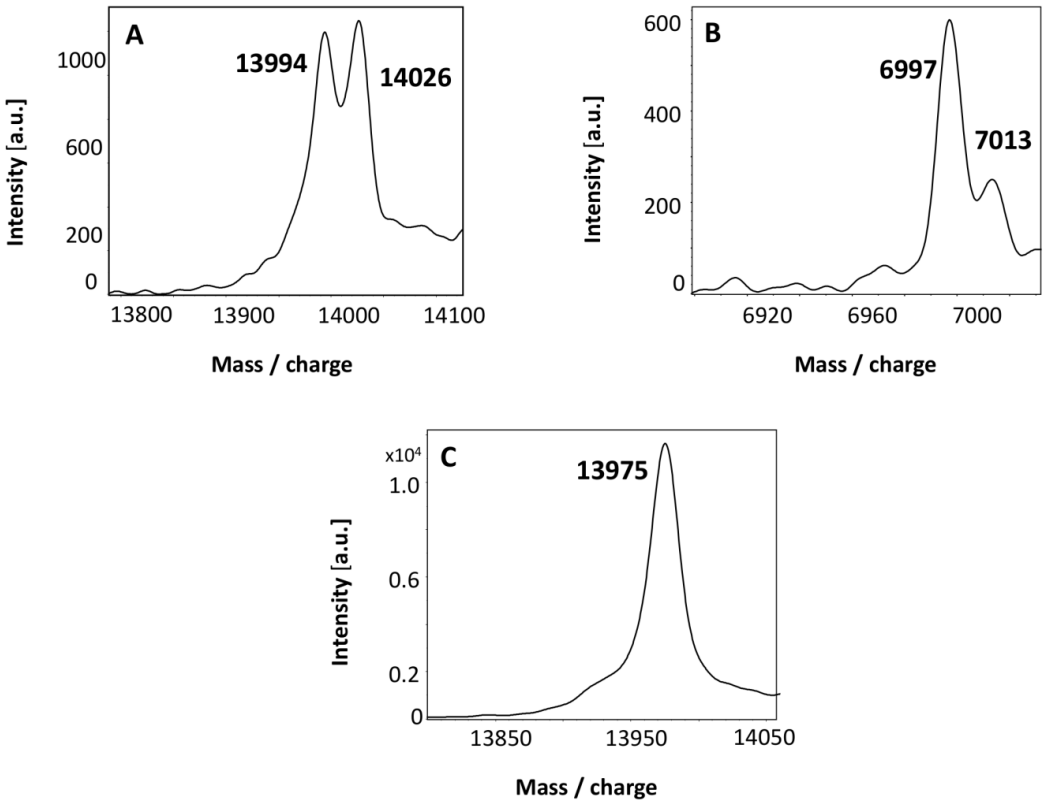

Figure III.23. MALDI-TOF spectrum of persulfurated Rhd_2599 proteins. 30 µM Rhd_2599 was incubated with 2 mM thiosulfate **(A)** and 0.5 mM GSSH **(B)**. The Rhd_2599-Cys64Ser variant protein is shown after incubation with 2 mM thiosulfate **(C)**. Note that in (B) the spectrum for the doubly charged protein is shown, the theoretically calculated molecular mass of His-tagged Rhd_2599 is 13,991 Da.

In the next step Rhd_2599 mutant proteins that carried Cys-Ser mutations of the probable active site cysteine, Cys64, and the second cysteine residue, Cys74, were incubated with thiosulfate. In case of Rhd_2599-Cys64Ser a mass increase was not observed (Figure III.23 C), whereas the Rhd_2559-Cys74Ser variant protein still exhibited the 32 Da mass addition after incubation with thiosulfate (not shown). The 1,5-IAEDANS method was not applicable to Rhd_2599. Neither wild type nor mutant proteins reacted with the reagent in the native or in their persulfurated states.

2.5.7 *In vitro* sulfur transfer reactions with Rhd_2599 as sulfur donor

Sturm described a transfer of sulfane sulfur from Rhd_2599 to TusA using thiosulfate as substrate (Sturm, 2009). Now it was of interest, whether Rhd_2599 would also transfer sulfur to DsrE2 and possibly directly to the sulfur-binding proteins of the Dsr system, DsrEFH and DsrC. To this end the protocol used by Sturm was slightly modified; the mobilization of sulfane sulfur by Rhd_2599 was allowed to take place in the absence of a possible sulfur acceptor protein and excess thiosulfate was removed from the protein solution either by gel filtration or dialysis before the acceptor protein was added.

The transfer to DsrE2 was assessed with 1,5-IADENS yielding a negative result. Rhd_2599 proved unable to transfer sulfur to DsrEFH which was verified with both mass spectrometry and the fluorescence method. A different result was obtained when DsrC served as the acceptor (Figure III.24). When persulfurated Rhd_2559 was incubated with DsrC and 1,5-IAEDANS treatment was performed afterwards, the fluorescence associated with DsrC was found to be noticeably decreased compared to the control containing only DsrC. Note that Rhd_2599 does not react with 1,5-IAEDANS (see above) and is therefore not visible under UV-light (upper panel). DsrC and Rhd_2599 have similar molecular masses, 14.6 kDa and 13.9 kDa, respectively, leading to similar migration patterns in the gel (Figure III.24, lower panel).

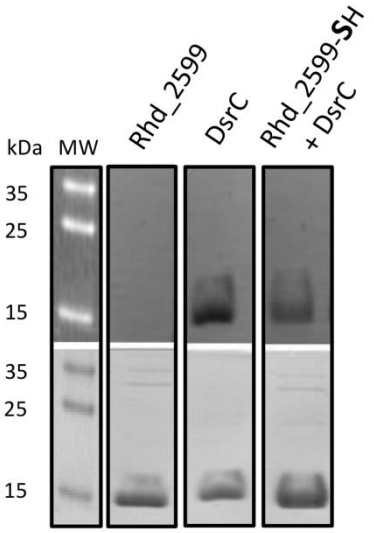

Figure III.24. Persulfuration of DsrC after incubation with persulfidic Rhd_2599. 200 pmol DsrC was added to 200 pmol Rhd_2599 which was incubated with 2 mM thiosulfate beforehand. After treatment with fluorescent 1,5-IAEDANS the protein solution was analysed via SDS-PAGE (15%).The upper panel shows the gel under UV-light, the gel after Coomassie staining is shown in the lower panel. Molecular weight (MW) of marker proteins is given in kDa.

2.6 Individual characterization of TusA

2.6.1 Analysis of the primary structure

Alc. vinosum TusA was first identified by Dobler (Dobler, 2008). It is a small protein with a molecular weight of 8.3 kDa (theoretically calculated molecular mass for recombinant TusA: 10,300 Da) that is encoded by Alvin_2600. Further homologues of the gene or genes encoding for the TusA-like proteins YeeD and YedF are not present in *Alc. vinosum*. Sequence analysis of the protein revealed that *Alc. vinosum* TusA shares higher similarities with TusA proteins from other sulfur oxidizing organisms than with the well characterized protein from *E.coli* (Dobler, 2008). With identities around 90% the protein sequence is highly conserved among the *Chromatiaceae*. In comparison the shared identity between *Alc. vinosum* and *E. coli* TusA is only 37%.

The characteristic motif for TusA proteins (cd00291) is Cys-Pro-X-Pro (Figure III.25). In *E. coli* the motif is located in helix α1. This helix exhibits a unique structural feature as it is stabilized by hydrogen bond between Arg18 and Glu21 rather than the typical N-capping box (Katoh *et al*., 2000). The acidic glutamic acid resides at the "X" position in the TusA specific motif and was shown to be crucial for the protein's function (Katoh *et al*, 2000). None of the TusA protein listed in Table III.1 carries glutamic acid in the motif. Instead, the amino acid is exchanged with a hydrophobic residue in bacterial TusA. The dominating substitution is leucine; TusA from *Hyphomicrobium denitrificans* carries an isoleucine residue and TusA from *Burkholderiales bacterium* JOSHI_001 a methionine residue. The only exception is *Magnetospirillum magneticum*. Here, the motif contains glycine, which can be either hydrophobic or hydrophilic. The glycine residue was also found in all analysed TusA proteins from archaeal sulfur oxidizers. It should be noted that a Φ Blast search with *E. coli* TusA and

the Cys-Pro-Glu-Pro motif as queries among the sulfur oxidizing prokaryotes only yielded results within the *Sulfolobaceae*.

In the *E. coli* homologue Cys19 is crucial for the protein's function, whereas the second cysteine, Cys56, is irrelevant for the the biosynthesis of 5-methylaminomethyl-2-thiouridine *in vivo* (Ikeuchi *et al*., 2006). The latter cysteine residue is not strictly conserved in sulfur oxidizers and was only found within the α-Proteobacteria, the *Aquificae*, and the endosymbiontic γ-Proteobacteria. In TusA from the Chlorobi proteins a second cysteine is preserved 5 amino acids downstream of the active site cysteine residue.

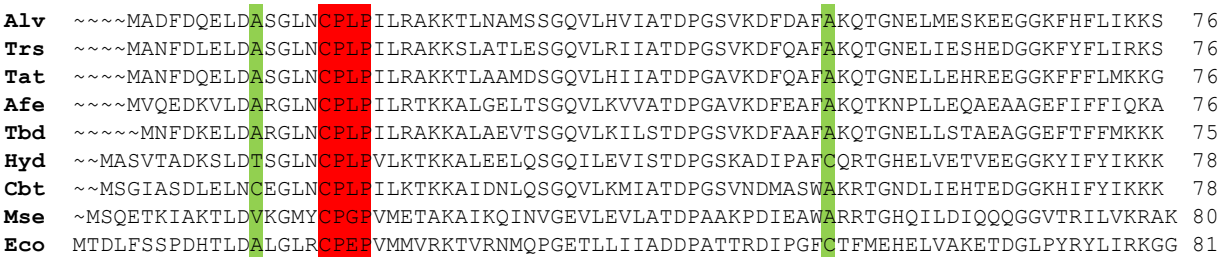

Figure III.25. Sequence alignment of TusA proteins from sulfur oxidizing microorganisms and *E. coli*. The characteristic Cys-Pro-X-Pro motif is highlighted in red. Other conserved cysteine residues are are highlighted in green. *Allochromatium vinosum* (Alv; YP_003444542), *Thiorhodospira sibirica* (Trs; WP_006787555), *Thioalkalibvirbio thiocyanoxidans* (Tat; WP_006745882), *Acidithiobacillus ferrooxidans* (Afe; YP_002426937), Candidatus *Ruthia magnifica* (Rum; YP_903482), *Rhodomicrobium vannielii* (Rva; YP_004010984) *Thiobacillus denitrificans* (Tbd; YP_314332), *Hydrogenivirga sp.* (Hyd; WP_008286378), *Chlorobaculum tepidum* (Cbt; NP_661741), *Metallosphaera sedula* (Mse; YP_001191627), *Escherichia coli* (Eco; NP_417927).

2.6.2 The interaction between TusA and DsrEFH *in vitro*

Alc. vinosum TusA has been shown to interact with DsrEFH in band shift assays. Upon incubation with each other the migration patterns of the proteins changed and two additional bands appeared (Figure III.26A). The presence of the active site cysteines DsrE-Cys78 and TusA-Cys15 are crucial for this reaction (Stockdreher, 2009). Considering the difference in the motifs of *Alc. vinosum* and *E. coli* TusA and the fact that an interaction between *E. coli* TusA and TusBCD was so far not detected (Ikeuchi *et al*., 2006; Numata *et al*., 2006) it was of interest to see whether *E. coli* TusA would evoke a reaction and change the migration patterns of DsrEFH in a band shift assay. As a control the reactions between TusBCD and the TusA proteins were also evaluated.

2.6.2.1 Cloning of *E. coli tusA* and *tusBCD*, heterologous overproduction and purification of recombinant EcTusA and TusBCD

The oligonucleotides EcTusA_for and EcTusA_rev were used to amplify *E. coli tusA* and introduce restriction recognition sites for *Nde*I and *Bam*HI. The *tusBCD* sequence was amplified with the oligonucleotides TusBCD_for and TusBCD_rev that also introduced *Nde*I and *Bam*HI recognition sites. Genomic DNA from *E. coli* K-12 served as template. After digestion with the respective restriction enzymes the amplicons were separately cloned into the corresponding sites of pET-15b. Heterologous overproduction of the EcTusA and TusBCD was carried out in *E. coli* BL21(DE3) hosting the plasmids pET15bEcTusA or pET15bTusBCD. The proteins were isolated from the soluble fraction via Ni-NTA affinity chromatography according to the manufacturer's instruction (see II.8.4.1; Figure III.26).

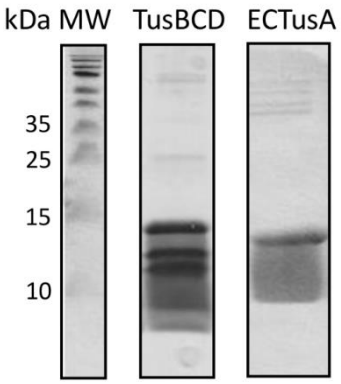

Figure III.26. Purification of TusBCD and EcTusA. **Both proteins eluted from Ni-NTA columns and were applied to SDS-PAGE (15%). Shown gels are Coomassie stained. EcTusA, *E. coli* TusA.** Molecular weight (MW) of marker proteins is given in kDa.

2.6.2.2 Analysis via band shift assay

For the assay the proteins were incubated as described in II.8.8 and afterwards applied to native PAGE to check for alterations in migration patterns that would indicate the formation of protein complexes *in vitro* (Figure III.27). The incubation of DsrEFH with *Alc. vinosum* TusA led to the appearance of two extra bands that migrated slower than DsrEFH alone and quicker than TusA alone. Attempts to recover the additional bands and determine the proteins that were contained in these bands was not possible due to the fact that DsrH and TusA have almost identical molecular weights (10.9 kDa and 10.3 kDa, respectively) and migrate at the same height. For Western blot analysis the protein concentrations transferred to the membrane proved to be too small. When DsrEFH was incubated with *E. coli* TusA the additional bands did not occur (Figure III.27A).

To evaluate whether this method is generally applicable to *E. coli* TusA, the protein was incubated with the DsrEFH homologue TusBCD in the next step. As evident from the Figure III.27B EcTusA and TusBCD indeed interacted and the reaction strongly resembled the one of their *Alc. vinosum* homologues, the incubation led to the formation of two additional bands. TusBCD was further incubated with each of the *Alc. vinosum* pendants of its interaction partners in *E. coli*, AvTusA and DsrC. The mutual incubation resulted in complexes that were stable during the electrophoretic run as one extra band was obviously visible for each protein combination (Figure III.27, lanes 6 and 7). The consumption of individually migrating TusBCD that was seen for the reaction with EcTusA did not come to pass. Taken together the results show that while the TusBCD reacted with proteins from *Alc. vinosum* TusA from *E.coli* was not interchangeable with TusA from *Alc. vinosum* for the reaction with DsrEFH.

The individual migration profiles of the two TusA proteins agree with this conclusion. *E. coli* TusA migrated mainly in one broad band whereas two almost equally broad bands were identified for *Alc. vinosum* TusA. The second band is most likely due to the formation of a dimeric form, because this band did not apparent in the absence of TusA-Cys15 (Stockdreher, 2009).

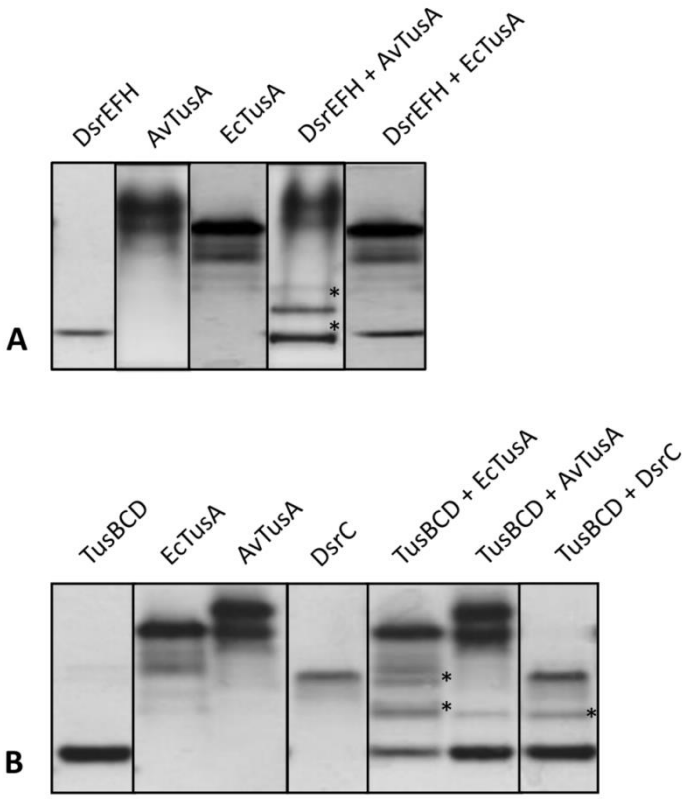

Figure III.27. Interaction of DsrEFH/TusBCD and AvTus/EcTusA analysed with native PAGE (7.5%). **(A)** Interaction of DsrEFH with *Alc. vinosum* and *E. coli* TusA. **(B)** Interaction of TusBCD with TusA from *E. coli* and *Alc. vinosum* and DsrC. Asterices indicate additional bands. Proteins were used in the following amounts: DsrEFH, 100 pmol; TusBCD, 200 pmol; *Alc. vinosum* TusA, 800 pmol; *E. coli* TusA, 800 pmol; DsrC, 400 pmol. The proteins were incubated in 5mM HEPES (pH 7.8), 0.1 M KCl, 0.01% (v/v) Tween 20 and 25 µM TCEP for 30 minutes at 30°C before electrophoresis. Av, *Alc. vinosum*. Ec, *E. coli*.

2.6.2.3 Analysis via Surface Plasmon Resonance (SRP)

For a more detailed analysis the interaction between TusA and DsrEFH should be further assessed with SRP. DsrEFH was covalently bound to a CM5 chip and TusA served as analyte. However, with this method no interaction was detected.

2.6.3 Thiosulfate / GSSH:cyanide sulfurtransferase activity of TusA

When TusA was tested as thiosulfate:cyanide sulfurtransferase and GSSH:cyanide sulfurtransferase, the results were negative. MADLI-TOF mass spectrometry verified that TusA was not able to mobilize sulfane sulfur from these low molecular weight thiols.

2.6.4 TusA as sulfur binding protein

TusA was reported to bind one sulfur atom via Cys15 after incubation with the L-cysteine desulfurase IscS and L-cysteine *in vitro* (Stockdreher, 2009). In order to persulfurate TusA for subsequent tests as sulfur donor, sulfide was now tested as a possible substrate for TusA. Figure III.28 shows two superimposed spectra obtained from MADLI-TOF mass spectrometry. In both spectra the peak for

non-persulfurated TusA is present. The additional mass of 32 Da was clearly identified in the first spectrum while a third peak, indicating extra 64 Da, was only insinuated. In the second spectrum, retrieved from another sample, the distinctiveness was reversed; here, the peak for additional 64 Da was clearly visible, while the peak representing 32 Da was merely denoted.

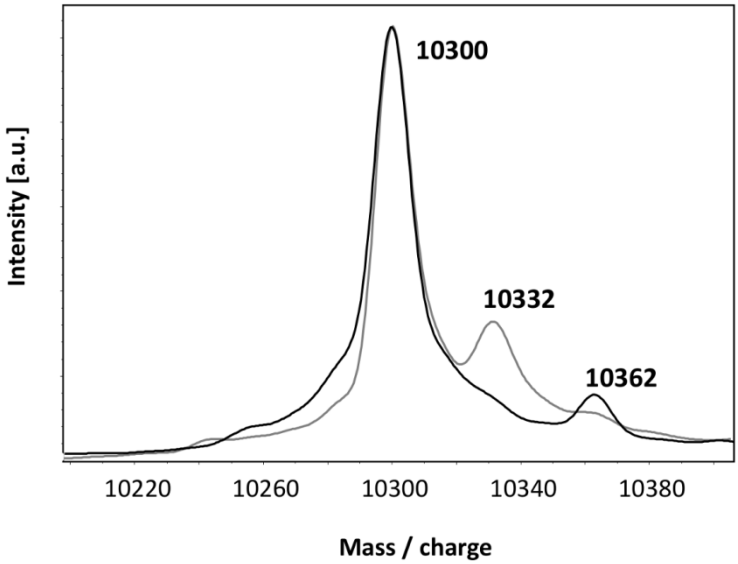

Figure III.28. Sulfur-binding capacity of TusA analysed with MALDI-TOF mass spectrometry. 30 µM of TusA was incubated with 2 mM sulfide before the protein was analysed via MALDI-TOF mass spectrometry. The theoretically calculated molecular weight of His-tagged TusA is 10,300 Da. Binding of sulfur atoms is indicated by an additional mass of 32 Da. The figure shows two superimposed spectra obtained from two individually prepared samples.

This experiment was then repeated using the 1,5-IAEDANS method to detect persulfide formation. TusA was successfully labelled with 1,5-IAEDANS. The 1,5-IAEDANS specific fluorescence was detected after incubation with the wild type protein whereas none was detected when the Cys15Ser variant was tested (Figure III.29A). The method was also applicable for the detection of persulfide formation on TusA (Figure III.29B). In consistence with the results for DsrEFH and DsrC the emission of light was significantly reduced after TusA was incubated with sulfide. Reduction of persulfurated TusA prior to 1,5-IAEDANS treatment recovered the thiol group only partially as evident from Figure III.29B.

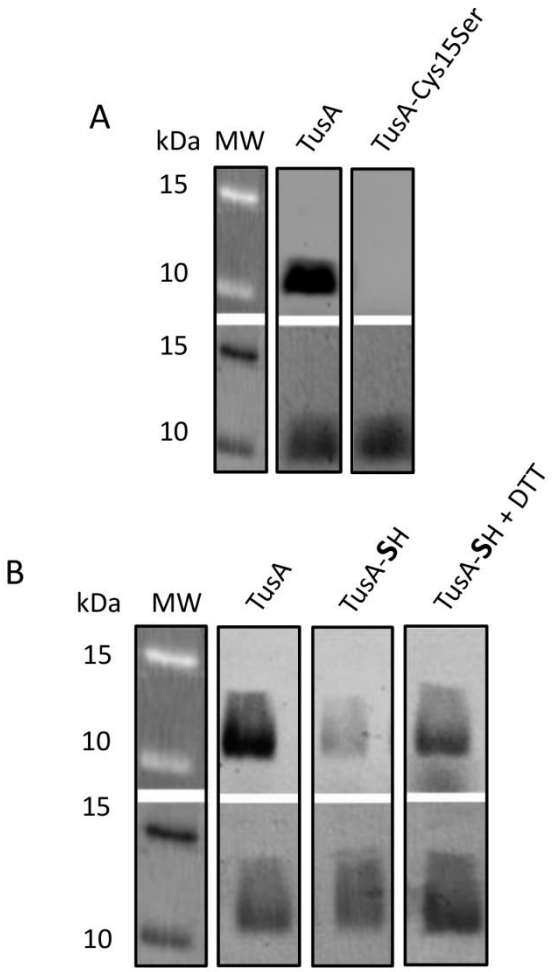

Figure III.29. **Labelling and sulfur binding capacity of TusA analysed with 1,5-IAEDANS. (A)** 200 pmol of reduced TusA and TusA-Cys15Ser was treated with 1,5-IAEDANS before electrophoresis. **(B)** TusA was reduced with DTT before incubation with 4 mM sulfide. After dialysis the persulfidic protein was again treated with DTT before it was subjected to 1,5-IAEDANS treatment. Afterwards the samples were reduced and 200 pmol of each sample was applied to electrophoresis (15%) The upper images show the gels under UV light, the lower after staining with Coomassie Molecular weight (MW) of marker proteins is given in kDa.

2.6.5 *In vitro* sulfur transfer reactions with TusA as sulfur donor

In the next step the sulfurtransferase capabilities of TusA were evaluated using MALDI-TOF mass spectrometry and the 1,5-IAEDANS method. While the reaction with DsrE2 was negative, Rhd_2599 was found to be persulfurated upon incubation with persulfidic TusA (not shown). Furthermore, the reaction between TusA and DsrEFH yielded positive results. After incubation with persulfidic TusA the fluorescence of DsrEFH was significantly reduced compared to the control sample while that of TusA became marginally more prominent again (Figure III.30A), thereby illustrating the transfer of sulfane sulfur from TusA to DsrEFH *in vitro*. Persulfide formation on DsrE was also observed when DsrEFH-Cys20Ser was used as acceptor protein. Additionally, the sulfur transfer was reversible (Figure III.30B). After TusA was incubated with persulfurated DsrEFH the formation of a persulfide was detected in TusA. While the fluorescence of TusA was reduced relative to the untreated sample

the fluorescence of DsrEFH was almost completely restored to the native state. As a control, persulfurated DsrEFH was also incubated with the mutant TusA protein lacking Cys15. In this case, the fluorescence of DsrEFH was unchanged (not shown).

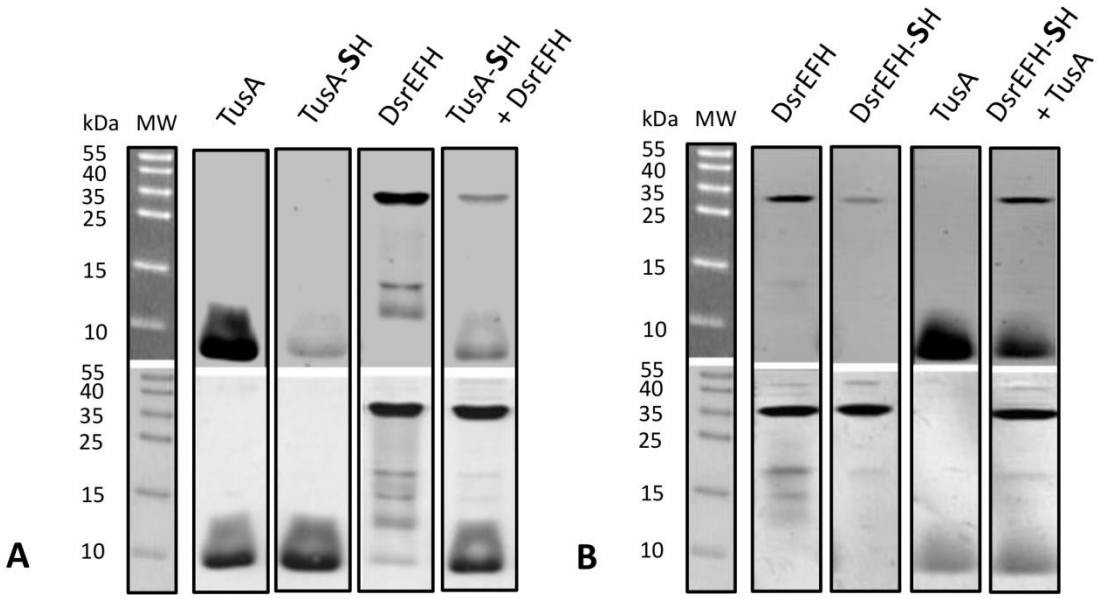

Figure III.30. Sulfur transfer between TusA and DsrEFH. For sulfur transfer reactions persulfurated donor protein was incubated with the acceptor protein for 60 minutes at 30°C before the reaction mixtures were treated with 1,5-IAEDANS as described. For analysis SDS-PAGE was performed (15%). The upper panels show the gels under UV light, the lower panels show the same gels after staining with Coomassie. Molecular weight (MW) of marker proteins is given in kDa.

In a further experiment persulfidic TusA was mixed with DsrC resulting in one sulfur atom bound to DsrC-Cys111 as shown in Figure III.31. When only DsrC-Cys100 was present this reaction did not occur (not shown).Unlike the reversible reaction between DsrEFH and TusA the transfer of sulfur atoms from DsrC to TusA was not detected (not shown).

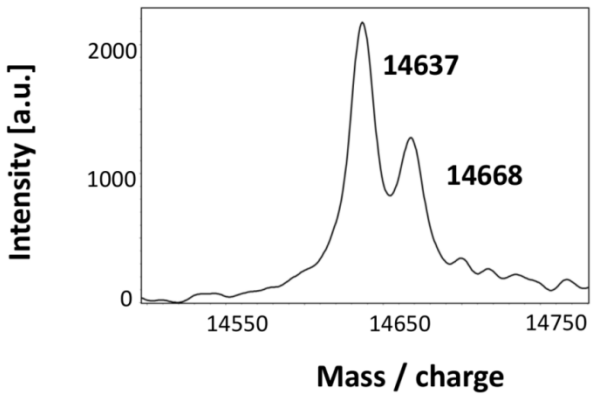

Figure III.31. Persulfuration of DsrC after incubation with persulfidic TusA 30 µM persulfidic TusA was incubated with 30 µM DsrC as acceptor molecule for one hour at 30°C before the reaction mixture was analysed via MADLI-TOF mass spectrometry.

2.7 Individual characterization of DsrE2

2.7.1. Analysis of the primary structure

The protein DsrE2 is encoded by Alvin_2601. It is annotated as hypothetical protein of unknown function and belongs to the DsrE/DsrF/DsrH superfamily (pfam13686). The protein was found to be strictly conserved within DsrAB containing *Chromatiaceae* with maximum identities ranging from 88 – 91%. Apart from the Chromatiales, the *Acidithiobacillaceae*, the Thiotrichales and the endosymbionts DsrE2 proteins were hardly found in other γ-Protobacteria.

From amino acid analysis two transmembrane helices were predicted that are arranged so that the carboxy-terminus is located in the cytoplasm. The carboxy-terminus carries three cysteine residues: Cys110, Cys120 and Cys156 (Table III.3). All cysteines are conserved in homologues within the *Chromatiaceae*, the *Ectothiorhodospiraceae* and the β-Proteobacteria. Cys120 is the only cysteine that was found in bacteria and archaea alike. Sequence alignments of all DsrE2 proteins listed in Table III.1 with *Alc. vinosum* DsrE and *E. coli* TusD revealed that this residue corresponds to active site cysteine residue, Cys78, in the latter two proteins. A quick overview of the presence of cysteine residues in DsrE2 proteins is given in Table III.3.

Table III.3. Distribution of cysteine residues in DsrE2 proteins from genome-sequenced sulfur oxidizers. Numbering is according to the sequence of *Alc. vinosum* DsrE2. * Only DsrE2 in *Magnetospirillum gryphiswaldense* contains Cys156.

	Cys110	**Cys120**	**Cys128**	**Cys140**	**Cys156**
α-Proteobacteria		●			*
β-Proteobacteria	●	●			●
Acidithiobacillaceae	●	●			
Chromatiaceae	●	●			●
Ectothiorhodospiraceae	●	●			●
Thiotrichaceae	●	●			●
Endosymbionts	●	●			
Chlorobi	●	●			
Aquificae DsrE2A	●	●			
Aquificae DsrE2B		●		●	
Sulfolobaceae DsrE2A		●	●		
Sulfolobaceae DsrE2B		●			

Apart from the omnipresent Cys120 a second cysteine was found in close distance in all DsrE2 proteins, which probably indicates a vital role for both cysteines. DsrE2 proteins from α-Proteobacteria are the only exceptions as only Cys120 was found. In the Aquificae and the *Sulfolobaceae* the situation is somewhat different. Two copies of the *dsrE2* gene are located next to *tusA* and the gene products vary in regard to the conserved cysteine residues (Figure III.32). Proteins containing two cysteines in close proximity (Cys-X_9-Cys for Aquificae and Cys-X_7-Cys for

Sulfolobaceae) were designated DsrE2A; the other residue was labelled DsrE2B. The least conserved cysteine is Cys156 and was found exclusively in the Chromatiales and the β-Proteobacteria.

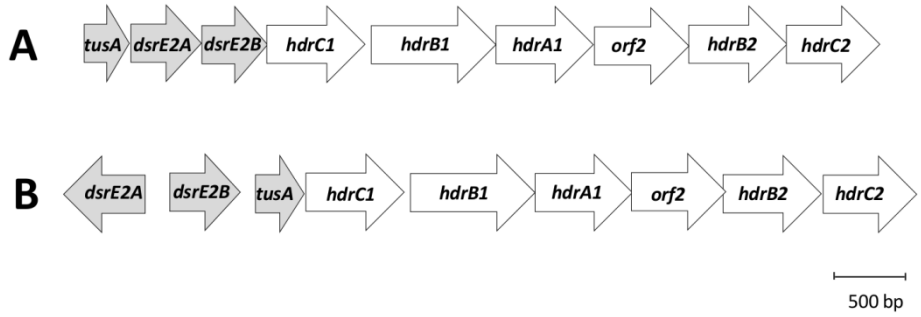

Figure III.32. Schematic overview of the genomic organisation of the *dsrE2-tusA-hdr* gene cluster in the *Aquificaceae* (A) and *Sulfolobaceae* (B).

2.7.2 Cloning of *dsrE2* wild type and mutated sequences, heterologous overproduction and purification of recombinant DsrE2 variants

DsrE2 was produced from three distinct overexpression plasmids: pET15bDsrE2, pET22bDsrE2 and pIBADsrE2. In a first approach recombinant DsrE2 was overproduced fused to a His-Tag. The *dsrE2* gene was amplified using DsrE2_for and DsrE2_rev as oligonucleotides and genomic DNA isolated from *Alc. vinosum.* The amplicon was subsequently cloned into the *Nde*I and *Bam*HI recognition sites of pET-15b and pET-22b, fusing it to an amino-terminal and carboxy-terminal His-tag, respectively. The amplicon for cloning *dsrE2* into pASK-IBA5plus was obtained via PCR with the oligonucleotides DsrE2_Strep_for and DsrE2_Strep_rev. After digestion with *Bsa*I the fragment was cloned into the corresponding site of pASK-IBA5plus and thereby fused to an amino-terminal Strep-tag. *E. coli* C41(DE3) and *E. coli* C43(DE3) cells served as hosts for the overproduction of the resulting plasmids. Conditions for overproduction were the same for all three plasmids and are described in II.6.2.5. The pellets were resuspended in lysis buffer before the cells were disrupted via sonication (1.5 min mL^{-1}, 50% intensity, 4°C, CeII Disruptor B15, Banson, Danbury, USA). Cell debris and whole cells were separated from the soluble fraction by a centrifugation step (25,000 x g; 4°C; 30 min). The membrane fraction was prepared by ultracentrifugation (145,000 x g; 2h; 4°C) of the soluble fraction. Solubilisation was carried out with the detergent Triton-X100 in a final concentration of 1% in lysis buffer for 3 hours or overnight under gentle stirring on ice. After another ultracentrifugation step the isolation of recombinant DsrE2 from the supernatant was performed via affinity chromatography according to the manufacturer's instruction. The carboxy-terminal His-tag proofed to be completely unsuitable for the purification of DsrE2 since the recombinant protein was located in insoluble inclusion bodies. Inclusion bodies are usually observed during overproduction in a heterologous hosts when the recombinant protein is misfolded and not proteolytically degraded (Baneyx & Mujacic, 2004). The binding of the amino-terminal His-tag to the Ni-NTA matrix was impaired so that the

majority of the DsrE2 already eluted during washing steps. These protein solutions were unusable for further experiments given that unspecifically bound proteins contaminated the solutions. The amount of DsrE2 that finally eluted during the actual elution steps was minor. The amino-terminal Strep-tag on the other hand resulted in the successful purification of recombinant DsrE2 (theoretically calculated molecular mass: 19,012 Da) (Figure III.33). The average protein yield from 1litre *E. coli* culture constituted 4 mg. UV-Vis spectroscopy showed no indications for cofactors bound to the protein.

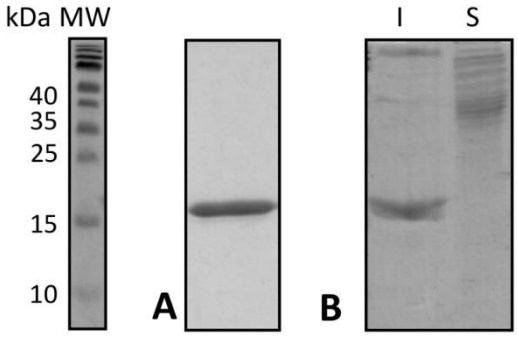

Figure III.33. Identification of DsrE2 in Coomassie stained SDS-PAGE (15%). (A) Elution fraction of amino-terminal Strep-tagged DsrE2. **(B)** Insoluble and soluble fraction of crude extract of *E. coli* C41 (DE3) cells after overproduction of carboxy-terminally His-tagged DsrE2 and cell disruption via sonication. I, insoluble fraction. S, soluble fraction. Molecular weight (MW) of marker proteins is given in kDa.

For the production of mutant proteins in which one of three cysteine residues was exchanged for a serine residue the plasmids pIBADsrE2-Cys110Ser, pIBADsrE2-Cys120Ser and pIBADsrE2-Cys156Ser were constructed. Site-directed mutagenesis of the cysteine residues was carried out (Horton, 1975) using the oligonucleotides DsrE2_C110S_for, DsrE2_C110S_rev, DsrE2_C120S_for, DsrE2_C120S_rev, DsrE2_C156S_for and DsrE2_C156S_rev. The protocol for overproduction and purification were exactly the same as described for the wild type protein. While DsrE2-Cys156Ser was easily produced and purified in amounts comparable to the unaltered protein, the exchange of Cys110 led to a significantly reduced protein yield (0.8 mg l^{-1} *E. coli* culture). The mutation of Cys 120 led to storage of the protein into inclusion bodies during overproduction as was observed for DsrE2 fused to a carboxy-terminal His-tag before (Figure III.33B).

2.7.3 Identification of intramolecular disulfide bonds

As stated above DsrE2 carries two highly conserved cysteine residues and a third cysteine that is conserved among the Chromatiales and the β-Proteobacteria. Together with the failed attempts to purify DsrE2 fused to a carboxy-terminal His-tag and DsrE2-Cys120Ser it was reasonable that intramolecular disulfide bonds would contribute to the stabilization and/or the function of DsrE2. To test this hypothesis DsrE2 and the mutated proteins DsrE2-Cys110Ser and DsrE2-Cys156Ser were incubated in an open reaction tube for 15 minutes at 30°C and subsequently analysed via non-reducing SDS-PAGE. Control samples were mixed with denaturing and reducing buffer and cooked for 5 minutes before they were subjected to electrophoresis (Figure III.34).

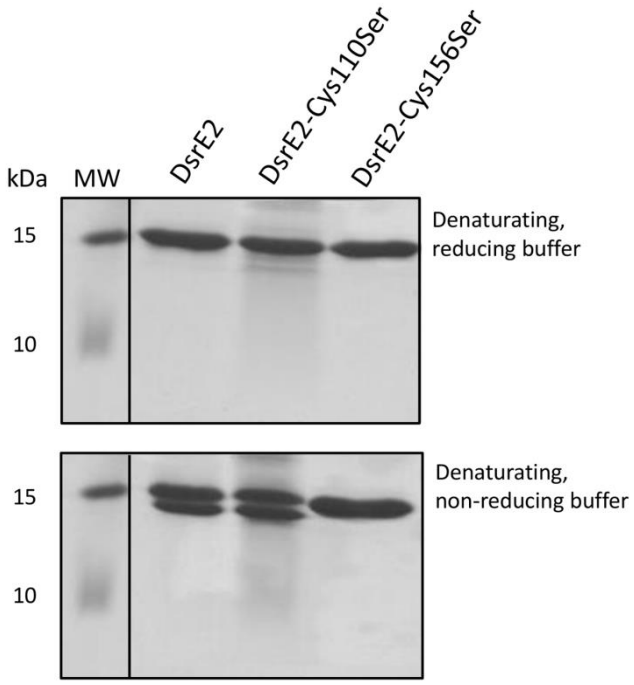

Figure III.34. Analysis of DsrE2 proteins under reducing and non-reducing conditions. 25 µg of DsrE2 wild type protein, DsrE-Cys110Ser and DsrE2-Cys156Ser was mixed with denaturing, reducing buffer or denaturing, non-reducing buffer and applied to 15% SDS-PAGE and subsequent Coomassie staining. Molecular weight (MW) of marker proteins is given in kDa.

As is evident from Figure III.34 all DsrE2 proteins migrated in a single distinct band after reduction and denaturation. However, under non-reducing conditions the wild type protein and DsrE2 lacking Cys110 migrated in a double-band, a second band migrated directly under the band that was already observed for the reduced proteins. However, the second band was not observed for DsrE2-Cys156Ser which adds support to the ideas that DsrE2 forms intramolecular disulfide bonds.

2.7.4 Thiosulfate / GSSH:cyanide sulfurtransferase activity of DsrE2

DsrE2 was tested negative for GSSH and thiosulfate:cyanide sulfurtransferase activity. This finding agrees with the the fact that DsrE2 does not contain any of the rhodanese motifs that were reported by Bordo & Bork (Bordo & Bork, 2002).

2.7.5 DsrE2 as sulfur-binding protein

The capacity of DsrE2 to act as sulfur carrier was tested by incubating the reduced protein with sulfide and further analysis with the 1,5-IAEDANS method. As shown in Figure III.35 1,5-IAEDANS bound to the protein enabling its detection under UV light. In its persulfurated state the fluorescence was significantly reduced, which was reversed by reduction with DTT prior to the 1,5-IAEDANS treatment, confirming that DsrE2 is a sulfur binding protein. Experiments with the mutant variants resulted in the same observations. The formation of a persulfide was verified for both proteins. However, when exposed to UV light DsrE2-Cys156Ser differed from the other variants as the fluorescence was equally spread between two bands. While the upper band was also visible in the gel after Coomassie staining, the faster migrating band was not detectable after Comassie staining. MALDI-TOF mass spectrometry was not applicable to DsrE2 as the protein was not detected.

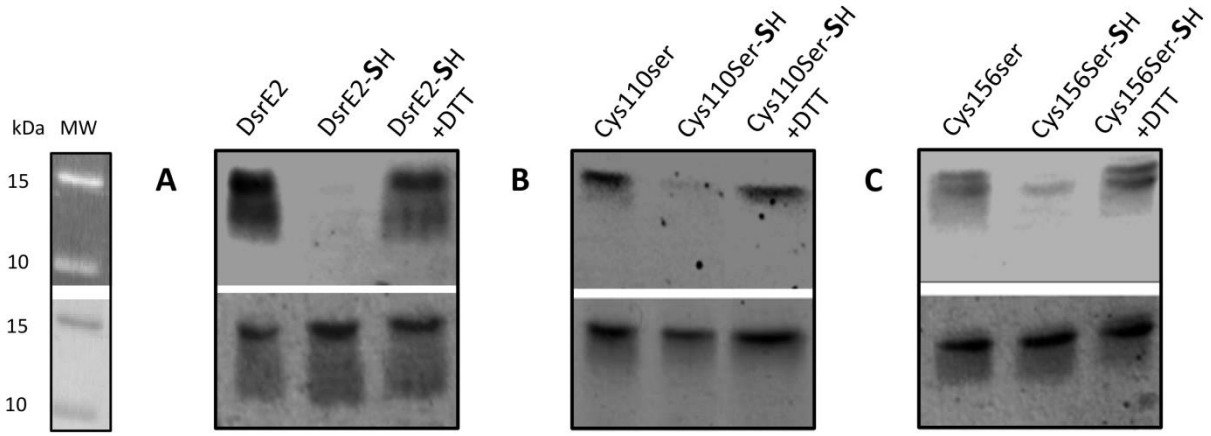

Figure III.35. Sulfur binding capacity of DsrE2 wild type and mutant proteins. After reduction DsrE2 wild type **(A)**, DsrE2-Cys110Ser **(B)** and DsrE2-Cys156Ser **(C)** was incubated with 4 mM sulfide. After dialysis the proteins were again treated with DTT before they were treated with 1,5-IAEDANS. Afterwards the samples were reduced and 200 pmol of each sample was subjected to electrophoresis (15%). Upper panels show the gels under UV light, the lower the gels after Coomassie staining. Cys110Ser, DsrE2-Cys110Ser; Cys156Ser, DsrE2-Cys156Ser. Molecular weight (MW) of marker proteins is given in kDa.

2.7.6 *In vitro* sulfur transfer reaction with DsrE2 as donor and acceptor protein

DsrE2 was not tested as sulfur donor protein, because the detergent Triton-X100 interfered with the obligatory dialysis that was used to remove excess sulfide from the protein solution. TusA, DsrEFH and DsrC were tested positive for persulfuration after incubation with the dialysis product of Triton-X100 and sulfide containing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl). When the sulfur accepting potential of DsrE2 was tested, no persulfide formation was identified after incubation with persulfidic Rhd_2599, TusA, DsrEFH and DsrC.

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IV Discussion

The main aim of this work was to explore the possibility of sulfur trafficking in *Alc. vinosum* during the degradation of sulfur globules with focus on cytoplasmic events and the identification of participating sulfurtransferases. While transient sulfur globules are located in the periplasm the oxidation of the stored sulfur takes place in the cytoplasm and is conducted by the proteins of the Dsr system. Similarities in sequence and structure with the sulfurtransferases TusBCD and TusE in *E. coli* led to the proposal that DsrEFH and DsrC might act as sulfurtransferases and supply DsrAB with sulfur (Cort *et al*., 2008). To verify this notion DsrEFH and DsrC were analysed in regard to their capacity to mobilize, bind and transfer sulfane sulfur. In addition the interaction between these proteins was analysed in detail to confirm the formation of a stable complex. The second part of the study was dedicated to the individual characterization of Rhd_2599, TusA and DsrE2 as sulfurtransferases. Furthermore, the putative involvement of the three proteins in the dissimilatory sulfur metabolism of *Alc. vinosum* was systematically explored.

DsrEFH and DsrC as sulfurtransferases

For the detection of a putative persulfide formation on DsrEFH and DsrC the proteins were incubated with either sulfide or IscS and cysteine. Both strategies resulted in the *in vitro* persulfuration of DsrEFH and DsrC and confirmed both proteins as sulfur carriers.

DsrEFH decomposed into its subunits and allowed the analysis of the individual subunits during MADLI-TOF mass spectrometry. The spectra of DsrF and DsrH showed no alterations whereas as two different species were detected for DsrE, persulfurated and native DsrE. Cys78 is the only cysteine residue present in DsrE and corresponds to the sulfur binding cysteine in TusD, TusD-Cys78. This leads to the conclusion that the cysteine in position 78 is likely the active site for the DsrE/DsrF-like family.

Cys20 in DsrH is located in a second putative active site of DsrEFH and should generally be available for potential substrate molecules as demonstrated by fluorescence labelling of DsrEFH variants with 1,5-IAEDANS. The fluorescent signal was reduced in proteins carrying single Cys-Ser substitutions for DsrE-Cys78 and DsrH-Cys20 and was completely lost in the DsrEFH variant that carried only DsrF-Cys40 and DsrH-Cys95. However, DsrH-Cys20 was not persulfurated. DsrH-Cys20 was also irrelevant for the sulfur binding capacity of DsrEFH since the mutant protein DsrEFH-Cys20Ser was efficiently persulfurated. The residue was further expendable for the interaction with DsrC and TusA *in vitro*. On the other hand, DsrH-Cys20 is essential for the degradation of sulfur globules; the complementation of a Δ*dsrE* mutant strain with the sequence carrying the *dsrH-Cys20Ser* substitution did not restore the wild type phenotype as was seen for the complementation with wild type *dsrEFH* (Stockdreher *et al*., 2012). This contradicts the complementation experiments of the *tusBCD* negative

E. coli mutant. Here, only Cys78 of TusD is relevant for the efficient biosynthesis of 5-methylaminomethyl-2-thiouridine (Numata *et al*., 2006). Therefore, TusBCD seemingly needs only one redox-active cysteine residue that covers the function of the protein while the catalytic cycle of DsrEFH demands a second cysteine in DsrH. The precise function of DsrH-Cys20 remains elusive.

The detection of the DsrEFH-persulfide with 1,5-IAEDANS unexpectedly yielded additional information. DsrEFH migrated mainly as a trimer in the gel and only a small portion ran as monomer. This portion was significantly decreased after incubation with sulfide pointing to conformational changes that are forced upon the protein by the binding of sulfane sulfur. A similar observation was made during the labelling experiment with DsrEFH mutant proteins; the monomeric forms were absent in the DsrEFH proteins lacking the cysteine in DsrE. This might explain why DsrEFH variants, that carry the DsrE-Cys78Ser mutation, exhibit slightly different migration patterns relative to wild type DsrEFH in native PAGE electrophoresis (Dahl *et al*., 2008).

Cys111 was identified as the binding site for sulfur compounds in DsrC. The protein was specifically persulfurated at Cys111; DsrC-Cys100 did not bind sulfur nor was it required for the persulfuration reaction of DsrC. DsrC-Cys111 occupies the penultimate position of the carboxy-terminus and is conserved in all "true" DsrC proteins (here defined by being encoded within the *dsr* operon) and TusE proteins. DsrC-Cys100 on the other hand is an indicator for a sulfur-driven metabolism. This cysteine is restricted to organisms that also contain DsrAB (Cort *et al*., 2008). The residue is buried in the globular part of DsrC (Cort *et al*., 2008) which makes it unlikely to be available for potential substrates. The fluorescent labelling with 1,5-IAEDANS of the DsrC mutant proteins agreed with this notion since the reagent did not bind to the DsrC protein carrying only Cys100.
While Cys111 is clearly dedicated to the sulfur binding in DsrC and TusE proteins the function of Cys100 is apparently related to the activity of DsrAB. The catalytic function of DsrC in sulfur oxidizers and sulfate reducers has been proposed to involve a thiol-disulfide exchange. The crystal structure of DsrC from *Dsv. gigas* (Hsieh *et al*., 2010) and *Dsv. vulgaris* (Oliveira *et al*, 2008) show DsrC bound to DsrAB. The cysteines corresponding to Cys111 were found either in close distance to the active site of DsrAB or within bonding distance to the previous cysteine. Since the last ten residues of these carboxy-termini are equal to that in *Alc. vinosum* DsrC the same level of flexibility can be expected here. In fact, for DsrC from *Alc. vinosum* and the sulfate reducer *Dsv. vulgaris* the oxidized versions have already been reported (Cort *et al*., 2008; Venceslau *et al*., 2013). Currently, the thiol-disulfide exchange for DsrC from sulfur oxidizers is supposed to release the final product of the Dsr cycle, sulfite. In this model DsrC leaves DsrAB after oxidation of sulfur with a sulfonate group bound to the carboxy-terminus (Cort *et al*., 2008; Stockdreher *et al*., 2012). The incubation of DsrC with sulfite led indeed to the formation of a sulfonate group at Cys111 *in vitro*. Since this reaction is reversible the sulfonate group could be reductively released as sulfite and then further oxidized to

sulfate (Bailey & Cole, 1959; Cecil & Wake, 1962). Interestingly, sulfite was not a suitable substrate for DsrEFH. Therefore, the requirements for binding a sulfonate group appear to be dif¬ferent from those needed for the formation of a persulfide and are present in DsrC, but not in DsrEFH.

Alc. vinosum encodes five *dsrC* homologues. While Alvin_1256 is part of the Dsr system, Alvin_0345 or Alvin_0732 might participate in the biosynthesis of 5-methylaminomethyl-2-thiouridine. Both proteins carry a cysteine in the penultimate position which is required for sulfur binding, Alvin_0345 even harbours the second cysteine in a distance of 10 amino acids. Alvin_0028 and Alvin_1508 on the other hand do not carry any of these cysteines. Hence, a function in sulfur metabolism is highly unlikely. Instead, a regulatory function for these proteins is imaginable. The globular part of DsrC proteins contains a helix-turn-helix motif, comparable to bacterial transcription regulator proteins (Cort *et al*., 2008). Grimm *et al.* showed that Alvin_1256 specifically binds to DNA sequences that contain the region upstream of *dsrA* (Grimm *et al*., 2010b). Hence, Alvin_0028 and Alvin_1508 might be involved in the regulation of the expression of other genes.

The results presented here allow assigning a function to DsrEFH in *Alc. vinosum* and most likely in other sulfur oxidizing prokaryotes using the Dsr system. The hypothesized sulfur transfer from DsrEFH to DsrC was verified *in vitro* using MADLI-TOF mass spectrometry and the 1,5-IAEDANS method. The collected data lead to the conclusion that DsrEFH is indeed a sulfurtransferase and conducts transfer of sulfur to DsrC *in vitro* and most likely *in vivo*. The reaction involved only the redox-active cysteines that are also essential for persulfide formation and interaction of DsrEFH and DsrC (Cort *et al*., 2008; Stockdreher *et al*., 2012). The transfer was directional which is evidenced by the incapacity of DsrC to transfer sulfur to DsrEFH. The transfer is in accordance with the observation for the corresponding Tus proteins from *E. coli* (Ikeuchi *et al*., 2006).

After incubation with persulfidic DsrEFH up to three sulfur atoms were bound to DsrC-Cys111. The question whether the formation of the polysulfide chain bound to DsrC-Cys111 is also occurring *in vivo* remains unsolved. A polysulfide chain is known from the Sud protein from *Wolinella succinogenes*: a chain of up to ten sulfur atoms built up on this periplasmic sulfurtransferase under experimental conditions (Klimmek *et al*., 1998). Another example for a protein binding polysulfide is the sulfide:quinone oxidoreductase from *Acidanus ambivales* in which a chain of three sulfur atoms bridges a pair of active site cysteine residues (Brito *et al*., 2009). If the polysulfide chain on DsrC indeed occured *in vivo*, sulfur atoms would have to be successively oxidized by DsrAB and released from the same. In the structure of DsrAB from *Dsv. vulgaris* (Oliveira *et al*., 2008) a channel for sulfite access to the active site was identified that is distinct from the large cavity that was originally proposed to serve as the access route for sulfite in DsrAB from the archaeal sulfate reducer *Archaeoglobus fulgidus* (Schiffer *et al*., 2008). The large cavity described for *Arg. fulgidus* DsrAB is almost completely occupied by the carboxy-terminal arm of DsrC in the structures where DsrAB co-

crystallized with DsrC (Oliveira *et al*., 2008, 2011; Hsieh *et al*., 2010). This was not the case for the *Archaeoglobus* protein (Schiffer *et al*., 2008). As an alternative, a second and narrower funnel was proposed as entrance for sulfite that is not blocked by DsrC and is also present in the other published DsrAB structures (Oliveira *et al*., 2011). If the same was true for *Alc. vinosum* DsrAB, sulfite molecules could in principle be successively released via this dedicated channel for sulfite. However, as explained above, sulfite is proposed to be released from sulfonated DsrC. A new catalytic cycle could then only start after reduction of this disulfide bond which would require the release of disulfidic DsrC from DsrAB. Taken together, it seems more reasonable that the chain of three sulfur atoms bound to DsrC is an artefact and is caused by the lack of sulfur-converting DsrAB that is present *in vivo*.

Beside the fact that DsrC and DsrAB are copurified along with other Dsr proteins from *Alc. vinosum* in a supercomplex (Dahl *et al.*, 2005) only little is known about the interaction between these two proteins. Nevertheless, the structures of DsrAB from the sulfate reducers *Dsv. vulgaris*, *Dsv. gigas* and *Dsm. norvegicum* provide insights into this topic. They show the carboxy-terminal flexible region of DsrC inserted into the cleft between DsrA and DsrB and thereby blocking the substrate channel for sulfite (Oliveira *et al*., 2008, Hsieh *et al*., 2010, Oliveira *et al*., 2011). DsrC from *Dsv. gigas* was observed bound to DsrAB in three different conformations. In the first conformation DsrC was covalently linked to the catalytic siroheme via the terminal cysteine. In the second one the linkage was broken and the cysteine was closer to the sulfite molecule, which was bound in the substrate pocket. The third conformation showed the flexible carboxy-terminus turned away from the siroheme and towards the other cysteine of DsrC. For *Dsv. vulgaris*, Oliveira and co-workers described a two-step mechanism for sulfite reduction. Sulfite is considered to be reduced to a S^0 intermediate consuming four electrons that are provided by an unknown donor. The sulfur atom is then transferred to the terminal cysteine DsrC-Cys104. Afterwards the flexible arm swings away from the catalytic site and comes in close proximity to DsrC-Cys93, which finally reduces the sulfur atom by forming a disulfide bond with DsrC-Cys104 (Oliveira *et al*., 2008). Though in *Alc. vinosum* DsrAB is supposed to work in the reverse direction, i.e. oxidizing sulfur to sulfite, the general arrangement of DsrC and DsrAB in the complex should be similar to the proteins in sulfate reducing bacteria. In the present work it was demonstrated that DsrC from *Alc. vinosum* accepts and binds sulfur that is delivered by DsrEFH to the terminal cysteine. This finding strongly supports the postulate that DsrC serves as substrate-binding protein for DsrAB in sulfur oxidizers. In its persulfidic state DsrC could bring sulfane sulfur in contact with the catalytic siroheme of DsrAB where it is then oxidized. Afterwards DsrC might dissociate from DsrAB in a sulfonated form. After releasing sulfite as terminal product of the Dsr cycle DsrC has to be regenerated.

The involvement of a sulfur transfer reaction in the Dsr mechanism resolves the inhibition of sulfur globule degradation by thiol-binding reagents such as iodoacetamide (Hurlbert, 1968). Transfer of sulfur atoms in the persulfidic state has the clear advantage of bypassing the toxicity of free sulfide. Though low concentrations of intracellular sulfide are tolerated to allow cysteine biosynthesis (Kessler, 2006), it seems reasonable that the constant and sudden flow of sulfur atoms from the sulfur globules to the cytoplasm under experimental conditions would severely affect the viability of individual cells. Redox-active thiol groups in proteins offer a sheltered environment for sulfane sulfur which averts the decomposition of the persulfides that can be observed in low molecular persulfides. Additionally, sulfur trafficking enables a controlled and specific transfer from protein to protein and optimizes substrate presentation (Kessler, 2006). The longevity of DsrEFH and DsrC persulfides was not monitored, but they were stable for at least 18 hours, which should ensure their physiological significance.

Neither DsrEFH nor DsrC showed thiosulfate or GSSH:cyanide sulfurtransferase activity. MALDI-TOF mass spectrometry verified that the proteins bound no sulfur to DsrE-Cys78 and DsrC-Cys111 after incubation with these substrates. This shows that DsrEFH and DsrC are not able to mobilize sulfane sulfur from thiosulfate and GSSH and probably other low molecular weight thiols by themselves. This incapacity agrees with the hypothesis that another sulfurtransferase is responsible for the introduction of sulfur into the Dsr system. DsrEFH and DsrC seem to act as shuttles that mediate the transfer of sulfur from a cytoplasmic donor to DsrAB where it is further oxidized. This assumption is supported by a study on the regulation of *dsr* genes during growth on sulfide using absolute quantitative real-time RT-PCR by Grimm *et al.* (Grimm *et al.*, 2010b). Relative to *dsrA* the transcription levels of *dsrEFH* and *dsrC* are significantly higher when *Alc. vinosum* is grown on sulfide. High copy numbers of DsrEFH and DsrC would guarantee a sufficient substrate supply for DsrAB and a turnover rate that is high enough for efficient sulfur oxdiation.

Heterodisulfide reductases in dissimilatory sulfur oxidation

Thiol-disulfide switches appear to be a common feature during the formation of sulfite in dissimilatory sulfur oxidation. The surveyed genomes revealed that the sulfur oxidizing prokaryotes are split into two groups. In the first group the Dsr proteins, including the heterodisulfide reductase homologous DsrK, are present while the second group contains the Hdr system. The latter is encoded by *hdrC1B1A1orf2hdrC2B2*. HdrABC is related to the soluble heterodisulfide HdrABC in methanogens without cytochromes (Hedderich *et al*. 2005). *orf2* is annotated as hypothetical protein and contains no conserved domains. Based on a transcriptomic study on aerobically grown *Atb. ferrooxidans* cells Quatrini and co-workers suggested that the HdrABC complex might operate in the direction reverse to that in methanogenic archaea and use the naturally existing proton gradient to oxidize disulfide

intermediates to sulfite. Unfortunately, significant information on the biochemistry of the Hdr complex is not yet available (Ossa *et al*., 2011; Liu *et al*, 2012). Given the nature of this enzyme the mechanism will probably involve a thiol-disulfide switch in proteins with redox-active cysteine residues, e.g. sulfurtransferases and sulfur carrier proteins. The mutual exclusion of the Dsr and Hdr proteins among the sulfur oxidizers is noteworthy and strongly supports the theory that Hdr indeed operates in the oxidative direction. Apart from *Tav. nitratireducens*, *Tav. sulfidophilus* and *Sfc. denitrificans* either the Dsr or the Hdr proteins are present. The *Hyphomicrobiaceae* and the *Ectothiorhodospiraceae* are the exception to the perception that only one system was acquired in each of the sulfur oxidizing families. In this regard *Chp. thalassium* is notable, too, as this green sulfur bacterium carries a truncated version of the *hdr* operon instead of the *dsr* genes like all other Chlorobi strains. This early branching member of the Chlorobi encodes only rudimentary parts of the systems that are required for the oxidative sulfur metabolism among which *dsrE2* (YP_001995564) is located in a small sulfur island. *fccAB* (YP_001995560/1), *soxYZ* (YP_001995563/2) and *dsrC* (YP_001995565) were also found in this sulfur island. It is therefore not surprising that *Chp. thalassium* reportedly degrades sulfur globules slowly (Gibson *et al*., 1984).

The transmembrane complex DsrMKJOP is currently supposed to provide electrons for the regeneration of thiolic DsrC (Grein *et al*., 2010a). The subunits DsrMK are related to HdrDE which is responsible for heterodisulfide reduction in *Methanosarcina barkeri*. The catalytic subunit HdrD corresponds to DsrK (Heiden *et al*, 1994; Künkel *et al*, 1997; Grein *et al*., 2010a, 2013). In methanogenic archaea Hdr complexes catalyse the reduction of the heterodisulfide CoM-S-S-CoB that is formed in the terminal step of methanogenesis (Hedderich *et al*., 2005; Thauer *et al*., 2008). While the disulfide consisting of coenzyme M and coenzyme B is restricted to the methanogenic archaea Hdr-like proteins are widely spread among prokaryotes hinting that energy conservation coupled to disulfide/thiol conversions might be a more common feature (Hedderich *et al*., 1998; Grein *et al*., 2013). Studies on the DsrMKJOP complex in sulfate reducing and sulfur oxidizing bacteria concluded that in both cases electrons are transferred from the periplasm into the cytoplasm although DsrAB in sulfur oxidizers and sulfate reducers operate in opposite directions. In both cases DsrK is supposed to be involved in thiol/disulfide chemistry and use DsrC in its oxidized state as "heterodisulfide" substrate (Pires *et al*., 2006; Cort et al., 2008; Grein *et al*., 2010a). For *Alc. vinosum* the interaction between DsrK and DsrC has been shown (Grein *et al*., 2010a).

Analysis of the *in vitro* interaction between DsrEFH and DsrC

The interaction between DsrEFH and DsrC and its dependency on the cysteine residues DsrE-Cys78 and DsrC-Cys111 was first shown via band shift assays in native polyacrylamide gels. After incubation with each other two additional bands appeared that migrated between DsrEFH and DsrC alone (Cort *et al*., 2008; Dahl *et al*., 2008). Data collected in the present study confirmed that both

bands contained four proteins, which is consistent with the presence of DsrC and the DsrEFH subunits. BN-PAGE revealed that the faster migrating complex contained DsrEFH and DsrC in a stoichiometric ratio of 1:1 whereas the slower migrating band exhibited an 1:2 ratio demonstrating that both DsrE subunits can simultaneously bind a DsrC molecule *in vitro*. Studies on the interaction that used Surface Plasmon Resonance further illustrated that the complex is very stable. Once the complex was formed no dissociation of the proteins was detected (Stockdreher *et al*., 2012).

Distribution of *rhd-tusA-dsrE2*

In the second part of this work several lines of evidence were combined to verify a possible function for Rhd_2599, TusA and DsrE2 in the dissimilatory sulfur metabolism of *Alc. vinosum*. First, genome-sequenced sulfur oxidizers were surveyed for the presence of *tusA* and the flanking genes, *rhd* and *dsrE2*. The cluster as a whole and the truncated versions, *tusA-dsrE2* and *rhd-tusA*, are indeed highly abundant within the sulfur oxidizing community, conserved even in complete families. They are conspicuously co-localized with genes encoding established or very probable major components of the sulfur oxidation pathways which strongly supports a role of the three gene products in sulfur oxidation. Besides *dsr* and *hdr* genes this includes genes for the sulfite oxidizing complex PSRLC3 (e.g. Chlorobi) and the flavocytochrome c sulfide dehydrogenase *fccAB* (e.g. *Akl. ehrlichii*). Sequence analysis of the individual proteins showed that the number of cysteines in DsrE2 and the motif in TusA are mostly consistent among the sulfur oxidizers and clearly separate them from TusA and DsrE2 proteins that are encoded in organisms without sulfur driven energy metabolism (discussed in detail below).

Among the phototrophic sulfur oxidizers the green sulfur bacteria were especially intriguing. In some of the Chlorobi, e.g. *Chl. luteolum*, proteins for sulfur oxidation and most probable for sulfite oxidation are encoded in the same genomic region and the *rtd* sequence is located directly between these gene clusters. Though this model organisation was not found in all Chlorobi and *dsr* and *psrlc3* were detected in completely different regions in some strains, each of the operons was still clustered with *rhd*, *tusA* or *dsrE2*. This indicates a strong genetic link between these three clusters rather than a random distribution of genes.

The gene encoded upstream of *rhd_2599*, Alvin_2598, is another clue towards a functional relation between the rhodanese, TusA and DsrE2 with proteins of the Dsr complex. Homologues of Alvin_2598 have just recently been acknowledged as members of the Dsr system in the Chlorobi. Here, the genes have been designated *dsrV* and *dsrW*, which are located directly downstream of *dsrP* (Holkenbrink *et al*, 2011). The gene products are annotated as sirohydrochlorin ferrochelatase and share 26-44% identity with the multifunctional siroheme synthase CysG from *E. coli*. CysG is soley responsible for the synthesis of siroheme from uroporphyrinogen III (Warren *et al*,, 1990; Spencer *et*

al., 2003). The amidated derivate of siroheme, siroamide, is the cofactor for DsrAB. It is therefore feasible that the enzyme in charge for siroheme biosynthesis is encoded within the *dsr* operon. Especially since the protein, that performs the amidation reaction is DsrN (Lübbe *et al*., 2006). Homologues of Alvin_2598 are conserved in all DsrAB containing *Chromatiaceae* and located directly upstream of the *rhd_2599* homologue in the reverse direction of transcription. It should be noted that the gene for siroheme synthase (WP_020504601) in *Lpc. purpurea* is encoded directly adjacent to a rhodanese gene (WP_020504602); both are located apart from *tusA* and *dsrE2*. If Alvin_2598 and its homologues were to be acknowledged as *dsrV/dsrW* this would link Alvin_2598-2601 directly to the *dsr* operon in the *Chromatiaceae*.

The lack of *rtd* in genome-sequenced Chlorobi species coincides with an impaired sulfur metabolism. *Chl. ferrooxidans* is incapable to live on reduced sulfur compounds as sole electron source and has lost most of the sulfur related genes probably due to adapting its energy metabolism to the oxidation of ferrous iron (Heising *et al*., 1999; Gregersen *et al*., 2011). Sulfide is a suitable substrate for C*hl. parvum* and it is oxidized to sulfur, but C*hl. parvum* is unable to convert this intermediate to sulfate which has been attributed to its *dsrEFH* deficiency (Kelly *et al*., 2008).

Among bacteria without a sulfur driven metabolism the gene cluster was found in members of the Bacilli, e.g. *Bacillus thuringiensis* and *Paenibacillus mucilaginosus*. The genes *tusA* and *dsrE2* are also present in *Bacillus subtilis* and are known as *yrkI* and *yrkE*, respectively. In all these strains the genes are clustered with genes for TusA/rhodanese (*yrkF*) and rhodanese/β-lactamase (*yrkH*) fusion proteins and *yrkJ* which encodes a TauE-like protein. In *Cupriavidus necator* TauE is a membrane-bound protein that supposedly transports sulfite from the cytoplasm to the periplasm as a step in the dissimilation of sulfoaliphatics (Weinitschke *et al*., 2007). It is feasible that such a high density of genes encoding for sulfur mobilizing and transferring proteins in the Bacillus strains indicates the conversion of sulfur containing molecules and the transport of the respective products across the cytoplasmic membrane. The sequence analysis of individual proteins showed that in these cases the number of conserved cysteines in DsrE2 and the TusA motif vary from that in sulfur oxidizers.

Transcription of *rhd_2599, tusA* and *dsrE2*

The transcription of *rhd_2599*, *tusA* and *dsrE2* in the presence of sulfide, sulfur and thiosulfate was analysed via relative qRT-PCR. The transcription responded to the switch of electron donors from malate to a reduced sulfur compound with elevated levels further hinting the involvement in the oxidative sulfur metabolism. While elevated mRNA levels for *tusA* and *dsrE2* had already been detected in the transcriptome study of *Alc. vinosum* (Weissgerber *et al*., 2013) *rhd_2599* was indifferent towards sulfur oxidizing conditions. Relative qRT-PCR analysis, however, showed that the gene was increasingly expressed when *Alc. vinosum* was grown on sulfide, thiosulfate and elemental

sulfur and thereby matched the results for *tusA* and *dsrE2*. In a macroarray study of *Atb. ferrooxidans* the homologous rhodanese was also found to be present in higher copy numbers under sulfur oxidizing conditions relative to iron oxidation (Acosta *et al*., 2005; the rhodanese was designated P11). In transcriptomic studies of *Atb. ferrooxidans* on the other hand *p11* was inconspicuous (Quatrini *et al*., 2009).

The highest mRNA levels were found in cells grown on sulfide and external sulfur. The lower levels triggered by thiosulfate may be explained by a comparison of the concentrations of internal sulfur that are yielded with different sulfur sources. The periplasmic Sox system in *Alc. vinosum* hydrolytically releases the sulfone sulfur of thiosulfate directly as sulfate. Only sulfane sulfur is fed into sulfur globules, which leads to low concentrations of intermediately stored sulfur and immediately emitted sulfate. Contrarily, sulfate that originates from the substrates sulfide and external sulfur is completely processed by DsrAB. The oxidation of volatile sulfide is a very rapid process relative to the oxidation of thiosulfate. While thiosulfate can be detected in the medium for over 10 hours sulfide is completely metabolized within a maximum of two hours and the formation of sulfur globules can be observed within minutes (Franz *et al*., 2009). Sulfate is first detected after sulfide is completely oxidized; this leads to high concentrations of transiently stored sulfur. External sulfur probably also results in high concentrations of sulfur globules. Since the separation of *Alc. vinosum* cells from insoluble sulfur so far proofed to be impossible, no exact information on the internal sulfur concentration is available. But judging from the presence of sulfur globules, which were reported after about three hours and significant amounts of sulfate hours later, it can be assumed that the concentration of sulfur globules is rather high. Taken together, these findings correlate the increased expression of *rhd_2599*, *tusA* and *dsrE2* on sulfide and elemental sulfur with high concentrations of intermediately stored sulfur.

Insights from *Alc. vinosum* Δ*rtd*::ΩKm and the *Alc. vinosum* transcriptome and proteome

The generation of a *rhd_2599-tusA-dsrE2* deficient *Alc. vinosum* strain resulted in a genetically instable mutant that was not maintainable over a time. The mutant strain was auxotroph for reduced sulfur compounds since no colonies were detected on solid medium, that contained the organic substrate malate, but was lacking a sulfur source. Supplementation with sulfide on the other side resulted in a massive accumulation of sulfur globules. The sulfur globule degradation negative phenotype is characteristic for the deletion of individual *dsr* genes (Lübbe *et al*., 2006; Sander *et al*., 2006; Dahl *et al*., 2008) and adds further support for the hypothesis that Rhd_2599, TusA and DsrE2 are involved in oxidation of intermediately stored sulfur. However, since the strain was not available for characterization or complementation this could not be verified beyond doubt. In fact, it is likely that the function of at least TusA is not exclusively dedicated to sulfur metabolism. The failed attempt to delete *tusA* was a first hint that TusA might be critical for the viability of *Alc. vinosum* (Dobler, 2008). In *E. coli* TusA was shown to be involved in the maintenance of the stability of the stress-

induced sigma factor RpoS, formation of the FtsZ-ring, the synthesis of 2-thiouridine and the molybdenum cofactor. Additionally, TusA affects the redox homeostasis, the FeS cluster biosynthesis via the ISC system and the expression of genes that are regulated by the transcriptional activator FNR (Yamashino *et al*., 1998; Ishii *et al*., 2000; Ikeuchi *et al*., 2006; Nakayashiki *et al*., 2013; Dahl, J. U. *et al*., 2013). The biosynthesis of FeS clusters and the molybdenum cofactor were also negatively influenced in a *tusA* (PA1006) deficient strain of *Pseudomonas aeruginosa.* The absence of PA1006 further affected the expression of nitrate-responsive, quorum sensing and iron-regulated genes. Additionally, differences in biofilm growth, virulence and the Type IV secretion system were observed (Filiatrault *et al*., 2013; Tombline *et al*., 2013). For the majority of the phenomena it is unclear whether the TusA proteins have a direct effect or the absence of the protein causes downstream effects.

The biosynthesis of 2-thiouridine in *E. coli* is so far the only process for which the direct and crucial involvement of TusA has been shown (Ikeuchi *et al*., 2006). Regarding a possible function for TusA in 2-thiouridine biosynthesis in *Alc. vinosum* it should be stressed that 5-methyl-2-thioruridine tRNA derivatives are universally present (Suzuki *et al*., 2005); however, the Tus proteins are not conserved in all domains of life or even Bacteria. This implies that their role is either exerted by completely different sulfurtransferases or the mechanism established for *E. coli* is modified in organisms lacking (some of) the Tus proteins (Ikeuchi *et al*., 2006). In *Alc. vinosum* only one copy for *tusA* and the *tusBCD* homologous *dsrEFH* are encoded whereas five homologues of *tusE/dsrC* are present. If DsrEFH participated in the biosynthesis of 5-methylaminomethyl-2-thiouridine the deletion of *dsrE* probably would have led to increased doubling times as reported for the tusBCD negative *E. coli* strain (Ikeuchi *et al*., 2006). Yet, this was not the case; under photoorganoheterotrophic conditions the growth of *Alc. vinosum* Δ*dsrE* was not different from the wild type (Dahl *et al*., 2008). Furthermore, the interaction of IscS and TusA in *Alc. vinosum* has not been shown yet. *Alc. vinosum* TusA is missing three amino acids that are relevant for the interaction with IscS in the *E. coli* protein, namely Arg31, Phe58 and Glu 21 (numbering according to *E. coli* TusA). While the substitution of the latter strongly reduced the interaction between TusA and IscS, the former two were absolutely essential for the interaction (Shi *et al*., 2010). In *Alc. vinosum* these residues are substituted with amino acids of a different chemical nature (Asn27, Gln54 and Leu17; *Alc. vinosum* counting) and might have an impact on the IscS/TusA interaction. Taken together the biosynthesis of 5-methylaminomethyl-2-thiouridine appears to be achieved by either a direct sulfur transfer from IscS to MnmA, as originally suggested for *E. coli* (Kambampati & Lauhon, 2003), or in conjunction with a cysteine-containing DsrC/TusE homologue (Alvin_0732 and Alvin_0345). The lack of *thiI, iscX, fdhD* and *cyaY*, interaction partners of IscS in *E. coli*, is consistent with modified pathways that are based on the activity of IscS in *Alc. vinosum*.

A role for TusA in molybdopterin biosynthesis and an impact of the protein on iron-sulfur cluster formation as described for *E. coli* (Dahl, J. U. *et al*., 2013) is possible in *Alc. vinosum* and would probably result in a strong phenotype since a balanced synthesis of iron-sulfur clusters is expected to be of essential importance for a photolithoautotrophic organism. The biosynthesis of high potential iron-sulfur protein (HiPIP), the primary electron donor to the reaction centre in *Alc. vinosum*, may serve as an example. It should be noted that the impact of the *tusA* deletion on the synthesis of the molybdenum cofactor in *E. coli* is stronger under anaerobic conditions and that it is currently not clear how TusA exactly contributes in this pathway (Dahl, J. U. *et al*., 2013).

To address the possibility that Rhd_2599, TusA and DsrE2 are involved in the biosynthesis of sulfur containing cofactors their transcriptional profiles were compared with those of genes related to the biosynthetic pathways. The transcription of the latter was mainly indifferent under photolithoautotrophic conditions indicating a constitutive transcription. Merely the gene expression of the *suf* genes increased during growth on elemental sulfur. This directly contradicts the results for *tusA* and *dsrE2*; enhanced mRNA levels have already been reported with sulfide and thiosulfate as electron donor. Relative qRT-PCR confirmed this and also revealed a higher transcription rate when *Alc. vinosum* was exposed to elemental sulfur as well as well as raised mRNA levels for *rhd_2599* under all tested conditions. But unlike the conflicting profiles of the *rtd* genes and the biosynthesis operons the transcription of the *rtd* genes matches that of the *dsr* operon. Further hints towards the function of TusA, Rhd_2599 and DsrE2 were gathered by analysing proteomic data for *Alc. vinosum* (Weissgerber *et al*., 2014, revision submitted). Here, TusA was found to be among the most abundant proteins in cells grown on reduced sulfur compounds. As an example, Figure IV.1 shows the situation for *Alc. vinosum* grown on elemental sulfur.

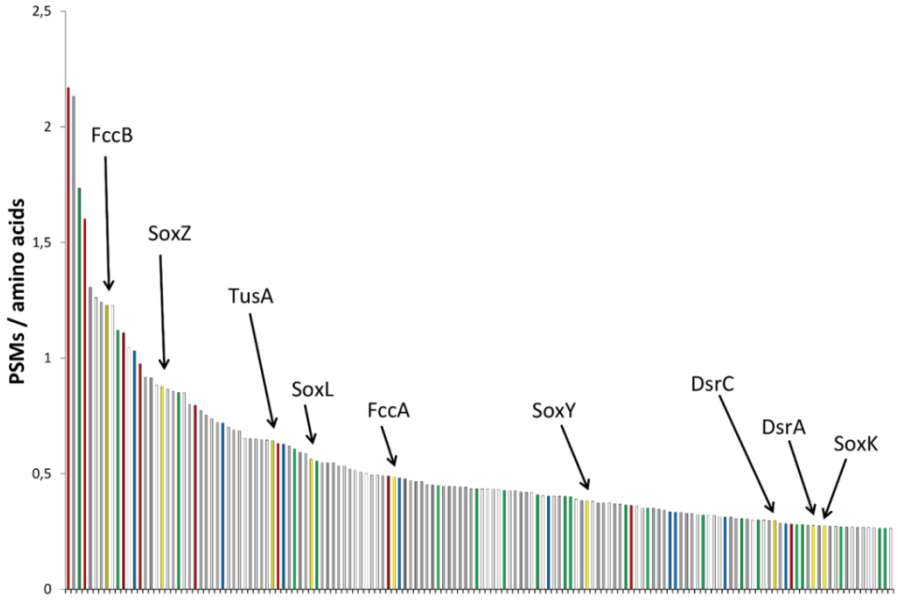

Figure IV.1. The 150 most abundant proteins in *Alc. vinosum* grown on elemental sulfur as electron donor. Proteins are ranked according to the ratio of their peptide spectrum matches and the number of amino acids; thus avoiding an underestimation of small and overestimation of large proteins. Proteins belonging to five functional groups were distinguished as follows; energy conservation (blue); C-metabolism (green); gene expression, replication, chaperones (grey); photosynthesis (red); sulfur metabolism (yellow). Data kindly provided by Thomas Weissgerber.

In accordance with the photolithoautotrophic growth mode, primarily enzymes involved in carbon dioxide fixation (RubisCo, phosphoribulokinase), photosynthesis (light harvesting complexes) and energy conservation (subunits of ATP synthase) were found among the 150 most abundant proteins. It is furthermore not surprising that enzymes of oxidative sulfur metabolism were found among the highest ranking proteins. These include both subunits of the sulfide dehydrogenase flavocytochrome *c* (FccAB), DsrA, DsrC and components of the periplasmic thiosulfate oxidizing Sox system (SoxL, SoxK and SoxYZ). TusA outranked most of these central players. Interestingly, proteins for the biosynthesis of sulfur containing cofactors, e.g. IscS and MoeB, were not present under the "Top 150".

Rhd_2599

The disruption of the *rhd_2599* reading frame did not result in a phenotype that differed from the wild type. This leads to the conclusions that Rhd_2599 is either not a key player in dissimilatory sulfur metabolism or the loss of Rhd_2599 was functionally compensated by another rhodanese. To approach this issue the deletion of other rhodanese genes was reviewed. GlpE and PspE are expamples for single-domain rhodaneses in *E. coli*. GlpE is part of the sn-glycerol 3-phosphate (*glp*) operon and PspE is part of the phage-shock protein (*psp*) operon. Though both enzymes were characterized thoroughly their physiological role is still unknown. The deletion of *glpE* and *pspE* resulted in a phenotype comparable to the wild-type, just like witnessed for the deletion of *rhd_2599* (Ray *et al*., 2000; Cheng *et al*, 2008). In fact, only for two rhodanese-like proteins a possible *in vivo* function was

established by the evaluation of the phenotype upon inactivation of the respective gene. The disruption of a rhodanese in *Saccharopolyspora erythraea* caused cysteine auxotrophy and the deletion of RhdA in *Azt. vinelandii* led to a heightened sensitivity towards oxidative events. A function as redox switch for RhdA was therefore concluded (Donadio *et al*., 1990; Cereda *et al*., 2009). Kessler noted the redundancy of rhodanese and rhodanese-domain containing proteins in an organism would hinder establishing the *in vivo* function by characterising a phenotype upon inactivation of the rhodanese gene (Kessler, 2006). For example, *E. coli* encodes nine proteins that contain a rhodanese domain; in *Alc. vinosum* eight proteins carry the domain. Besides Rhd_2599 for three other proteins a cytoplasmic location has been predicted: Alvin_0866, Alvin_0868 and Alvin_1587. Alvin_0866 and Alvin_1587 exhibited elevated transcription levels under sulfur-oxidizing conditions and would thus be prime candidates for replacing Rhd_2599 (Weissgerber *et al*., 2013). In this context the functional replacement of Rhd_2599 in *Alc. vinosum rhd_2599*::ΩSm appears to be likely. The fact that the rhodanese is conserved as part of the *tusA* gene cluster in the *Hydrogenophilaceae*, *Acidithiobacillaceae*, *Chromatiaceae*, Chlorobi and some species of the *Ectothiorhodospiraceae*, but not in all the sulfur oxidizers on the other hand challenges the relevance of Rhd_2599 *in vivo* and would in turn raise again the question how sulfur is initially introduced to the sulfur oxidation machinery. The successive deletion of all rhodanese genes would aid to elucidate this question.

Yet, the opposing phenotypes of *Alc. vinosum rhd_2599*::ΩSm and *Alc. vinosum rtd*::ΩKm confirmed the two promoter sites that were predicted for the *rtd* gene cluster: the first upstream of *rhd_2599*, the second upstream of *tusA*. Otherwise the insertion of the Ω-cassette in the rhodanese gene would have led to an equally severe phenotype. The extra promoter site for *tusA* supports the idea that the transcription of *tusA* is not only induced in response to sulfur oxidation, but is also regulated by signals for biosynthetic pathways such as the biosynthesis of the molybdenum cofactor. Under sulfur oxidizing conditions *rhd_2599*, *tusA* and *dsrE2* were co-transcribed. PCR with cDNA obtained from mRNA of sulfide grown cells yielded amplicons for *rhd_2599*/*tusA* and *tusA*/*dsrE2*. Yet, the signal for the *tusA*/*dsrE2* fragment was more pronounced than the *rhd_2599/tusA* fragment. This further supports the assumption that the promoter upstream of *tusA* might be induced by an additional signal. *dsrC* is also regulated by a secondary promoter while residing in the *dsr* operon. The gene is constitutively expressed on a basal level and is supposed to have an extra, regulatory function (Grimm *et al*., 2010b).

Since the deletion of *rhd_2599* had no impact on the viability or dissimilatory sulfur metabolism the overall thiosulfate:cyandide sulfurtransferase activity of *Alc. vinosum* wild type and *Alc. vinosum rhd_2599::*ΩSm grown on either malate or sulfide were compared. To assess the relevance of Rhd_2599 for the degradation of sulfur globules sulfide grown cells were harvested eight hours after application of sulfide to ensure that the metabolism was fully adapted to sulfur oxidation. Overall, the

rhodanese activity was increased in wild type cells that were grown on sulfide relative to the activity in malate grown cells. The activity in the membrane fraction was almost insignificant on malate, but increased 6-fold under sulfur oxidizing conditions. Thus, rhodanese proteins generally participate in the heteroorganotrophic growth mode of *Alc. vinosum*, even more so under photolithoautotrophic conditions as evidenced by the increased enzyme activity in sulfide grown cells. The reduced activity in the membrane fraction compared to the soluble fraction is attributed to the uneven distribution of rhodaneses within in the cell; only one enzyme is predicted to be membrane bound (Alvin_3028) while seven rhodanese-domain containing proteins are predicted to be soluble. The absence of Rhd_2599 was not relevant for the rhodanese activity during growth on malate as no significant difference to the wild type was detected. But under sulfur oxidizing conditions the activity in the soluble fraction of the mutant strain reached only 58% of the wild type activity and the increase in activity compared to malate grown cells was below 10%. The activity in the membrane fraction on the other hand was ~6-fold enhanced which equates the increase seen in wild type cells. Tough it is not clear which other rhodaneses are active under the applied conditions the data indicate that Rhd_2599 probably accounts for a major part of the overall rhodanese activity during the degradation of sulfur globules.

The active site motif Cys-Arg-Ser-Gly-Ala-Arg is conserved in all Rhd_2599 homologues in the *Chromatiaceae* and is conform with the thiosulfate:cyanide sulfurtransferase motif Cys-Arg-X-Gly-X-[Arg/Thr] (Bordo & Bork, 2002). Accordingly, thiosulfate:cyanide sulfurtransferase activity was shown by Sturm (Sturm, 2009). Since thiosulfate is oxidized in the periplasm by the Sox proteins it is an unlikely substrate for cytoplasmic rhodaneses (Hensen *et al*., 2006). A possible substrate for Rhd_2599 is persulfidic glutathione amide (GASSH), which is currently supposed to be the organic carrier molecule for sulfur atoms from sulfur globules to the cytoplasm (Cort *et al*., 2008). Bartsch and co-workers demonstrated that some members of the *Chromatiaceae* produce this amidated derivate instead of glutathione. As shown for *Marichromatium gracile* glutathione amide is produced during heteroorganotrophic growth, but when grown on sulfide it is converted to the corresponding persulfide (Bartsch *et al*., 1996). Additionally, glutathione amide is a candidate for the organic residue that terminates the polysulfanes inside the sulfur globules. Taken together this led to the hypothesis that persulfurated glutathione amide might be the carrier molecule that transfers sulfane sulfur from the periplasm to the cytoplasm. Under this aspect it is intriguing that Rhd_2599 could use persulfidic glutathione as substrate. However, it should be noted that the data of the *Alc. vinosum* metabolome do not indicate the presence of glutathione amide or differences in the concentration of glutathione in cells grown on malate or reduced sulfur compounds (Weissgerber *et al*., 2014, submitted).

In vitro persulfuration of Rhd_2599 with thiosulfate or GSSH resulted in an additional mass of 32 Da which corresponds to exactly one sulfur atom. This is in good accordance with the characteristic ping-

pong mechanism of rhodaneses (Vazquez *et al*., 1987). It also indicates that the binding of three sulfur atoms after incubation with sulfide is probably an artefact (Sturm, 2009). Rhd_2599 contains two cysteine residues within close distance (Cys64-X_9-Cys74) that are conserved in homologous proteins in the Chromatiales. Cys64 is located in the active site and only this cysteine was identified as the sulfur mobilizing and binding site while the partly conserved Cys74 was not required *in vitro*. Nonetheless, the conservation of two cysteine residues within close vicinity appears to be a common theme among cytoplasmic sulfur carriers and sulfurtransferases in *Alc. vinosum*, apart from Rhd_2599 this pattern was also detected in DsrE2 and DsrC. For DsrC the formation of an intramolecular disulfide bond for reductive release of the sulfonate group would be a part of the protein's *in vivo* cycle. The significance of putative intramolecular disulfide bonds in Rhd_2599 and DsrE2 are elusive at this point. It is, however, tempting to speculate that the oxidation of Cys64 and Cys74 is the reason why Rhd_2599 was not labelled with 1,5-IAEDANS. Yet, this assumption is unlikely. Rhd_2599 was reduced by DTT prior to the 1,5-IAEDANS treatment, so a potential disulfide bond should have been reduced to the thiolic equivalents. Furthermore, the reagent could not bind to proteins carrying only one cysteine. Since the formation of dimers was not observed it can be concluded that both cysteine residues are buried inside the protein rather than surface exposed and are thereby not available for 1,5-IAEDANS labelling.

TusA

In this study TusA was evalutated as a possible interaction partner and sulfur donor for DsrEFH in analogy to the sulfur relay system that was established for 5-methylaminomethyl-2-thiouridine biosynthesis in *E.coli*. Ikeuchi *et al*. detected *in vitro* sulfur transfer from TusA to TusBCD, but could not show the direct interaction between the proteins via analytical gel filtration (Ikeuchi *et al*., 2006). Surface plasmon resonance also yielded negative results (Numata *et al*., 2006). The SRP results of DsrEFH with *Alc. vinosum* TusA confirmed this finding. With DsrEFH immobilized and covalently bound to the chip and TusA as analyte interaction was not observed. Band-shift assays on the other hand showed that DsrEFH and TusA affect each other's migration in native PAGE. Two extra bands appeared in the gel upon mutual incubation. To gain this effect the presence of TusA-Cys15 and DsrE-Cys78 is required (Stockdreher, 2009). This pattern has a striking resemblance with the pattern that occurs as the consequence of the complex formation of DsrEFH and DsrC and strongly indicates interaction between TusA and DsrEFH. The assay was also applicable to the homologous *E. coli* proteins. The incubation of TusBCD with *E. coli* TusA resulted in two extra bands as well. In the next step, proteins from *E. coli* and *Alc. vinosum* were mixed to check whether the homologous proteins can replace each other *in vitro*. The incubation of TusBCD with DsrC or *Alc. vinosum* TusA led indeed to a modified migration pattern. Though only one additional band was identified, the proteins clearly interacted. Contrarily to this finding, DsrEFH and *E. coli* TusA did not interact with each other

under the tested conditions. Apparently, the two TusA proteins behave differently *in vitro* and this could also be the case *in vivo*.

The common motif for TusA-like proteins is Cys-Pro-X-Pro. In *E. coli* the "X" position is inhabited by acidic glutamic acid and the residue is vital for its function. Substitution with an alanine residue led to a significantly reduced interaction with IscS *in vitro* (Shi *et al*., 2010). The physiological effect was shown in the *E. coli sirA1* mutant. Here, TusA contains a Glu-Lys replacement in the "X" position which resulted in filamentation of the cells (Katoh *et al*., 2000). This is the same phenotype that was reported for the deletion of the complete gene (Yamashino *et al*., 1998; Ishii *et al*., 2000). YeeD and YedF are homologues of TusA that present in *E. coli*. Again, the glutamic acid residue in the X position in the motif is substituted; both proteins harbour hydrophobic aromatic amino acids: Cys-Pro-Phe-Pro and Cys-Pro-Tyr-Pro, respectively. Neither YeeD nor YedF were able to replace TusA in a *tusA* deficient *E. coli* mutant (Dahl, J. U. *et al*., 2013).

The NMR structure of *E. coli* TusA showed that Glu21 is directly involved in the stabilization of the protein structure by forming a hydrogen bond with Arg18. Substitution of this residue might lead to conformational changes which in turn could affect the *in vivo* function. Not one of the TusA proteins listed in Table III.1 carries the Cys-Pro-Glu-Pro motif. The Cys-Pro-Leu-Pro and Cys-Pro-Gly-Pro motifs, however, are highly conserved in bacteria and archaea, respectively, and indicate a function that is common for all these TusA proteins which might not be conform to that of *E.coli* TusA. *tusA* genes are widespread and often numerous copies are conserved in the genome of a single organism; e.g. *Hydrogenobaculum* sp. HO contains four *tusA* homologues. A Φ-Blast search revealed that none of the sulfur oxidizers listed in Table III.1 encode a single TusA protein that contains the *E. coli* motif. The *Sulfolobaceae* are the only exception. Within in the *Chromatiaceae* the *E. coli* motif was found only in *Rheinheimera* strains which lack both, sulfur oxidizing systems and DsrE2. Taken together this suggests that the amino acid in the "X" position could be the deciding element in regard to potential interaction partners and thereby regulate the *in vivo* function of TusA.

In this context the aforementioned study on *Psm. aeruginosa* is of interest to demonstrate that TusA in *Alc. vinosum* could indeed be involved in the biosynthesis of sulfur containing cofactors. Apart from PA1006 which carries the Cys-Pro-Leu-Pro motif a second TusA is also encoded in *Psm. aeruginosa* and it harbours the *E. coli* motif (PA1564). Nonetheless, the deletion of PA1006 caused a severe phenotype including disturbances in the FeS and molybdenum cofactor biosynthesis (Filitrault *et al*., 2013; Tombline *et al*., 2013). Thus, PA1564 could not compensate the loss of PA1006. The most likely function for PA1564 is the biosynthesis of 5-methylaminomethyl-2-thiouridine as TusBCDE are also encoded (PA2605-08).

DsrE2

DsrE2 is closely associated with TusA homologues that carry sulfur oxidizer motif. As mentioned above DsrE2 is absent in the *Rheinheimera* strains that contain exclusively TusA proteins with the *E. coli* motif. In fact, this pattern applies to all γ-Proteobacteria. Apart from the sulfur oxidizers listed in Table III.1 individual *dsrE2* genes are only conserved in an uncultured SUP05 cluster bacterium (Taxonomy ID 655186) which is a member of the *Oceanospirillaceae*. Here, two *dsrE2* homologues are present (EEZ79954 and EEZ79773) and in both gene products cysteines corresponding to Cys110 and Cys120 are present. Genes for *tusA* were found directly adjacent (EEZ79953 and EEZ79772). A truncated *dsr* operon was detected as well (*dsrLABEFHCMK*; EEZ79530-39), again underlining the strong connection between TusA/DsrE2 and the Dsr proteins.

Alc. vinosum DsrE2 was successfully overproduced and purified for the first time from the membrane fraction of *E. coli* C41(DE3) and C43(DE2) via the amino-terminal Strep-Tag. The carboxy-terminus, especially Cys120, is vital for DsrE2 as shown by the failed attempts to purify DsrE2 either fused to a carboxy-terminal His-tag or carrying the serine substitution in position 120.

Site-directed mutagenesis of Cys110 yielded a protein amount that was reduced 5-fold relative to wild type DsrE2. Given that a second cysteine is conserved in close distance to the ubiquitously present Cys120 (Cys-X_9-Cys in bacteria and Cys-X_7-Cys in archaea) in almost all analysed sulfur oxidizers (in DsrE2 proteins from α-Proteobacteria soley Cys120 is present) led to the conclusion that these cysteines might be involved in the formation of an intramolecular disulfide bond, though disulfide bonds in cytoplasmic proteins are rare (Ritz and Beckwith, 2001). However, this could not be verified via non-reducing SDS-PAGE. Under the tested conditions the observed disulfide bond involved the least conserved cysteine residue, Cys156. It is possible that air exposure alone did not suffice to induce the oxidation of Cys110 and Cys120. A recent study on DsrC from *Dsv. vulgaris* required the presence of L-arginine to generate a disulfide bond between the conserved cysteine residues Cys93 and Cys104 at the carboxy-terminus (Venceslau *et al.*, 2013). The mutant protein lacking Cys156 displayed an intriguing migration pattern when analysed with 1,5-IAEDANS. Here, DsrE2-Cys156Ser migrated in a double band; thus, indicating the formation of a disulfide bond between the remaining cysteine residues. However, the double band was only visible under UV-light. The formation of oligomeric DsrE2 under was not detected.

DsrE2 is able to bind sulfur *in vitro*. Incubation with sulfide significantly reduced the level of fluorescence compared to DsrE2 in its thiolic state. Yet, DsrE2 did not accept sulfur from persulfidic Rhd_2599, TusA, DsrEFH and DsrC. The question whether Triton X-100 interfered with the reaction or the transfer reaction *per se* does not exist cannot be satisfactorily addressed at this point.

Though it was not proven experimentally, Cys120 most likely provides the redox active thiol group to which sulfane sulfur was bound. Neither the substitution of Cys110 nor that of Cys156 impaired the sulfur binding capacity of DsrE2. Moreover, Cys120 aligns with the sulfur binding cysteine, Cys78, in DsrE and TusD. The involvement of Cys110 and Cys156, however, cannot be excluded. DsrE2 could not be tested as sulfur donor since Triton X-100 interfered with dialysis obligatory for the 1,5-IAEDANS method and prevented the detection of proteins using MALDI-TOF mass spectrometry.

DsrE2 was tested as thiosulfate/GSSH:cyanide sulfurtransferase activity negative. Nonetheless, the capacity of DsrE2 to mobilize sulfane sulfur and a possible transfer to TusA should be further investigated. So far, no evidence exists that TusA itself can mobilize sulfur from a low molecular weight thiol. Given that TusA is the direct sulfur donor for the Hdr and Dsr systems this raises the question how sulfur is transferred from an organic carrier molecule to TusA in organisms that lack the rhodanese. A possible answer is that DsrE2 fills this role and feeds sulfane sulfur to TusA via a mechanism that differs from the one established for rhodaneses. This might be another explanation to why the disruption of *rhd_2599* had no effect on the degradation of internally stored sulfur. Based on their structures members of the DsrE/DsrF/DsrH family (DsrEFH from *Alc. vinosum*, YchN and TusBCD from *E. coli*, MTH1491 from *Mtb. thermoautotrophicum* and Tm0979 from *Thermotoga maritima*) have originally been proposed to function as peroxiredoxins, dehydrogenases, oxidoreductases or hydrolases (Shin *et al*., 2002; Christendat *et al*., 2002; Gaspar *et al*., 2005; Numata *et al*., 2006; Dahl *et al*., 2008). So far, redox-active cysteines were established in DsrEFH and TusBCD. In this study DsrE2 was shown to contain at least one catalytic cysteine. Therefore, these proteins fall under the definition “thiol enyzmes”, that were defined by Nagahara by the presence of redox-active cysteines. These cysteines exhibit a lower pK_a as unperturbed cysteine residues and he distinguished between single- or double-catalytic site cysteine residues. While transferases harbour only one redox-active cysteine, oxidoreductases and isomerases contain double-catalytic active site cysteine residues (Nagahara, 2011). The transfer from DsrEFH/TusBCD to DsrC/TusE requires only the presence of Cys78 in TusD/DsrE and penultimate cysteine in TusE/DsrE. Thus, DsrEFH and TusBCD fall into the transferase group. With highly conserved Cys110 and Cys120 DsrE2 could indeed belong to the oxidoreductase/isomerase group and mobilize sulfur in manner that would not be detected using the rhodanese assay after Ray that takes advantage of the rhodanese specific ping-pong mechanism (Ray *et al*., 2000).

Possible role of Rhd_2599, TusA and DsrE2

Considering a possible function for Rhd_2599, TusA and DsrE2 the collected data disaccord with the hypothesis that TusA and DsrE2 are responsible for efflux of H_2S that is generated by the reduction of elemental sulfur by SreABC as suggested for *Atb. ferrooxidans* (Osorio *et al*., 2013). The abundance of *rhd*, *tusA* and *dsrE2* in sulfur oxidizing prokaryotes has to be brought into an agreement with a

catalytic step that is shared by all these organisms. Usually reduced sulfur compounds are oxidized to the highest oxidation state possible, e.g. sulfate in *Alc. vinosum*. Sulfide in high concentrations is usually quickly oxidized to sulfur in the periplasm of *Alc. vinosum* by three different enzymes, namely the soluble flavocytochrome c and the membrane-bound sulfide:quinone oxidoreductases SqrD and SqrF. The last two are both predicted to be oriented towards the periplasm (Reinartz *et al*., 1998; Gregersen *et al*., 2011; Weissgerber *et al*., 2011). The transport via TusA and DsrE2 in *Atb. ferrooxidans* would translocate H_2S into the periplasm where the membrane-bound sulfide:quinone oxidoreductase (AFE_1792) is supposed to oxidize sulfide to elemental sulfur (Rohwerder & Sand, 2003; Wakai *et al*., 2007). Finally, in the course of H_2S emerging as a gaseous signalling molecule in humans (Wang, 2002; Lefer, 2007) the permeability of lipid membranes for H_2S was examined and the resistance of these membranes was shown to be negligible (Mathai *et al*., 2009). The export of H_2S via two different proteins would therefore appear to be redundant. Overall, a function in H_2S efflux seems unlikely. Instead, the obtained results correlate Rhd_2599, TusA and DsrE2 with the oxidation of sulfur globules and the production of sulfite. Therefore, a role as direct and indirect sulfur donors for DsrAB and HdrABC, respectively, appears likely and would be a unifying element for sulfur oxidizers using DsrAB or HdrABC.

Sulfur transfer reactions and a new model for sulfur globule degradation

Figure IV.2 summarizes successful sulfur transfer reactions among Rhd_2599, TusA, DsrEFH and DsrC. On the acceptor site Rhd_2599 and DsrC are remarkable; both proteins accepted sulfur from all tested donor proteins. Regarding Rhd_2599 it should be taken into consideration that this was the only protein that mobilized sulfane sulfur from low molecular weight thiols. Therefore, it can be assumed that persulfidic proteins represent a further substrate for this rhodanese. Persulfurated DsrC on the other hand likely serves as the direct substrate for DsrAB *in vivo* (Cort *et al*., 2008; Oliveira *et al*., 2011; Stockdreher *et al*., 2012). It would be feasible that thiolic DsrC acts as sulfur trap to ensure the constant flow of sulfur atoms necessary for a high turnover rate of DsrAB. The fact that the persulfide of DsrC is very stable falls in line with this assumption. Given that the sulfane sulfur is bound to DsrC-Cys111 in the penultimate position of carboxy-terminus of DsrC, which extends from the globular part of the protein, this persulfide should be the most exposed persulfide and thus, most vulnerable to a nucleophilic attack by another redox-active cysteine. The transient formation of a trisulfide bond might explain why sulfur was not transferred to other proteins apart from Rhd_2599. Mobilization of sulfur from a trisulfide bond by a rhodanese has not been reported, though. Analysis of undigested DsrC via MALDI-TOF mass spectrometry that was performed for this study would not distinguish between a persulfide and the putative trisulfide, because it only detects the additional mass, which is the same in both cases. Digestion with trypsin of persulfidic DsrC prior to mass spectrometry

should yield the information as demonstrated for methionyl human growth hormone (Canova-Davis *et al*., 1996).

The transfer reactions TusA→ DsrEFH→ DsrC and TusA→ DsrC agree with the observations made for the Tus proteins from *E. coli*, although the latter was not integrated into the proposed model for 2-thiourdine biosynthesis (Ikeuchi *et al*., 2006). It should be stressed that TusA was the only protein that was capable to transfer sulfur to DsrEFH. Furthermore, TusA accepted sulfur from persulfurated DsrEFH. Thus, the transfer reaction is reversible. A potential transfer from TusBCD to TusA could not tested in the Ikeuchi study since TusBCD was not persulfurated by IscS.

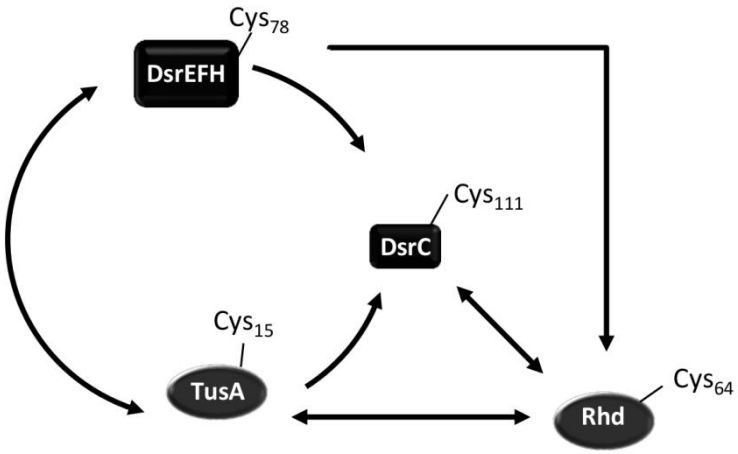

Figure IV.2. Summary of detected sulfur transfer reactions *in vitro*. Cysteine residues responsible for sulfur binding are depicted.

The number of successful transfer reactions raises the question of specificity. It can be ruled out that the persulfuration of the acceptor proteins is an artefact that might be attributed to the chemical reduction of persulfides or short polysulfide chains that were bound to the donor protein. Otherwise DsrEFH and TusA should both have been persulfurated after incubation with persulfidic DsrC and Rhd_2599. The fact that all these transfer reactions were detected *in vitro* does not inevitably mean they also occur *in vivo*, though. The interaction between all the proteins could be tested *in vitro*, but the results appear to depend on the method and might yield false negative results. The interaction study of TusA with TusBCD and TusA with DsrEFH using SRP may serve as example for this. A better approach would be the identification of *in vivo* interaction partners of each protein under sulfur oxidizing conditions. The tandem affinity purification would serve this purpose (Puig *et al*., 2001). For now, it is not possible to decide whether the cytoplasmic transfer of sulfane sulfur is executed in an orderly and highly specific fashion from protein A to protein B to protein C or if the system operates on a flexible basis that allows reversible reactions and multiple reaction partners as indicated in Figure IV.2.

Based on the data gathered here, in both scenarios the rhodanese would be the entry point for individual sulfur atoms since only this enzyme mobilized sulfane sulfur from the GSSH. The terminal sulfur acceptor is also identical: DsrC. DsrEFH and TusA would mediate the sulfur transfer between Rhd_2599 and DsrC. In either case, the final sulfur transfer to DsrC would generate the direct substrate for DsrAB. After oxidation of the sulfane sulfur DsrC dissociates from the active site of DsrAB with a sulfonate group bound to DsrC-Cys111. By formation of an intramolecular disulfide bond between DsrC-Cys111 and DsrC-Cys100 sulfite would be reductively released from DsrC as final product of the cycle. In the concluding step the thiol groups of DsrC would be regenerated via reduction by DsrK. And the cycle could start again. Figure IV.3 gives an overview of the model that favours the relay system.

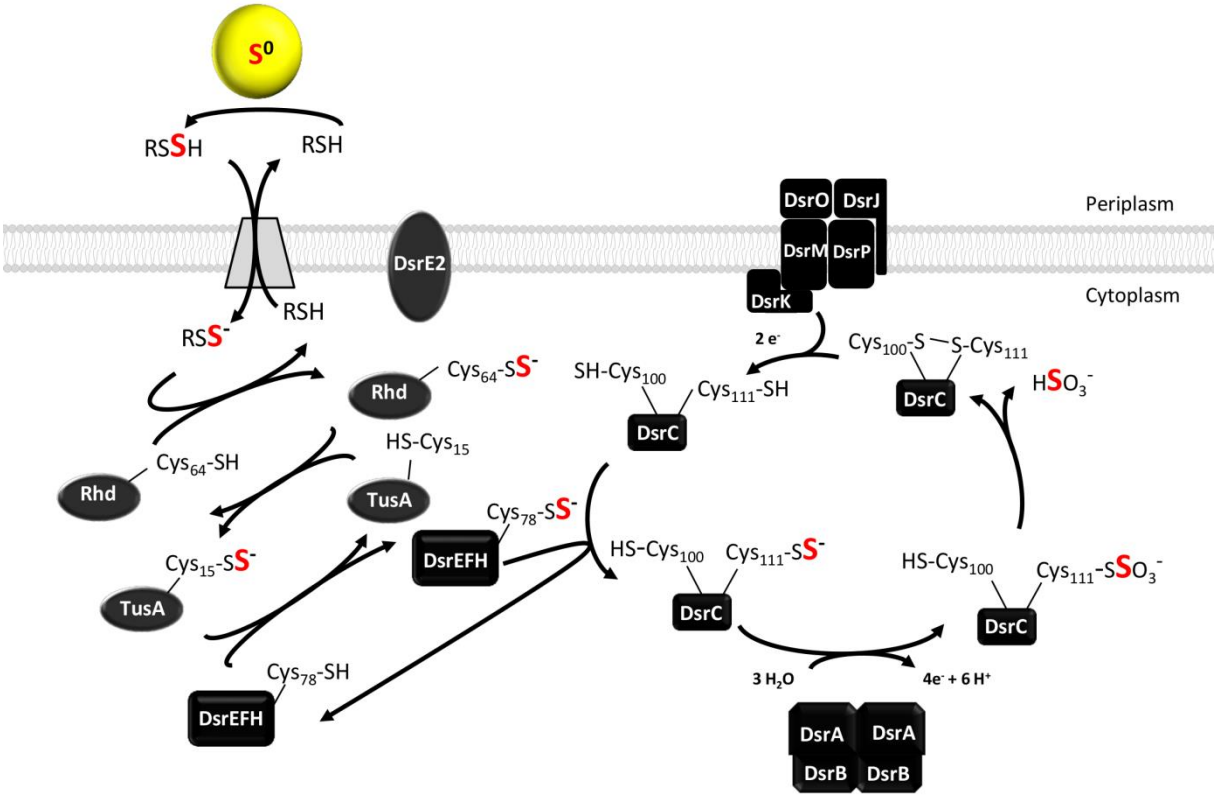

FIGURE IV.3. Proposed model for sulfur oxidation in *Alc. vinosum* introducing Rhd_2599, TusA, DsrEFH and DsrC as sulfur donating proteins for DsrAB. Thiol groups and persulfides are shown in the ionized or protonated state according to their supposed pK_a values of around 8.5 (Riederer, 2009) and 6.2 (Everett *et al.*, 1994) respectively. Since persulfides are 1 to 2 pK_a units more acidic than their thiol equivalents the pK_a of the assumed carrier molecule glutathione amide persulfide was calculated on the basis of the pK_a for glutathione (Tajc *et al.*, 2004) to be around 7.2.

V Outlook

The present study provides significant insights into cytoplasmic sulfurtransferases and their function in *Alc. vinosum*. However, unanswered questions remain and the obtained data led to new questions which makes this topic an interesting objective for future research.

The inactivation of *rtd* in *Alc. vinosum* led to a drastic phenotype, but the influence of each gene product can hardly be evaluated at this point. Therefore, the generation of more mutant strains should be pursued. Subsequent deletion of genes for cytoplasmic rhodaneses (especially Alvin_1587 and Alvin_0866) would allow answering the question if Rhd_2599 was functionally replaced in *Alc. vinosum rhd_2599::*ΩSm or if this rhodanese does not participate in the degradation of sulfur globules. Analysing the impact of DsrE2 would also be of interest. The generation of a *tusA* negative mutant appears to be mandatory. Instead of deleting the complete gene it might be possible to generate mutants that carry either a mutation in TusA-Cys15 or in the start codon of *tusA*. As positive selection marker the Ω-Km cassette could be inserted into the existing *EcoRI* restriction site between *rhd_2599* and *tusA.*

The sulfur binding site of DsrE2 could not be identified, though Cys120 appears to be the redox-active cysteine residue. The issue could be approached by generating a DsrE2 protein that carries only Cys120. It would also be compelling to determine if DsrE2 indeed acts as oxidoreductase.

The identification of the *in vivo* interaction partners of Rhd_2599, TusA, DsrE2, DsrEFH and DsrC via tandem affinity assays would clarify the question whether all observed sulfur transfer reactions are possible *in vivo*.

Metabolomic data of *Alc. vinosum* do not support the theory that persulfidic glutathione or glutathione amide act as carrier molecule for sulfur across the cytoplasmic membrane. Disturbance in glutathione maintenance might settle this question. Alvin_1430-34, which encode a putative glutathione transporter that is homologous to YliABCD in *E. coli*, and Alvin_1323/24, encoding a possible glutathione disulfide reductase, are ideal candidates for deletion.

So far, the interaction of DsrC with DsrAB and the mechanism of the dissimilatory sulfite reductase have mostly been hypothesized in analogy to results from sulfate reducing prokaryotes. Yet, DsrAB from sulfur oxidizing and sulfate reducing bacteria are supposed to work in opposite directions and will most likely differ in the details. Addressing this issue in the future appears be imperative.

The research on dissimilatory sulfur metabolism would benefit from the elucidation of the Hdr system since these proteins are conserved in major parts of sulfur oxidizing prokaryotes that concomitantly lack the *dsr* genes. This would include the generation of deletion mutants to determine the *in vivo* function as well as the characterization of the individual gene products of the *hdr* operon. It would also be interesting to see whether Dsr and Hdr are interchangeable.

Summary

The subject of this thesis was the exploration of the possibility that the process of sulfur globule degradation in *Alc. vinosum* involves sulfur trafficking. Additionally, potential participants in the cytoplasmic events of this mechanism should be identified. Indeed, the obtained data provide the first experimental proof that the oxidation of intermediary stored sulfur includes sulfurtransferase activity and that individual sulfur atoms are passed on towards DsrAB by a pool of proteins via persulfidic intermediates.

DsrEFH was verified as sulfurtransferase. Sulfane sulfur was exclusively bound to Cys78 in the DsrE subunit and could be transferred to DsrC and TusA. Cys111 was identified as the unique binding site for sulfur species in DsrC; apart from sulfane sulfur DsrC also binds sulfite. The results present DsrC as sulfur trap rather than a sulfurtransferase and add support to the notion that DsrC might serve as substrate donor for DsrAB in *Alc. vinosum*. The band shifts that were observed in the native PAGE upon incubation of DsrEFH with DsrC were ascribed to the formation of stable complexes between the two proteins in the stoichiometry 1:1 and 1:2 (DsrEFH:DsrC).

Several independent lines of evidence indicate a function for Rhd_2599, TusA and DsrE2 in the oxidation of sulfur globules in *Alc. vinosum*. Apart from the ubiquitous presence of the genes in all major sulfur oxidizing families and their genomic link to genes encoding major components of the sulfur oxidation machinery, the transcriptomic patterns of *rhd_2599*, *tusA* and *dsrE2* paralleled the transcriptional upregulation of the *dsr* operon. The three genes form a transcriptional unit, although a secondary promoter upstream of *tusA* was detected. The disruption of the *rtd* locus led to an instable mutant with a severe and sulfur oxidizing negative phenotype. The insertion of an Ω-streptomycin cassette into the *rhd_2599* reading frame on the other hand was of no consequence for the mutant strain, although the enzyme accounts for the majority of rhodanese activity under sulfur oxidizing conditions.

Characteristic patterns in the sequence of TusA and DsrE2 were identified and can be used to distinguish these proteins from homologues in prokaryotes with a non-sulfur based energy metabolism. TusA proteins encoded together with the rhodanese and DsrE2 contain hydrophobic residues in the "X"-position of the TusA motif. Leucine is dominating within bacterial TusA; glycine is conserved in archaeal TusA. With few exceptions all DsrE2 proteins contain two cysteine residues in short distance; Cys120 and Cys110 in DsrE2 from bacteria and Cys120 and Cys128 in DsrE2 from archaea. Rhd_2599 also carries two cysteine residues; however only the cysteine within the rhodanese motif, Cys64, was essential for sulfurtransferase activity. Rhd_2599, TusA and DsrE2 all bind sulfur, though Rhd_2599 alone was able to mobilize sulfur from an inorganic sulfur compound. Data collected from sulfur transfer experiments confirmed the flow of sulfur atoms between Rhd_2599, TusA, DsrEFH and DsrC *in vitro*.

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