Biochemical and genetic analysis of the Sox multienzyme complex in the purple sulfur bacterium *Allochromatium vinosum*

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn 2006

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

Diese Dissertation ist auf dem Hochschulschriftenserver

der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online

elektronisch publiziert.

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Tag der Promotion: 2006

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Abbreviations

Amp	ampicillin
bp	base pair(s)
BSA	bovine serum albumine
CBB	Coomassie Brilliant Blue
CDP-Star	disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxethane-3,2´-(5´-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl)-1-phenyl phosphate
CIAP	calf intestine alkaline phosphatase
Cm	chloramphenicol
Da	Dalton
DAB	3´-3´diaminobenzidine
dig	digoxigenin
DMSO	dimethylsulfoxide
EDTA	ethylendiamintetraacetate
Em	erythromycin
FPLC	fast protein liquid chromatography
g	earth acceleration / gravitational constant
HEPES	N-[2-hydroxyethyl]-piperazine-N´-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-S-thiogalactoside
kb	kilo base pairs
kDa	kiloDalton
Km	kanamycin
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
Rif	rifampicin
sox	sulfur oxidation
TEMED	tetramethylendiamine
T_m	melting temperature
Tris	trishydroxymethylaminomethane

A. Introduction

Thiosulfate, together with sulfide, is a very abundant reduced sulfur compound and can be degraded to different products, mostly by bacterial action. Being of intermediate oxidation state, it plays an important role in the sulfur cycle. In dissimilatory sulfur metabolism thiosulfate can be used as electron donor or as electron acceptor. Thiosulfate can be produced by chemical oxidation of H_2S, FeS and FeS_2 (Chen and Morris, 1972; Goldhaber, 1983). A biological source for thiosulfate is the fermentation of taurine, together with the potential fermentation of other organosulfonates in anoxic environments (Denger *et al.*, 1997).

A number of ways for the degradation of thiosulfate have been discussed in the literature (Brune, 1989; Brune, 1995b).
One possibility is the oxidation to tetrathionate catalysed by a thiosulfate:acceptor oxidoreductase. This enzyme has been isolated from organisms like *Allochromatium vinosum*, thiosulfate-oxidizing strains of *Chlorobium limicola* and from *Rhodopseudomonas palustris*. The first enzyme isolated originated from *A. vinosum* (Smith, 1966) and exhibited tetrathionate formation activity only under slightly acidic conditions with an optimal pH value of pH 5.0. The enzyme was inactive at a neutral pH value of pH 7.0. The high-potential iron-sulfur-protein (HiPIP) was found to be an effective electron acceptor for this enzyme (Fukumori and Yamanaka, 1979). A seemingly different thiosulfate:acceptor oxidoreductase was also isolated from *A. vinosum*, which catalysed the same reaction, but with an optimal pH value of pH 8.0 and a c-type cytochrome c_{550} as optimal electron acceptor. The exact reaction mechanism, however, remained unclear (Schmitt *et al.*, 1981). Recently, the thiosulfate:acceptor oxidoreductase responsible for tetrathionate formation in *A. vinosum* has been characterized in detail (Sperling, 2001). The protein is constitutively produced and soluble in the periplasm. It has been purified from the organism, together with a protein homologous to nucleoside diphosphate kinases, and contains a heme c_{554} as prosthetic group. The catalysed reaction follows a ping-pong-bi-bi mechanism, first releasing the produced tetrathionate before the transfer of the obtained electrons from the enzyme takes place. This enzyme is in all probability identical to the enzymes described by Smith and Fukumori and Yamanaka.

The enzyme isolated from *C. limicola* exhibited similarity to both enzymes from *A. vinosum*, as its pH optimum was in the slightly acidic region (pH 6.0) and a complex of two c-type cytochromes was used as effective electron acceptor (c_{551} and c_{555}) (van Grondelle *et al.*, 1977). Thiosulfate:acceptor oxidoreductases catalyse the oxidation of thiosulfate to tetrathionate in a two electron step without the release of protons. However, this reaction mechanism potentially is not the initial step for a complete degradation of thiosulfate to sulfate, which would yield eight electrons altogether and release ten protons.

Rhodaneses and thiosulfate reductases, the second enzyme group potentially responsible for thiosulfate degradation, are quite similar in their reaction mechanism. Both enzymes function as thiosulfate:acceptor sulfur transferases, releasing sulfite from thiosulfate and transferring the sulfane sulfur to an acceptor molecule (Le Faou *et al.*, 1990). The first step is the release of sulfite from the thiosulfate molecule and the production of persulfides under the participation of thiol acceptors. The persulfide then reacts with a second SH - group under the formation of a disulfide and the release of H_2S. The difference between rhodanese and thiosulfate reductase lies in the ability to use cyanide (CN^-) as a thiophilic acceptor. While for rhodaneses CN^- works well, thiosulfate reductases are unable use CN^-. Rhodaneses are fairly widespread (e.g. found in plants, animals as well as in bacteria) and are thought to function either in cyanide detoxification or in the formation of sulfide for Fe-S-clusters in iron-sulfur proteins (Cerletti, 1986; Cereda *et al.*, 2003). For the rhodanese purified from bovine liver mitochondria cyanide is the preferred acceptor forming thiocyanide (SCN^-). Thiosulfate reductases are far more difficult to examine because of their instability, with the one exception of the thiosulfate reductase isolated from yeast, which uses glutathion as a thiophilic acceptor (Chauncey *et al.*, 1987).

A third possibility for thiosulfate degradation would be a hydrolytic cleavage, which is quite favourable under the energetic point of view. This reaction was originally suggested by Trüper and Pfennig (1966) and would lead to the direct formation of H_2S and SO_4^{2-}. The bacterium *Desulfuvibrio sulfodismutans* derives energy from this reaction, although in this organism it presumably takes place in two steps (Bak and Pfennig, 1987). The first hydrolytic step leads to the formation of H_2S and SO_3^{2-} from thiosulfate. The sulfite is then further oxidized to sulfate by APS reductase and ADP sulfurylase. At present no enzyme is known to catalyse the direct hydrolysis of thiosulfate to sulfate and H_2S in a one step reaction.

A fourth pathway to degrade thiosulfate produces sulfate as the only end product and is catalysed by a periplasmic multienzyme system. This protein complex was first detected and examined in two chemotrophic *Paracoccus* strains (*Paracoccus versutus* by Lu and Kelly (1983b) and *Paracoccus pantotrophus* by Friedrich and coworkers (Chandra and Friedrich, 1986)). The purified and reconstituted enzyme system from *P. versutus*, which *in vitro* catalyses a c-type cytochrome dependent thiosulfate oxidation, consists of four different subunits: enzyme A, enzyme B and the two c-type cytochromes c_{551} and $c_{552.5}$ (Lu and Kelly, 1983c; Kelly *et al.*, 1997). Early experiments suggested the involvement of a rhodanese to obtain thiosulfate oxidation by the reconstituted complex *in vitro* (Lu and Kelly, 1983b). Further purification, however, achieved a separation of rhodanese activity and the enzyme activity essential for a functional enzyme complex (Lu and Kelly, 1983a).

The group of Friedrich performed extensive analyses of thiosulfate oxidation in the closely related organism *P. pantotrophus*, detecting a multienzyme system similar to that of *P. versutus*, which is responsible for thiosulfate oxidation to sulfate. It is encoded by a *sox* gene cluster consisting of 15 open reading frames (Wodara *et al.*, 1994; Wodara *et al.*, 1997) (see Figure A1).

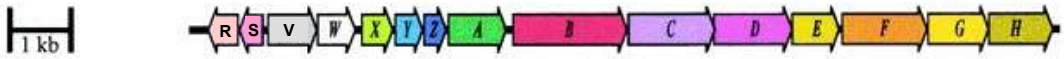

Figure A1: The *sox* gene cluster in *P. pantotrophus* GB17 (modified after Friedrich *et al.*, 2001)

Four of the encoded proteins (SoxXA, SoxYZ, SoxB and SoxCD) could be reconstituted *in vitro* to form a functional multienzyme system. The Sox complex in *P. pantotrophus* is essential for thiosulfate and sulfide oxidation *in vivo* (Chandra and Friedrich, 1986) and catalyses an *in vitro* reduction of cytochrome c coupled to the oxidation of thiosulfate, sulfide, sulfite and elemental sulfur (Friedrich *et al.*, 2000; Rother *et al.*, 2001). The reconstituted periplasmic Sox multienzyme system oxidizes its sulfur substrates to sulfate without the formation of free intermediates.

The heterodimeric SoxYZ has been identified as the substrate binding molecule of the complex (Quentmeier and Friedrich, 2001). The sulfur compound is coupled to a conserved cysteine residue, located in a new consensus motif at the C-terminus of SoxY. The substrate

coupling takes places under participation of SoxXA. SoxXA is a heterodimeric c-type cytochrome, that contains three heme binding sites. SoxX is a monoheme protein subunit, while the second subunit SoxA is a diheme. SoxXA is reduced while oxidatively coupling the sulfur compound to the substrate binding molecule SoxYZ. The monomeric protein SoxB has been identified as a manganese-containing sulfate thiol esterase or sulfate thiol hydrolase (Friedrich *et al.*, 2005; Epel *et al.*, 2005) and is responsible for hydrolytic cleavage of a sulfate group from the bound sulfur substrate. If thiosulfate is the bound substrate, the release of the outer sulfone sulfur as sulfate leaves the inner sulfane sulfur behind, still bound to the cysteine residue of SoxY. The sulfane sulfur is then oxidized by SoxCD, that acts as a sulfur dehydrogenase. SoxCD is a $\alpha_2\beta_2$ tetramer, composed of the molybdoprotein subunit SoxC and the diheme cytochrome c subunit SoxD (Quentmeier *et al.*, 2000). Further action of SoxB finally releases the sulfane sulfur in form of a second sulfate molecule. Thereby the substrate binding molecule SoxYZ is restored, and the circular reaction mechanism can start again with a new substrate molecule (Friedrich *et al.*, 2001). The model proposed for the reaction mechanism is demonstrated in Figure A2.

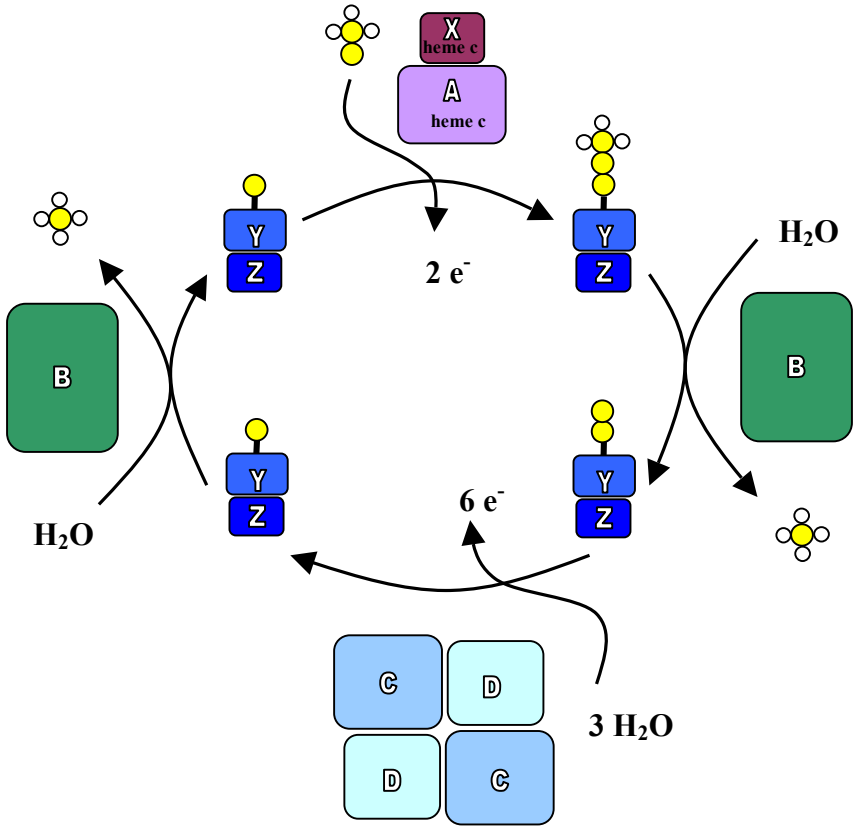

Figure A2: Proposed mechanism for the Sox multienzyme complex in *P. pantotrophus* (modified after Friedrich *et al.*, 2001).

Apart from the four proteins forming the *in vitro* reconstituted complex, *in vivo* also other Sox proteins play a more or less essential role in thiosulfate oxidation in *P. pantotrophus*.
The open reading frame *soxV* encodes a protein with homology to CcdA in *P. pantotrophus*, involved in c-type cytochrome formation. The inactivation of *soxV*, however, led to a lack of growth on thiosulfate or molecular hydrogen, while cytochrome c formation remained unaffected. The inactivation of *soxV* also caused an inhibition of *soxW* expression, the latter encoding a periplasmic thioredoxin (Bardischewsky and Friedrich, 2001).
The inactivation of *soxF*, encoding a periplasmic flavoprotein, had a less substantial effect, as thiosulfate oxidation remained possible (Rother *et al.*, 2001). SoxF exhibited sulfide dehydrogenase activity *in vitro* (Quentmeier *et al.*, 2004). Additionally, a novel activity has been proposed for the protein, as it appears to have an activating effect on the *in vitro* reconstituted Sox enzyme system. If the SoxYZ component has been separately inactivated by reduction before the reconstitution, the potentially redox-balancing function of SoxF leads to an active enzyme complex (Friedrich *et al.*, 2005).
The proteins encoded by *soxR* and *soxS*, predicted to be a transcriptional regulator and a periplasmic thioredoxin, respectively, are involved in the regulation of *sox* gene expression. The protein SoxR has been shown to act as a repressor for *sox* gene expression, while on the other hand the protein SoxS is essential for full expression (Rother *et al.*, 2005). Whether the proteins encoded by *soxE*, *soxG* and *soxH* play an essential role in the thiosulfate-oxidizing Sox multienzyme complex still remains to be examined in detail.

The Sox system appears to be widespread. A phylogenetic and distributional examination of the Sox system in different prokaryotes was performed with *soxB* as the indicator gene (Petri *et al.*, 2001). It is found in green sulfur bacteria as well as in different groups of proteobacteria. The thermophilic bacterium *Aquifex aeolicus* also contains the gene *soxB*. Because of the increasing amount of partial or complete genome sequences, more *sox*-gene containing organisms will in all probability be identified. The presence of other *sox* genes has been demonstrated in a couple of organisms, some of which exhibit quite significant differences in comparison to the "model organism" *P. pantotrophus*, either concerning presence or absence of genes or the arrangement of the *sox* genes (Friedrich *et al.*, 2001; Friedrich *et al.*, 2005).

Differences can also be observed on the basis of specific protein characteristics. When a closer look is taken at the heterodimeric protein SoxXA, two major groups of organisms can be established, based on the heme content of the SoxA subunit. One group exhibits two heme binding sites in the SoxA sequence (like *P. pantotrophus* (Friedrich *et al.*, 2000) or *Rhodovulum sulfidophilum* (Appia-Ayme *et al.*, 2001)), the other group only contains one heme binding site in SoxA, e.g. in *Starkeya novella*. Only a fairly conserved cysteine residue remains of the second heme binding site in SoxA, which has been demonstrated to form a disulfide bridge (Kappler *et al.*, 2004). The protein SoxXA has been crystallized from the phototrophic bacterium *R. sulfidophilum* (Bamford *et al.*, 2002a; Bamford *et al.*, 2002b). Based upon the obtained structure, a model for the putative electron transfer route through the protein has been proposed. The heme-heme distances have been determined in the heterodimer, thereby demonstrating, that one heme group of SoxA is potentially not involved in electron transport. The distance between the heme in SoxX and the C-terminal heme in SoxA is small enough for electron tunnelling effects. The N-terminal heme in SoxA, however, is in too great a distance to allow electron tunnelling. This heme group, that is potentially not involved in electron transfer, represents the one, whose binding site is missing in one group of SoxA proteins.

The organism *Allochromatium vinosum* is a Gram-negative rod that belongs to the family *Chromaticeaea* in the group of γ-proteobacteria. The natural habitats of this purple sulfur bacterium are environments with fresh or salt water, that contain hydrogen sulfide (Pfennig and Trüper, 1989). Under anoxic conditions *A. vinosum* grows phototrophically in the light, but also has the possibility to grow facultatively chemoautotrophic or mixotrophic under micro- to semioxic conditions (Kämpf and Pfennig, 1980). The bacterium contains a vesicular photosynthetic membrane system and performs anoxygenic photosynthesis. As electron donors for this process a broad range of substrates can be used. Apart from reduced sulfur compounds like thiosulfate, sulfide, polysulfides, sulfur and sulfite also substrates like molecular hydrogen, formiate, acetate, propionate, pyruvate, fumarate, malate and succinate are used (Pfennig and Trüper, 1989). As the organism is accessible for genetic manipulation via conjugation (Pattaragulwanit and Dahl, 1995) and electrotransformation (Hensen, unpublished) and utilizes a broad substrate spectrum, it presents a rewarding goal for further examination of sulfur metabolism.

Concerning the degradation of the different reduced sulfur compounds in *A. vinosum*, several pathways have already been proposed, supported by more or less conclusive data.
The enzyme flavocytochrome c_{552} has been postulated to be involved in sulfide oxidation, but an insertional inactivation of the corresponding genes *fccAB* refuted this assumption. No effect on sulfide oxidation could be observed (Reinartz *et al.*, 1998), and data about a slight effect on thiosulfate and sulfite oxidation are of questionable significance (Reinartz, 1997). A sulfide:quinone oxidoreductase (SQR) has been postulated as another enzyme potentially responsible for sulfide oxidation. This flavoprotein is quite common in prokaryotes (Dahl *et al.*, 2002) and catalyses the electron transfer from sulfide to the quinone pool in the membrane. As a product of the SQR activity polysulfides have been postulated, that are quite unstable, resulting in the disproportioning to sulfide and elemental sulfur (Brune, 1989; Dahl *et al.*, 2002). Nevertheless, the presence of polysulfides has been demonstrated in *Rhodobacter capsulatus* as the product of SQR activity (Griesbeck *et al.*, 2002). In *A. vinosum* polysulfides have also been identified as the first detectable product of sulfide oxidation (Prange *et al.*, 2004). A sulfide:quinone oxidoreductase activity has been observed in *A. vinosum* (Reinartz *et al.*, 1998), but the activity could not be related to a specific enzyme so far. Therefore, the enzyme responsible for sulfide oxidation in *A. vinosum* still remains to be identified.

Sulfur globules are an obligate intermediate of sulfide and thiosulfate oxidation in *A. vinosum*. These highly refractive structures have been shown to be located in the periplasm (Pattaragulwanit *et al.*, 1998). Earlier examinations already led to the assumption that the sulfur inside of these globules is not present as liquid elemental sulfur in a S_8 ring structure (Hageage, Jr. *et al.*, 1970; Guerrero *et al.*, 1984). Instead more recent *in situ* studies of the sulfur globules with XANES (X-ray near edge absorption structure) spectroscopy demonstrated, that the main part of the sulfur is potentially present in form of bis-organylsulfanes in a structure of R-S_n-R ($n \geq 4$) (Prange *et al.*, 1999; Prange *et al.*, 2002). The sulfur globules are coated by an unimolecular layer, consisting of the three proteins SgpA, SgpB and SgpC. As the proteins exhibit similarity to structural proteins like keratin (Brune, 1995a), a purely structural function was also proposed for the sulfur globule proteins in *A. vinosum*. An enzymatic function is thought to be unlikely (Pattaragulwanit *et al.*, 1998). The inactivation of *sgpA* or *sgpB* had no effect on sulfur globule formation, indicating that both proteins are able to replace each other. The inactivation of *sgpC*, however, led to the

formation of considerably smaller sulfur globules. The double mutant *sgpBC* was not able to from sulfur globules at all (Prange *et al.*, 2004).

The oxidation of sulfur, stored in the periplasmic globules, to sulfite is catalysed by proteins encoded in the *dsr* operon (Pott and Dahl, 1998; Dahl *et al.*, 2005). The genes *dsrAB* encode a cytoplasmic siroamid-containing dissimilatory sulfite reductase, that has been demonstrated to be essential for the degradation of sulfur globules (Pott and Dahl, 1998). A direct oxidation of the sulfur stored in the periplasm seems unlikely, leading to the assumption of a preceding substrate transport over the membrane. Thereby the sulfur is potentially reduced to sulfide, which corresponds with the catalytic action of a reverse sulfite reductase (a six-electron step from sulfide to sulfite). Downstream of *dsrAB* 13 open reading frames have also been identified to be a part of the larger *dsr* transcription unit. Apart form the membrane complex DsrMKJOP all other encoded Dsr proteins are located in the cytoplasm. A lot of research has gone into the attempt to clear up the function of the different Dsr proteins (Grimm, 2004; Dahl *et al.*, 2005; Lübbe, 2005; Schulte, 2005; Sander, 2005), but a definitive reaction mechanism still has to be established.

For the oxidation of sulfite to sulfate two different pathways have been postulated. A two-step pathway takes place with the intermediary formation of APS (adenosine 5´- phosphosulfate), catalysed by the enzyme APS reductase. APS is then further degraded to sulfate by the action of ATP sulfurylase. But the insertional inactivation of the APS reductase demonstrated, that this enzyme is not essential for sulfite oxidation in batch culture (Dahl, 1996). Examinations in continuous culture, however, showed that the APS reductase activity is still beneficial under certain growth conditions (Sanchez *et al.*, 2001). The second pathway for sulfite oxidation is the direct oxidation to sulfate. The enzyme responsible for this reaction, a sulfite:acceptor oxidoreductase (SOR), has been isolated from *S. novella* as a periplasmic heterodimeric protein with a molybdenum cofactor and a heme c_{552} as prosthetic groups (Kappler *et al.*, 2000). Tungstate, as a specific antagonist of molybdate, inhibits sulfite oxidation when added to *A. vinosum* cultures (Dahl, 1996), thereby indicating the involvement of a molybdoprotein in sulfite oxidation. However, up until now neither the enzyme SOR nor another potentially involved molybdoprotein has been successfully isolated from *A. vinosum*. The question therefore remains which enzyme is essential for sulfite oxidation.

For the degradation of thiosulfate in *A. vinosum* different pathways have been postulated. Depending on the medium pH, thiosulfate is either oxidized to tetrathionate or to sulfate (Smith, 1966). The amount of produced tetrathionate rises with a falling pH in the medium, with tetrathionate as the main product at pH 6.25. The enzyme responsible for tetrathionate formation in *A. vinosum* has been identified as a thiosulfate:acceptor oxidoreductase (see above; Sperling, 2001). Formation of tetrathionate, however, is not necessarily disadvantageous, as abiotic reactions lead to the production of substances that are again accessible to enzymatic degradation. According to Kelly *et al.* (1969) and Suzuki (1999) several chemical conversions of tetrathionate are possible:

(i) a reaction with thiosulfate to obtain pentathionate and sulfite,
(ii) a reaction with sulfite to obtain trithionate and thiosulfate
(iii) a reaction with thiosulfate to obtain pentathionate and trithionate

The renewed production of thiosulfate from tetrathionate by abiotic means has also been postulated by Hansen (1974), assuming a reaction of tetrathionate and sulfide (in environments rich in sulfide) to elemental sulfur and thiosulfate. This reaction has been demonstrated for the heterotrophic tetrathionate-producing bacterium *Catenococcus thiocyclus* (Sorokin *et al.*, 1996). The formation of trithionate and potentially pentathionate from tetrathionate has been demonstrated in *A. vinosum* (Sperling, 2001). The sulfite and thiosulfate produced by these abiotic reactions can eventually be further oxidized to sulfate.

A long-held conviction has been the participation of a rhodanese or thiosulfate reductase in thiosulfate oxidation in *A. vinosum*. The proteins responsible for this activity, however, could not be purified so far (Smith and Lascelles, 1966; Hashwa, 1975; Schwarz, 2001; Schulte, 2005).

The oxidation of thiosulfate to sulfate involves the formation of sulfur globules as obligate intermediates. In the oxidation to sulfate the sulfane and sulfone sulfur of the thiosulfate molecule have different fates, demonstrated by radioactively labelling the sulfone $[S\text{-}^{35}SO_3]^{2-}$ and sulfane sulfur $[^{35}S\text{-}SO_3]^{2-}$, respectively. While the sulfone sulfur directly appears as sulfate, the sulfane sulfur is first transferred to the sulfur globules and only then oxidized to sulfate (Smith and Lascelles, 1966; Trüper and Pfennig, 1966).

During my diploma thesis three *sox* genes (*soxBXA*) and two potentially *sox*-related genes (ORF9 and *rhd*) could be identified in *A. vinosum* (Hensen, 2001, see also "Results"). They

indicated the presence of a Sox multienzyme system in *A. vinosum*, potentially comparable to the complex found in *P. pantotrophus*.

The goal of this work has been the further examination of the influence of the *sox* encoded proteins on the thiosulfate oxidation in the phototrophic sulfur oxidizing γ-proteobacterium *A. vinosum*. The work was based on the results obtained during the work on my diploma thesis. Additional genomic sequence was analysed on the lookout for the so far absent, but seemingly important genes *soxYZ* and *soxCD*. The detection of the proteins encoded by the *sox* genes present in *A. vinosum* was an aim of this work, together with the purification and potential further analysis of these proteins. The *sox* mutant strains produced during the work on my diploma thesis were phenotypically characterized concerning the degradation of thiosulfate and sulfide. Additional *in frame* mutations of further identified *sox* genes was aimed for, again including the subsequent phenotypic analysis. To trace back potentially observed mutant phenotypes back to the inactivated genes, the establishment of complementation strains was also aimed for. On the basis of the obtained results a model for the function a potential Sox complex in *A. vinosum* should be developed.

B. Materials and Methods

B.1 Chemicals, materials and software

B.1.1 Chemicals

30 % acrylamide / bisacrylamide	Roth (Karlsruhe, Germany)
4 x Rotiload 1	Roth (Karlsruhe, Germany)
4-chloro-1-naphthol	Sigma (Taufkirchen, Germany)
anti-digoxigenin-AP	Roche (Mannheim, Germany)
blocking reagent	Roche (Mannheim, Germany)
CDP-Star	Roche (Mannheim, Germany)
developer	Kodak (Rochester, USA)
dig-dUTP	Roche (Mannheim, Germany)
fixer	Kodak (Rochester, USA)
HEPES	Sigma (Taufkirchen, Germany)
methanesulfonic acid	Merck (Langenfeld, Germany)
monobromobimane	Fluka (Taufkirchen, Germany)
phtalic acid	Merck (Langenfeld, Germany)
skim milk powder	Töpfer GmbH (Dietmannsried, Germany)

All other chemicals were obtained from the companies Sigma (Taufkirchen, Germany), Fluka (Taufkirchen, Germany), Merck (Langenfeld, Germany) and Roth (Karlsruhe, Germany).

B.1.2 Enzymes

alkaline phosphatase (CIAP)	MBI Fermentas (St.Leon-Rot, Germany)
horse heart cytochrome c	Sigma (Taufkirchen, Germany)
lysozyme	Fluka (Taufkirchen, Germany)
Pfu DNA polymerase	MBI Fermentas (St.Leon-Rot, Germany)
restriction enzymes	MBI Fermentas (St.Leon-Rot, Germany) or Invitrogen GmbH (Karlsruhe, Germany)

ribonulease A	Sigma (Taufkirchen, Germany)
T4 DNA ligase	MBI Fermentas (St.Leon-Rot, Germany)
Taq DNA polymerase	MBI Fermentas (St.Leon-Rot, Germany)

B.1.3 Standards for DNA and protein gel electrophoresis

1-kb DNA ladder	Invitrogen (Karlsruhe, Germany)
PageRuler Prestained Protein Ladder	MBI Fermentas (St.Leon-Rot, Germany)

B.1.4 Kits

BCA Protein Assay	Perbio (Bonn, Germany)
QIAprep Spin Miniprep Kit	Qiagen (Hilden, Germany)
QIAquick Gel Extraction Kit	Qiagen (Hilden, Germany)
SuperSignal West Pico Chemiluminescent Substrate	Perbio (Bonn, Germany)

B.1.5 Other materials

Anaerocult A	Merck (Darmstadt, Germany)
Anaerotest	Merck (Darmstadt, Germany)
Centriplus Centrifugal Filter Device	Millipore (Schwalbach, Germany)
dialysis tube (MWCO 6000 to 8000)	Serva (Heidelberg, Germany)
Immobilon-P (PVDF membrane)	Millipore (Schwalbach, Germany)
Protran BA 85 cellulose nitrate membrane	Schleicher & Schuell (Dassel, Germany)
Sartolon nylon membrane	Sartorius (Göttingen, Germany)
Whatman 3MM paper	Millipore (Schwalbach, Germany)
X-ray films X-OMAT AR	Kodak (Rochester, USA)

B.1.6 Software and online tools

BioEdit	sequence alignment editor	www.mbio.ncsu.edu/BioEdit/page2.html
BLAST	comparison of nucleotide or protein sequences with data bank entries	www.ncbi.nlm.nih.gov/

Clonemanager	sequence processing	Scientific &Educational Software
ClustalW	program for sequence alignments	www.ebi.ac.uk/clustalw/
Cream 4.1	photodocumentation	Kem-En-Tec (Kopenhagen, DK)
ExPasy	DNA and protein analysis	www.expasy.ch
LEO	german-english dictionary	www.dict.leo.org
PC1000	HPLC control software	Thermo Electron (Dreieich, D)
UV Winlab	control software for Perkin-Elmer spectrometer Lambda 11	Perkin-Elmer

B.2 Microorganisms, plasmids and primers

B.2.1 Bacterial strains

strain	genotype or phenotype	source or reference
E. coli strains		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1endA1 hsdR17* (r_k^- m_k^-) *supE44* λ^- *thi-1 gyrA relA1*	(Hanahan, 1983)
SM10	Km^r *supE44 thi-1 thr-1 recA leuB6 lacY1 tonA21* RP4-2-Tc::Mu-Km::Tn7 in chromosome	(Simon *et al.*, 1983)
S17.1	C600 ::*recA thi prohsdR-M*+ [RP4:2-Tc::Mu::Km:Tn7]	(Simon et al., 1983)
BL21(DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm met* (DE3)	Novagen
A. vinosum strains		
DSMZ 180^T	type strain	(Pfennig and Trüper, 1971) (Imhoff *et al.*, 1998)
185SM50	Sm^r, spontaneous streptomycin-resistant mutant of *A. vinosum* DSMZ 185	(Prange, unpublished)
Rif50	Rif^r, spontaneous rifampicin-resistant mutant of *A. vinosum* DSMZ 180^T	(Lübbe, unpublished)
Δ*soxX*	Km^r, *soxX*::KmΩ in DSMZ 180^T	(Hensen, 2001)
Δ*soxB*	Km^r, *soxB*::KmΩ in DSMZ 180^T	(Hensen, 2001)
Δ*soxBX*	Km^r, *soxBX*::KmΩ in DSMZ 180^T	(Hensen, 2001)
Δ*rhd*/ORF9	Sm^r, Km^r, *rhd*/ORF9::KmΩ in 185SM50	(Hensen, 2001)
Δ*soxY*	Rif^r, *in frame* deletion of *soxY* in Rif50	This work
Δ*soxX*+*X*	Km^r, Em^r, complementation of Δ*soxX* with plasmid pΔ*soxX*+*X*	This work
Δ*soxY*+*Y*	Rif^r, Km^r, complementation of Δ*soxY* with plasmid pΔ*soxY*+*Y*	This work

B.2.2 Plasmids

plasmid	genotype	source or reference
pGEM7 Zf(+)	Ap^r, *lacZ´*, f1 *ori*	Promega
pET-11a	Ap^r	Novagen
pET-22b	Ap^r, His Tag (C-terminal)	Novagen
pBBR1-MCS	Cm^r, Mob^+, *rep*, *lacZα*	(Kovach *et al.*, 1994)
pBBR1-MCS-2	Km^r, Mob^+, *rep*, *lacZα*	(Kovach *et al.*, 1995)
pHP45ΩKm	Ap^r, Km^r	(Fellay *et al.*, 1987)
pHP45ΩEm	Em^r	(Prange, unpublished)
pK19*mobsacB*	Km^r, Mob^+, *sacB*, *oriV*, *oriT*, *lacZα*	(Schäfer et al., 1994)
pGEM-SoxB	Ap^r, 4.5-kb *Nco*I fragment (*soxB* to ORFc) in pGEM5 Zf(+)	(Hensen, 2001)
pDHEcoYZ	Ap^r, 1.5-kb *Eco*RI fragment (ORFd to *soxZ*) in pGEM7 Zf(+)	This work
pDHClaYZ	Ap^r, 2.5-kb *Cla*I fragment (*soxY* to ORFf) in pGEM7 Zf(+)	This work
pExSoxA	Ap^r, 800-bp PCR fragment of *soxA* (*Nde*I/*Bam*HI) in *Nde*I/*Bam*HI of pET-11a	This work
pExSoxB	Ap^r, 1.8-kb PCR fragment of *soxB* (*Nde*I/*Xho*I) in *Nde*I/*Xho*I of pET-22b	This work
pExSoxYZ	Ap^r, 840-bp PCR fragment of *soxYZ* (*Nde*I/*Hin*dIII) in *Nde*I/*Hin*dIII of pET-22b	This work
pΔ*soxX+X*	Cm^r, Em^r, 4.5-kb *Apa*I/*Spe*I fragment from pGEM-SoxB in *Apa*I/*Spe*I of pBBR1-MCS, Em^r cartridge (*Sma*I) in *Eco*RV of construct	This work
pΔ*soxY+Y*	Km^r, 1.5-kb PCR fragment of *soxYZ* (*Xba*I) in *Xba*I of pBBR1-MCS2	This work

B.2.3 PCR primers

ClimYfor	5´- TTT CGT GCC AGT AAC GGT –3´
ClimZrev	5´- AGC ATG TCG CCT GCC TTG –3´
Km1	5´- TTG ATC CCC TGC GCC AT –3´
soxXforward	5´- AAC GTC AAT GAT CGA GAG –3´
soxXreverse	5´- GGT GGC GAT CCG TTC AGA –3´
Yforward	5´- AGG CCG TCT AGA ATT TCC GTG ACA CAT TGC –3´
Yreverse	5´- TGC GCC TCT AGA GGC TGG TTT CGA ATT CTA –3´
Ysoe-forward	5´- AGA GGA GAT AAA TCA ACA AGC TCT ATA AGA –3´
Ysoe-reverse	5´- TCT TAT AGA GCT TGT TGA TTT ATC TCC TCT –3´

Primer for gene expression in *E. coli*:

sABam	5´- GCG TTT CGG GAT CCT CAT TT –3´
sANde	5´- ACA TCG TCC ATA TGA CCA AG –3´
sBNde2	5´- TGC CCA GGC ATA TGT GTC CCA TGT –3´
sBXho	5´- TGC CCA GGC ATA TGT GTC CCA TGT –3´
soxZHindIII	5´- CGT TCC GAA GCT TGC TGA TCT C –3´
sYNde	5´- AGG AGA TAA CAT ATG ATC GAT GCC A –3´

Sequencing primer:

Y1for1	5′- ACG AGA CGA CCG TCA AGT –3′
Y1rev1	5′- ACC AGA TCA CCG AGG ATG –3′
Y2rev1	5′- ACT GAT TCT TGG CCG CGA –3′

B.3 Cultivation

B.3.1 PFENNIG medium (modified after Pfennig and Trüper, 1992)

PFENNIG medium was used for the cultivation of *A. vinosum* under photolithoautotrophic conditions. The medium consisted of four different solutions. The following quantities equal an amount of 10 litres of medium.

Solution 1: salt solution in 10 litre carboy

KCl	3,3	g
$MgCl_2$ x 6 H_2O	3,3	g
$CaCl_2$ x 2 H_2O	4,3	g
NH_4Cl	3,3	g
trace element solution SL 12 (10x)	10	ml
demineralised water	9250	ml

If the PFENNIG medium was used for the cultivation of *A. vinosum* 185SM50 and the corresponding mutant, 1% (w/v) NaCl was added to solution 1.

Solution 2: phosphate solution

KH_2PO_4	3,3	g
demineralised water	250	ml

Solution 3: carbonate solution

$NaHCO_3$	15	g
demineralised water	250	ml

Solution 4: sulfide solution

HNaS x H_2O	4 g
demineralised water	250 ml

Phosphate, carbonate and sulfide solution were autoclaved in tightly closed, brimful bottles. The solutions 1 to 4 were autoclaved separately. After sterilization solution 1 was cooled to room temperature. In the next step the solutions 2 to 4 were added under nitrogen atmosphere, resulting in a slightly pink and cloudy appearance of the medium. CO_2 was applied to the medium, until it lost its colour and cloudiness, corresponding to a pH value of pH 6.5 to 7.0. The completed medium was filled into brimful, tightly closed bottles and could be stored in the dark for several months.

If the PFENNIG medium was prepared without the addition of sulfide solution, it was referred to as 0 medium. For the production of 0 medium the pH value was adjusted towards the lower limit of the above mentioned pH span to prevent the carbonate from precipitating.

Trace element solution SL 12 (10 x) :

EDTA-Na x 2 H_2O	3	g
$FeSO_4$ x 7 H_2O	1,1	g
$ZnCl_2$	42	mg
$MnCl_2$ x 4 H_2O	50	mg
H_3BO_4	300	mg
$CoCl_2$ x 6 H_2O	190	mg
$CuCl_2$ x H_2O	2	mg
$NiCl_2$ x 6 H_2O	24	mg
Na_2MoO_4 x 2 H_2O	18	mg
demineralised water	ad 1000 ml	

The solution was stored unsterilised at 4°C.

B.3.2 Thiosulfate medium (Sperling, 2001)

Thiosulfate medium was used as an alternative to PFENNIG medium for photolithoautotrophic growth of *A. vinosum*. This medium was used to produce the large amounts of cell material necessary for protein purification. Thiosulfate medium consisted of two solutions, the following amounts needed for 10 l medium.

Solution 1:	100 x macro element solution	100 ml
	demineralised water	ad 9500 ml
Solution 2:	Na_2CO_3	26,5 g
	$NaHCO_3$	21 g
	$Na_2S_2O_3$ x $5H_2O$	31 g
	sulfide solution	25 ml
	demineralised water	ad 500 ml

100 x macro element solution:	KH_2PO_4	100 g
	NH_4Cl	70 g
	$MgSO_4$ x $7H_2O$	40 g
	$CaCl_2$ x $2H_2O$	10 g
	trace element solution SL 12 (10x, see B.3.1)	100 ml
	HCl (37 %)	193 ml
	demineralised water	ad 500 ml
Sulfide solution:	HNaS x $1H_2O$	7,4 g
	demineralised water	ad 100 ml

Solutions 1 and 2 were autoclaved separately. After cooling, solution 2 was added under stirring and nitrogen atmosphere. The medium pH was at a value of pH 7.5 without further titration.

B.3.3 RCV medium (Weaver *et al.*, 1975)

RCV medium was used for photoorganoheterotrophic cultivation of *A. vinosum*. To prepare this medium, 5 % (v/v) solution A (20 x) was mixed with 0,5 % yeast extract and 0,19 % NaOH, adjusted to a pH value of pH 7.0 and autoclaved. For cultivation of *A. vinosum* 185SM50 and the corresponding mutant 1 % (w/v) NaCl was added to this first solution. 5 % (v/v) solution B (20 x) was autoclaved separately and added after cooling.

20 x solution A:

malate	60	g
NH_4Cl	25	g
$MgSO_4$ x 7 H_2O	4	g
$CaCl_2$ x 2 H_2O	1,4	g
trace element solution SL12 (10x, see B.3.1)	20	ml
demineralised water ad	1000	ml

20 x solution B : potassium phosphate buffer

K_2HPO_4	180	mM
KH_2PO_4	180	mM
pH 7.0		

B.3.4 RCV solid medium

For cultivation of *A. vinosum* on solid medium 0,5 % NaCl and 1 % phytagel as a gelling agent was added to RCV medium and autoclaved separately from solution B. After a short cooling time the RCV medium was completed. Additionally 0,25 % (v/v) feeding solution, 0,2 % (v/v) sodium acetate and 0,2 % (v/v) thiosulfate solution were added before pouring the plates. The solid medium was prepared directly before use because of the volatile sulfide.

Solutions: 1 M sodium acetate, pH 7.0
0,4 M sodium thiosulfate
feeding solution (3,1 g HNaS x H_2O, 5 g $NaHCO_3$, ad 100 ml demineralised water)

The solutions were autoclaved and stored at room temperature.

B.3.5 Luria Bertani medium (LB medium, (Sambrook *et al.*, 1989))

LB medium was used for the cultivation of *Escherichia coli*.

tryptone	10 g
yeast extract	5 g
NaCl	5 g
demineralised water	ad 1000 ml
pH 7.5	

The liquid medium was aliquoted before sterilisation, usually in volumes of 5 ml. For the preparation of LB solid medium 1,5 % agar was added. For the selection of antibiotic-resistant mutants of *E. coli*, antibiotics were added after sterilisation and cooling to approximately 55°C (antibiotic concentrations see B.3.8).

B.3.6 2 x YT medium (Sambrook *et al.*, 1989)

E. coli was cultivated in 2 x YT medium for the preparation of competent cells used for transformation (see B.3.7).

tryptone	16 g
yeast extract	10 g
NaCl	5 g
demineralised water	ad 1000 ml
pH 7.0	

B.3.7 Preparation of competent *E. coli* cells

Solutions: 2 x YT medium

$CaCl_2$ / $MgCl_2$ solution (70 mM $CaCl_2$, 20 mM $MgCl_2$)

Competent cells of *E. coli* for transformation were prepared using the calcium chloride method after Dagert and Ehrlich (1974).

In the first step 5 ml of 2 x YT medium were inoculated with *E. coli* and incubated (over night, 180 rpm on a shaker at 37°C). 700 µl of this starting culture were used to inoculate 70 ml of 2 x YT medium in a shaking flask. The preparation was incubated as before, until an optical density at 600 nm of OD_{600}= 0,3 to 0,5 was reached. In the next step the culture was harvested (1900 x g, 4°C, 6 min). The resulting pellet was resuspended in 21 ml of $CaCl_2$ / $MgCl_2$ solution, incubated on ice for 30 to 45 minutes and harvested again. After resuspending the pellet in 7 ml of $CaCl_2$ / $MgCl_2$ solution, it was once more incubated on ice for 30 to 45 minutes. Finally, the preparation was mixed with 1750 µl of sterile glycerol and stored at –70°C in aliquots of 250 µl. This procedure for the preparation of competent cells was applied to all *E. coli* strains used in this work.

B.3.8 Antibiotic concentrations

For the selection of antibiotic-resistant clones of *A. vinosum* and *E. coli*, respectively, the following antibiotic concentrations were applied:

A. vinosum:	ampicillin	10 µg/ml
	erythromycin	10 µg/ml
	kanamycin	10 to 25 µg/ml
	rifampicin	50 µg/ml
E. coli:	ampicillin	100 µg/ml
	chloramphenicol	50 µg/ml
	erythromycin	100 µg/ml
	kanamycin	50 µg/ml

B.3.9 Production of recombinant Sox proteins in *E. coli*

For the production of recombinant proteins the *E. coli* strain BL21(DE3) was used. The respective gene, encoding for the protein to be produced, was ligated into a derivate of the pET plasmid series. The respective gene was amplified with the help of primers, that inserted restriction sites into the 5´ and 3´ end of the gene (applied primer pairs: SoxA→ sANde/sABam, SoxB→ sBNde2/sBXho, SoxYZ→ sYNde/soxZHindIII (see B.2.3)). All *sox* genes were amplified including the respective predicted signal peptide sequence. The plasmid-containing *E. coli* was first cultivated in 5 ml of LB medium with the appropriate antibiotic over night at 37°C as a starting culture. This culture was harvested (15700 x g, 5 min), and the pellet was resuspended in 0,5 ml of LB medium. This preparation was used to inoculate 100 ml of LB medium in a shaking flask, that was incubated at 37°C and 180 rpm, together with the appropriate antibiotic. After an optical density of OD_{600} = 0,5 was reached, 100 µl of 0,1 M IPTG solution was added to induce the gene expression. After an incubation period of 2 h the culture was harvested (16000 x g, 4°C, 15 min), and the pellet was stored at –20°C for potential further use. An *E. coli* culture without the pET plasmid derivative, that was used as a negative control, was treated the same way. Samples for the control of protein production were taken before induction and directly before harvesting. These samples were mixed with an equivalent volume of Rotiload 1, heated to 95°C for 10 min and subjected to SDS-PAGE.

B.3.10 Conservation of *A. vinosum* and *E. coli*

Solutions:

	A. vinosum	10 % (v/v) dimethylsulfoxide (DMSO)
	E. coli	glycerol

Both solutions were autoclaved and stored at room temperature.

A culture of *A. vinosum* was cultivated photoorganoheterotrophically on RCV medium (see B.3.3) for approximately 5 days. 50 ml of this culture were harvested (2500 x g, 20 min) and resuspended in 5-10 ml of RCV medium. This preparation was mixed with an equal volume of DMSO solution and stored in liquid nitrogen in Nunc reaction tubes (Wiesbaden, Germany).

The *E. coli* culture to be stored was grown over night on LB medium (see B.3.5), mixed with an equal volume of glycerol solution and stored at –70°C.

B.4 Isolation of DNA

B.4.1 Isolation of genomic DNA from *A. vinosum* liquid culture

Solutions:	TES buffer	100 mM NaCl, 10 mM TrisHCl, 1mM EDTA, pH 8.0
	saccharose TES buffer	20 % saccharose in TES buffer
	lysozyme RNAse solution	20 mg/ml lysozyme, 1mg/ml RNAse
	sarcosine solution	10 % (w/v) laurylsarcosine, 250 mM EDTA
	TE buffer	10 mM TrisHCl, 1mM EDTA, pH 8.0

For the isolation of genomic DNA from liquid culture, the sarcosyl lysis method after Bazaral and Helsinki (1968) was applied. *A. vinosum* was grown on RCV medium for approximately 5 days and harvested afterwards (2500 x g, 4°C, 10 min). The pellet was washed in 50 mM TrisHCl, pH 8.0 and used as starting material for DNA isolation. 50 to 80 mg of the cell material were resuspended in 2 ml ice-cold TES buffer. After centrifugation (15700 x g, 4°C, 10 min) 250 µl of saccharose TES buffer was added to the pellet, and the preparation was incubated on ice for 30 minutes. 250 µl of lysozyme RNAse solution was added, followed by incubation at 37°C (30 min, slight shaking) to achieve a disruption of the cells. After the addition of 100 µl of sarcosine solution, the material was drawn up into a sterile syringe (cannula: 1,2 x 49 mm), to achieve a shearing of the DNA. 300 µl of sterile water was added, and the DNA was subsequently purified via phenol / chloroform extraction (see B.4.3). After dialysis against TE buffer (3 h and 15 h, respectively) and sterile water (2 h), performed at 4°C in the dark, the isolated DNA was stored at 4°C.

B.4.2 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* using the QIAPrep Spin Miniprep Kit, provided by Qiagen (Hilden, Germany). For the procedure 3 to 9 ml of the respective plasmid-containing *E. coli* culture, grown over night on LB medium together with the appropriate antibiotic, was subjected to plasmid isolation. The procedure was carried out as described by the manufacturer. The DNA was eluted with 30 to 50 µl of sterile water and stored at –20°C.

B.4.3 Phenol / chloroform extraction

A phenol / chloroform extraction was carried out to remove contaminating protein from a DNA sample. One volume of phenol / chloroform / isoamylalcohol (25:24:1) was added to the DNA solution, and the latter was extracted by powerful shaking. After a centrifugation step (15700 x g, 5 min) the upper DNA-containing phase was removed for further use. This step was repeated with the DNA-containing fraction, until no further protein precipitation occurred. To remove traces of phenol from the DNA solution, a final extraction with chloroform / isoamylalcohol (24:1) was performed.

B.4.4 Purification of DNA after PCR or restriction digestion

The purification of DNA fragments could be accomplished by two different methods.
The first possibility was the direct purification from the digestion preparation with the help of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The procedure was performed as described by the manufacturer. The DNA was eluted in 30 µl of sterile water.
The second possibility was the electrophoretic separation of the digested DNA from unwanted DNA fragments or enzymes on an agarose gel (see B.7.1). The desired DNA fragment was cut out of the gel, the band visible in UV light after the preceding ethidium bromide stain. The DNA was isolated from the gel fragment using the above mentioned QIAquick Gel Extraction Kit.

B.4.5 Photometric determination of DNA concentration and purity

To determine the concentration and purity of isolated DNA, the absorption of a diluted sample was measured at 260 nm and 280 nm, corresponding to the absorption maxima of DNA and protein at the respective wavelengths (Sambrook *et al.*, 1989). The DNA concentration was calculated as follows: an absorption of 1 at 260 nm equals 50 ng of DNA per ml concerning double stranded DNA (Sambrook *et al.*, 1989). The DNA purity was determined by the ratio of the absorption at 260 nm and 280 nm. A ratio of 1,8 to 2 is desirable, values below indicate a protein contamination, values above a contamination with RNA.

B.5 Amplification of DNA by PCR

B.5.1 PCR standard protocol

The use of PCR (polymerase chain reaction) allows the amplification of great amounts of DNA fragments with a defined length from known DNA sequences (Mullis *et al.*, 1986).
The primers used as starting points for the polymerase should be chosen to be complementary to the ends of the DNA fragment to amplified, with the 3´-ends running towards each other. Furthermore, a GC content of approximately 50 % and a lack of potential hairpin structures is aimed for. With the template DNA as a model, a thermostable DNA polymerase from either *Thermococcus aquaticus* (*Taq* polymerase) or *Pyrococcus furiosus* (*Pfu* polymerase) carries out an *in vitro* synthesis of new defined DNA strands. In contrast to the *Taq* polymerase, the *Pfu* polymerase exhibits a 3´-5´ exonuclease activity (proofreading). This leads to a decrease in the rate of falsely integrated nucleotides (a factor of about 12), compared to the *Taq* polymerase with a mistake rate of $8{,}5 \times 10^{-6}$ nucleotides /cycle, at the cost of a slower amplification rate.
A standard PCR was carried out in a total volume of 50 µl in a PCR tube of 500 µl volume using a Biometra TRIO-Thermoblock (Göttingen, Germany). A typical PCR reaction contained the following:

template DNA	0,1-0,5 µg
primer 1	50 pmol
primer 2	50 pmol
nucleotides (dATP, dTTP, dCTP, dGTP)	200 µM each
10 x polymerase buffer	5 µl

When using the *Pfu* polymerase no additional $MgCl_2$ (necessary for *Taq* polymerase) was needed, because the polymerase buffer provided everything necessary. For the *Taq* polymerase $MgCl_2$ concentrations between 1 and 2 mM were applied. After adding up to 50 µl with sterile demineralised water, the preparation was overlaid with mineral oil to minimize evaporation. The PCR reaction was started by the addition of the Polymerase (*Taq*: 2,5 U; *Pfu*: 1,25 U) during the first denaturing step (“hot start”).
A PCR program starts with the initial denaturation of the DNA double strand at 95°C. After another short denaturation step (first part of the repeated cycle) the primer annealing takes

place. The annealing temperature is dependent on the GC and AT content of the applied primers: $T_m = 4 \times (G + C) + 2 \times (A + T)$ °C

The actual annealing temperature should be about 4 degrees lower than the calculated primer melting temperature T_m.

The PCR stringency can be influenced by the annealing temperature and, using *Taq* polymerase, the $MgCl_2$ content. The following elongation of the DNA strands by the polymerase takes place at 72 °C. The duration of this step is dependent of the size of the fragment to be amplified and the speed of the applied polymerase. Because of the exercised proofreading function, the *Pfu* polymerase exhibits only half the speed of the *Taq* polymerase (the latter with approximately 1 kb/min). Because of the cycle repetition an exponential amplification of the DNA occurs.

Standard PCR program:

initial denaturation	95°C	3 min	
denaturation	95°C	30 sec	
annealing	y °C	30 sec	x 30-35
elongation	72°C	z min	
final elongation	72°C	z + 5 min	
storage	4°C	hold	

[y dependent on primer composition, z dependent on fragment size]

B.5.2 Colony PCR

The colony PCR represents a slightly modified PCR in so far, as not precedingly purified DNA was used as template, but cell material from *A. vinosum*. The material was taken either from liquid culture (50µl, precipitated by centrifugation (15700 x g, 5 min) and resuspended in 50 µl of demineralised water) or from solid medium (colony material resuspended in 50 µl RCV medium, further treatment as described above). From both preparations a volume of 1 µl was applied as a PCR template. The initial denaturation time was extended to 10 minutes (for better cell destruction), and the polymerase was added after this initial step was finished.

B.5.3 Construction of a digoxigenin-labelled probe for Southern hybridisation

When a digoxigenin-labelled DNA probe was needed for southern hybridisation, a part of the nucleotide dTTP in the PCR reaction was replaced by digoxigenin-dUTP. This nucleotide was inserted into the growing DNA strand instead of dTTP. After the PCR the preparation was electrophoretically separated. After staining and documentation (see B.7.2) the desired DNA fragment was excised and eluted from the gel. The isolated DNA was used as a probe for Southern hybridisation (see B.8).

B.5.4 Splicing by overlap extension (SOEing) PCR

Gene SOEing (gene splicing by overlap extension) is used for site directed mutagenesis and recombination of DNA molecules (Horton, 1995). The method of SOEing PCR was applied to insert *in frame* mutations into the *A. vinosum* genome. This method guarantees the sole deletion of one gene, without the polar effects caused by the inserted resistance cassettes, because the reading frame stays intact. The method was used for the *in frame* deletion of *soxY*. This method is based on the possibility to modify PCR products by adding sequences at the 5´ end of the applied primers. Thereby overlapping fragments are produced that can be successively extended.

DNA fragments upstream and downstream of *soxY* were amplified. To the primers flanking the region to be deleted (Ysoe-reverse and Ysoe-forward, see Figure B1) a 15 bp sequence was added at the 5´ end, that was complementary to the other respective primer. This resulted in an overlapping region of 30 bp. The DNA fragments of PCR1 and PCR 2 could function as their respective primers in the following extension reaction in PCR 3. By choosing the primers it had to be taken care to maintain the reading frame of the genes downstream of the deletion. *Xba*I restriction sites were inserted into the outer primers (Yforward and Yreverse) for successive cloning of the PCR fragment into the plasmid pK19*mobsacB*.

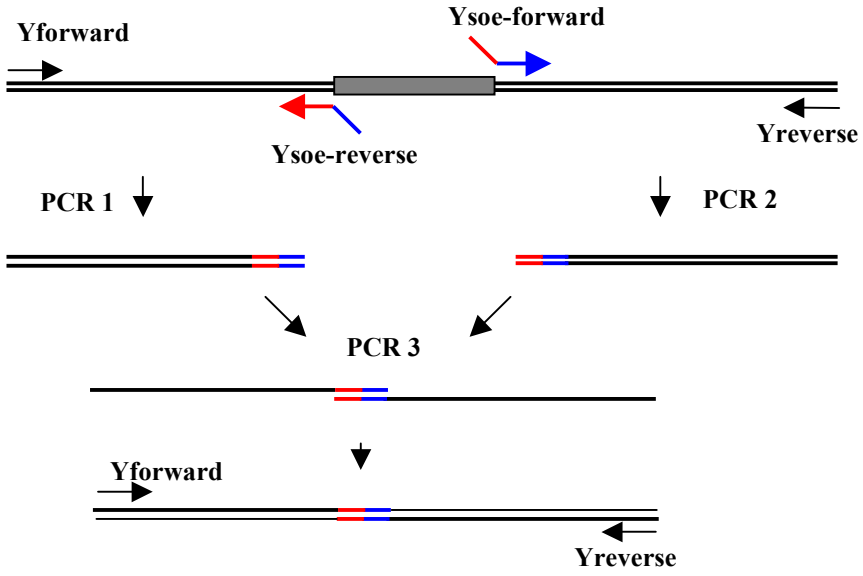

Figure B1: Gene splicing by overlap extension (SOEing) PCR to obtain the *in frame* deletion of *soxY*. The sequence to be deleted is depicted in gray. The complementary sequences of the inner primers are colour-coded.

The first two PCR reactions were performed after the standard protocol (PCR 1: Yforward /Ysoe-reverse, PCR 2: Ysoe-forward / Yreverse). For the third PCR reaction an amount of 1,5 µl fragment of PCR 1 and PCR 2, respectively, was added to the preparation, together with the two outer primers. PCR 3 took place in two separate reactions. In the first reaction the fragments from PCR 1 and PCR 2 served as their respective primers. The annealing temperature corresponded to the overlapping region. In the second part of the PCR the joined fragment was amplified, making use of the outer primers and therefore using a different annealing temperature. The program for PCR 3 (for the specific case of *soxY*) was chosen as follows:

initial denaturation	95°C	5 min	
denaturation	95°C	1 min	
annealing (PCR fragments)	68°C	1 min	x 14
elongation	72°C	3 min	
denaturation	95C	1 min	
annealing (outer primers)	65°C	1 min	x 30
elongation	72°C	3 min	
prolonged elongation	72°C	10 min	
storage	4°C	hold	

B.6 Enzymatic DNA modification

B.6.1 Digestion with restriction enzymes

All DNA digestions using restriction endonucleases were performed after Sambrook *et al.* (1989). The restriction buffers were used according to the instructions provided by the manufacturer (MBI Fermentas, St.Leon-Rot, Germany). The total preparation volume was between 20 µl and 100 µl with the use of 1-2 U of enzyme per µg DNA. The digestion was performed for 1,5 to 3 h with plasmid DNA and 4 to 6 h with genomic DNA at a temperature recommended by the manufacturer.

B.6.2 Heat inactivation

When the successive use of different enzymes was aimed for in a DNA digestion preparation, the preceding heat sensitive enzymes were inactivated at 65°C for 15 minutes. This step was necessary to minimize negative effects on the performance of the following enzymes.

B.6.3 Dephosphorylation

A dephosphorylation leads to the removal of 5´-phosphate groups from DNA fragments. Thereby the rate of undesired re-ligations within a DNA fragment could be minimized. For the reaction 1 U of CIAP (calf intestine alkaline phosphatase) was added to the preparation and incubated at 37°C for 1h.

B.6.4 Ligation

The ligation of digested DNA fragments into an equally digested plasmid was performed in a total volume of 10 to 20 µl. The preparation contained digested plasmid and insert DNA (in a ratio of approximately 1 to 3), 1 Weiss unit of T4 DNA ligase and the corresponding amount of the provided 10 x ligase buffer. One Weiss unit is equivalent to approximately 200 cohesive-end ligation units (with one cohesive-end ligation defined as the amount of enzyme required to give 50 % ligation of *Hin*dIII fragments of lambda DNA under given conditions, for specifications see manual of the manufacturer (MBI Fermentas, ST.Leon-Rot, Germany)). The preparation was incubated at room temperature for 3 h or over night at 16°C.

B.6.5 DNA sequencing

DNA sequencing was performed at the facilities of Sequiserve (Vaterstetten, Germany).

B.7 Electrophoretic DNA separation

B.7.1 Agarose gel electrophoresis

Solutions:	50 x TAE buffer	2 M Tris, 1 M acetic acid, 0,5 M EDTA, pH 8.0
	10 x sample buffer	0,25 % bromphenol blue, 40 % saccharose

For DNA fragment separation gels with an agarose concentration of 1 % (w/v) were prepared with 1 x TAE buffer, that was also used as electrophoresis buffer. The electrophoresis was performed in the gel chambers Horizon 58 or Horizon 11-14, obtained from Gibco BRL (Eggenstein, Germany). Before the application on the gel, 0,1 volume of 10 x sample buffer was added to the preparation. The included saccharose provided the necessary weight, and bromphenol blue indicated the running front. The separation in the small gel chamber (Horizon 58) was finished after approximately 2 h at 70-100 V, larger gels (Horizon 11-14) were run over night at 15-20 V.

B.7.2 Staining and documentation of agarose gels

Ethidium bromide was used for the staining of agarose gels, which intercalated into the DNA and thereby making it visible in UV radiation. The staining was carried out with a 0,1 % ethidium bromide solution for 5 to 10 minutes. A transilluminator, using UV light, was used for documentation with the help of a video documentation device of the company INTAS (Göttingen, Germany).

B.8 Southern hybridisation

B.8.1 Southern transfer

Solutions:	20 x SSC	3 M NaCl, 0,3 M sodium citrate, pH 7.0
	blotting buffer	0,4 M NaOH

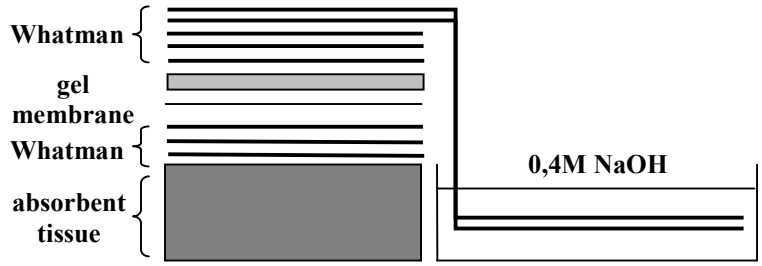

Figure B2: Assembly used for Southern transfer

The digested and electrophoretically separated DNA was transferred onto a Sartolon nylon membrane from Sartorius (Göttingen, Germany) by capillary blot. After staining with ethidium bromide and a short washing step with demineralised water, the gel was incubated for 10 min in 0,25 M HCl. Onto a batch of absorbent tissue were placed three layers of Whatman 3MM paper, soaked in blotting buffer. On top were successively stacked the soaked membrane, the gel, three more layers of soaked Whatman paper and two larger soaked layers of Whatman paper as a liquid bridge (see Figure B2). After two hours of DNA transfer the membrane was shortly rinsed in 2 x SSC, and the transferred DNA was covalently linked to the membrane by UV crosslinking (UV Stratalinker 1800, Stratagene). The membrane was either directly used for hybridisation or stored shrink-wrapped on Whatman paper at 4°C until further use.

B.8.2 Hybridisation

Solutions:	buffer 1	0,1 M maleic acid, 0,15 M NaCl, pH 7.5
	prehybridisation solution	2 % (v/v) buffer 1, 2,5 % (v/v) 20x SSC, 0,3 mM N-lauroyl sarcosine, 0,06 mM sodium lauryl sulfate (SDS) 2 % blocking reagent

For hybridisation with the DNA fragments immobilized on the nylon membrane, a single stranded digoxigenin-labelled DNA probe was used (see B.5.3).
To prevent unspecific binding of the DNA probe to the membrane, the first step after Southern transfer was the saturation of the membrane with blocking reagent. This was achieved by at least 4 hours incubation with 20 ml of prehybridisation solution in a Hybaid Mini hybridisation oven. The temperature for prehybridisation was chosen according to the temperature applied for the later hybridisation step. The temperature was dependent on the use of either a heterologous (→ 60°C) or a homologous DNA probe (→ 68°C). When employing a heterologous DNA probe, the temperature was lowered to decrease the stringency of hybridisation. After prehybridisation the DNA probe was heated to 100°C for 15 min, added to the membrane and incubated over night for 16 to 18 h at the chosen temperature. Afterwards the probe was stored at –20°C for further use.

B.8.3 Chemiluminescence detection

Solutions:	2 x SSC + 1 % SDS	
	0,1 x SSC + 1 % SDS	
	washing buffer	0,3 % (v/v) Tween 20 in buffer 1
	buffer 2	1 % (w/v) blocking reagent in buffer 1
	buffer 3	0,1 M Tris, 0,1 M NaCl, pH 9.5

Preceding the detection of the hybrid DNA strands on the membrane, unspecifically bound probe first had to be removed from the membrane by several washing steps. The membrane was twice incubated in 2 x SSC + 1 % SDS solution for 5 min at room temperature. To increase the stringency, the two following washing steps were carried out using

0,1 x SSC + 1 % SDS solution at the chosen hybridisation temperature. All the subsequent steps were performed at room temperature.

After 5 min incubation with washing buffer, buffer 2 was applied to the membrane and incubated for 30 min to once more saturate the unspecific binding sites of the membrane before incubation with the antibody. The first step in the actual detection of the hybrid DNA strands was the addition of anti-dig-AP in buffer 2 (0,013 %(v/v)) for 30 min. This anti-digoxigenin alkaline phosphatase antibody conjugate binds to the dig-dUTP used for DNA probe labelling. Unbound and unspecifically bound antibody was removed from the membrane by twice washing it in washing buffer for 15 min. The membrane was prepared for the chemiluminescence substrate by 5 min incubation in buffer 3, thereby adjusting the membrane pH to the desired value of pH 9.5. The substrate solution (CDP-Star, at a dilution of 1:1000 in buffer 3) was added to the membrane and incubated for 20 min. Afterwards the membrane was shrink-wrapped on Whatman 3MM paper. The alkaline phosphate linked to the antibody splits the CDP star, resulting in an emittance of light detectable on X-ray film. The film was exposed for 10 min up to several hours, depending on the signal strength, to obtain the desired signals.

B.9 DNA transfer

B.9.1 Plasmid transfer to *E. coli* by transformation

This method was applied for the transfer of plasmid DNA into competent cells of *E. coli* (see B.3.7). Competent cells of *E. coli* stored at –70°C were first thawed on ice. The whole ligation preparation or 1 µl of an already prepared plasmid were added to 100 µl of the thawed cell material and incubated on ice for 30 min. After heat shock at 42°C for 90 sec, the cells were immediately cooled on ice for 2 min, mixed with 500 µl of 2 x YT medium (see B.3.6) and incubated at 37°C for 1 h to recover from the procedure. The cell material was brought out onto selective LB solid medium (see B.3.4) containing the appropriate antibiotic for selection of positive clones (see B.3.8).

B.9.2 Plasmid transfer to *A. vinosum*

Two methods for plasmid transfer into *A. vinosum* were applied: electrotransformation and conjugation. The former was established during my diploma thesis (Hensen, 2001).

B.9.2.1 Electrotransformation

Solutions: RCV medium (B.3.3)
10 % (v/v) dimethylsulfoxide (DMSO)
1 M sodium acetate
0,4 M sodium thiosulfate

To produce electrocompetent cells, a culture of *A. vinosum* was grown photoorganoheterotrophically on RCV medium to an optical density of approximately 0,6 at 690 nm, corresponding to the exponential growth phase. Cell material equivalent to a total cell number of about 2 x 10^{10} (approximately 45 ml) was harvested (7500 x g, 4°C, 15 min). The pellet was washed three times in 2 ml of ice cold DMSO solution, precipitated again (5900 x g, 4°C, 10 min) and finally resuspended in 1 ml of the same solution. This cell material was used directly for electrotransformation, as further storage significantly decreased the transformation efficiency. The plasmid transfer was performed in electrotransformation cuvettes with 2 mm width, using a Gene Pulser II together with a Pulse Controller Plus (Biorad, München). One preparation contained 50 µl of cell solution and 0,1 to 0,5 µg of purified plasmid DNA. After mixing and incubation on ice for 15 min, the electrical pulse was applied (200 Ω, 25 µF; equally good results obtained with 9 kV cm^{-1} and 12,5 kV cm^{-1}, respectively). The time constant τ should be about 5 ms under the chosen conditions. Directly after exposure to the electric pulse the cuvette was placed on ice. The cell material was resuspended in 2,5 ml of RCV medium, supplied with 10 mM sodium acetate and 4 mM sodium thiosulfate, and incubated in the light for 18 hours in brimful, tightly closed glass vials. For the selection of transformants *A. vinosum* was plated on selective RCV solid medium, containing the appropriate antibiotic, and was incubated anaerobically in the light.

B.9.2.2 Plasmid transfer from *E. coli* to *A. vinosum* by conjugation

The method of plasmid transfer in *A. vinosum* by conjugation was established in the mid nineties (Pattaragulwanit and Dahl, 1995). Donor strain for the conjugative plasmid transfer was either *E. coli* SM10 or *E. coli* S17.1. On LB solid medium with the appropriate antibiotic (see B.3.4 and B.3.8) an over night culture of the plasmid-harbouring *E. coli* strain was cultivated at 37°C. The recipient strain *A. vinosum* Rif50 (a spontaneous rifampicin resistance mutant of *A. vinosum* DSM180) was grown on RCV medium (see B.3.3) to the stationary phase (OD_{690} ~ 1,5). The total cell number was determined by the measured optical density at 690 nm (Pattaragulwanit, 1994). A culture volume containing approximately 12 x 10^8 cells of *A. vinosum* was harvested by centrifugation at 9300 x g for 5 min. The resulting pellet was washed twice in 0,5 ml RCV medium and finally resuspended in 0,5 ml of the same solution. *E. coli* cell material was suspended in 3 ml RCV medium to an optical density at 600 nm of approximately 0,8. An OD_{600} of 0,1 is equivalent to a total cell number of 1 x 10^9 cells/ml (Sambrook *et al.*, 1989).

Equal amounts of *A. vinosum* and *E. coli* suspension (0,5 ml each) were carefully mixed, resulting in a three times higher cell number of *A. vinosum* compared to *E. coli*. The mixture was sedimented (9300 x g, 5 min) and resuspended in a little volume of the supernatant. The cell suspension was applied onto a sterile cellulose nitrate filter (0,45 µm, Sartorius (Göttingen, Germany)), lying on RCV solid medium without added antibiotics. After two days of anaerobic incubation in the light, the filter was transferred to a reaction tube to wash the cells from the filter with 1 ml RCV medium. For the selection of transconjugants the cell material was brought out onto the appropriate selective RCV solid medium and incubated anaerobically in the light.

B.10 Determination of protein concentration

B.10.1 Determination of protein concentration: Lowry

Solutions:	solution A	2 % Na_2CO_3 in 0,1 M NaOH
	solution B	0,5 % $CuSO_4$ x 5 H_2O
	solution C	1 % potassium sodium tartrate
	solution D	48 ml A + 1 ml B + 1 ml C
	solution E	Folin Ciocalteu reagent (diluted 1: 2)

Protein determination by the method of Lowry was partly used for liquid culture of *A. vinosum*. A sample of 200 µl liquid culture was mixed with 1 ml ice-cold acetone / methanol (7:2) on ice for at least 10 min to decolourise the cells and precipitate the protein. After centrifugation (15700 x g, 4°C, 10 min) the pellet was dried and resuspended in 100 µl demineralised water. 1 ml of freshly prepared solution D was added, and the preparation was incubated in the dark for 10 min. After the addition of 100 µl of solution E and a second incubation for 30 min, the absorption of the supernatant (15700 x g, 5 min) was determined at 500 nm against a reagent blank. A calibration curve was recorded at a range of 0 to100 µg BSA per 200 µl preparation.

B.10.2 Determination of protein concentration: BCA

Protein determination by the BCA protein assay (Perbio (Bonn, Germany)) was partly used for fermenter samples, but mostly for the determination of protein concentration after chromatographic purification steps. The analysis was performed in small volumes in 96-well plates and essentially as described by the manufacturer. 25 µl of protein sample was mixed with 200 µl working reagent and incubated at 37°C for 30 min. Afterwards the absorption was measured at 550 against a reagent blank. A calibration curve was recorded at a range of 0 to 500 µg BSA ml^{-1}.

B.10.3 Determination of protein concentration: Bradford

The use of Bradford reagent was the method of choice for the determination of protein concentration in liquid culture of *A. vinosum* (e.g. fermenter samples). An amount of 100 µl

liquid culture was centrifuged (15700 x g, 10 min) and resuspended in 33 µl of demineralised water. 1 ml of Bradford reagent (Sigma (Taufkirchen)) was added to the preparation, mixed and incubated for 10 min. The absorption was measured at 595 nm against a reagent blank. A calibration curve was recorded at a range of 0 to 500 µg BSA ml^{-1}.

B.11 Electrophoretic protein separation

B.11.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions :	solution A	1,5 M TrisHCl, 0,3 % SDS, pH 8.8
	solution B	0,5 M TrisHCl, 0,4 % SDS, pH 6.8
	5 x electrophoresis buffer	15 g Tris, 72 g glycine, 5 g SDS, ad 1000 ml demineralised water

Protein electrophoresis was performed using a discontinuous SDS polyacrylamide gel, consisting of a stacking gel for sample focussing and a running gel for the actual separation. The amount of the 30 % acrylamide / bisacrylamide solution (Roth (Karlsruhe, Germany)) was varied, dependent on the size of the protein to be analysed (the higher the molecular weight of the protein, the lower the acrylamide / bisacrylamide concentration). The following table summarizes the composition of different gels:

	10 %	12,5 %	15 %	4,5 %
demineralised water	5 ml	4 ml	3 ml	3 ml
solution A	3 ml	3 ml	3 ml	-
solution B	-	-	-	1,25 ml
Acrylamide / bisacrylamide	4 ml	5 ml	6 ml	0,75 ml
10 % (w/v) APS[1]	100 µl	100 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl	5 µl

[1] : ammoniumperoxosulfate

The gels were prepared by mixing the chemicals in the order stated above, the polymerisation was started by the addition of APS and TEMED. The recipe provided enough solution to cast two gels (thickness of 0,75 or 1 mm) in a Mini Protean II or 3 gel chamber (BioRad

(München, Germany)), respectively. Both running and stacking gel needed about 60 min for polymerisation. Protein samples were mixed with 0,25 x volumes of 4 x sample buffer (Rotiload 1, Roth (Karlsruhe, Germany)) and heated to 95°C for 5 min. After cooling on ice the samples were loaded onto the stacking gel. The electrophoresis was performed with 1 x electrophoresis buffer at 60 to 100 V. The PageRuler Prestained Protein Ladder by MBI Fermentas (St.Leon-Rot, Germany), that could also be used for Western blotting (see B.11.3) was used as a marker for molecular weight determination.

B.11.2 Coomassie stain

Solutions:	Coomassie staining solution	0,25 % Coomassie Blue R250, 50 % methanol, 10 % acetic acid, 40 % demineralised water
	decolourising solution / fixer	10 % acetic acid, 20 % methanol, 70 % demineralised water

After protein electrophoresis the stacking gel was removed, and the running gel was transferred into Coomassie staining solution. After at least 30 min incubation the staining solution was replaced by decolourising solution / fixer. The latter was exchanged several times, until the protein bands were clearly visible. The gel was conserved by vacuum drying on an Aldo-Xer gel dryer (Schütt (Göttingen, Germany)).

B.11.3 Electroblotting (Western blotting)

Solution:	Towbin blotting buffer	1,52 g Tris, 7,2 g glycine, 100 ml methanol, ad 500 ml demineralised water

The protein transfer from the SDS gel onto a cellulosenitrate membrane (Protran BA 85 by Schleicher & Schuell (Dassel, Germany)) was accomplished with the help of a Transblot SD Semi-Dry transfer call (Biorad (München, Germany). After electrophoresis and discarding of the stacking gel, the running gel was transferred to Towbin blotting buffer and incubated for 15 min. A gel-sized nitrocellulose membrane was also incubated in Towbin blotting buffer for an equal amount of time. For the transfer three layers of Whatman 3MM paper, slightly larger than membrane and gel and soaked in blotting buffer, were placed on the anode. Membrane,

gel and another three layers of soaked Whatman 3MM paper (this time gel-sized) were stacked onto the first layer of Whatman 3MM paper. The cathode was placed on top. The protein transfer was performed at 15 V. The transfer time was dependent on the size of the protein to be transferred (15 min for SoxYZ, 30 min for SoxXA, 45 min for SoxB).

B.12 Protein detection methods

The immunological detection of proteins after the transfer onto a nitrocellulose membrane was performed with antibodies raised in rabbits. The applied antigen was either a synthetically produced and potentially immunogenic peptide of the protein in case of SoxA or the complete proteins of *P. pantotrophus* in case of SoxB (Friedrich *et al.*, 2000) and SoxYZ (Quentmeier *et al.*, 2003). All antisera were produced at the facility of Eurogentec (Seraing, Belgium). The antisera against SoxB and SoxYZ were thankfully provided by the group of Cornelius Friedrich in Dortmund. Depending on the strength of the obtained signal, the detection was either performed with 4-chloro-1-naphthol (SoxA and SoxB, see B.12.1) or with the SuperSignal® West Pico Chemiluminescent substrate (SoxYZ, see B.12.2).

B.12.1 Immunological protein detection with 4-chloro-1-naphthol

Solution:	10 x PBS	80 g NaCl, 2 g KCl, 6,1 g Na_2HPO_4, 2 g KH_2PO_4, ad 1000 ml demineralised water, after dilution to 1x PBS check pH (approx. pH 7.3)

Directly after Western transfer the membrane was incubated over night in 100 ml 1 x PBS with 5 % (w/v) skim milk at 4°C. After 1 h incubation at room temperature the membrane was washed five times with 1 x PBS for 5 min, respectively. The primary antibody was applied at a concentration of 1:1000 in 20 ml 1 x PBS with 0,1 g BSA for three (SoxA) and four (SoxB) hours, respectively. After a triple washing step (1 x PBS, 5 min) the secondary antibody was applied. An anti-rabbit antibody with a coupled horse radish peroxidase was used in a dilution of 1:5000 in 20 ml 1x PBS with 0,1 g BSA and incubated for 1 h. After two more washing steps the blot was developed. The membrane was transferred to 43 ml demineralised water, 7 ml ethanol containing 30 mg 4-chloro-1-naphthol was added and the reaction was started by the addition of 20 µl H_2O_2. After appearance of the desired black bands, the reaction was

stopped by repeatedly washing the membrane with demineralised water. Except for the last developing step, all incubations were performed while shaking.

B.12.2 Immunological protein detection with SuperSignal® substrate

Solutions:	20 x TBS	160 g NaCl, 1 M TrisHCl, ad 1000 ml demineralised water
	1 x TBS + Tween	50 ml 20 x TBS, 0,05 % (w/v) Tween 20, ad 1000 ml demineralised water, check pH (approx. pH 7.5)

After Western transfer the membrane was incubated for 1 h in 100 ml 1 x TBS + Tween with 5 % (w/v) skim milk. With preceding washing steps not necessary, the primary antibody was applied at a concentration of 1:1000 in 20 ml 1 x TBS + Tween with 0,1 g BSA and incubated over night. After washing the membrane five times with 1 x TBS + Tween for 5 min, respectively, the secondary antibody was applied at a concentration of 1:5000 in 20 ml 1 x TBS + Tween with 0,1 g BSA and incubated for 1 h. After washing the membrane as before, the Western blot was developed using the SuperSignal West Pico chemiluminescent substrate as specified by the manufacturer (Perbio (Bonn, Germany)). Apart from the developing step, all incubations were performed while shaking. The X-ray film was exposed until the bands were clearly visible (10 min to 18 h).

B.12.3 Detection and characterisation of cytochromes

Two methods for heme staining with different sensitivity were applied. While the method for heme detection performed directly in the gel after SDS-PAGE is normally sufficient, the more sensitive staining after blotting the proteins on a membrane could also be used in more difficult cases.

Detection of cytochromes in polyacrylamide gels:

Solutions:	fixer	10 % acetic acid, 20 % methanol
	neutralising solution	100 mM TrisHCl, pH 7.0
	staining solution 1	0,05 % 3´3´-diaminobenzidine in 100 mM TrisHCl, pH 7.0
	staining solution 2	0,1 % 3´3´-diaminobenzidine and 2 % H_2O_2 in 100 mM citrate buffer, pH 4.0
	stopping solution	7 % acetic acid

The identification of cytochromes directly in the polyacrylamide gel was performed after McDonnel and Staehelin (1981), using the peroxidase activity of heme groups for detection. The gel was incubated in the fixer for 30 min and adjusted to a pH value of pH 7.0 by a short incubation in neutralising solution. The gel was saturated with diaminobenzidine by a 30 min incubation in staining solution 1. The actual staining took place over night at 4°C with the use of staining solution 2. The proteins containing heme should be visible as brown bands. Horse heart cytochrome c was applied as a positive control. The staining was stopped by incubation in the stopping solution.

Detection of cytochromes after Western blotting

For the detection of cytochromes after Western blotting, the proteins were blotted onto a PVDF membrane, that was prepared as described by the manufacturer (Immobilon-P from Millipore (Schwalbach, Germany)). The detection was performed using the SuperSignal substrate, also making use of the peroxidase activity of heme groups. Because of the use of chemiluminescence instead of colorimetric methods (see above) the sensitivity of the detection was significantly increased. After the blotting 0,125 ml of working solution per cm^2 were applied onto the membrane. The working solution was prepared as described by the manufacturer. After 5 min incubation the solution was discarded, and the membrane was shrink-wrapped. A X-ray film was exposed immediately for 15 sec up to several hours, depending on the signal strength. The cytochrome-containing proteins were visible as black bands on the film. Horse heart cytochrome c was again used as a positive control.

Recording of pyridine spectra

Solutions:	pyridine solution	200 mM NaOH, 40 % (v/v) pyridine
	ferricyanide solution	100 mM $K_3Fe(CN)_6$

The recording of pyridine spectra was performed after Berry and Trumpower (1998). A protein sample (0,5 ml, heme concentration below 5 µM) was mixed with 0,5 ml pyridine solution and 3 µl ferricyanide solution, and the hemichrome spectrum was recorded. After the addition of a small amount of dithionite, the hemochrome spectrum was recorded. The spectra were recorded using a Perkin Elmer Lambda 11 spectrometer. A solution of 0,5 ml gel filtration stabilising buffer (see B.13.5.3), 0,5 ml pyridine solution and 3 µl ferricyanide solution was used as a reference. The heme concentration was determined using the extinction coefficient table by Berry and Trumpower.

UV/vis spectra of cytochrome-containing protein samples

To determine the presence of c-type cytochromes in protein samples during and after purification, spectra were recorded using either the Diode Array Spectrometer Agilent 8453 (during purification) or the Perkin Elmer Lambda 11 spectrometer (after the final gel filtration). The cytochromes were identified by their characteristic spectrum.

B.13 Protein purification

B.13.1 Cell harvesting and disruption

Solutions:	phenylsepharose buffer	50 mM potassium phosphate buffer, pH 7.5
	stabilising buffer PS	50 mM potassium phosphate buffer, 2 mM sodium thiosulfate, 1 mM magnesium sulfate, 1 µM phenylmethylsulfonylfluoride (PMSF), pH 7.5

A culture of *A. vinosum*, grown photolithoautotrophically on 10 l thiosulfate medium (see B.3.2), was harvested by centrifugation (10000 x g, 4°C, 20 min) after approximately five

days. The pellet was stored at –20°C until further use. The thawed cell material was resuspended either in phenylsepharose buffer or in stabilising buffer PS (depending on the applied purification strategy) at a ratio of 3 ml buffer per 1 g wet weight. After homogenisation the cells were disrupted by ultrasonic treatment (Cell Disruptor B15, Branson) at 50 % intensity and a duration of 1 min ml^{-1}. The following centrifugation step (27000 x g, 4°C, 30 min) was used to remove cell debris. The supernatant was referred to as crude extract. It was subjected to ultracentrifugation to separate membrane and soluble fraction. The crude extract was centrifuged at 145000 x g and 4°C for 3 h. The obtained supernatant was referred to as soluble fraction, while the pellet, resuspended in the respective buffer, was referred to as membrane fraction.

B.13.2 Ammonium sulfate precipitation

The precipitation of proteins with ammonium sulfate was used as a first purification step and as a preparation for the hydrophobic interaction chromatography (see B.13.5). The protein solution was stirred on ice and finely crushed powder of $(NH_4)_2SO_4$ was successively added up to a final saturation of 40 %. The amount of substance needed was determined by using tables by Wood (1976). After the complete amount of ammonium sulfate was added, the solution was stirred at 4°C over night. Precipitated proteins were removed the next day by centrifugation (27000 x g, 4°C, 30 min). The supernatant was subjected to hydrophobic interaction chromatography. The pellet, if needed, was resuspended in a small volume of buffer.

B.13.3 Dialysis of protein samples

To remove undesired salts from protein samples and to change buffers, the protein solution was dialysed over night against 4 l of the appropriate buffer at 4°C. The proteins were placed in dialysis tubes with a molecular weight cut off of 6000 to 8000 Da (Serva (Heidelberg, Germany)). To prepare the dialysis tubes for further use, they were autoclaved for 10 min in 1 mM EDTA, pH 8.0, 2 % Na_2CO_3, washed in demineralised water and autoclaved again for 10 min in 1 mM EDTA, pH 8.0.

B.13.4 Concentration of protein samples

Protein samples were concentrated using either polyethylenglycol (PEG 20000) or ultrafiltration centrifugation. Using the first method, the protein sample was transferred to a dialysis tube, that was covered with PEG 20000 and stored at 4°C until the desired reduction in volume was achieved. For the second method, ultrafiltration tubes (Centriplus YM 10, Millipore, Bedford, USA) were used with a molecular weight cut off of 10 kDa. The concentration was performed as described by the manufacturer.

B.13.5 Chromatography methods

The chromatographic protein separation was performed using the Fast-Performance-Liquid-Chromatography (FPLC) system and the HiLoad system of Pharmacia (Uppsala, Sweden). The utilised buffers were degassed and filtrated before use (0,45 µm). The columns were cooled to 4°C. Before applying the protein sample to a column, particulate matter was removed by filtration (0,45 µm). This filtration was omitted before the gel filtration, because of the small sample volume. Instead a guard column was used to keep potentially present debris away from the chromatography column. All columns (Pharmacia, Uppsala, Sweden) were treated as described by the manufacturer. If the ordinary buffer or the corresponding stabilising buffer was used for the chromatography depended on the purification strategy.

B.13.5.1 Hydrophobic interaction chromatography (HIC)

Column : Phenyl Sepharose 6 Fast Flow low substitution, gel volume 70 ml

Buffer A: 50 mM potassium phosphate buffer, pH 7.5

or

50 mM potassium phosphate buffer, 2 mM sodium thiosulfate, 1 mM magnesium sulfate, 1 µM phenylmethylsulfonylfluoride (PMSF), pH 7.5 (→ stabilising buffer PS)

Buffer B: buffer A with 40 % saturation of $(NH_4)_2SO_4$

Before use, the HIC column was equilibrated with buffer B. The supernatant after ammonium sulfate precipitation and centrifugation was loaded onto the column with a flow rate of 2 ml min^{-1}. After the sample was applied, the column was washed with a defined $(NH_4)_2SO_4$

saturation, depending on the protein to be purified. When no more unbound protein was washed from the column, the protein elution was achieved either via a linear or a stepwise decrease in $(NH_4)_2SO_4$ saturation, performed with a flow rate of 2,5 ml min^{-1}. After the gradient was finished, the column was washed with buffer A until no more protein was eluted. The protein amount was measured at 280 nm, and the salt gradient was recorded by conductivity. Fractions with a volume of 5 ml were collected.

B.13.5.2 Anionic exchange chromatography (IEX)

Column: MonoQ HR 5/5, gel volume 1 ml

Buffer A: 10 mM TrisHCl, pH 7.5

or

10 mM TrisHCl, 2 mM sodium thiosulfate, 1 mM magnesium sulfate, 1 µM PMSF, pH 7.5

(→ stabilising buffer MQ)

Buffer B: buffer A containing 1 M NaCl

The fractions after HIC, that contained the desired protein, were combined and dialysed over night against buffer A (see B.13.3). The MonoQ column (containing a strong anionic exchange material) was equilibrated with buffer A before use. The protein sample was applied to the column with a flow rate of 1 ml min^{-1}. The column was washed with a defined NaCl concentration (100 or 200 mM), until all unbound proteins were removed. The proteins were eluted using an increasing linear salt gradient up to 600 mM NaCl at a flow rate of 1 ml min^{-1}. The remaining proteins were eluted with 1 M NaCl. The protein amount was monitored at 280 nm and the theoretical salt gradient was recorded. Fractions with a volume of 1 ml were collected.

B.13.5.3 Gel filtration chromatography (GF)

Column:	HiLoad 16/60 Superdex TM 200, gel volume 126 ml
Buffer:	50 mM TrisHCl, pH 7.5
	or
	50 mM TrisHCl, 2 mM sodium thiosulfate, 1 mM magnesium sulfate, 1 µM PMSF, pH 7.5
	(→ stabilising buffer GF)
Standard proteins:	Sigma Marker low-range; molecular mass: 6500 to 66000 Da

To determine the apparent molecular mass of a protein via gel filtration chromatography, the column was calibrated with standard proteins. Before use the column was equilibrated with the gel filtration buffer. The protein sample, whose volume should not exceed 2 ml, was applied to the column with a flow rate of 0,2 ml min^{-1}. The protein was eluted isocratically with a flow rate of 0,5 ml min^{-1}. The protein amount was measured at 280 nm. Fractions with a volume of 1 ml were collected.

B.14 Determination of Sox enzyme activity

Solutions:	stabilising buffer GF, pH 7.5 (see B.13.5.3)
	100 mM sodium thiosulfate
	4 mM horse heart cytochrome c (Sigma)

The enzymatic activity of the Sox protein complex was determined as a thiosulfate-dependent reduction of horse heart cytochrome c as the artificial electron acceptor (ε_{550} = 29,5 $mM^{-1}cm^{-1}$). The reaction rate was quantified by monitoring the increase in extinction at 550 nm with a Perkin-Elmer Lambda-11 spectrometer. The reaction was started by the addition of the enzyme solution, and the extinction at 550 nm was recorded every 2 seconds for 10 min.

Composition of the preparation:	50 µl stabilising buffer GF
	100 µl thiosulfate solution (→ 10 mM)
	25 µl cytochrome c (→ 100 µM)
	enzyme solution
	demineralised water (ad 1 ml)

B.15 Phenotypic characterisation of *A. vinosum* mutants

B.15.1 Cultivation of *A. vinosum* wild type and mutant strains

Solutions: PFENNIG and 0 medium, respectively (see B.3.1)
0,4 M thiosulfate solution (10 % (w/v) $Na_2S_2O_3$ x $5H_2O$)
2 M sulfide solution (11,2 g NaHS x H_2O ad 100 ml demineralised water)
1 M sulfite solution (12,6 g Na_2SO_3 ad 100 ml demineralised water)
1 M HCl
1 M Na_2CO_3 solution

To characterise a phenotype potentially evoked in *A. vinosum* by gene inactivation, the corresponding mutant was grown photolithoautotrophically at 30°C under anaerobic conditions with the addition of defined amounts of reduced sulfur compounds. A glass fermenter with a volume of 1,5 l was used, together with an autoclavable pH electrode and sterile HCl and Na_2CO_3 solutions to maintain a pH equilibrium at pH 7.0±0.1. The fermenter is depicted in Figure B3.

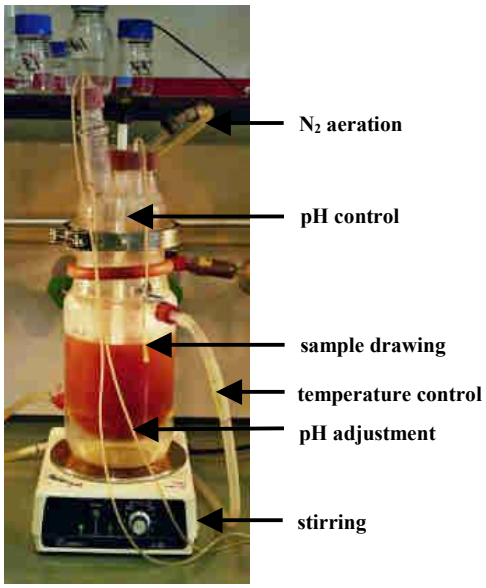

Figure B3: The assembly used for phenotypic characterisation of *A. vinosum* strains. The individual parts are labelled in the picture.

As a medium for phenotypic characterisation Pfennig as well as 0 medium was applied (see B.3.1). The advantage of 0 medium was the better defined amount of reduced sulfur compound present in the fermenter, as it was added separately. Medium sterilisation and storage often changed the sulfide concentration in Pfennig medium.
Mutants and corresponding wild types were cultivated in 250 ml RCV medium (see B.3.3) for approximately three days and harvested (10000 x g, 18°C, 20 min). The pellet was resuspended in a small volume of the supernatant. Medium and culture were filled into the fermenter under nitrogen aeration. Depending on the applied medium, the experiment was started either by the addition of culture (Pfennig medium) or the addition of defined amounts of reduced sulfur compounds (0 medium). During the experiment samples were taken in regular intervals to determine the following:

- culture for determination of optical density at 690nm 1 ml
- culture for derivatisation with MBB (see B.15.3) 50 µl
- culture pellet (15700x g, 3 min) for elemental sulfur determination (see B.15.2) 200 µl
- culture for protein determination etc. (see B.10) 1 ml
- culture supernatant (15700 x g, 3min) for the determination of sulfate and tetrathionate (see B.15.2 and B.15.3) 1 ml

The optical density was observed during the experiment as an indicator for culture growth and sulfur globule formation. The other parameters were determined after the conclusion of the experiment. The duration was dependent on quantity and number of sulfur compounds the culture was subjected to. Generally a second substrate was only applied if the first was no longer detectable. After the conclusion of the experiment, the culture was harvested (10000 x g, 10°C, 30 min) and the pellet was stored at –20°C until further use.

B.15.2 Analytical determination of sulfur compounds by wet chemical methods

Sulfur compounds were determined with material obtained during a fermenter experiment of *A. vinosum* wild type and mutant strains (see B.15.1).

B.15.2.1 Elemental sulfur via cyanolysis (modified after Kelly *et al.*, 1969)

Solution:	ferric nitrate reagent	30 g $Fe(NO_3)_3$ x 9 H_2O, 40 ml 55 % HNO_3 (or 34 ml 65 % HNO_3), ad 100 ml demineralised water

A cell pellet with up to 200 nmol sulfur was resuspended in 200 µl demineralised water. After the addition of 100 µl 0,2 M sodium cyanide solution, the preparation was incubated for 10 min at 100°C. 650 µl demineralised water and 50 µl ferric nitrate reagent were added, and after centrifugation (15700 x g, 2 min) the extinction at 460 nm was measured against a reagent blank. A calibration curve was recorded with sodium thiocyanate (rhodanide) at a range of 0 to 300 nmol per preparation of 200 µl.

B.15.2.2 Tetrathionate via cyanolysis (Kelly *et al.*, 1969)

Solutions:	ferric nitrate reagent	see 2.13.1
	Tris acatete buffer	1 M Tris acetate, pH 8.7

425µl of culture supernatant was mixed with 25 µl Tris acetate buffer and 25 µl 0,2 M sodium cyanide solution and incubated at 30°C for 30 min. After the addition of 25 µl ferric nitrate reagent the extinction at 460 nm was measured against a reagent blank. The calibration curve obtained during elemental sulfur determination was also used for tetrathionate determination.

B.15.2.3 Sulfite (modified after Pachmayr, 1960)

Solutions:	2 % (w/v) zinc acetate in demineralised water
	0,04 % (w/v) fuchsin in 10 % (v/v) H_2SO_4
	formaldehyde (37 %)

690 µl of culture supernatant was diluted with demineralised water to fit into the calibration curve. The diluted sample was mixed with 200 µl zinc acetate and 100 µl fuchsin reagent and incubated for 10 min at room temperature. After the addition of 10 µl formaldehyde and another 10 min incubation time, the extinction at 570 nm was measured against a reagent

blank. A calibration curve was recorded with sodium sulfite at a range of 0 to 40 nmol per preparation of 690 µl.

B.15.3 Analytical determination of sulfur compounds using HPLC

For the determination of sulfur compounds, apart from wet chemical methods, a HPLC by Thermo Electron (Dreieich, Germany) was used, consisting of a degasser SCM1000, the pumps P200 and P4000, the autosampler AS3000 or a manual Rheodyne valve, the column oven, the UV detector UV 150 or UV 6000 and the fluorescence detector FL3000. The chromatograms were analysed using the software provided with the device. All columns were provided by Merck (Langenfeld, Germany). The columns were treated according to the instructions provided by the manufacturer. Buffers (except for HPLC grade methanol) were filtrated with a 0,22 µm filter before use.

B.15.3.1 Determination of thiols using HPLC

Solutions:	HEPES buffer	50 mM HEPES, 5 mM EDTA, pH 8.0 (titrated with NaOH)
	monobromobimane solution (MBB)	96 mM monobromobimane in acetonitrile
	65 mM methanesulfonic acid	

Derivatisation:

The detection of thiols like sulfide, polysulfides, thiosulfate and sulfite was performed after derivatisation with the fluorescent dye monobromobimane (Rethmeier *et al.*, 1997). 50 µl of cell material was mixed with 50 µl HEPES buffer and 55 µl acetonitrile. After the addition of 5 µl monobromobimane solution the preparation was mixed and incubated in the dark for 30 min. The reaction was stopped by the addition of 100 µl methanesulfonic acid, and the preparations were stored at -20°C until the measurement.

HPLC analysis of derivatised thiols:

Column: LiChrospher® 100 RP 18 ec (250-4, 5 µm)
flow rate 1 ml / min, temperature 35°C
Detection: fluorescence detection (excitation 380 nm, emission 480 nm)
Solutions: A: 0,25 % acetic acid, pH 4.0 (titrated with 10 M NaOH)
B: methanol
Elution: binary gradient

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

The detection of derivatised thiols was performed by reversed phase HPLC with binary gradient elution and fluorescence detection. For the sedimentation of particulate components the preparation was centrifuged (15700 x g, 5min). Because of the sensitivity of the detector, the sample was diluted 1:20 with the solvent mixture (85 % A, 15 % B). 200 µl of the diluted sample was filled into HPLC vials. The autosampler injected 50 µl of each sample into a 100 µl sample loop. Calibration curves were recorded with sodium sulfite, sodium thiosulfate and sodium sulfide at a range of 50 µM to 1 mM, respectively. As no standard for polysulfides was available, the amount and composition of these measured substances remains relative. The retention times for the respective substances were 4 min for sulfite, 9 min for thiosulfate, 39 min for polysulfide I, 40 min for sulfide and 44 min for polysulfide II.

B.15.3.2 Determination of elemental sulfur using HPLC

Column:	LiChrospher® 100 RP 18 (125-4, 5 µm)
	flow rate 1,2 ml / min, temperature 35°C
Detection:	UV detection (263 nm)
Solution:	95 % methanol, 5 % demineralised water
Elution:	isocratic

For the determination of elemental sulfur 100 µl culture were mixed with 100 µl chloroform, mixed vigorously and left untouched, until the two phases reappeared. The lower phase contained the elemental sulfur extractable in chloroform. The sulfur concentration was determined using reversed phase HPLC and UV detection (Rethmeier *et al.*, 1997). 50 µl of the chloroform phase were injected into a Rheodyne valve with a 20 µl sample loop. A calibration curve was recorded with elemental sulfur dissolved in chloroform from 25 µM to 5 mM. The retention time for sulfur was 4,5 min.

B.15.3.3 Determination of sulfate using HPLC

Column:	Polyspher IC AN 1
	flow rate 1,3 ml / min, temperature 35°C
Detection:	UV detection (indirect, 254 nm)
Solution:	1,5 mM phtalic acid, 1,38 mM Tris, 300 mM boric acid, pH 4.2 (do not titrate)
Elution:	isocratic

Sulfate was determined using anionic exchange HPLC with indirect UV detection. The solvent exhibits a stronger absorption than sulfate, therefore resulting in a negative sulfate peak. Sulfate was measured from culture supernatant, that was centrifuged before use (15700 x g, 5 min). The autosampler injected 60 µl in a 100 µl sample loop. The calibration curve was reported from 50 µM to 8 mM with sodium sulfate. The retention time was approximately 12 min.

C. Results

C.1 Sequence analysis

C.1.1 First set of *sox* genes

During the work on my diploma thesis three *sox* genes and two potentially *sox*- related genes were identified. They are shown in Figure C1, surrounded by genes in all probability not involved in the Sox system.

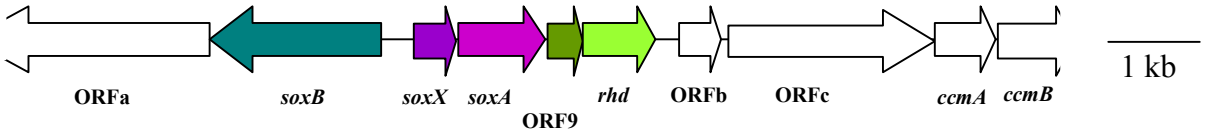

Figure C1: Gene region containing the first set of *sox* genes and *sox*-related genes in *A. vinosum*. The relevant open reading frames referred to in the text are depicted in colour.

soxB: For the *soxB* gene (597 aa, 65377 Da) sequence analysis predicts a putative signal peptide of 29 amino acids. It contains a double arginine motif (Brüser and Sanders, 2003), that suggests a transport to the periplasm via the Tat pathway. Sequence comparison reveals a domain typical for 5´-nucleotidases and high similarity to several *soxB* genes found in chemo- and phototrophic organisms (including *P. pantotrophus* and *C. tepidum*). In accordance with results obtained for SoxB from *P. pantotrophus* the putative cofactor would be a dinuclear manganese cluster (Epel *et al.*, 2005).

***soxX*:** The *soxX* gene (128 aa, 13629 Da) is orientated divergently from *soxB* on the 5´-strand and separated from it by a 325 bp intergenic region, containing two putative promoter sequences. The putative *soxB* promoter is situated 72 bp upstream of the corresponding start codon, the putative *soxX* promoter 244 bp upstream of the corresponding start codon. Sequence analysis revealed a putative signal peptide for SoxX (24 aa), typical for Sec-dependent transport to the periplasm. One heme binding motif (CxxCH) indicates, that the protein is a monoheme c-type cytochrome. SoxX exhibits similarity to several other SoxX proteins from different organisms, all of which also probably contain one heme cofactor.

***soxA*:** The *soxA* gene encodes a protein (281 aa, 31730 Da), that includes a putative signal peptide of 20 amino acids, typical for Sec-dependent transport. SoxA contains one heme binding motif (CxxCH), that suggests it to be a periplasmic monoheme c-type cytochrome. SoxA exhibits similarity to several other SoxA proteins from different organisms, which contain either one heme binding site as in *S. novella* (Kappler *et al.*, 2004) or two heme binding sites as in *P. pantotrophus* (Friedrich *et al.*, 2000). Whether this difference leads to a different function is not yet known.

Different SoxA proteins are shown in the alignment in Figure C2. *C. tepidum* SoxA was chosen as a representative of other SoxA proteins encoded in the green sulfur bacteria, that exhibit the same characteristics (e.g. *Chlorochromatium aggregatum*). The C-terminal heme binding motif is conserved in all the depicted sequences. The N-terminal heme binding motif, however, is missing in *A. vinosum*, *S. novella* and *C. tepidum*, only one of the cysteine residues remains. For SoxA in *S. novella* (Kappler *et al.*, 2004) and *R. sulfidophium* (Cheesman *et al.*, 2001) an unusual heme ligand has been postulated (His/Cys instead of His/Met). The cysteine residues involved in heme coordination (marked in yellow) are also conserved in *A. vinosum*. For the C-terminal heme the cysteine is the potential second ligand in addition to histidine. As the N-terminal heme binding motif was destroyed, the formation of a disulfide bond between the remaining heme binding cysteine and the ligand cysteine was observed in *S. novella* (Kappler *et al.*, 2004), a situation also possible in *A. vinosum*.

```
AvinSoxA            ---MTKHGFLLATLVLAGATLPIGPVTAAT-----PEEEQAAFQAYFKQR
SnovSoxA            -MRRFAAGCLALALLVLPFVLTGARAAEDES-------------------
CtepSoxA            MKKTIQRGLFTGALVLMTAMTAK-PANAEVNYQALVDADVKAFQGFFRKE
PpanSoxA            -MPRFTKTKGTLAATALGLALAGAAFADPVEDGLVIETDSGPVEIVTKTA
RsulSoxA            -MKTMTG-RLVAAALVCGGAFSGAAVSAGPDDPLVIN---GEIEIVTRAP
Clustal Consensus                  :        . .            . .    .

AvinSoxA            FPNVPEDEFKNG--TYAIDPVTRENWEAIEEFPPYENAISQGETLWNTPF
SnovSoxA            ----------------EKEIERYRQMIEDPMANPGFLNVDRGEVLWSEPR
CtepSoxA            FPDVKLEDFGNG--VYALDEDARKQWKEMEEFPPYELDVEAGKALFNKPF
PpanSoxA            PPAFLADTFDTIYSGWHFRDDSTRDLERDDFDNPAMVFVDRGLDKWNAAM
RsulSoxA            TPAHLADRFDEIRSGWTFRTDDTQALEMDDFENSGMVFVEEARAVWDRPE
Clustal Consensus    .    .                .          .    :. .   :. .

AvinSoxA            AD-GQGYADCFP-----DGPAIMNHYPRWDRERGQVMTLPLALNACRTAH
SnovSoxA            GTRNVSLETCDLGEGPGKLEGAYAHLPRYFADTGKVMDLEQRLLWCMETI
CtepSoxA            AN-GKSLASCFP-----NGGAVRGMYPYFDEKRKEVVTLEMAINECRVAN
PpanSoxA            GVNGESCASCHQGPE-S-MAGLRAVMPRVDEHTGKLMIMEDYVNACVTER
RsulSoxA            GTEGKACADCHGAVD-DGMYGLRAVYPKYVESAGKVRTVEQMINACRTSR
Clustal Consensus   .   . .   *          .     *       ::  :   :  *
```

```
AvinSoxA            -GETPLKYKKG---------PIADLLAYIAFESRGQITRVEIPQDDPRAL
SnovSoxA            QGRDTKPLVAKPFSGPGRTSDMEDLVAFIANKSDGVKIKVALATP--QEK
CtepSoxA            -GEKPYAWEKG---------DIARVSAYIASISRGQ--KVDVKVKSKAAY
PpanSoxA            MGLEKWGVTSD---------NMKDMLSLISLQSRGMAVNVKIDG---PAA
RsulSoxA            MGAPEWDYIGP---------DMTAMVALIASVSRGMPVSVAIDG---PAQ
Clustal Consensus    *                   :  : : *:  * *    * :   .

AvinSoxA            AAYEQGKRFYFARRGQLNFACAHCHLATSGTKLRTETLSPA-------YG
SnovSoxA            EMYAIGEALFFRRSSINDFSCSTCHG-AAGKRIRLQALPQLDVPGKDAQL
CtepSoxA            DAYMKGKKFFYAKRGQLNMSCSGCHMEYAGRHLRAEIISPA-------LG
PpanSoxA            PYWEHGKEIYYTRYGQLEMSCANCHEDNAGNMIRADHLS---------QG
RsulSoxA            STWEKGREIYYTRYGQLDLSCASCHEQYFDHYIRADHLS---------QG
Clustal Consensus     :  *. ::: : .  :::*: **    .  :* : :.

AvinSoxA            HTTHWPVYRSEWGEMGTLHRRFAGCNEQVRAKAFEPQGEEYRNLEYFLTY
SnovSoxA            TMATWPTYRVSQSALRTMQHRMWDCYRQMRMPAPDYASEAVTALTLYLTK
CtepSoxA            HTTHFPVFRSKWGEIGTLHRRYAGCSNNIGAKPFAPQSEEYRDLEFFQTV
PpanSoxA            QINGFPTYRLKDSGMVTAQHRFVGCVRDTRAETFKAGSDDFKALELYVAS
RsulSoxA            QINGFPSYRLKNARLNAVHDRFRGCIRDTRGVPFAVGSPEFVALELYVAS
Clustal Consensus       :* :* . . : : : *  .* .:    .    .     *  : :

AvinSoxA            MNNGLELNGPGARK
SnovSoxA            QAEGGELKVPSIKR
CtepSoxA            MSNGLKYNGPASRK
PpanSoxA            RGNGLSVEGVSVRH
RsulSoxA            RGNGLSVEGPSVRN
Clustal Consensus     :* . :   . :.
```

Figure C2: Alignment of different SoxA amino acid sequences. Conserved amino acids are marked in gray, the conserved heme binding motifs and the corresponding remains are marked in red. The conserved cysteine residues applied as heme ligands are marked in yellow. Organisms: *A. vinosum*, *S. novella*, *C. tepidum*, *P. pantotrophus*, *R. sulfidophilum*. All sequences were obtained from GenBank with the exception of the *A. vinosum* sequence taken from (Hensen, 2001).

ORF9: This ORF (114 aa, 12194 Da) encodes a presumably periplasmic protein with a signal peptide of 29 amino acids for Sec-dependent transport. The protein exhibits no conserved domains, but shows similarity to hypothetic proteins in *C. limicola* as well as in *C. tepidum*. In both organisms the corresponding open reading frame is located in a *sox* gene cluster. In *C. tepidum* a similar open reading frame CT1020 is located between *soxA* and *soxB*. However, a function of the putative protein was not obvious.

rhd: The encoded protein (249 aa, 27178 Da) contains a putative signal peptide of 50 amino acids for Sec-dependent transport, indicating a periplasmic location. The protein contains a conserved domain typical for rhodaneses (RHOD-PspE2: rhodanese homology domain (Bordo and Bork, 2002)), enzymes responsible for sulfur group transfer, that are found in all three domains of life. The function may be detoxification of cyanide by addition of sulfur to form non-toxic thiocyanide (rhodanide). Based upon this similarity the open reading frame is named *rhd*. A gene encoding a putative rhodanese is also found in the *sox*

gene cluster of *Aquifex aeolicus* (GenBank accession number: AAC07633). As sulfur group transfer could be part of the thiosulfate oxidation pathway, the presence of a rhodanese in the vicinity of *sox* genes may be of importance.

C.1.2 Second set of *sox* genes

Taking the model for the *P. pantotrophus* Sox system into account, two proteins essential for its function were still missing: the substrate binding protein SoxYZ and the sulfur dehydrogenase SoxCD.

C.1.2.1 Cloning of the gene region containing soxYZ

The first step in the lookout for *soxYZ*, encoding the substrate binding protein, was the utilisation of the already published *soxYZ* gene sequence of *C. limicola* (Verte *et al.*, 2002). Using the primer pair ClimYfor / ClimZrev , a DNA fragment containing both *soxY* and *soxZ* was amplified with genomic DNA of *C. limicola* as template. After digoxigenin-labelling it was used as a heterologous probe for Southern hybridisation. The examination of *A. vinosum* DNA, digested with different restriction enzymes, revealed two fragments suitable for cloning (a 2,5 kb *Cla*I fragment and a 1,5 kb *Eco*RI fragment), that produced a positive signal. These fragments were ligated into an equally digested plasmid pGEM7 Zf(+), respectively, yielding the two plasmids pDHEcoYZ and pDHClaYZ. The corresponding inserts were sequenced and proved to be overlapping. This led to the detection of the second set of *sox* genes in *A. vinosum*, which are located independently from the genes identified during my diploma thesis that are discussed above. Therefore the model of a continuous *sox* gene cluster, as found in *P. pantotrophus*, appeared not to be correct for the situation in *A. vinosum*. The five open reading frames of the second gene region are depicted in figure C3.

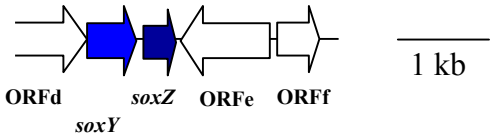

Figure C3: Gene region containing the second set of *sox* genes in *A. vinosum*. The *sox* genes are highlighted in colour. Nucleotide and amino acid sequence see appendix (G.1 and G.2).

C.1.2.2 Sequence analysis

ORFd: This incomplete open reading frame encodes a protein with unknown cellular location, as the N-terminal sequence is missing. The protein exhibits a TPR domain, which is usually involved in protein-protein interaction (Sikorski *et al.*, 1990). The TPR domain usually appears in cytoplasmic proteins, giving a hint towards the localisation of the protein encoded by ORFd. Sequence comparison reveals similarity to either putative peptidase-family-M48-proteins or putative Zn-dependent proteases from different eubacteria. No obvious connection to the thiosulfate metabolism could be concluded.

soxY: The ORF *soxY* encodes a putative periplasmic protein (154 aa, 15796 Da) with a signal peptide of 31 amino acids. A promoter search reveals a potential promoter responsible for the transcription of *soxYZ* inside of ORFd. The –10 box is located 299 bp upstream of the *soxY* start codon. The location of the genes *soxYZ* independent from the other *sox* genes presents the possibility for independent regulation of gene expression.
The protein SoxY contains a double arginine motif typical for a Tat-dependent transport to the periplasm. The transport via the Tat pathway as an already folded holoprotein normally hints towards a cofactor-containing protein. But in the case of SoxY no sequence motifs for cofactor binding could be identified. Still, this type of transport would be needed, if SoxY was actually responsible for the transportation of SoxZ to the periplasm. The latter did not exhibit a signal peptide at all, therefore being potentially a cytoplasmic protein. This kind of hitchhiker transport was already predicted for the SoxYZ protein in *P. pantotrophus* (Friedrich *et al.*, 2001).
Similarity search reveals homologies to several other SoxY proteins from different *sox* gene containing bacteria. The similarity of the *A. vinosum* protein to *C. tepidum* SoxY is quite large ($E=2.2e^{-23}$) compared to SoxY from *P. pantotrophus* ($E=3.2e^{-13}$), corresponding with other parallels between *C. tepidum* and *A. vinosum*. Preceding examination of SoxY revealed the presence of a consensus motif at the C-terminus of the protein, surrounding the cysteine residue involved in substrate binding (Quentmeier and Friedrich, 2001). An alignment of the predicted amino acid sequence of *A. vinosum* SoxY with some already published sequences (see Figure C4) demonstrated the presence of the same motif in the *A. vinosum* sequence. The Tat transport signal could also be identified in all the examined SoxY sequences.

```
AvinSoxY            MIDAKRRTLVKGSLAAGAVVGAGLITP-RAFADWNAAAFQAKDIPTAMTG
SnovSoxY            MQLRSWTPSRRETMGLGLTAMLAAALA-------PRLVMATEEMVAAELK
CtepSoxY            -MGISRRDFCRTIAGSAASFAVLAVMPGRLLASWNEKAFSASKLDEAIAA
PpanSoxY            MIL-----SRREALWIGFGGLAAAALP-------GKAVMAST--VDELTA
RsulSoxY            MEF-----TRRGAMALSAGLAAALMLP-------ASAVRAA---VEDKIA
Clustal Consensus       .     :     .          .    :.    .  : .

AvinSoxY            LLGSDAAEVSDRIKIKAPDIAENGAVVPVTVETDLEG-----VTSISLIA
SnovSoxY            KLYGDKSMADGKIKLDLPEIAENGLVVPLNIDVDSPMTEQDYVKAVHVFA
CtepSoxY            KFGSLPIEDSTAIQIKAPEIAENGAFVPVSVSTTIPG-----ATNISIFT
PpanSoxY            AFTGGAATGEGGLTLTAPEIAENGNTVP--IEVKAPG-----AVAIMLLA
RsulSoxY            EFTGGADAGADGITLTTPEIAENGNTVP--IEVEAPG-----AVEIMVVA
Clustal Consensus    : .        : :  *:*****  ** .:..         .  : :.:

AvinSoxY            AKNQSPLIASFEFVDPSVIPFVATRIKMAETADVIAVVKAG-DKLYKNAK
SnovSoxY            DGNPLPQVVTYKFTPQSGKAAASIRMRLAQTQNVIAVAEMSNGALYTTKA
CtepSoxY            PANFSPMIASFD-VLPRMIPDVSLRMRMAKTSNLVVIVQAG-GKLYRATR
PpanSoxY            AGNPEPAVATFNFGPAAADQRAATRIRLAQTQDVIALAKMADGSVVKAQT
RsulSoxY            SANPTPDVARVSFGPLAGAQRLSTRIRLGGTQDVIAVAKMGDGSFRRAAN
Clustal Consensus     *  * :.  .          : *:::. * :::.:.: . . .

AvinSoxY            SVKVTIGGCGG
SnovSoxY            QVKVTIGGCGG
CtepSoxY            EVKVTIGGCGG
PpanSoxY            TVKVTIGGCGG
RsulSoxY            TVKVTIGGCGG
Clustal Consensus    **********
```

Figure C4: Alignment of different SoxY amino acid sequences. The C-terminal consensus sequence and other conserved amino acids are marked in gray, the conserved cysteine residue (the potential substrate binding site) is marked in red. The double arginine motif for Tat transport is marked in green. Organisms: *A. vinosum*, *S. novella*, *C. tepidum*, *P. pantotrophus*, *R. sulfidophilum*. All sequences were obtained from GenBank with the exception of the *A. vinosum* sequence.

soxZ: Although SoxZ in *P. pantotrophus* has been detected in the periplasm, the protein has no signal peptide. The same appears to be true for *A. vinosum* SoxZ, that is predicted to be cytoplasmic. The protein (104 aa, 11231 Da) exhibits homology to several SoxZ proteins from different organisms. As could be observed for SoxY, the similarity of SoxZ was bigger between *A. vinosum* and *C. tepidum* (E=2.7e^{-18}) than between *A. vinosum* and *P. pantotrophus* (E=9.6e^{-8}). An alignment of SoxZ amino acid sequences from different organisms (see Figure C5) revealed no obvious consensus motif, that could give a hint towards the protein function. As the substrate binding is performed by the cysteine residue in the SoxY subunit of a SoxYZ heterodimer, the role of the SoxZ subunit remains to be elucidated.

```
AvinSoxZ            -MSDIKIRAKLE-----GDETTVKCLMSHPMETGLRKDSKTNEVIPAHFI
SnovSoxZ            MAIKSKPRVKVPSEAKAGEIIEIKTLISHEMESGQRKD-SSGKTIPRAII
CtepSoxZ            ----MKIKAVVQ-----NNIVSVKVLIPHPMDTGRVKD-QAGALIPAHFI
PpanSoxZ            MADDAKPRVKVPSSAKAGETVTVKALISHKMESGQRKD-ADGKLIPRSII
RsulSoxZ            MAEGVKARVKAPRTVAAGETVVIKTLISHQMESGQRKD-SDGSLIPRSII
Clustal Consensus        * :.        .:   :* *:.* *::*  **:  .  **  :*

AvinSoxZ            REVVCKVKGAVVMKTSWSGGVSKNPYLSFKFKGGAVGDPIEIAWTDNTGE
SnovSoxZ            NTFTASFNGKPFFEANWFTAVSANPYQAFFYKASESGE-FTFTWKDDDGS
CtepSoxZ            TEVTATIGGDTVFHAELGSGVSKDPYLSFQFKGAKAGDMLKVSWVDNKGA
PpanSoxZ            NRFTCELNGVNVVDVAIDPAVSTNPYFEFDAKVDAAGE-FKFTWYDDDGS
RsulSoxZ            NRFAVAYNGQNVIDVALAPAISTNPYFEFEAVIPEAGD-MVFTWYDDDGS
Clustal Consensus     ..    *  ....    .:* :**  *       *: : .:* *: *

AvinSoxZ            SQSATAEISG-
SnovSoxZ            EQSATAKLTVA
CtepSoxZ            SETAEAAITAM
PpanSoxZ            VYEDVKPIAVA
RsulSoxZ            VYEEVKSIAIG
Clustal Consensus         ::
```

Figure C5: Alignment of different SoxZ amino acid sequences. Conserved amino acid residues are marked in gray. Organisms: *A. vinosum*, *S. novella*, *C. tepidum*, *P. pantotrophus*, *R. sulfidophilum*. All sequences were obtained from GenBank with the exception of the *A. vinosum* sequence.

ORFe: The open reading frame ORFe is located divergently from *soxZ*, pointing towards an independent transcription. The encoded protein (221 aa, 25895 Da) exhibits no signal peptide. The cytoplasmic protein shows a SAM (S-adenosyl-L-methionine) binding motif, a conserved domain typical for methylases. The function would be a transfer of a methyl group from SAM to either nitrogen, oxygen or carbon, thereby achieving a modification of DNA, RNA and proteins. This function is frequently employed in diverse organisms ranging from bacteria to plants and mammals. Sequence comparison also indicated high homology to protein-L-isoaspartate o-methyltransferases, that occur in a broad range of bacteria and play a role in the repair and/or degradation of damaged proteins.

ORFf: This open reading frame encodes a putative cytoplasmic protein with a conserved domain typical for GTP-cyclohydrolases I. These proteins catalyse the biosynthesis of formic acid and dihydroneopterin triphosphate from GTP, the first step in the biosynthesis of tetrahydrofolate in prokaryotes. The protein is also found in eukaryotic sources.

A summary of the genetic results gained so far (see Table C1) indicate the presence of five *sox* genes in *A. vinosum* (*soxBXA* and *soxYZ*), encoding the three proteins SoxB, SoxXA and SoxYZ. Preceding experiments performed by Ulrike Kappler, including a thorough search on genetic and protein basis, made the presence of *soxCD* and the corresponding proteins in *A. vinosum* very unlikely .

gene	bp	protein	MW [Da]	cofactor	function
soxB	1791	SoxB	65377,30 / 62077,28	manganese[1]	sulfate thiohydrolase
soxX	384	SoxX	13628,60 / 10750,21	heme	cytochrome c
soxA	843	SoxA	31729,65 / 29254,62	heme	
soxY	464	SoxY	15796,34 / 12702,61	-	substrate binding
soxZ	314	SoxZ	11230,87 / -	-	

Table C1: The *sox* genes so far identified in *A. vinosum*, together with the encoded proteins (including molecular weight for the unprocessed and processed form), their putative cofactor content and the postulated function. [1]Postulated because of results obtained in *P. pantotrophus* (Epel *et al.*, 2005).

C.2 Production of heterologous Sox proteins in *E. coli* for antisera testing

The next goal after the identification of five putative *sox* genes in *A. vinosum* was the detection of the encoded proteins in the organism. For the detection of SoxA, SoxB and SoxYZ the respective antisera were applied. The SoxA antiserum was raised against a potentially highly immunogenic synthetic peptide derived from the nucleotide sequence in *A. vinosum*. In contrast, the SoxB and SoxYZ antisera were raised against the respective complete proteins from *P. pantotrophus*. These antisera were thankfully provided by the group of Cornelius Friedrich in Dortmund. As none of the antisera was directed against an already known *A. vinosum* protein, they all needed to be tested for their functionality. The test was performed with the respective recombinant proteins produced in *E. coli* as an expression system.

C.2.1 SoxA

The protein SoxA was produced in *E. coli* BL21(DE3) under aerobic conditions. As seen in Figure C6, SoxA was successfully produced and could be detected only in the induced culture of *E. coli*.

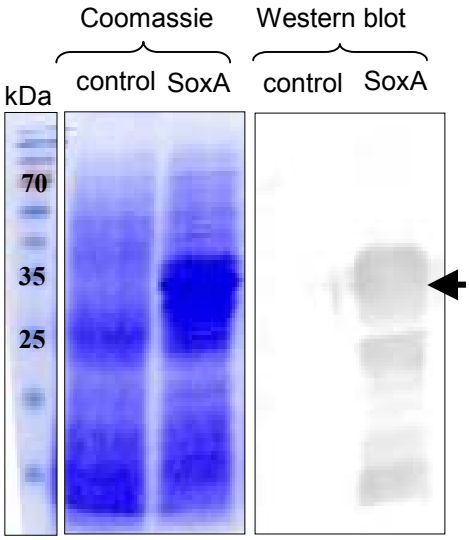

Figure C6: Production of recombinant SoxA in *E. coli* BL21(DE3). Comparison of negative control and SoxA-producing culture in Coomassie-stained SDS-PAGE and Western blot incubated with SoxA antiserum. The arrow indicates the position of SoxA at approximately 30 kDa.

The strongest signal was produced at the molecular weight of approximately the size predicted for SoxA (unprocessed: 32 kDa / processed: 30 kDa). Wether the signal peptide remained attached to the protein could not be determined from the SDS-PAGE. There was no signal in the control experiment. Nevertheless, a lot of background noise appeared in the preparation containing SoxA. The additional signals were potentially due to the heterologous protein origin, as the antiserum provided unequivocal signals with *A. vinosum* cell material. The use of *E. coli* BL21(DE3) for the production of a recombinant c-type cytochrome is not ideal concerning the functionality of the protein, as this *E. coli* strain is not intended for this kind of expression. The *E. coli* strain HM125 contains the *ccm* gene cluster, encoding for proteins necessary for correct c-type cytochrome maturation. However, the joint expression of *soxX* and *soxA* in this *E. coli* strain was not successful (data not shown). As for the desired purpose of antibody testing the protein did not have to contain a correctly inserted heme, the produced SoxA was sufficient.

C.2.2 SoxB

The production of recombinant SoxB in *E. coli* BL21(DE3) was also successful (see Figure C7). As the difference between processed (62 kDa) and unprocessed SoxB (65 kDa) was not distinguishable in the SDS-PAGE, it could not be determined whether the signal peptide remained attached to the protein.

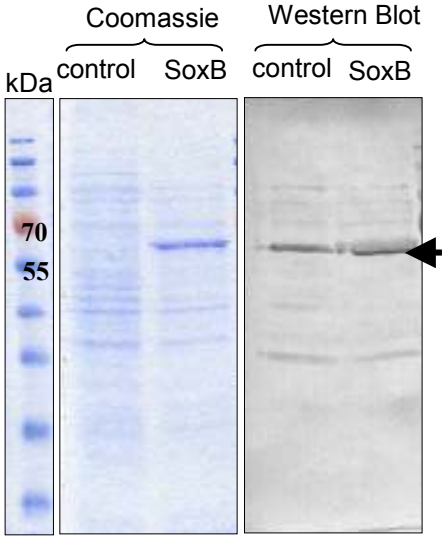

Figure C7: Production of recombinant SoxB in *E. coli* BL21(DE3). Comparison of negative control and SoxB-producing culture in Coomassie-stained SDS-PAGE and Western Blot incubated with Anti-SoxB antiserum. The arrow indicates the putative signal for SoxB at approximately 62 kDa, right below the additional background signal.

The antiserum against SoxB provided some difficulties, as at first glance there were identical signals in the negative control as well as with the produced protein. But after taking a closer look a second band directly below the contaminating signal could be identified in the SoxB-containing lane, that is not present in the negative control. Later experiments with *A. vinosum* made clear, that this problem did not arise solely from the background of protein production in *E. coli* (as was the case for SoxA). This experiment rather provided a preview of the difficulties in detecting SoxB in and purifying it from *A. vinosum*, caused by the presence of a background signal at almost the exact molecular weight of SoxB. The problem was potentially due to the origin of the antigen, that was used for antibody production. Antibodies directed against SoxB from *P. pantotrophus* seem to bind not only to SoxB but also to a second protein from *A. vinosum*, that was accidentally purified in addition to the actual SoxB (see C.3.3).

C.2.3 SoxYZ

The production of heterologous SoxYZ in *E. coli* BL21(DE3) was also successful (see Figure C8), even though some questions remain about the identity of the produced proteins.

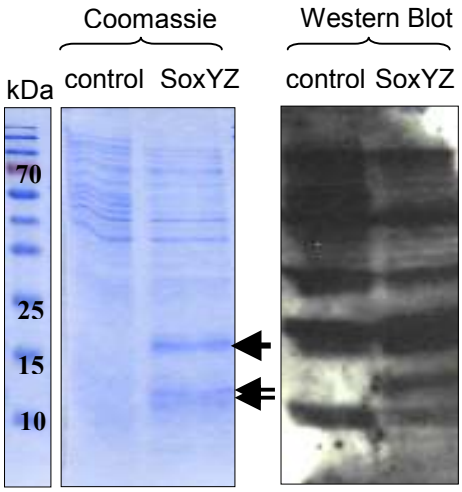

Figure C8: Production of recombinant SoxYZ in *E. coli* BL21(DE3). Comparison of negative control and SoxYZ-producing culture in Coomassie-stained SDS-PAGE and Western Blot. The upper arrow indicates the putative unprocessed SoxY at approximately 15 kDa. The middle and lower arrow indicate the potentially processed SoxY at 12,7 kDa and the putative SoxZ at approximately 11 kDa.

SoxY in its unprocessed form has a molecular weight of 15,8 kDa, corresponding to the band slightly above the 15 kDa marker band (indicated by the upper arrow). The two bands at a lower molecular weight could represent the potentially processed SoxY (12,7 kDa) and the putative SoxZ (11,2 kDa). The identification of the proteins with the corresponding antibody provided several difficulties. The protein band of the putative unprocessed SoxY produced a signal in the Western Blot, but that signal also appeared in the negative control. Regarding the results obtained for SoxB, that does not rule out an identification of SoxY. The two bands at lower molecular weight both provided a signal, in this case without a corresponding signal in the negative control. So this could be a positive signal for the detection of SoxYZ from *E. coli*.

C.3 Purification of Sox proteins from *A. vinosum*

C.3.1 Preceding experiments

Before the start of the Sox protein purification from *A. vinosum*, the first questions to be answered concerned the localisation of the proteins in the cell and a potential induction of the *sox* gene expression by the putative substrate(s). As the SoxA antiserum provided the best results, it was used for these first experiments, on the assumption that the other Sox proteins are in all probability equally localised and regulated.

C.3.1.1 Protein localisation and inducibility

To clarify the points in question, *A. vinosum* was cultivated either photo-organoheterotrophically on malate or photolithoautotrophically on thiosulfate and sulfide. The gained cell material was then subjected to ultrasound treatment and ultracentrifugation to separate the membrane and soluble fraction. The presence of SoxA was examined in crude extract, membrane and soluble fractions of both growth conditions (see Figure C9).

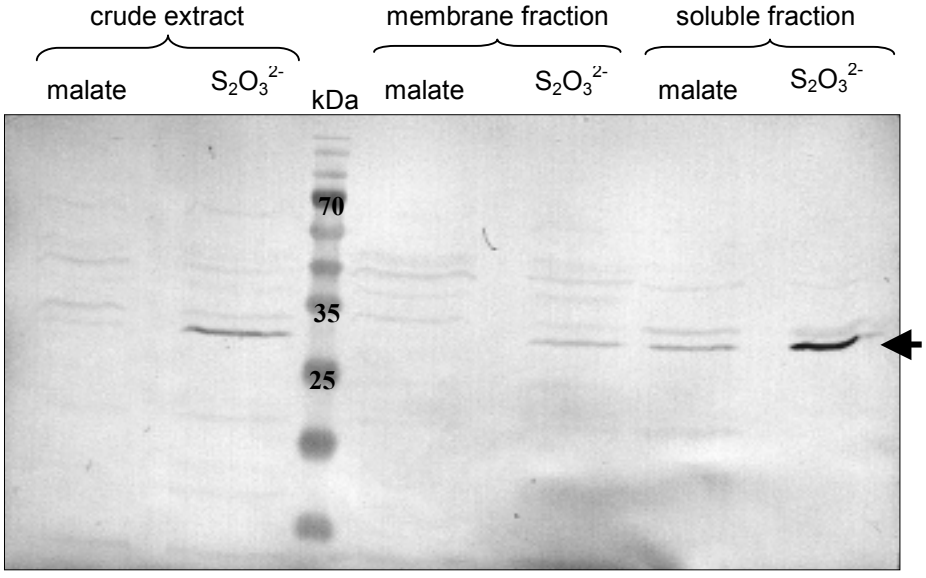

Figure C9: Detection of SoxA by Western blot analysis in crude extract, membrane fraction and soluble fraction of *A. vinosum* cell material, grown on medium containing malate or thiosulfate / sulfide (labelled only with "$S_2O_3^{2-}$"). The arrow indicates the signal for SoxA at approximately 30 kDa. Each lane contained 90 µg of protein.

SoxA was detected in all fractions, when *A. vinosum* was grown on thiosulfate and sulfide. But the strongest signal appeared in the soluble fraction, in correspondence with the predicted periplasmic location of the protein. When grown without the potential substrate(s) SoxA was nevertheless detectable, but at a significantly lower intensity. The strongest signal appeared again in the soluble fraction, a weak signal was perceptible in the crude extract. No signal for SoxA could be identified in the membrane fraction. Therefore, thiosulfate and/or sulfide provide an induction of gene expression above a low constitutive level. As the applied medium contained thiosulfate and sulfide, that are both potential substrates for the Sox multienzyme complex, the actual inducer could not be identified. As the thiosulfate concentration is five times higher than that of sulfide, the former is the more likely candidate. For best results in protein purification *A. vinosum* was grown on thiosulfate / sulfide, and the soluble fraction was subjected to the purification procedure.

C.3.1.2 Ammonium sulfate precipitation

40 g of cell material resuspended in the corresponding buffer was applied to ultrasound treatment and ultracentrifugation. The first following step in purification was protein precipitation from the centrifugation supernatant with ammonium sulfate. The goal was to keep the Sox proteins soluble in the supernatant. As a first experimental shot a 40 % saturation with ammonium sulfate was chosen. After resuspending the pellet, both fractions were tested concerning their SoxA content, again as a model for the other Sox proteins (see Figure C10).

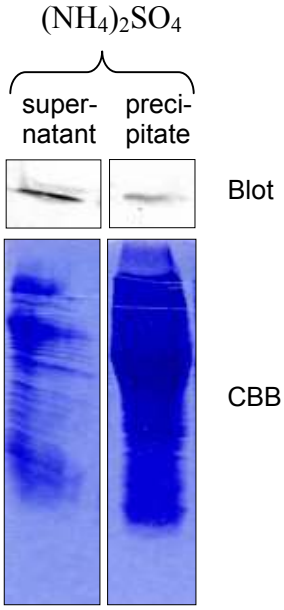

Figure C10: Ammonium sulfate precipitation of thiosulfate-grown *A. vinosum* cell material: Coomassie-stained SDS-PAGE with supernatant and resuspended precipitate, together with the corresponding Western blot signal for SoxA.

Even with the much higher protein concentration in the resuspended precipitate (discernible from the Coomassie stain), the signal for SoxA was still significantly stronger in the supernatant. The slight loss of Sox protein to the precipitate was acceptable. So a 40% saturation with ammonium sulfate was the first step in all Sox protein purifications.
The high salt concentration in the supernatant after ammonium sulfate precipitation predestined the preparation for hydrophobic interaction chromatography, that needs a high salt concentration as a starting condition.

C.3.2: Purification of SoxXA

Even if the applied antiserum was only directed against SoxA, the joint purification of SoxA and SoxX was assumed, taking into account the results achieved in other organism like *P. pantotrophus* (Friedrich *et al.*, 2000), *R. sulfidophilum* (Appia-Ayme *et al.*, 2001) and *S. novella* (Kappler *et al.*, 2004). SoxXA could be purified from these organisms as a functional heterodimer according to the Sox system model. Therefore, the reference to SoxXA in the purification description implies the detection of the subunit SoxA, while the other subunit SoxX was not detected but assumed to be present.

C.3.2.1 Different purification strategies

Different strategies were tested for the purification of SoxXA from *A. vinosum* in the first chromatographic step (HIC: hydrophobic interaction chromatography). It was examined, whether a linear or a step gradient of decreasing ammonium sulfate concentration provided better results concerning protein purity. In later experiments the applied buffer was also modified. While in the first experiments the buffer composition was quite simple (just the buffer substances and the elution-active substance, adjusted to the correct pH value), for later experiments additional substances were added (sodium thiosulfate, magnesium sulfate, PMSF) to obtain a better environment concerning protein stability. Therefore the respective buffers were referred to as stabilising buffers and were also applied in the other purification steps and for the other Sox proteins. The results obtained for the different purification strategies were comparable in protein yield and purity. Therefore, only the latest attempt using a step gradient and stabilising buffer in HIC is presented. Figure C11 shows the chromatogram of the hydrophobic interaction chromatography.

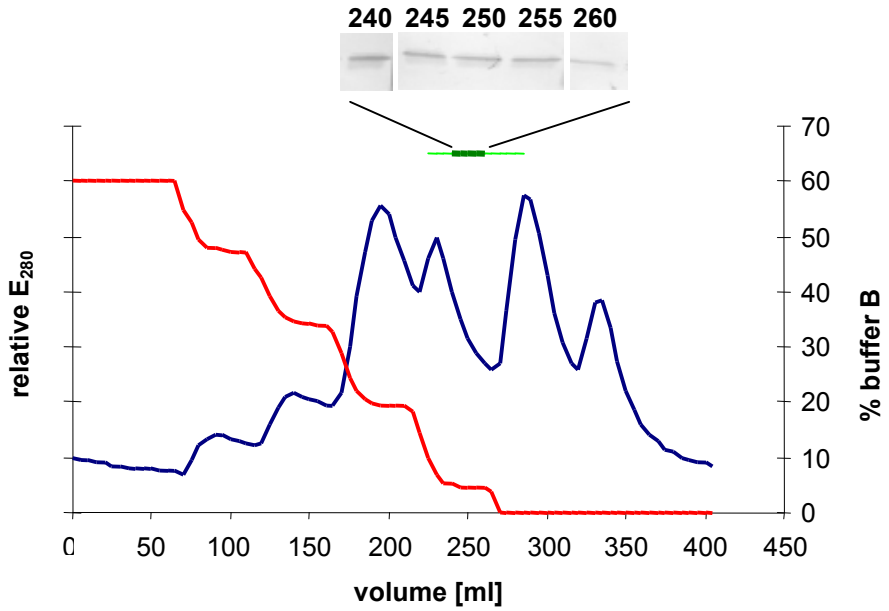

Figure C11: Chromatogram of the hydrophobic interaction chromatography to purify SoxXA, using a Phenylsepharose matrix and a step gradient. The gradient was started at 60 % buffer B, followed by a stepwise decrease of 10 % every 50 ml. Protein measurement at 280 nm (blue) is depicted as relative units. The salt gradient (red), depicted as % buffer B, was measured by conductivity. The fractions containing SoxXA are marked in light green, the fractions pooled and used for the following purification step in dark green. The SoxA signals for the latter are depicted above the chromatogram.

SoxA was detectable over a relatively broad range of fractions after the hydrophobic interaction chromatography. However, for further purification steps only fractions, that exhibited a strong signal in the Western blot (elution volume 240 to 260 ml), were pooled and dialysed over night against the low salt buffer for anionic exchange chromatography (→ stabilising buffer MQ).

The second method used for purification was a strong anion exchange column (MonoQ). The dialysed protein sample was applied to the column and washed with 10 % buffer B. The protein elution was achieved by a linear gradient of increasing NaCl concentration up to 60 % buffer B. The resulting chromatogram is depicted in Figure C12.

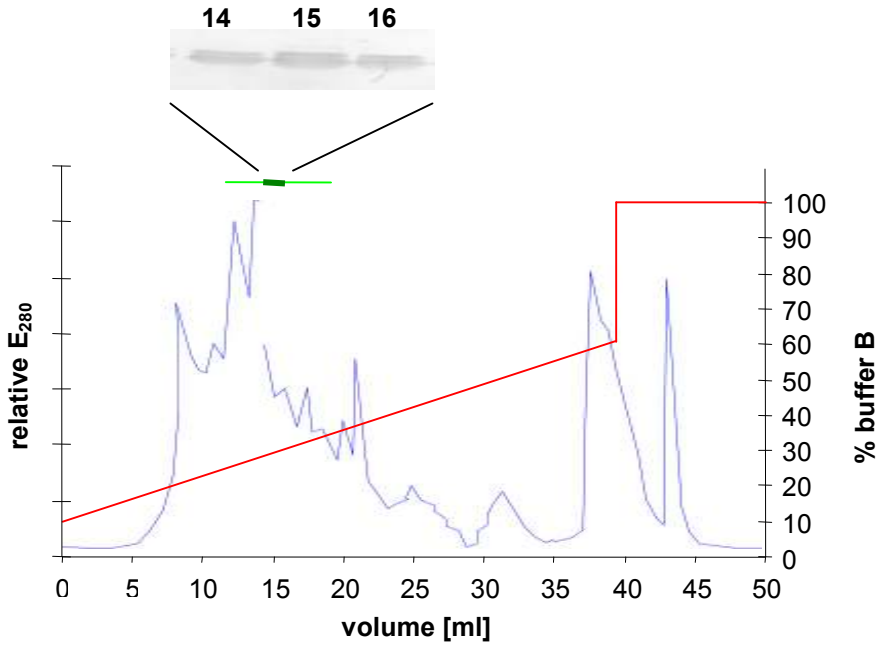

Figure C12: Chromatogram of the anionic exchange chromatography to purify SoxXA using a MonoQ column and a linear gradient. The gradient was started at 10 % buffer B and increased to 60 % in a volume of 40 ml. Residual protein was eluted with 100 % buffer B. Protein measurement at 280 nm (blue) is depicted as relative units. The theoretical salt gradient (red) is depicted as % buffer B. The fractions containing SoxXA are marked in light green, the fractions pooled and used for the following purification step in dark green. The SoxA signals for the latter are depicted above the chromatogram.

After the ion exchange chromatography (IEX) SoxXA was again detectable in a range of fractions. The anionic exchange chromatography led to a concentration of the protein, so that the SoxXA-containing fractions 14 to 16 ml exhibited a slightly pink colour typical for cytochromes.

The last purification step was a gel filtration chromatography using a Superdex 200 column. Because of a preceding calibration with proteins with known molecular weight (dextran blue: 2000 kDa, bovine albumin: 66 kDa, carboanhydrase: 29 kDa, horse heart cytochrome c: 12,4 kDa, aprotenin: 6,5 kDa), it was possible to obtain the molecular weight of an unknown protein. The logarithmic calibration curve for the Superdex 200 is depicted in figure C13.

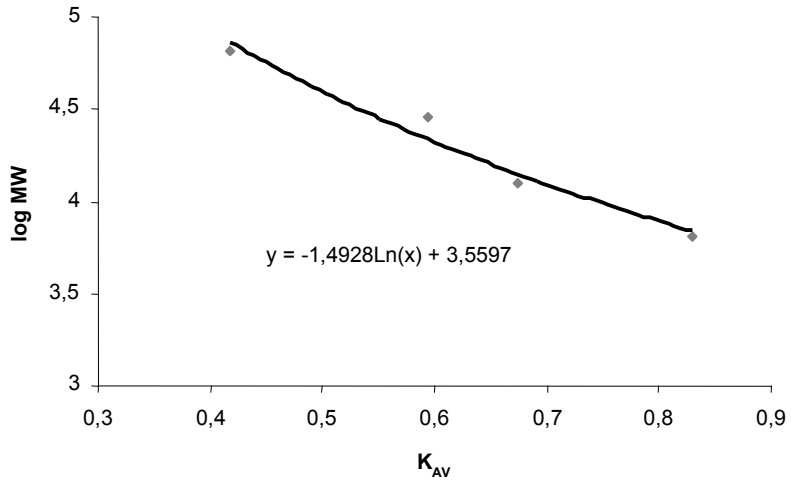

Figure C13: Calibration curve for the gel filtration column Superdex 200. The void volume (v_0) was set at 43 ml, corresponding to the elution volume of dextran blue. The total volume (v_t) was 126 ml. $K_{AV} = (v_e - v_0) / (v_t - v_0)$

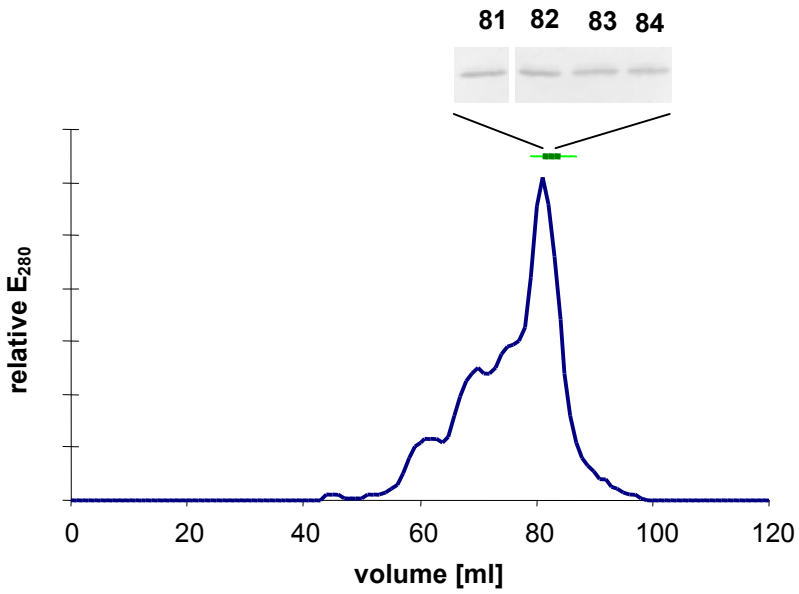

Figure C14: Chromatogram of gel filtration to purify SoxXA using a Superdex 200 column. Protein measurement at 280 nm (blue) is depicted as relative units. The fractions containing SoxXA are marked in light green, the fractions pooled and used for the following purification step in dark green. The SoxA signals for the latter are depicted above the chromatogram.

After the gelfiltration the strongest signals for SoxA corresponded to an elution volume of 81 to 84 ml, an average of v_e= 82,5 ml. This elution volume of 82,5 ml corresponds to a molecular weight of approximately 46,6 kDa, that is in accordance with the postulated heterodimer of SoxA (30 kDa) and SoxX (11 kDa). Therefore, the subunit structure already described for SoxXA proteins of other organism and predicted for SoxXA of *A. vinosum* could be confirmed.

The SoxXA purification was followed by observing the protein content of the SoxXA-containing fractions after the separate chromatographic steps by SDS-PAGE (see figure C15).

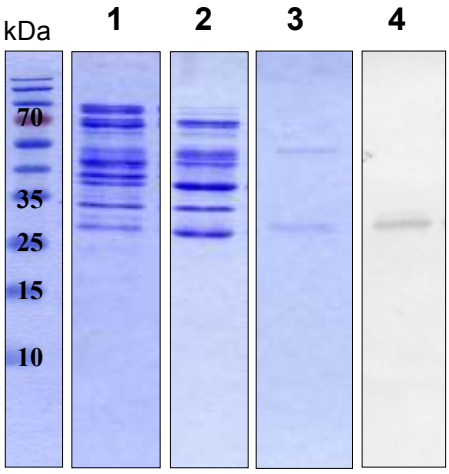

Figure C15: Purification of SoxXA from *A. vinosum*, followed by SDS-PAGE. Lanes: 1. HIC fraction eluted at 250 ml, 2. IEX fraction eluted at 15 ml, 3. GF fraction eluted at 83 ml, 4. Western blot (of GF fraction at 83 ml) with SoxA antiserum.

The SDS-PAGE demonstrated, that SoxXA was purified almost to homogeneity. However, only the subunit SoxA is clearly visible, SoxX not discernible at the expected size of 11 kDa. According to the expected and verified subunit structure of the heterodimeric SoxXA the subunits SoxA and SoxX should be present in equimolar amounts. However, the Coomassie-staining of SoxX is too weak in relation to the actual protein concentration. This effect has also been observed for SoxX of *P. pantotrophus* (Friedrich *et al.*, 2000), *R. sulfidophilum* (Appia-Ayme et al., 2001) and *S. novella* (Kappler *et al.*, 2004), confirming, that there is no need to question the subunit structure obtained by gel filtration. Nevertheless, the yield of SoxXA is fairly low, as even the strongly stained SoxA is hardly visible in the SDS-PAGE.

When the presented purification is compared to the preceding purification attempts, it exhibits a slight difference, as no protein bands below 25 kDa are visible in the Coomassie-stained SDS-PAGE. With the only real difference between the preceding attempts and this purification being the buffer composition, a closer look at it could deliver the desired explanation for the obtained results. Potentially the protein bands with a small molecular weight, apart from SoxX, were only fragments of larger proteins, whose degradation was prevented in the presented purification by the addition protease inhibitor PMSF and / or sodium thiosulfate and magnesium sulfate.

C.3.2.2 Further analysis of the purified SoxXA

The protein yielded after gel filtration was used for further examination of SoxXA concerning its heme content. The protein SoxXA was fairly diluted after gel filtration, with the SoxX subunit hardly visible. Therefore, the SoxXA containing fractions 80 to 89 after gel filtration were pooled and concentrated about 17 fold before the following experiments. Thereby contaminating proteins were once again introduced into the preparation for the sake of an increased SoxXA content. The analysis of SoxXA included the measurement of a spectrum, two heme staining methods differing in sensitivity, and the quantification of the heme content by the measurement of pyridine spectra. In the two latter cases horse heart cytochrome c was used as positive control.

Spectrum:

When recording a spectrum of the fractions containing SoxXA, a typical spectrum for a c-type cytochrome could be obtained (see Figure C16).

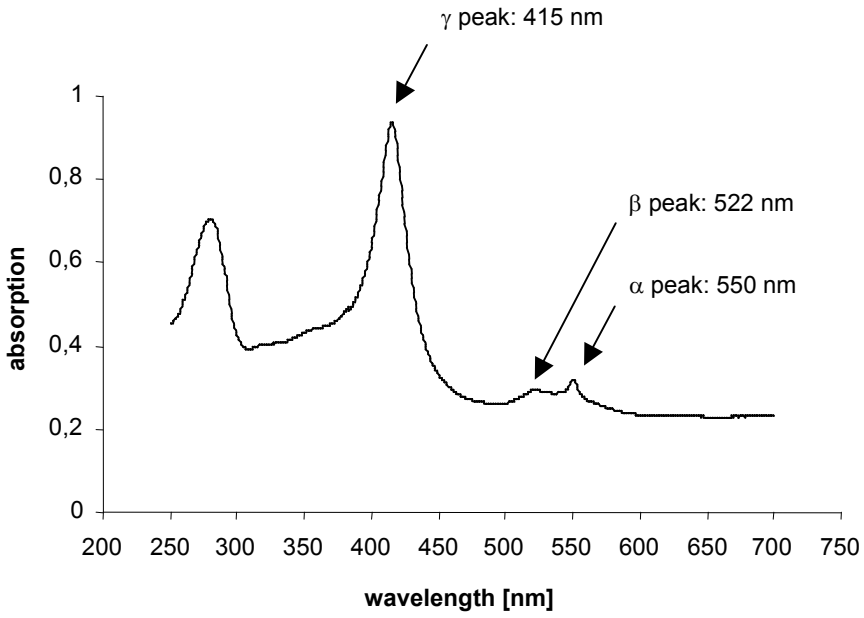

Figure C16: Spectrum of purified and concentrated SoxXA after gel filtration (as prepared). Identified peaks: α: 550 nm, β: 522 nm, γ: 415 nm. Protein concentration: 486 µg/ml. The absorptions at 280 nm (0,7042) and 415,5 nm (0,9392) result in a ratio of 0,75.

While the spectrum for concentrated SoxXA exhibited all characteristics of the postulated c-type cytochrome, the surprising fact was the detection of the reduced form. Without the addition of reductants like dithionite SoxXA presented the reduced spectrum of a cytochrome c_{550} with a α peak at 550 nm, a β peak at 522 nm and a γ peak (soret band) at 415 nm. Potentially the shoulder on the left side of the soret band represented the δ-peak at about 365 nm. Despite the chosen conditions of aerobic protein purification, under which related cytochromes become air-oxidised (Friedrich *et al.*, 2000; Appia-Ayme *et al.*, 2001; Kappler *et al.*, 2004), the reduced form seemed to be quite stable. The spectrum of a reduced c-type cytochrome was also recorded during the purification procedure in the SoxXA-containing fractions after HIC and IEX.

The quotient of the absorption at 280 nm (representing the total protein content) and the absorption at 415 nm (representing the heme content) gives information about the achieved purity of SoxXA. With a quotient of $E_{280} / E_{415} = 0{,}7042 / 0{,}9392 = 0{,}75$ the achieved purity is not as good as was assumed because of the SDS-PAGE results. However, it must be taken into account, that gel filtration fractions containing proteins apart from SoxXA have been pooled for concentration. Therefore, the actual purity is in all probability much better, as was also observed in the SDS-PAGE.

Heme staining:

Now that the SoxXA heterodimer was confirmed to be a cytochrome c_{550}, the actual heme content remained to be determined. By heme group detection after denaturing SDS-PAGE the subunits SoxX and SoxA were examined separately for the presence of heme as a prosthetic group. Two different methods were applied with differing sensitivity. The results are shown in figure C17, together with the positive control.

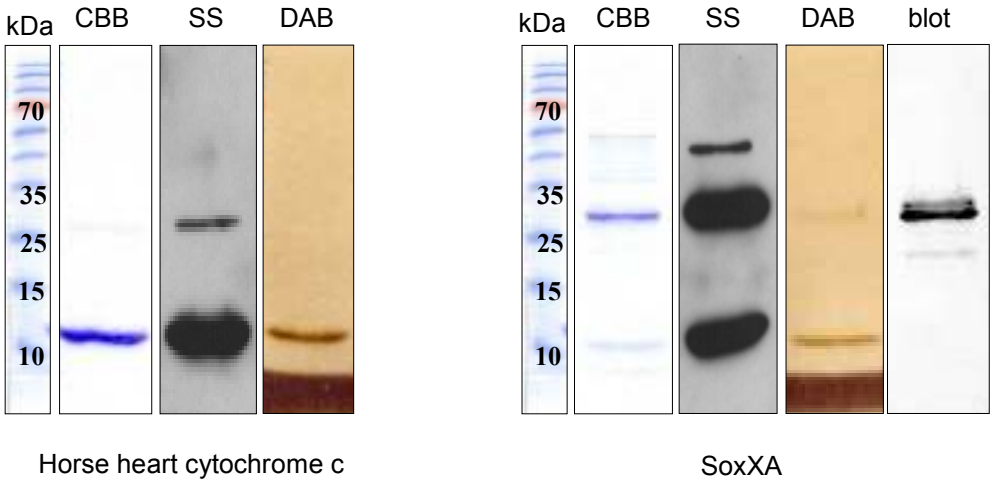

Figure C17: Heme group detection in horse heart cytochrome c and concentrated SoxXA.
CBB: SDS-PAGE stained with Coomassie Brilliant Blue, SS: heme detection with SuperSignal substrate, DAB: heme staining with diaminobenzidine, blot: Western blot with SoxA antiserum. Protein concentration: horse heart cytochrome c 333 µg/ml, SoxXA 486 µg/ml.

The positive control performed with horse heart cytochrome c provided an excellent example for the difference in sensitivity concerning the two heme detection methods. The Coomassie stain showed the protein at its expected size of 12,4 kDa, but also a potential aggregate at a higher molecular weight. The less sensitive method applying diaminobezidine only stained the protein band at 12,4 kDa. The sensitive detection with SuperSignal substrate, however, using the heme peroxidase activity to produce a chemiluminescent signal, was able to produce signals for both protein bands. After the concentration of the gel filtration fractions both subunits of SoxXA were visible after Coomassie staining, together with a slight background of contaminating proteins. Diaminobenzidine stained both subunits, SoxX and SoxA, therefore confirming the postulated presence of heme in each subunit. The reason for the weaker stain in SoxA than in SoxX is not known. The heme detection by chemiluminescence provided equally strong signals for both SoxX and SoxA, with an additional signal at a higher molecular weight. Because of its size of approximately 40 kDa it could be a SoxXA heterodimer not denatured during sample preparation. However, the Western blot analysis showed SoxA only to be present in the expected protein band at 30 kDa. Therefore, the additional signal is potentially due to another c-type cytochrome in the preparation apart from SoxXA, even if at significantly lower concentration. Nevertheless, SoxXA was the major cytochrome in the preparation

Pyridine spectra:

To obtain the actual amount of heme in the protein pyridine spectra were recorded. As the concentrated protein was not completely homogeneous, the obtained results were less of a quantitative, but more of a qualitative value to confirm the results already obtained by other methods. Horse heart cytochrome c was used as a positive control for correct experimental procedures. The results are presented in figure C18.

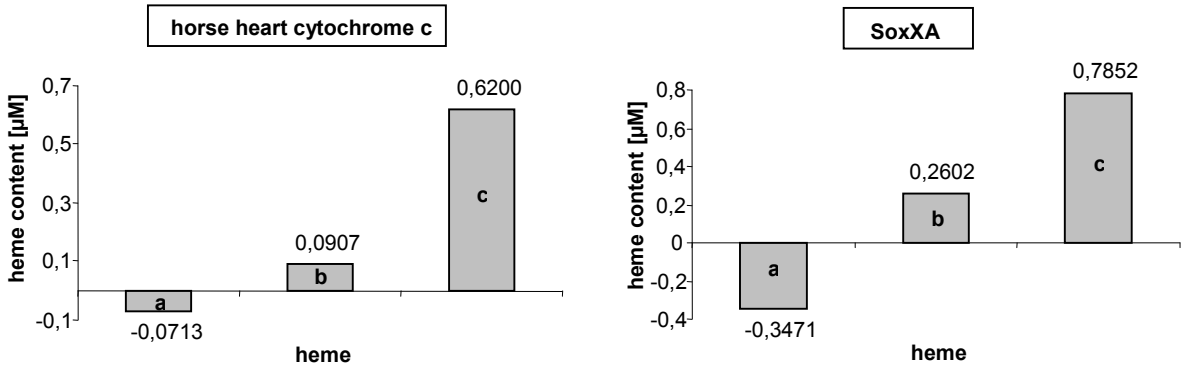

Figure C18: Determination of protein heme content by recording of pyridine spectra for defined protein concentrations of horse heart cytochrome c (as positive control) and SoxXA. Protein concentration: horse heart cytochrome c 1,24 µg/ml, SoxXA 48,6 µg/ml.

Looking at the results in a strictly qualitative way, they confirm the identity of horse heart cytochrome c and SoxXA as c-type cytochromes. Concerning the quantification of the heme content, the results varied in their value. For horse heart cytochrome c a heme content of 0,62 µmol heme c per µmol protein was obtained, representing approximately the one heme group known to be present in one protein molecule. For SoxXA a heme content of 0,67 µmol heme c per µmol protein was obtained. As the heterodimeric protein should contain one heme per subunit, two heme molecules per protein SoxXA, the determined heme content is about 50 % of the expected amount. This could be due to the additional contaminating proteins that artificially raise the protein concentration, but not the heme content. Therefore approximately only half of the examined protein would be SoxXA.

C.3.3 Purification of SoxB

C.3.3.1 Different purification strategies

For the purification of SoxB the same strategy used for SoxXA was applied concerning the order of chromatographic methods. For SoxB only the step gradient was used in hydrophobic interaction chromatography with and without stabilising buffer. As the obtained results for both attempts were equivalent, only the purification using stabilising buffer is presented.. The chromatogram for the hydrophobic interaction chromatography to purify SoxB is depicted in Figure C19. As SoxB and SoxXA could be purified from the same hydrophobic interaction chromatography, the same chromatography was used to obtain both proteins.

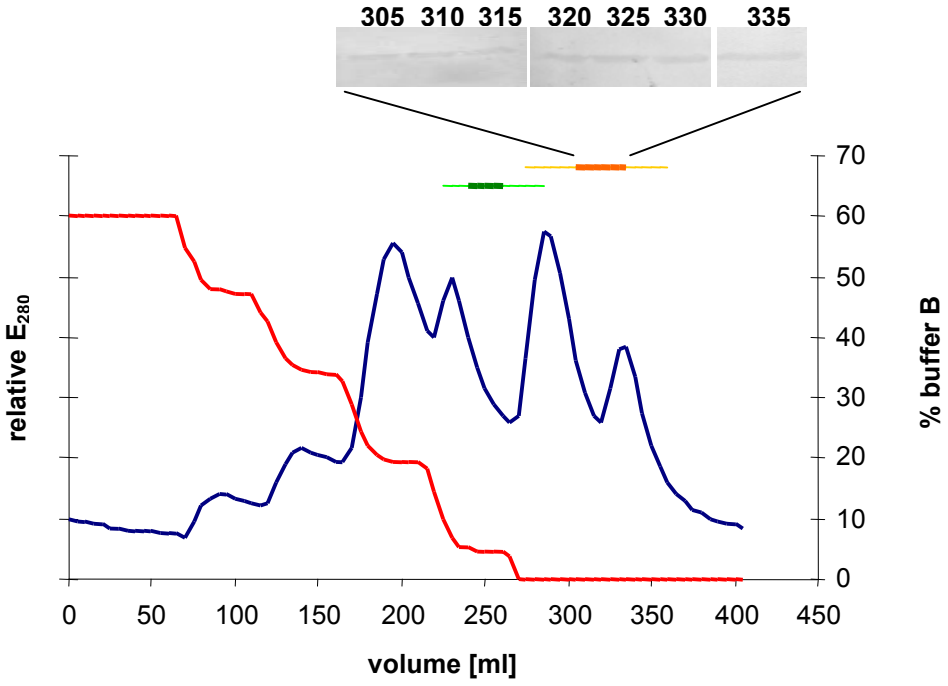

Figure C19: Chromatogram of the hydrophobic interaction chromatography to purify SoxB, using a Phenylsepharose matrix and a step gradient. The gradient was started at 60 % buffer B, followed by a stepwise decrease of 10 % every 50 ml. Protein measurement at 280 nm (blue) is depicted as relative units. The salt gradient (red), depicted as % buffer B, was measured by conductivity. The fractions containing SoxXA are marked in green. The fractions containing SoxB are marked in light orange, the fractions pooled and used for the following purification step in dark orange. The SoxB signal for the latter is depicted above the chromatogram.

The SoxB antiserum indicated the presence of the protein in a broad range of fractions, that also overlapped with the SoxXA containing fractions depicted in green. Because of the results obtained for antiserum testing, a slight uncertainty remained concerning the identity of the detected protein. Nevertheless, the fractions with the strongest potential signal for SoxB (an elution volume of 305 to 335 ml) were pooled, dialysed against low salt MonoQ buffer and subjected to anionic exchange chromatography. The obtained chromatogram is depicted in Figure C20.

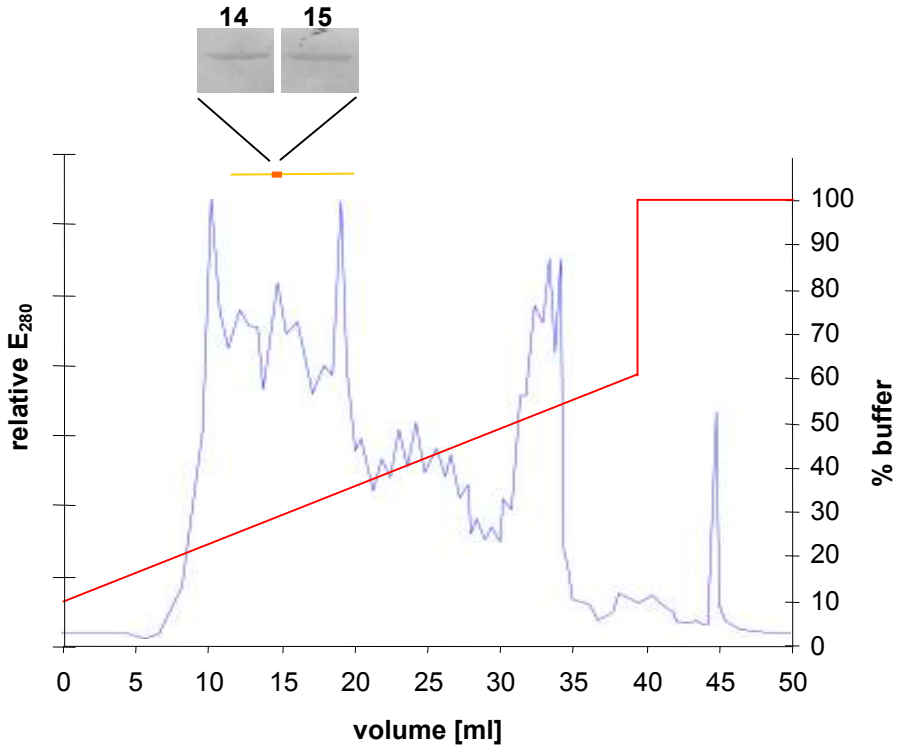

Figure C20: Chromatogram of the anionic exchange chromatography to purify SoxB using a MonoQ column and a linear gradient. The gradient was started at 10 % buffer B and increased to 60 % in a volume of 40 ml. Residual protein was eluted with 100 % buffer B. Protein measurement at 280 nm (blue) is depicted as relative units. The theoretical salt gradient (red) is depicted as % buffer B. The fractions containing SoxB are marked in light orange, the fractions pooled and used for the following purification step in dark orange. The SoxB signal for the latter is depicted above the chromatogram.

The results of the anionic exchange chromatography provided several problems concerning the accuracy of the SoxB antibody binding. When taking earlier experiments into account, SoxB was detectable in almost every fraction from 20 % up to 100 % buffer B. This could be due to an interaction of protein and column material. A second possibility would be the additional detection of another protein of the same size as SoxB, as observed in *E. coli* crude

extract (see Figure C7). The problem was to distinguish between the two proteins. In the first attempt, the fractions corresponding to the strongest Western blot signal were subjected to gel filtration. However, the protein eluted shortly after the void volume, representing a large protein aggregate of either SoxB or an altogether different protein. Further experiments confirmed, that these fractions do not contain SoxB. Therefore, a closer look was taken at the Western blot results. Directly below the would-be SoxB signal in the MonoQ fractions 14 and 15 ml appeared a second band, exactly as observed for the heterologous SoxB produced in *E. coli*. So these fractions were subjected to gel filtration. The resulting chromatogram is shown in Figure C21.

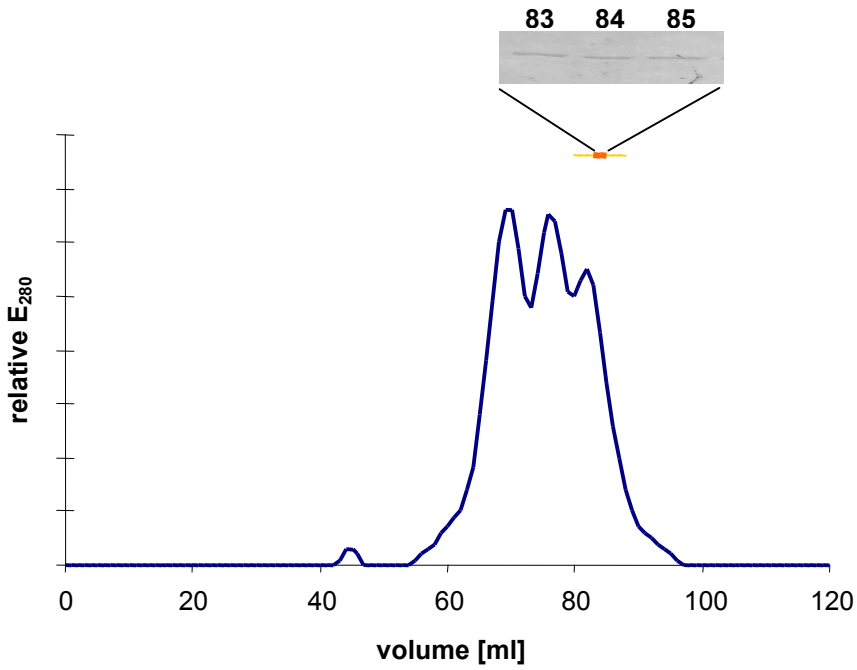

Figure C21: Chromatogram of gel filtration to purify SoxB using a Superdex 200 column. Protein measurement at 280 nm (blue) is depicted as relative units. The fractions containing SoxB are marked in light orange, the fractions with the strongest Western blot signal in dark orange. The SoxB signals for the latter are depicted above the chromatogram.

The gel filtration resulted in an elution of SoxB in a fairly small range of fractions. The strongest Western blot signals were obtained for the fractions corresponding to an elution volume of 83 to 85 ml. The mean elution volume of v_e = 84 ml is equivalent to a molecular weight of 41 kDa. Even if the measured protein size differs from the predicted protein size (62 kDa), this purified protein was far more likely to be SoxB than the protein eluting shortly

after the void volume. The identity of SoxB was confirmed by MALDI-TOF analysis (see C.3.3.2).

The purification of SoxB was quite successful, as only one other protein was visible on the SDS-PAGE after gel filtration (see Figure C22). The SDS-PAGE revealed a discrepancy between the molecular weight determined on the protein gel and the molecular weight determined by gel filtration. While according to the gel filtration SoxB would have a size of 41 kDa, the protein appeared as a band of approximately 60 kDa on the denaturing gel. The latter would be very close to the predicted protein size of 62 kDa. When applying the would-be SoxB to SDS-PAGE, a comparable protein band at approximately 60 kDa appeared. Perhaps this protein actually eluted from the gel filtration column in a large aggregate, that was degraded in the sample preparation for SDS-PAGE. In contrast, SoxB potentially exhibited an interaction with the column material, which resulted in a slight delay of elution.

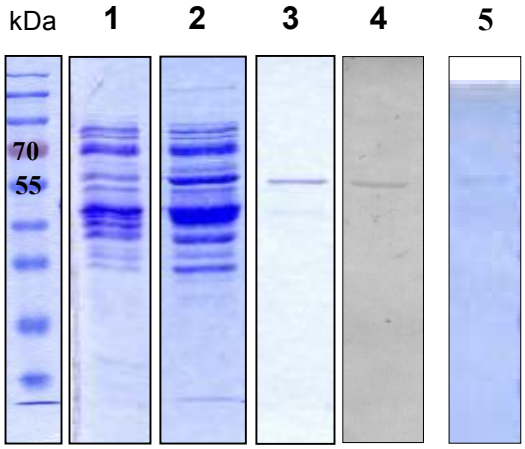

Figure C22: Purification of SoxB from *A. vinosum*, followed by SDS-PAGE. Lanes: 1. HIC fraction eluted at 320 ml, 2. IEX fraction eluted at 14 ml, 3. GF fraction eluted at 84 ml, 4. Western blot (of GF fraction 84 ml) with SoxB antiserum, 5. GF fraction not corresponding to SoxB, but detected with the antiserum.

When comparing the different attempts to purify SoxB, the results were equally good. In the presented purification there was only one other protein present apart from SoxB. Also the protein yield appeared to be slightly bigger in the presented approach, as judged by the Coomassie-stained SDS-PAGE. The use of stabilising buffer during purification had the same effect already observed during SoxXA purification. Compared to preparations without the stabilising agents, there were almost no small contaminating proteins present. Perhaps this is

again due to an increase in protein stability achieved by the addition of protease inhibitor, sodium thiosulfate and magnesium sulfate.

C.3.3.2 MALDI-TOF analysis of SoxB

Regarding the less-than-perfect performance of the SoxB antiserum, the purified protein was subjected to MALDI-TOF analysis for further proof. As the protein was quite diluted after gel filtration, the SoxB-containing fractions 81 to 90 ml were pooled and concentrated. After SDS-PAGE SoxB was cut out and analysed by MALDI-TOF after tryptic digestion. The obtained peptide fragments, together with the amino acid sequence and size of predicted fragments, are presented in Table C2.

peptide sequence	predicted mass [Da]	obtained mass [Da]
EEALFDYR	1042,4840	1042,3822
FGAVGGFAHLK	1103,5996	1103,4786
LAIAGELLYR	1118,6568	1118,5409
EPNVNLGIGSALGR	1396,7543	1396,6082
VHDAQISLSPGFR	1426,7437	1426,5041
LLPVFSNLIEPDR	1512,8420	1512,6846
FIPDWTFGIEDGR	1552,7430	1552,5818
NIAEFQGAFVAQNVR	1663,8550	1663,6901
→ ERPSDPYEIDAFGQVR	1860,9238	1860,7369
VAVIGQAFPYTPIANPSR	1901,0279	1900,8384
LLHITDTHAQLNPIYFR	2052,1025	2051,9053
SLLLDGGDTWQGSGTAYWTR	2184,0356	2183,8257
LGVDVMTGHWEFTYGDEEVIR	2453,1441	2452,9089
FGIAPGGLEAHAFTYLDFAAAAER	2495,2353	2494,9949
GIDVILGGHTHDGVPTPILVENPGGK	2592,3779	2592,1509

Table C2: MALDI-TOF analysis of purified SoxB. Amino acid sequence of tryptic peptide fragments predicted for SoxB (based upon the nucleotide sequence), their predicted mass [Da] and the respective mass of detected peptide fragments [Da]. The arrow indicates the peptide fragment, that confirms the signal peptide cleavage position.

MALDI-TOF analysis provided 15 peptide fragments that could be assigned to predicted tryptic fragments of SoxB. This unequivocally confirmed the identity of the purified protein as SoxB. Additionally, the proposed position for signal peptide cleavage could be confirmed,

as the fragment with a mass of 1860,737 Da only appears, when the protein has been correctly processed. This provides additional proof for the predicted periplasmic localisation of SoxB.

C.3.4 Purification of SoxYZ

C.3.4.1 Purification strategy

The first step in the chromatographic purification of SoxYZ was hydrophobic interaction chromatography, as performed for SoxXA and SoxB, but exclusively with the use of stabilising buffer. Because of the poor performance delivered by the SoxYZ antiserum in fractions after HIC, the identification of SoxYZ-containing fractions was fairly difficult. A linear gradient starting with 100 % buffer B was used in this case. The chromatogram for the hydrophobic interaction chromatography is depicted in Figure C23.

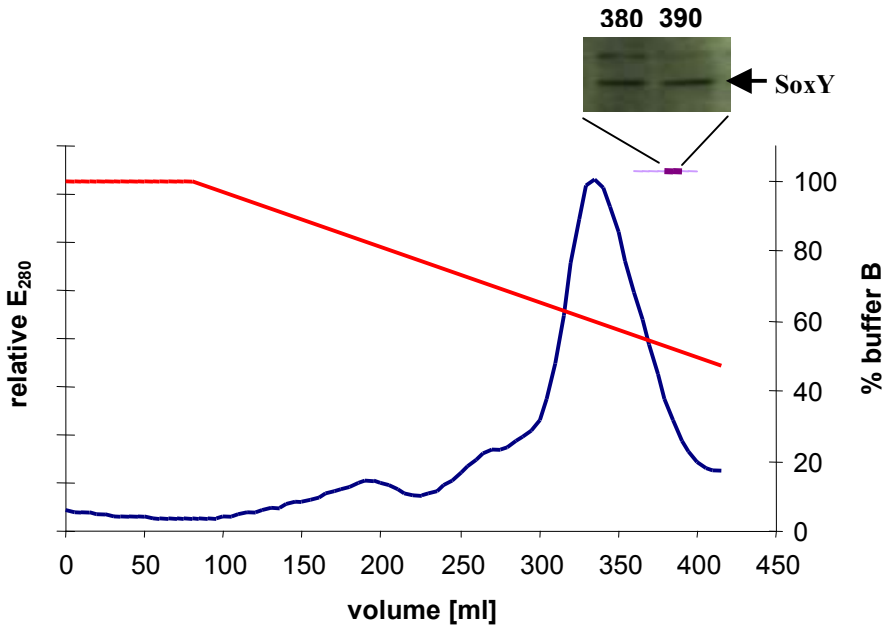

Figure C23: Chromatogram of the hydrophobic interaction chromatography to purify SoxYZ, using a Phenylsepharose matrix and a linear gradient. The gradient was started at 100 % buffer B, followed by a linear decrease to 50 % over 335 ml. Protein measurement at 280 nm (blue) is depicted as relative units. The salt gradient (red), depicted as % buffer B, was measured by conductivity. The fractions containing SoxYZ are marked in light purple, the fractions pooled and used for the following purification step in dark purple. The SoxYZ signals for the latter are depicted above the chromatogram.

A Western blot signal potentially assigned to SoxYZ was detectable in HIC fractions from 360 to 400 ml, with the strongest signal corresponding to an elution volume of 380 to 390 ml. The additional signal appearing at a higher molecular weight was in all probability due to the weak accuracy of the antiserum, the effect visible with *A. vinosum* cell material and heterologous protein produced in *E. coli*. The SoxYZ-containing fractions 380 to 390 ml were used for further purification. Because of the already low protein content (as observed in SDS-PAGE), the anionic exchange chromatography step was omitted. Before subjecting the protein sample to gel filtration, it needed to be concentrated to an appropriate volume of 2 ml. The following gel filtration was performed using the respective stabilising buffer. The resulting chromatogram is depicted in figure C24.

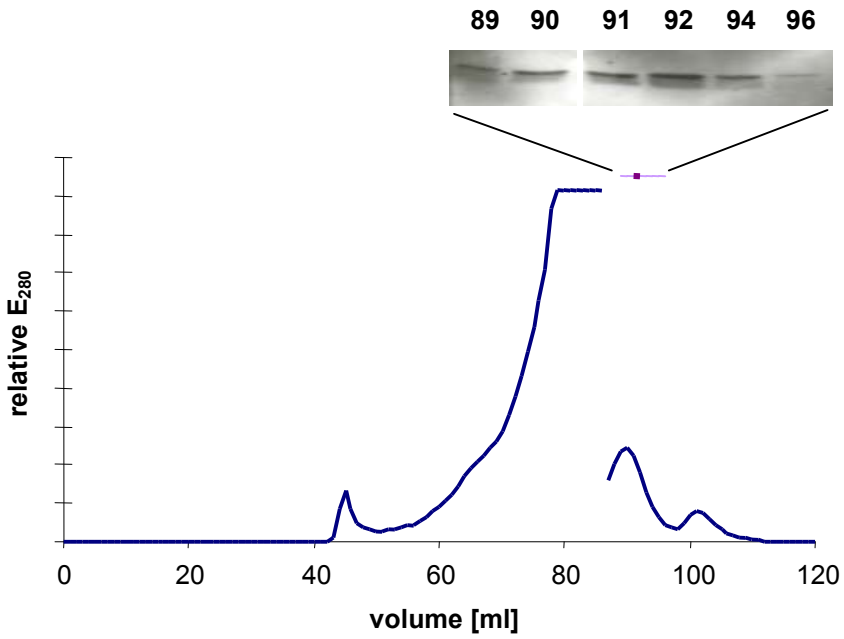

Figure C24: Chromatogram of gel filtration to purify SoxYZ using a Superdex 200 column. Protein measurement at 280 nm (blue) is depicted as relative units. The fractions containing SoxYZ are marked in light purple, the fractions with the strongest Western blot signal in dark purple. The SoxYZ signals for the latter are depicted above the chromatogram.

With less contaminating protein present in the preparation, the background noise in Western blot analysis was reduced, so that interpretation of the results was easier. During gelfiltration the protein responsible for the second signal in Western blot analysis after the hydrophobic interaction chromatography has been separated from SoxYZ, it appeared at a lower elution volume. SoxYZ eluted from the gel filtration column at an elution volume from 89 to 96 ml

with the strongest signals in fractions 91 and 92 ml. The mean elution volume of v_e = 91,5 ml corresponded to a molecular weight of 23 kDa. With SoxY at a size of 12,7 kDa and SoxZ at a size of 11,2 kDa, the determined molecular weight matched a heterodimeric subunit structure of SoxYZ.

The purification of SoxYZ was followed by SDS-PAGE, the results are shown in figure C25.

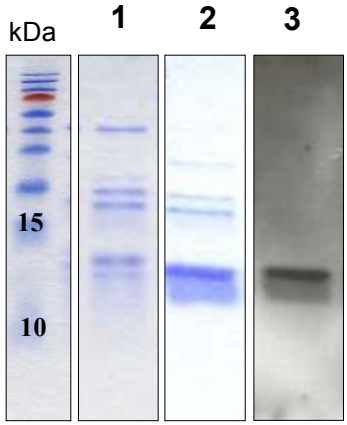

Figure C25: Purification of SoxB from *A. vinosum*, followed by SDS-PAGE. Lanes: 1. HIC fraction eluted at 380 ml, 2. GF fraction eluted at 92 ml, 3. Western blot (of GF fraction 92 ml) with SoxYZ antiserum.

The first chromatographic step (HIC) resulted in a considerable reduction of the protein content, thereby justifying the omission of the intermediate IEX step. After the gel filtration, however, several contaminating proteins apart from SoxYZ were still present in the preparation. The protein bands corresponding to SoxY and SoxZ were identified by the Western blot signal. But the actual identification of SoxY (upper band) and SoxZ (lower band) in the SDS-PAGE by their molecular weight could be deceiving. SoxY and SoxZ from *P. pantotrophus* have a molecular weight of approximately 11 kDa and 11,7 kDa, respectively. Nevertheless, SoxZ is found at a molecular weight of 16 kDa in SDS-PAGE (Friedrich *et al.*, 2000). Therefore a definite assignment of the observed protein bands to the respective Sox proteins was difficult. Additionally, the lower protein band was quite blurred, potentially due to the enhanced protease sensitivity of SoxYZ compared to the other Sox proteins that was observed in *P. pantotrophus* (Friedrich, personal communication).

C.3.4.2 MALDI-TOF analysis of SoxYZ

To shed more light on the identity of the purified proteins, the protein band corresponding to SoxY was subjected to MALDI-TOF analysis. After a preceding concentration of the SoxYZ-containing gel filtration fractions (87 to 96 ml) and SDS-PAGE, the protein was cut out of the gel, tryptically digested and analysed by MALDI-TOF. The obtained peptide fragments, together with the amino acid sequence and size of predicted fragments, are presented in Table C3.

peptide sequence	predicted mass [Da]	obtained mass [Da]
VTIGGCGG	633,3130	775,3874
DIPTAMTGLLGSDAAEVSDR	2018,9699	2018,9595
NQSPLIASFEFVDPSVIPFVATR	2534,3289	2534,3196
TNEVIPAHFIR	1296,7059	1296,6992

Table C3: MALDI-TOF analysis of purified SoxYZ. Amino acid sequence of tryptic peptide fragments predicted for SoxY and SoxZ (based upon the nucleotide sequence), their predicted mass [Da] and the respective mass of detected peptide fragments [Da]. The first three fragments are assigned to SoxY, the last to SoxZ.

The MALDI-TOF analysis of SoxYZ provided additional data to verify the identity of the protein. Fragments for SoxY as well as for SoxZ could be identified. Three fragments could be assigned to SoxY. The smallest of these fragments has potentially been modified. The predicted SoxY fragment (663 Da) contains the conserved C-terminus, together with the cysteine residue as the potential substrate binding site (VTIGG**C**GG). When a thiosulfate molecule (112 Da) was attached to the cysteine, the observed fragment size of 755 Da would be obtained. This modification could have taken place, as the proteins were purified from thiosulfate-grown *A. vinosum* cell material and the purification buffers also contained thiosulfate. This modification confirms the function of SoxY as the substrate binding subunit. One peptide fragment could be assigned to SoxZ. Therefore, the analysed protein sample must have been a mixture of both subunits, additionally verifying the interaction of SoxY and SoxZ.

C.3.5 Determination of enzymatic activity of Sox proteins in *A. vinosum*

The activity of the Sox system was determined as the thiosulfate-dependent reduction of horse heart cytochrome c, measured as an increase in extinction at 550 nm. Horse heart cytochrome c was applied, to differentiate between thiosulfate oxidation by the Sox system and thiosulfate oxidation by the thiosulfate:acceptor oxidoreductase. While ferricyanide was a very effective electron acceptor for the latter enzyme, and cytochrome c from *Saccharomyces cerevisiae* also performed fairly well, no activity of the thiosulfate:acceptor oxidoreductase was measured with horse hearty cytochrome c (Sperling, 2001). Therefore, any activity measured in the present enzyme assay should only be due to Sox enzyme activity. A blank was recorded using a preparation without the addition of enzyme solution. This blank was subtracted from all measured samples. Potential Sox enzyme activity was examined in the supernatant after ammonium sulfate precipitation and in three preparations with different concentrations of the purified and concentrated proteins SoxXA, SoxB and SoxYZ. The results are depicted in figure C26.

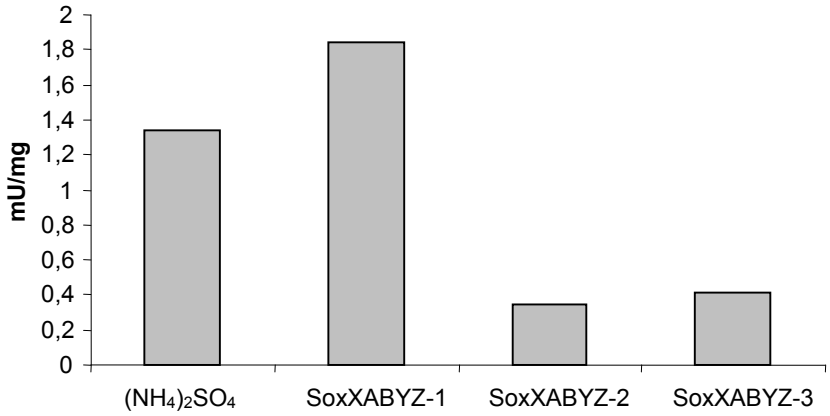

Figure C26: Determination of Sox enzyme activity in the supernatant after ammonium sulfate precipitation and with purified proteins in different compositions. Enzyme activity is presented in mU and mU/mg, respectively. Protein concentration in 1 ml assay: $(NH_4)_2SO_4$: 770 µg, SoxXABYZ-1: 3 µg of each protein; SoxXABYZ-2: SoxXA 4,86 µg, SoxB 3,05 µg, SoxYZ 29 µg ; SoxXABYZ-3: 1 µmol of each protein (SoxXA: 40,8 µg, SoxB: 62,2 µg, SoxYZ: 23,9 µg).

In the first preparation ($(NH_4)_2SO_4$) the supernatant obtained after ammonium sulfate precipitation was added to the assay. A thiosulfate-dependent reduction of horse heart cytochrome c of 1,34 mU/mg, significantly above the blank level, was detected. The reconstitution of the Sox multienzyme system with the purified proteins appeared to be partly

successful, as the first preparation (SoxXABYZ-1) exhibited an activity of 1,85 mU/mg. The other two preparations were significantly less active with 0,35 mU/mg (SoxXABYZ-2) and 0,41 mU/mg (SoxXABYZ-3).

C.4 *A. vinosum* mutant strains

During the work on my diploma thesis four different mutants in *sox* genes and potentially *sox*-related genes were produced (Hensen, 2001). The gene inactivation was achieved by the insertion of a kanamycin resistance cassette into the respective genes. The produced mutants are summarized in Table C4.

mutant strain	Characteristics	corresponding wild type
Δ*soxX*	insertion of a resistance cassette into *soxX*	DSM 180
Δ*soxB*	insertion of a resistance cassette into *soxB*	DSM 180
Δ*soxBX*	simultaneous insertion of a resistance cassette into *soxB* and *soxX* by removal of the intergenic region and insertion of a resistance cassette	DSM 180
ΔORF9/*rhd*	simultaneous insertion of a resistance cassette into ORF9 and *rhd*, thereby removing parts of both open reading frames	185SM50

Table C4: Characteristics and corresponding *A. vinosum* wild types of *A. vinosum* mutant strains produced during the work on the diploma thesis (Hensen, 2001).

C.4.1 The *in frame* mutant Δ*soxY*

C.4.1.1 Mutant construction

In addition to the already established insertional mutants, an *in frame* deletion of the *soxY* open reading frame was performed. The advantage of an *in frame* deletion is the preservation of the reading frame, thereby maintaining the transcription of the open reading frames situated downstream of the deletion. In contrast to that, the insertion of a resistance cassette can be cause for polar effects due to the destroyed reading frame. The corresponding wild type for this *in frame* mutant was a spontaneous rifampicin-resistant mutant of *A. vinosum* DSM 180, named *A. vinosum* Rif50 (Lübbe, personal communication). The *soxY* gene containing the

deletion was produced by SOEing PCR and inserted into the suicide vector pK19mobsacB. Because of the sensitivity of *A. vinosum* to the saccharase products, the mutation could be established in Rif50, leading to the mutant strain Δ*soxY*.

C.4.1.2 Mutant confirmation by PCR and Southern blot

The deletion of almost the complete *soxY* gene was confirmed by PCR and Southern blot analysis. The first primer pair for PCR analysis consisted of the two outer SOEing PCR primers (Yforward / Yreverse, PCR a), the second pair of one outer SOEing PCR primer and one primer with complementary sequence inside of the deletion (Yforward /Y2rev1, PCR b). The primer binding positions and the obtained PCR fragments are depicted in Figure C27.

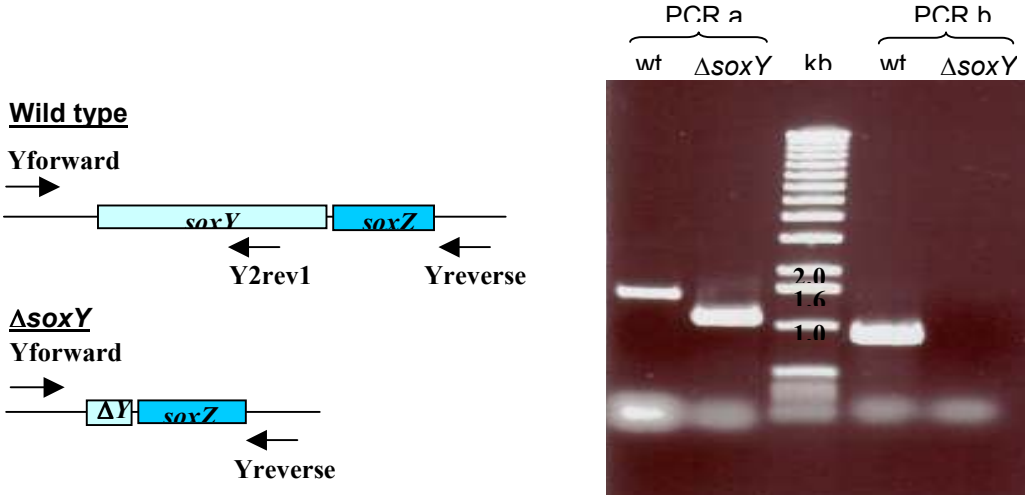

Figure C27: PCR control of the mutant strain Δ*soxY* in comparison to the corresponding wild type Rif50. The primer binding sites are depicted on the left, the obtained PCR products after gel electrophoresis on the right. PCR a: Yforward / Yreverse, PCRb: Yforward / Y2rev1.

The PCR analysis confirmed the correct deletion of almost the complete *soxY* gene in the mutant strain Δ*soxY*. For the wild type the first PCR (PCR a) produced a fragment of 1,5 kb. This fragment was reduced to a size of 1 kb in the mutant due to the deletion in the *soxY* gene. The second PCR (PCR b) could only obtain a 900 bp fragment in the wild type, as one of the primer binding site was deleted in the *soxY* mutant.

The Southern blot analysis was performed with digested genomic DNA of mutant and wild type strain. A digoxigenin-labelled *soxYZ* probe, produced with the SOEing PCR primer pair Yforward / Yreverse, was used for hybridisation. The obtained fragments are shown in Figure C28.

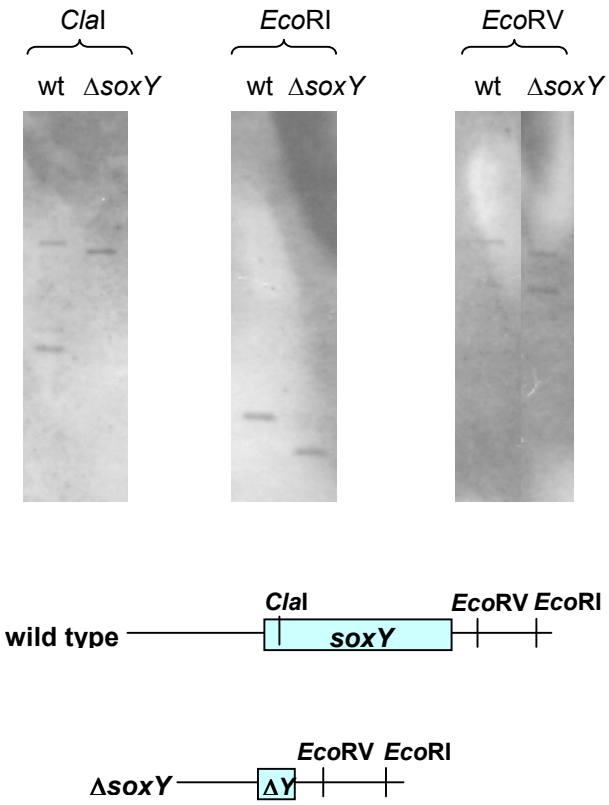

Figure C28: Southern blot analysis of the Δ*soxY* mutant strain and the corresponding wild type Rif50. The genomic DNA was digested with the restriction enzymes *Cla*I, *Eco*RI and *Eco*RV. The respective cutting positions are presented schematically. A *soxYZ* probe was used for hybridisation.

The Southern blot analysis confirmed the identity of the Δ*soxY* mutant in addition to the PCR results. The enzyme *Cla*I cut inside of *soxY*, thereby producing two detectable fragments in the wild type. The *in frame* deletion of *soxY* removed the *Cla*I recognition sequence, resulting in only one remaining detectable fragment. The enzymes *Eco*RI and *Eco*RV both cut inside of *soxZ* (the former right at the end of the nucleotide sequence). Accordingly, the detected fragment in the *Eco*RI digestion and one of the detected fragments in the *Eco*RV digestion, respectively, were reduced in size in the mutant strain.

C.4.2 Detection of SoxA in wild type and mutant strains

None of the produced mutants in *A. vinosum* contained a direct inactivation of the *soxA* open reading frame. Nevertheless, the polar effect caused by the insertion of an omega cassette must be taken into account. The insertion destroys the reading frame and inhibits the transcription of genes situated downstream of the target gene in the same transcription unit. Before their phenotypic characterisation the mutants Δ*soxX*, Δ*soxB* and Δ*soxBX* were tested for their ability to produce SoxA. With the corresponding gene located directly downstream of *soxX*, these two genes are very likely to be in one transcription unit. For these experiments the presence of SoxA was examined in crude extract of thiosulfate-grown *A. vinosum* wild type and mutants. The results are depicted in Figure C29.

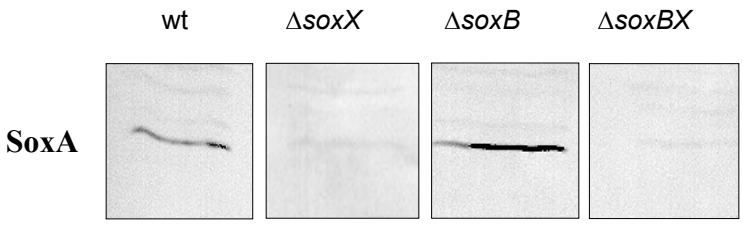

Figure C29: Comparison of SoxA production by Western blot analysis in thiosulfate-grown *A. vinosum* wild type and mutant strains Δ*soxX*, Δ*soxB* and Δ*soxBX*. The detection was performed in the respective crude cell extract. Protein amount in each lane: 186 µg

The Western blot analysis demonstrated, that SoxA was of course present in the wild type, but also detectable in the mutant Δ*soxB*. The inactivated gene *soxB* is located on the DNA strand complementary to the strand containing *soxX* and *soxA*. Therefore, a joint expression of *soxB* and *soxA* was already assumed to be very unlikely, an assumption verified by the presence of SoxA in the mutant. The situation was different in the other examined mutant strains. As soon as the *soxX* open reading frame was destroyed, either alone (Δ*soxX*) or together with *soxB* (Δ*soxBX*), the protein SoxA was no longer produced. The disruption of the *soxX* reading frame obviously had an effect on the expression of *soxA*. Therefore, these two genes appear to be part of one transcription unit. This is in correspondence with the already observed fact, that the two encoded proteins are both subunits of one heterodimeric protein.

The poor performance of the antisera against SoxB and SoxYZ in crude extract prevented the successful realization of comparable experiments concerning the presence of these two proteins in wild type and mutant strains.

C.4.3 Phenotypic characterisation of the different *A. vinosum* mutants

To determine, whether the insertional inactivation and *in frame* deletion of *sox* genes and *sox*-related genes had any effect on the degradation of reduced sulfur compounds, these were added to the different mutant strains separately and in defined concentrations. As the *in vivo* substrate spectrum of the Sox multienzyme complex usually includes thiosulfate and sulfide (Friedrich *et al.*, 2001; Appia-Ayme *et al.*, 2001), these two substrates were tested concerning their degradability. The *in vitro* substrate spectrum could be much broader, but was not taken into account so far. The phenotypic characterisations were performed using Pfennig and 0 medium. The difference was (i) in the presence of sulfide in Pfennig medium before sterilisation, in contrast to the separate addition of the sulfur compound in 0 medium directly before the start of the experiment, and (ii) the amount of sulfide in the media. The mutant strains Δ*soxB* and Δ*soxY* were characterised using exclusively 0 medium. The depicted results for all strains are always representatives of duplicate or triplicate experiments.

C.4.3.1 Thiosulfate oxidation

To determine, whether the inactivation of *sox* genes had an effect on thiosulfate oxidation in *A. vinosum*, 2 mM of thiosulfate were added to a fermenter containing the mutant strain or the respective wild type strain.

Mutant strain Δ*soxX*:

The mutant Δ*soxX* contained an insertional mutation in the *soxX* gene. This resulted in an inactivation not only of *soxX*, but also of *soxA* due to polar effects (see C.4.2). Therefore, any observable phenotype was at least due to the lack of both subunits of SoxXA in *A. vinosum*. The results obtained for thiosulfate degradation in Pfennig medium and 0 medium are presented in Figure C30.

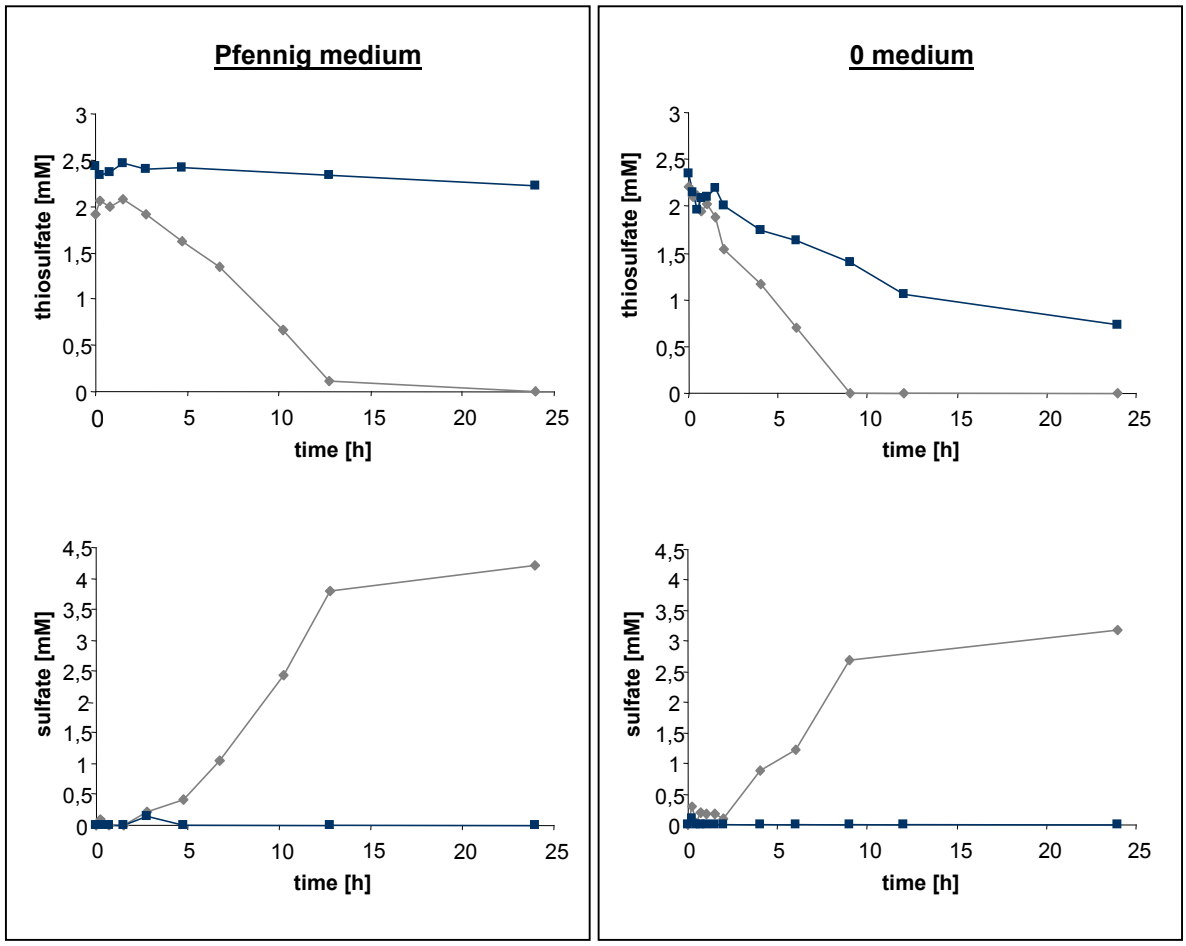

Figure C30: Thiosulfate oxidation in Δ*soxX* (dark blue) and wild type (grey). Compared are growth experiments on Pfennig and 0 medium, presenting thiosulfate concentration and sulfate produced from thiosulfate. Protein concentrations: Δ*soxX*-Pfennig: 130 µg/ml, wild type-Pfennig: 260 µg/ml, Δ*soxX*-0 medium: 51 µg/ml, wild type 0-medium: 61 µg/ml [Pfennig → Lowry, 0 → Bradford].

Obviously, thiosulfate oxidation in the *A. vinosum* strain Δ*soxX* was significantly impaired. While the wild type oxidised the added thiosulfate in approximately 10 h time, the mutant showed either no decrease at all (Pfennig) or significantly less decrease (0 medium) in thiosulfate concentration. This decline, however, is not due to oxidation to sulfate. While the wild type completely oxidised thiosulfate to sulfate, the mutant strain produced no sulfate at all in both approaches. Therefore, thiosulfate must be degraded to another sulfur compound. Even with the potential pathway via the Sox system blocked by gene inactivation, the pathway via the thiosulfate:acceptor oxidoreductase, oxidising thiosulfate to tetrathionate, should still be functional. When growing Δ*soxX* on Pfennig medium, no tetrathionate

formation could be detected, in accordance with the constant amount of thiosulfate. The determination of tetrathionate for the approach on 0 medium is depicted in Figure C31.

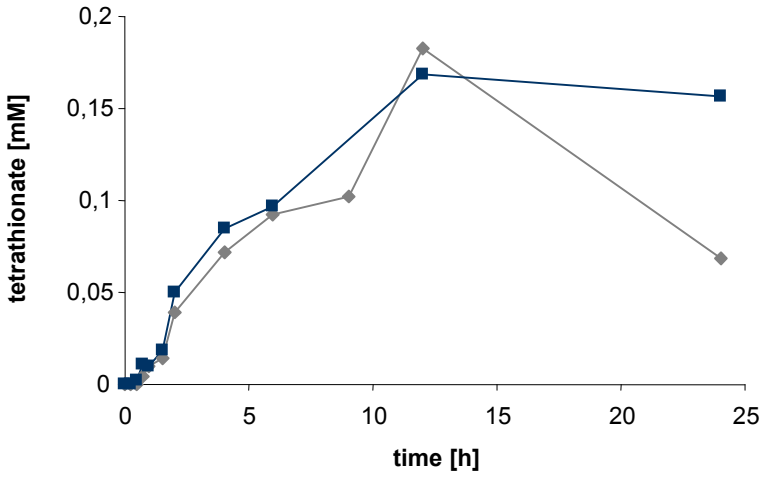

Figure C31: Tetrathionate formation on 2 mM thiosulfate in Δ*soxX* (dark blue) and corresponding wild type (grey), cultivated on 0 medium. Protein concentration: Δ*soxX*-0 medium: 51 µg/ml, wild type-0 medium: 61 µg/ml [Bradford].

Both in wild type and mutant strain tetrathionate formation was observed, being responsible for a decrease in thiosulfate concentration. The effect is not visible in the wild type, being only an addition to the oxidation of thiosulfate to sulfate. In the mutant, however, with tetrathionate formation potentially the only alternative to the impaired oxidation to sulfate, the decrease in thiosulfate concentration can be partly explained by the oxidation to tetrathionate. That the formation of tetrathionate was no complete compensation for the thiosulfate decrease, is in all probability due to the simultaneous and subsequent formation of significant amounts of trithionate from thiosulfate and tetrathionate, respectively. This effect has already been demonstrated in *A. vinosum* (Sperling, 2001) with the biggest amount of trithionate detected after the tetrathionate maximum.

Mutant strain Δ*soxB*:

The mutant Δ*soxB* contained an insertional mutation in the *soxB* gene. As the other *sox* genes in the vicinity (*soxXA*) are situated on the complementary strand, the transcription is not impaired (confirmed for the example of SoxA). Therefore, a mutant phenotype would be due only to an inhibition in expression of *soxB* and genes potentially in the same transcription

unit. The results obtained for thiosulfate degradation in 0 medium are presented in Figure C32.

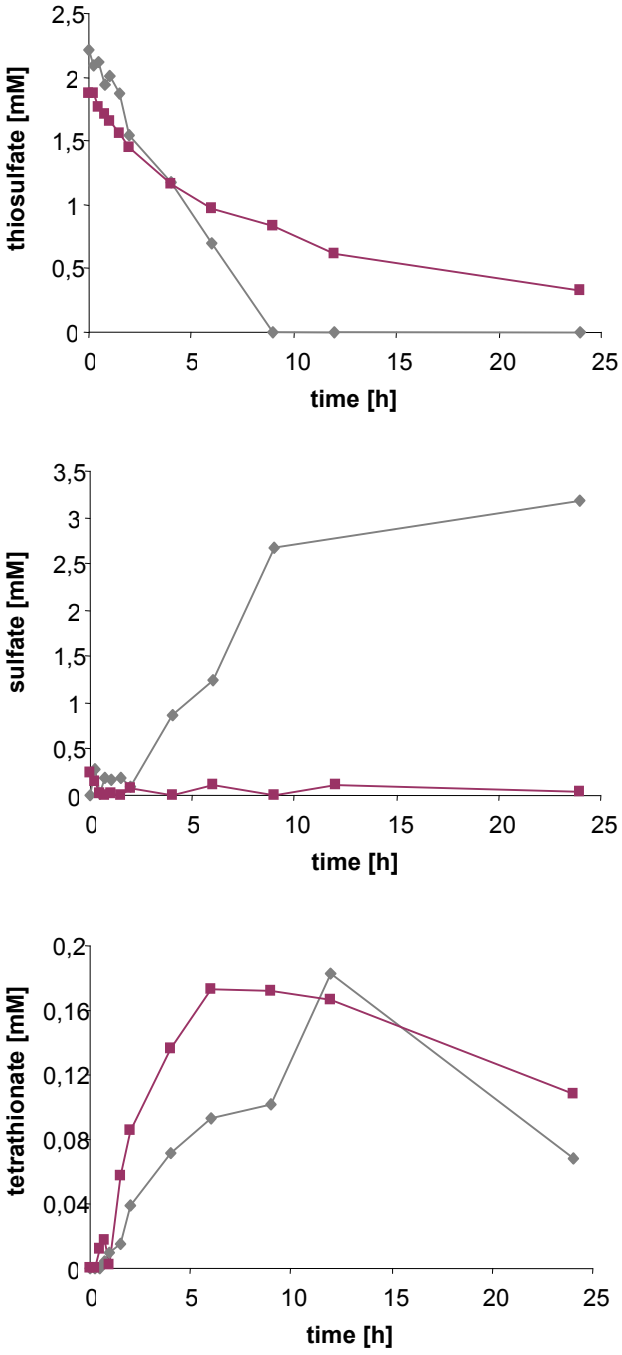

Figure C32: Thiosulfate oxidation in Δ*soxB* (purple) and wild type (grey). Development of thiosulfate concentration is presented together with sulfate and tetrathionate produced from thiosulfate. Protein concentration: Δ*soxB*-0 medium: 51 µg/ml, wild type 0-medium: 61 µg/ml [Bradford].

Thiosulfate oxidation in Δ*soxB* appeared to be impaired, but neither on a significant basis nor resulting in a complete inhibition, when observing only the development of thiosulfate concentration on 0 medium. However, the lack of sulfate production in the mutant confirmed

the complete inhibition of the pathway from thiosulfate to sulfate, as observed for Δ*soxX*. Both encoded proteins appear to be equally essential for the oxidation of thiosulfate to sulfate. The oxidation to tetrathionate, however, remained unaffected, as the formation took place as in the wild type. Again, the gap in sulfur compound quantity is potentially due to trithionate formation.

Mutant strain Δ*soxBX*:

To determine, whether the joint inactivation of *soxB* and *soxX* resulted in a phenotype differing from the respective single mutants, the double mutant was examined on Pfennig and 0 medium. The results obtained for the oxidation of 2 mM thiosulfate are presented in Figure C33, together with the corresponding wild type.

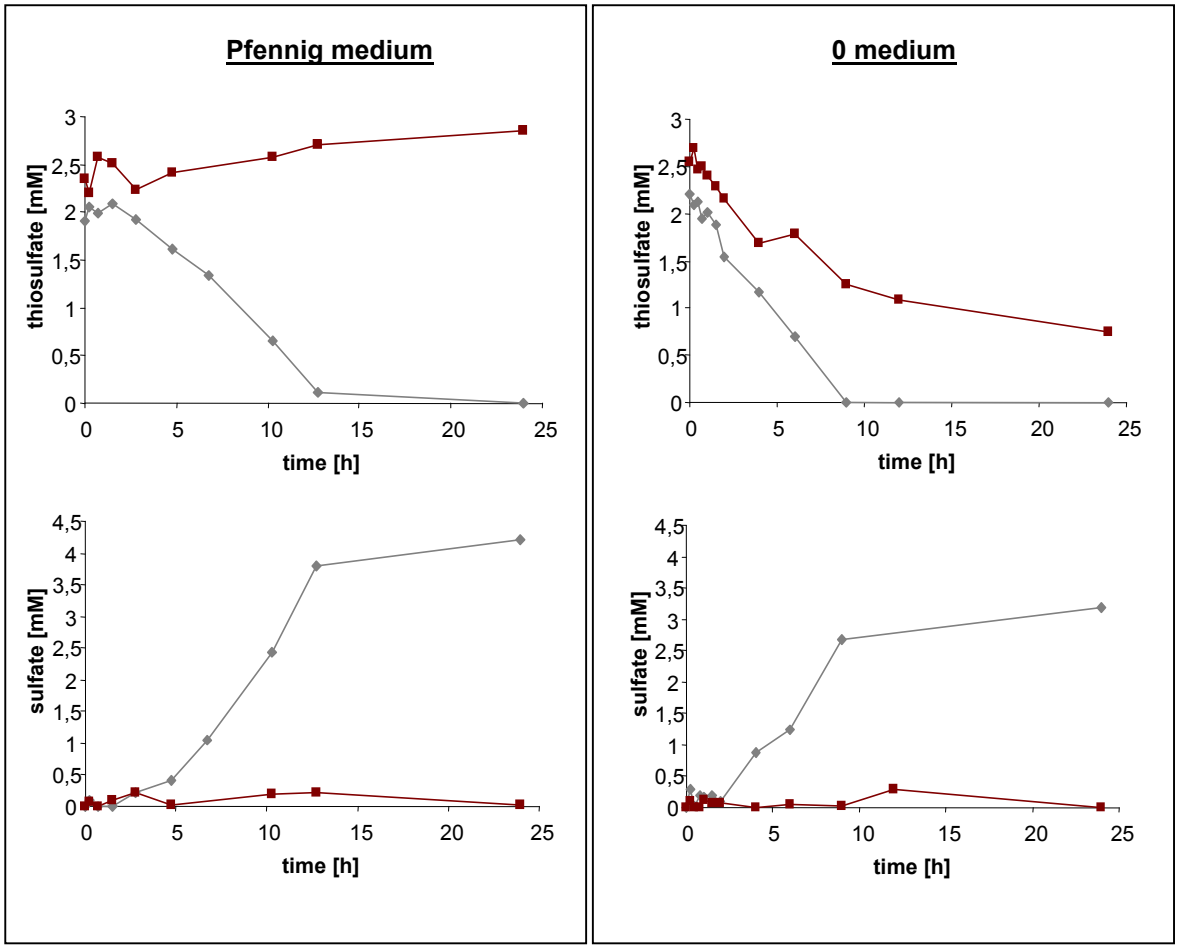

Figure C33: Thiosulfate oxidation in Δ*soxBX* (dark red) and wild type (grey). Compared are growth experiments on Pfennig and 0 medium, presenting thiosulfate concentration and the sulfate produced from thiosulfate. Protein concentration: Δ*soxBX*-Pfennig: 91 µg/ml, wild type Pfennig: 260 µg/ml, Δ*soxBX*-0 medium: 55 µg/ml, wild type 0-medium: 61 µg/ml [Pfennig → Lowry, 0 → Bradford].

As observed for both single mutants, the double mutant Δ*soxBX* was impaired in thiosulfate oxidation. Again the difference between Pfennig and 0 medium could be observed, as thiosulfate oxidation was completely inhibited in the former and only impaired in the latter. However, regarding the sulfate formation is was obvious that the pathway from thiosulfate to sulfate was completely inactivated in Δ*soxBX*. The oxidation to tetrathionate remained unaffected in the mutant strain, as can be observed in Figure C34. A significant trithionate production is again postulated to fill the gap between the decrease in thiosulfate and the produced tetrathionate. No tetrathionate was produced on Pfennig medium.

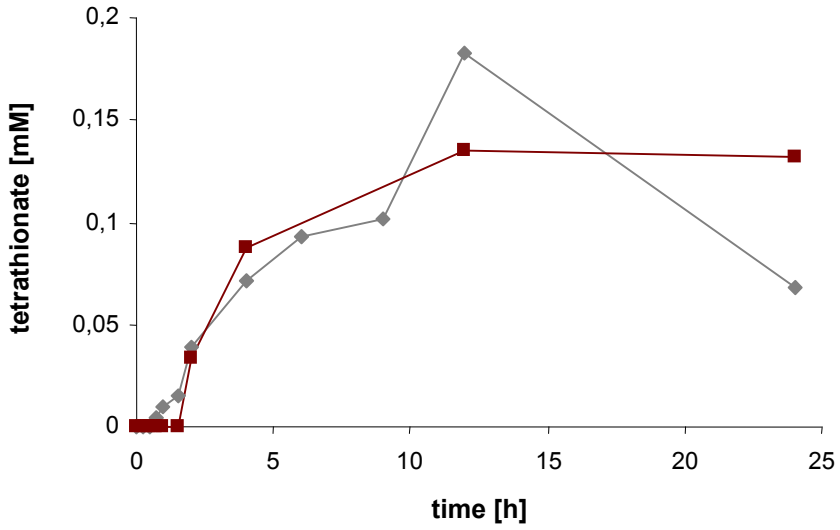

Figure C34: Tetrathionate formation on 2 mM thiosulfate in Δ*soxBX* (dark red) and corresponding wild type (grey), cultivated on 0 medium. Protein concentration: Δ*soxBX*: 55 µg/ml, wild type: 61 µg/ml [Bradford].

Mutant strain ΔORF9/*rhd*:

The gene products of the open reading frames ORF9 and *rhd* are not part of the basic Sox proteins SoxB, SoxXA and SoxYZ. Nevertheless, they could be involved in the degradation of thiosulfate. As the open reading frames are located downstream of *soxA*, all basic Sox proteins should in all probability be present in the mutant strain. The results obtained for the oxidation of 2 mM thiosulfate are presented in Figure C35, together with the corresponding wild type.

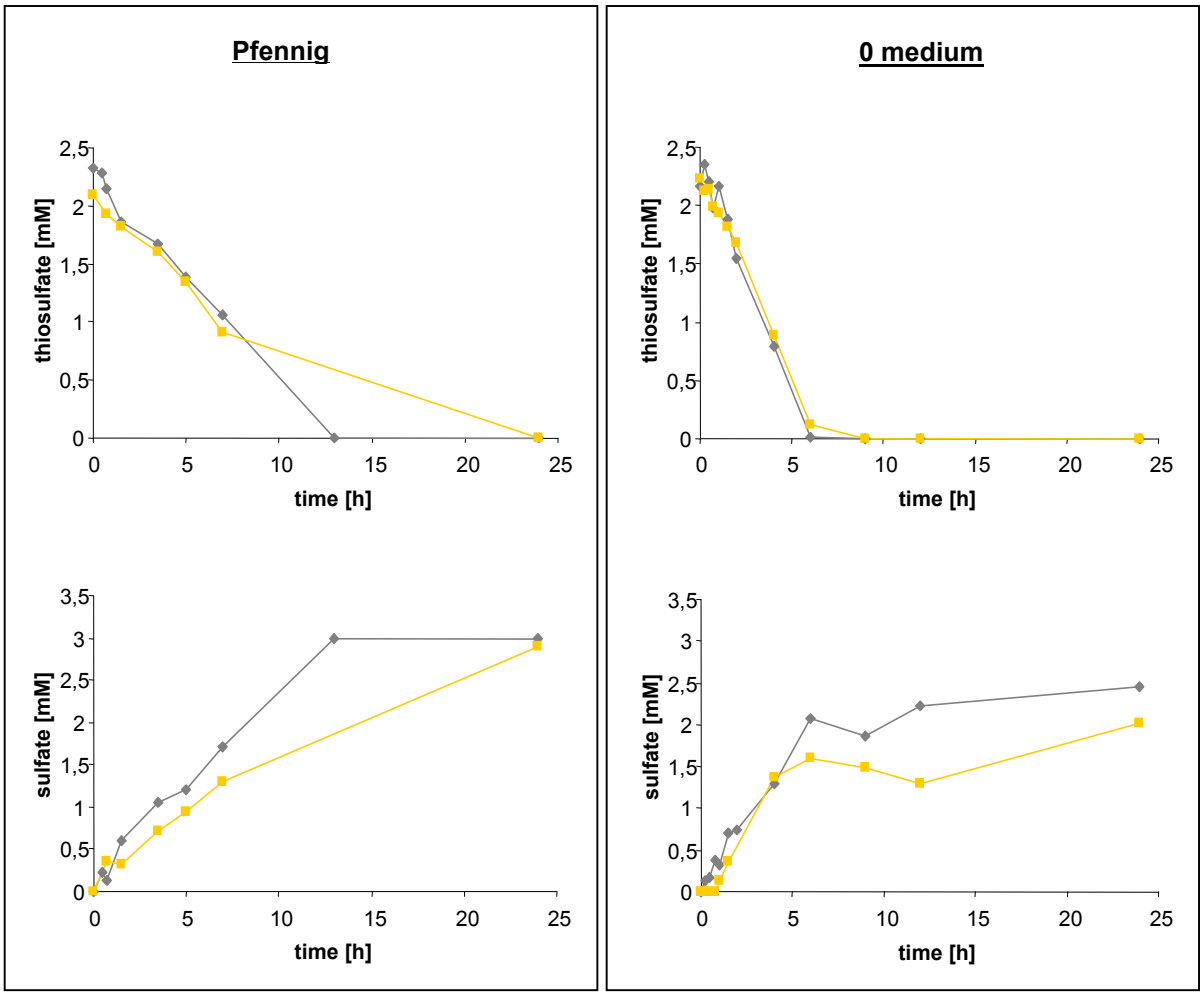

Figure C35: Thiosulfate oxidation in ΔORF9/*rhd* (orange) and wild type (grey). Compared are growth experiments on Pfennig and 0 medium, presenting thiosulfate concentration and the sulfate produced from thiosulfate. Protein concentration: ΔORF9/*rhd*-Pfennig: 86 µg/ml, wild type Pfennig: 260 µg/ml, ΔORF9/*rhd*-0 medium: 72 µg/ml, wild type 0-medium: 79 µg/ml [Pfennig → Lowry, 0 → Bradford].

The mutant strain ΔORF9/*rhd* was in all means comparable to the wild type. It oxidised thiosulfate to sulfate in both experimental approaches. This also included tetrathionate

formation, both pathways for thiosulfate degradation (to sulfate or to tetrathionate) intact. The decrease in tetrathionate was attributed to trithionate formation. The results for tetrathionate determination are depicted in Figure C36. At least under the chosen experimental conditions the inactivation of ORF9 and *rhd* had no effect on thiosulfate degradation.

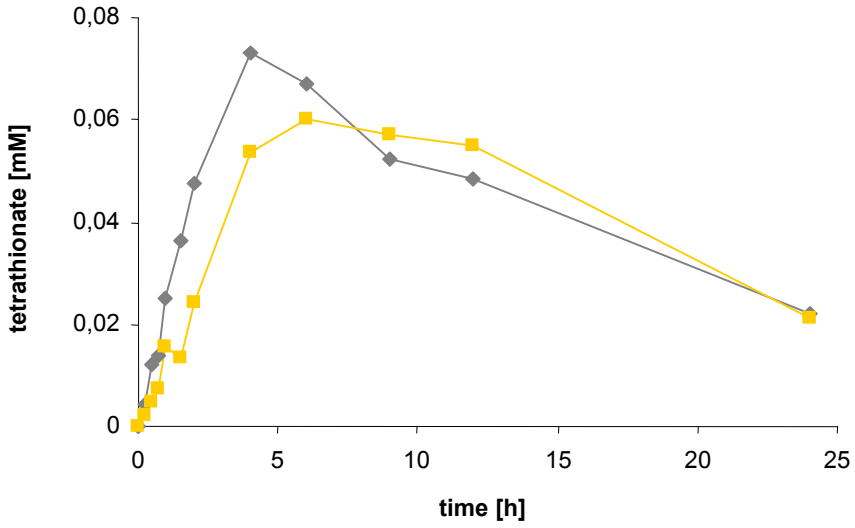

Figure C36: Tetrathionate formation on 2 mM thiosulfate in ΔORF9/*rhd* (orange) and corresponding wild type (grey), cultivated on 0 medium. Protein concentration: ΔORF9/*rhd*: 72 µg/ml, wild type: 79 µg/ml [Bradford].

Mutant strain Δ*soxY*:

In contrast to the preceding mutants the mutant strain Δ*soxY* was produced by *in frame* deletion of almost the complete gene *soxY*. Therefore, any observed phenotype can potentially be traced back to this sole source. The inactivation of *soxY*, however, could result in a special problem, concerning *soxZ*. SoxZ is thought to be co-transported to the periplasm with SoxY in a hitchhiker fashion. Without SoxY the protein SoxZ is potentially not correctly located in the cell. Therefore, a mutant phenotype is in all probability due to the lack of SoxY and a potentially inactive SoxZ. The results obtained for thiosulfate oxidation in Δ*soxY* are presented in Figure C37.

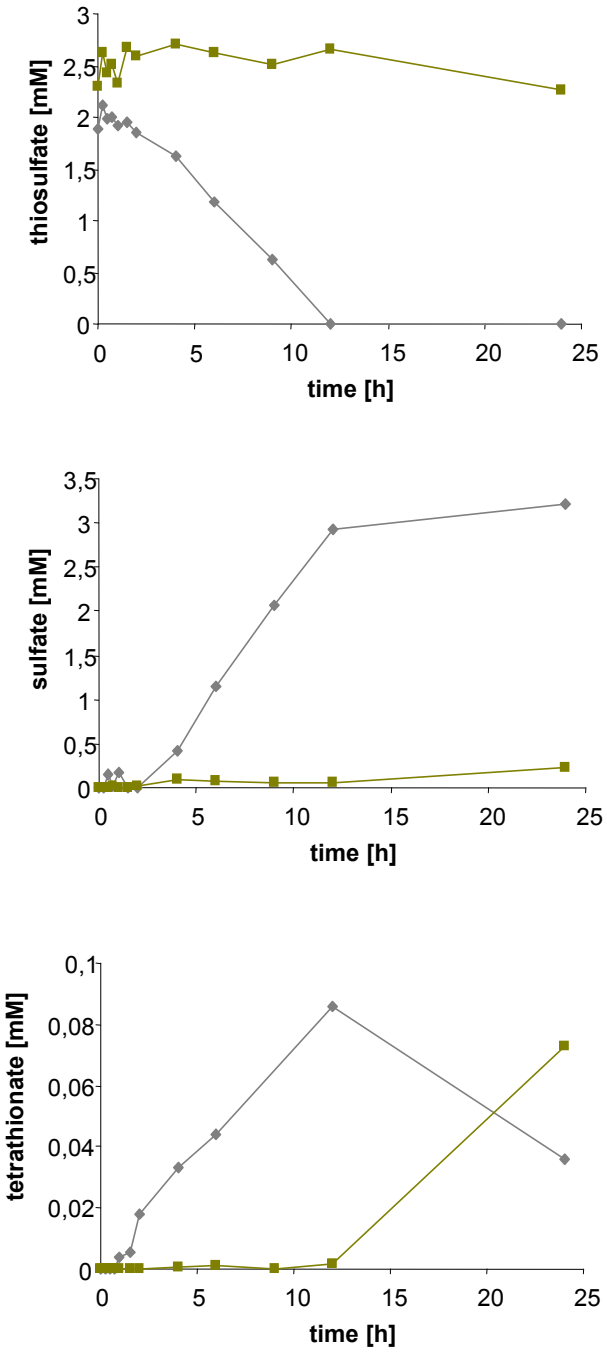

Figure C37: Thiosulfate oxidation in Δ*soxY* (green) and wild type (grey). Development of thiosulfate concentration is presented together with sulfate and tetrathionate produced from thiosulfate. Protein concentrations: Δ*soxY*-0 medium: 58 µg/ml, wild type 0-medium: 79 µg/ml [Bradford].

When observing thiosulfate concentration in the mutant strain, there was no significant decrease. This complete inhibition in oxidation of thiosulfate to sulfate was confirmed by the complete lack of sulfate production in Δ*soxY*. In contrast to the other mutants on 0 medium, that were impaired in thiosulfate oxidation, the thiosulfate concentration remained quite stable. This was due to a delayed production of tetrathionate compared to wild type and the

other mutants. The reason for this delay is explained, when dealing with the oxidation of sulfide by this mutant strain (see C.4.3.2), as thiosulfate was added to the culture with sulfite still present from sulfide oxidation (time t=48 h in Figure C41).

To summarise the results obtained for thiosulfate as substrate for the different mutant strains, all Sox proteins appear to be equally important for the oxidation of thiosulfate to sulfate. The proteins encoded by the open reading frames ORF9 and *rhd*, however, are in all probability not involved in thiosulfate oxidation. The formation of tetrathionate remained possible in all mutant strains.

C.4.3.2 Sulfide oxidation

The inactivation of the Sox multienzyme complex in *P. pantotrophus* and *R. sulfidophilum* resulted in an inhibition of sulfide oxidation. Therefore, the *A. vinosum* mutant strains were tested concerning their ability to oxidise sulfide. The characterisation was performed on Pfennig medium, that already contained approximately 7 mM of sulfide, and on 0 medium with the separate addition of approximately 2 mM of sulfide. The results for sulfide oxidation in different mutants are combined in Figure C38.

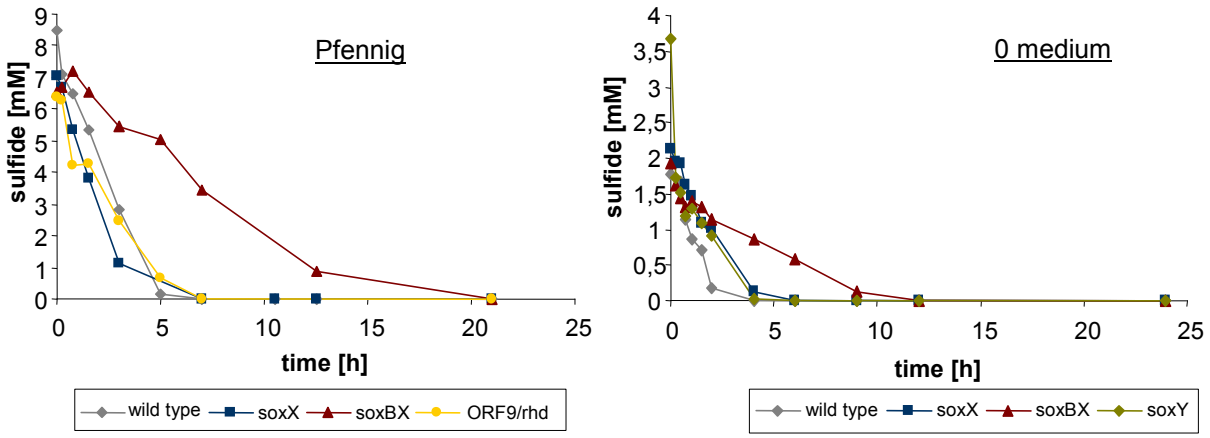

Figure C38: Sulfide oxidation in different mutants of *A. vinosum*, cultivated on Pfennig or 0 medium. The mutants Δ*soxB* and ΔORF9/*rhd*, cultivated on the latter, are not shown, but exhibited results comparable to the mutant Δ*soxX*. Protein concentrations: wild type-Pfennig: 260 µg/ml, Δ*soxX*-Pfennig: 130 µg/ml, Δ*soxBX*-Pfennig: 91 µg/ml, ΔORF9/*rhd*-Pfennig: 86 µg/ml, wild type-0 medium: 79 µg/ml, Δ*soxX*-0 medium: 51 µg/ml, Δ*soxBX*-0 medium: 55 µg/ml, Δ*soxY*-0 medium: 58 µg/ml [Pfennig → Lowry, 0 → Bradford].

Nearly all of the mutant strains oxidised the different amounts of sulfide as observed for the wild type. Only the mutant Δ*soxBX* appeared to be slightly impaired in sulfide oxidation, as approximately double the amount of time was needed to oxidise the presented sulfur compound.

The first products of sulfide degradation were two polysulfides, named polysulfide (PS) 39 and PS 42 after their retention time in HPLC thiol analysis. As no standards were available to record calibration curves, the results for polysulfide detection are presented in area counts. Some exemplary results for polysulfide determination are presented in Figure C39.

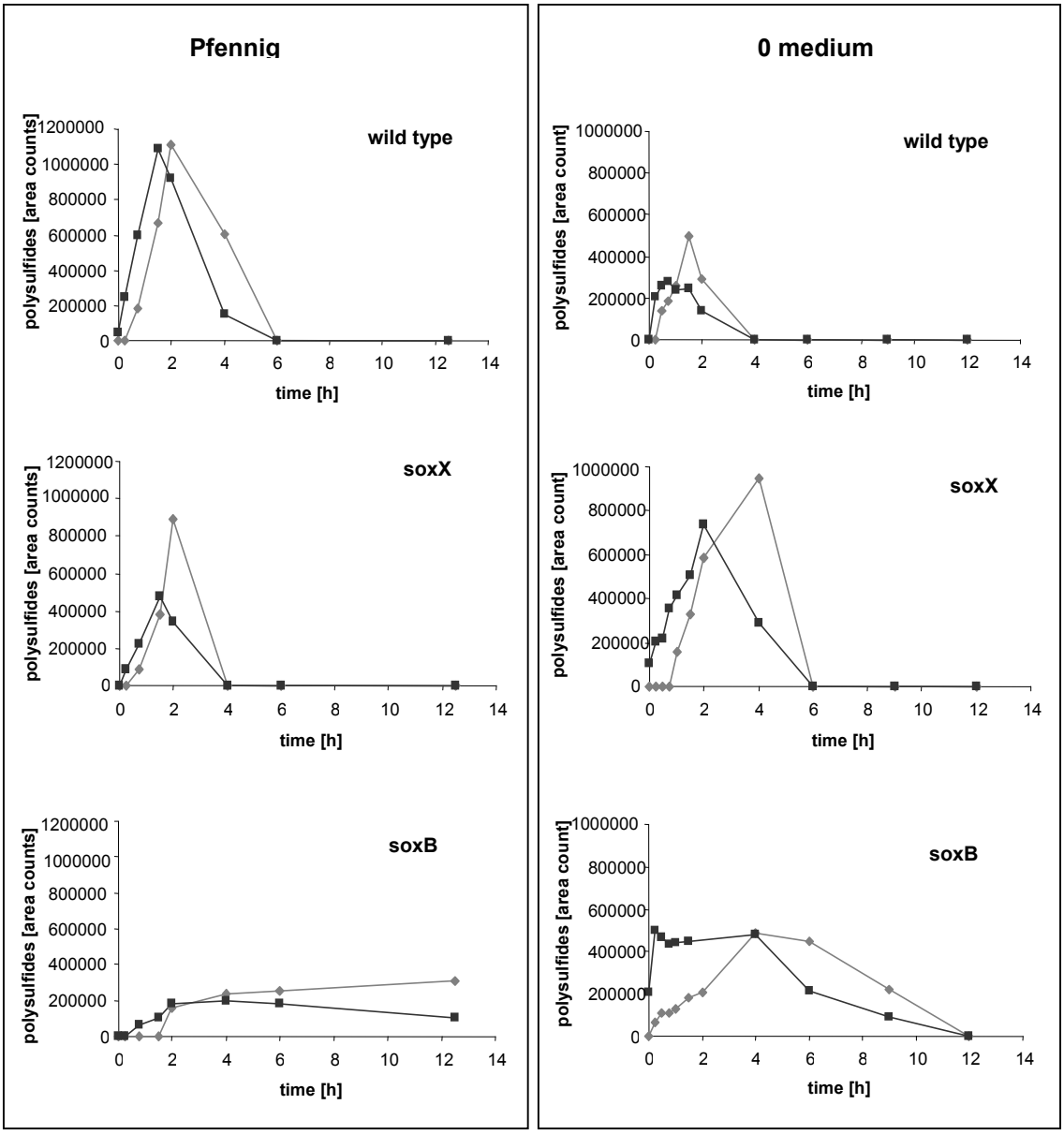

Figure C39: Polysulfide development from sulfide in wild type, mutant Δ*soxX* (as a representative for the other mutants apart from Δ*soxBX*) and mutant Δ*soxBX*, compared on Pfennig medium and 0 medium. Polysulfides are recorded as area counts, due to a lack of standards. Protein concentrations: wild type-Pfennig: 260 µg/ml, Δ*soxX*-Pfennig: 130 µg/ml, Δ*soxBX*-Pfennig: 91 µg/ml, wild type-0 medium: 79 µg/ml, Δ*soxX*-0 medium: 51 µg/ml, Δ*soxBX*-0 medium: 55 µg/ml [Pfennig → Lowry, 0 → Bradford].

As observed for sulfide oxidation, nearly all the mutant strains exhibit polysulfide development as observed for the wild type, regardless whether Pfennig or 0 medium was used. Again, the mutant Δ*soxBX* represents the only exception. Both approaches (Pfennig and

0 medium) resulted in a significantly slowed polysulfide formation and degradation, this being either the result of or the cause for the slowed sulfide oxidation.

Both polysulfides are then further oxidised to sulfur and stored in periplasmic sulfur globules. The exemplary results for sulfur detection in Figure C40 are in accordance with the exemplary results presented for sulfide and polysulfide determination.

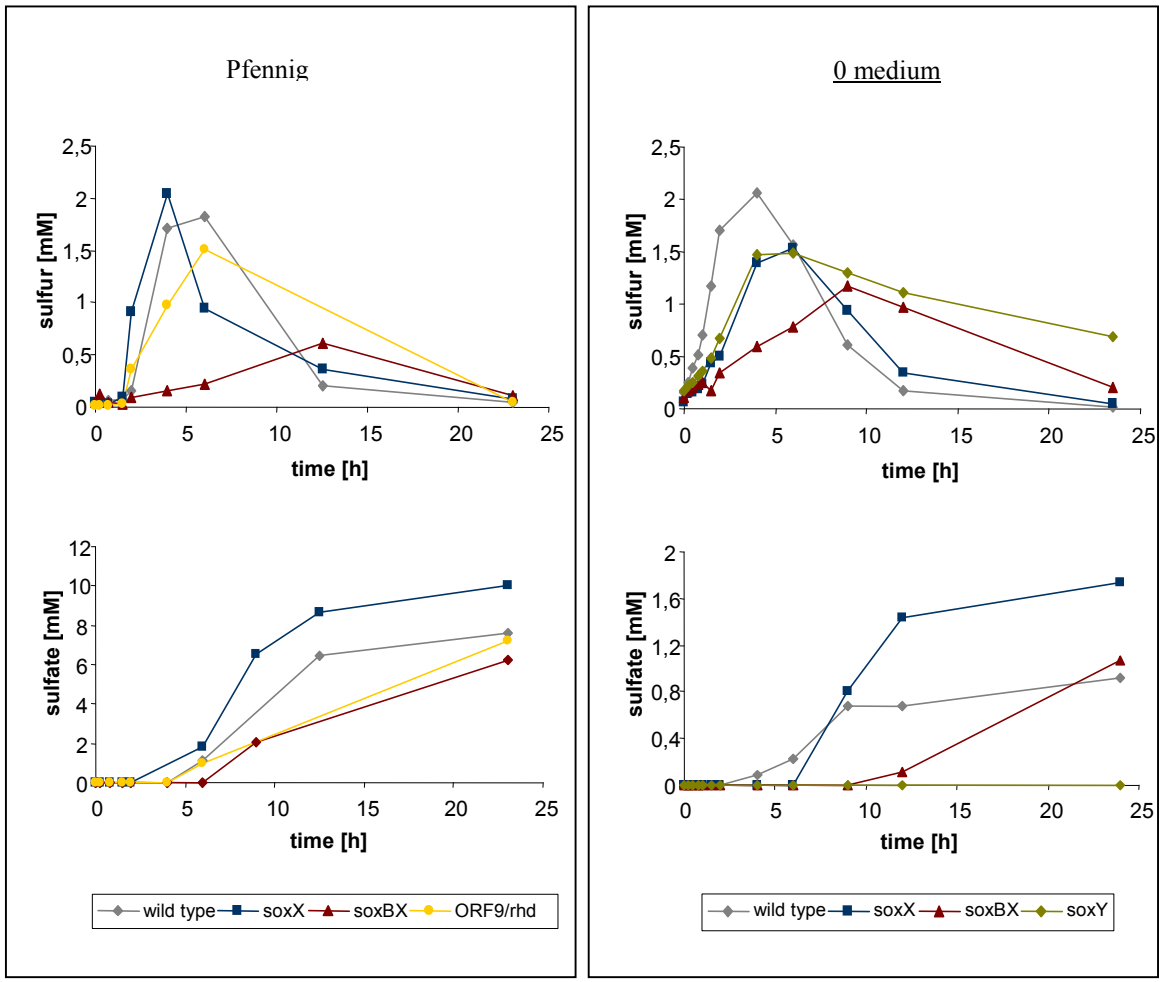

Figure C40: Development of sulfur and sulfate from sulfide in different mutants of *A. vinosum*, cultivated on Pfennig or 0 medium. The mutants Δ*soxB* and ΔORF9/*rhd*, cultivated on the latter, are not shown, but exhibited results comparable to the mutant Δ*soxX*. Sulfur was determined either by HPLC analysis (Pfennig) or by cyanolysis (0 medium). Protein concentrations: wild type-Pfennig: 260 µg/ml, Δ*soxX*-Pfennig: 130 µg/ml, Δ*soxBX*-Pfennig: 91 µg/ml, ΔORF9/*rhd*-Pfennig: 86 µg/ml, wild type-0 medium:79 µg/ml, Δ*soxX*-0 medium: 51 µg/ml, Δ*soxBX*-0 medium: 55 µg/ml, Δ*soxY*-0 medium: 58 µg/ml [Pfennig → Lowry, 0 → Bradford].

The first thing that attracted attention was the difference in the two sulfur determination methods. Both approaches resulted in comparable sulfur concentrations, even though the starting sulfide concentration in the Pfennig medium approach was about three times higher. This result confirmed, that the sulfur extractable by chloroform does not represent the whole amount of sulfur present in the cell. The use of cyanolysis, however, resulted in the detection of all the sulfur present in the cell.

When comparing all the mutant strains concerning sulfur development and sulfate production, two mutants stood out. The mutant Δ*soxBX*, as observed before for sulfide oxidation and polysulfide development, was significantly slowed in sulfur formation and degradation. However, the production of sulfate was comparable to the wild type and the other mutants. Therefore, the observed problems in sulfide degradation and the subsequent steps are possibly due to a slowed growth. The mutant Δ*soxY* exhibited sulfur formation as observed in the wild type. Sulfur oxidation, however, was significantly impaired, the complete amount only oxidised after 48 h (twice as long as all the other strains). Even though part of the sulfur was degraded during the observed 25 h, no sulfate was produced in the meantime. This discrepancy was due to a feature only observed in the Δ*soxY* mutant. During sulfur degradation significant amounts of sulfite were accumulated in the culture. In all the other strains, wild types and mutants, sulfite was hardly detectable as the intermediate of sulfur oxidation to sulfate. The situation observed in Δ*soxY* is presented in Figure C41.

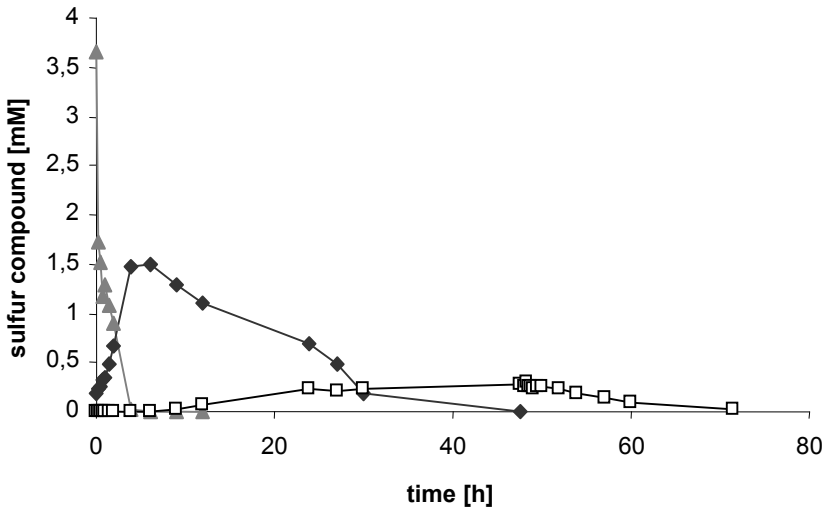

Figure C41: The oxidation of sulfide (grey triangles) to sulfur (black diamonds) and sulfite (open squares) in the mutant Δ*soxY*. Protein concentration Δ*soxY*: 58 µg/ml [Bradford].

After a sulfide oxidation comparable to the wild type, the mutant Δ*soxY* also exhibited sulfur formation as observed in the wild type. Sulfur degradation, however, was significantly slowed, in all probability due to the formation of significant amounts of sulfite. Altogether, approximately 800 µM of sulfate were detectable after the end of the experimental period. The presence of sulfite in the medium at the time thiosulfate was added (t=48 h) results in a delay of tetrathionate formation due to an inhibition of thiosulfate:acceptor oxidoreductase by sulfite (50 % inhibition of TAOR activity by 80 µM sulfite (Sperling, 2001)).

The examination of sulfide oxidation in the different *sox* mutants showed the Sox proteins potentially not be essential for sulfide oxidation (with a slight uncertainty concerning the situation in Δ*soxBX*). Surprisingly, the inactivation of *soxY* resulted in a significantly impaired sulfite oxidation.

C.4.4 Construction of complemented mutants

To determine, whether the phenotypes observed in the mutants could be traced back to the inactivation of a small set of genes or even one gene, two of the mutant strains (Δ*soxX* and Δ*soxY*) were supplemented with a plasmid, containing the intact version of the inactivated genes.

C.4.4.1 Complementation strains Δ*soxX*+*X* and Δ*soxY*+*Y*: construction and check

Construction of Δ*soxX*+*X*:

In the mutant Δ*soxX* a resistance cassette was inserted into *soxX*, thereby destroying this gene and at the same time inhibiting the expression of the following gene *soxA*. In order to complement the observed mutant phenotype, at least these two genes had to be reintroduced into the mutant strain, including the putative promoter situated upstream of *soxX*. Therefore, the complete insert of the plasmid pGEM-SoxB was ligated into the vector pBBR1-MCS, together with an additional erythromycin resistance cassette from pHP45ΩEm. The basic vector pBBR1-MCS was transferable to *A. vinosum* by conjugation and replicable in the organism. This was necessary, as the original goal was not the exchange of inactivated and intact gene by double cross over. The desired situation was a genome-based defective gene and a plasmid-based intact counterpart. Therefore, the need arose for an additional resistance to select against plasmid loss, as the already existing chloramphenicol resistance cassette was of no use in *A. vinosum*. The 4,5 kb insert of pGEM-SoxB contained part of *soxB*, the intergenic region, the genes from *soxX* to *rhd* and a small part of ORFb. The vector pBBR1-MCS was digested with *Apa*I/*Spe*I, as was pGEM-SoxB to cut out the insert. After ligation the construct was digested with *Eco*RV, and the erythromycin cassette was cut from pHP45ΩEm with *Sma*I. The produced blunt ends were again used for ligation to obtain the final complementation plasmid pΔ*soxX*+*X* (depicted in figure C42).

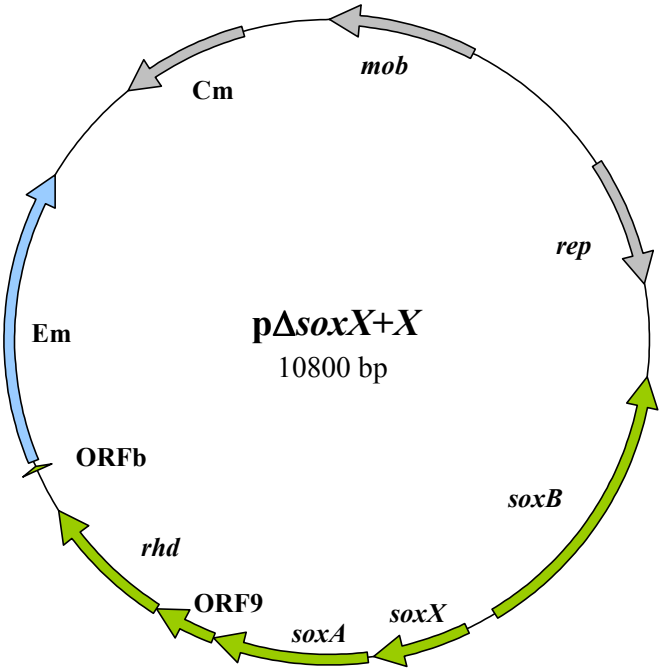

Figure C42: Map of the plasmid pΔ*soxX+X*, used for the complementation of the mutant strain Δ*soxX*.

The plasmid was successfully transferred into the mutant strain Δ*soxX*, thereby producing the complementation strain Δ*soxX+X*. This strain was characterized as in the preceding experiments, concerning the degradation of sulfide and thiosulfate.

Construction of Δ*soxY+Y*:

In the mutant Δ*soxY* only the gene *soxY* was deleted *in frame*. But according to the special nature of the joint transport of SoxY and SoxZ, both *soxY* and *soxZ* were reintroduced together into the mutant strain. With a plasmid-encoded SoxY and a genome-encoded SoxZ, difficulties could arise concerning the production of functional protein. The spatial distance and/or timing of the protein subunit production could be problematic as well as the production of equimolar amounts. Even if the putative natural promoter, located upstream of *soxY*, was applied for gene expression from the plasmid, potential regulatory regions could be missing. This would hamper a balanced regulation of the expression from both genome and plasmid. Therefore, to avoid these potential problems, *soxY* and *soxZ* were reintroduced as one transcription unit. The genes *soxYZ*, together with their natural promoter, were amplified by PCR with the primer pair Yforward/Yreverse. The fragment was digested with *Xba*I and

ligated into the equally digested vector pBBR1-MCS2, that already contained a kanamycin resistance, used for the selection against plasmid loss. This resulted in the construction of the complementation plasmid p∆*soxY+Y* (shown in figure C43).

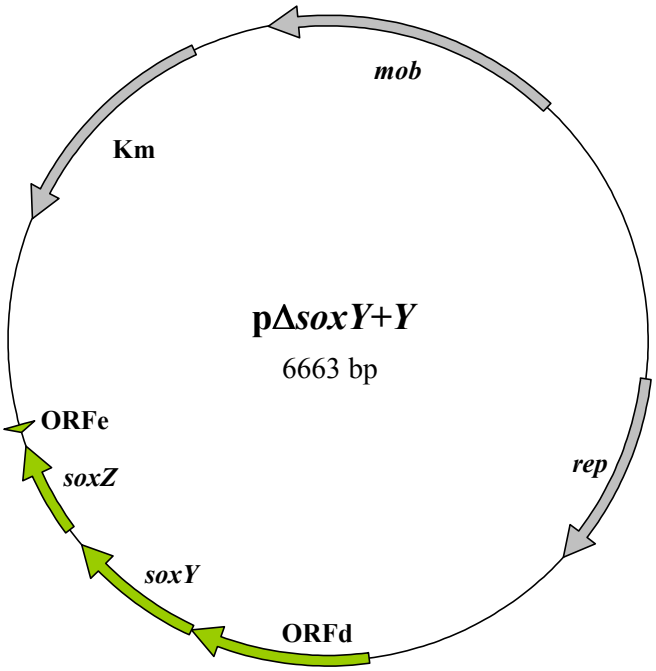

Figure C43: Map of the plasmid p∆*soxY+Y*, used for the complementation of the mutant strain ∆*soxY*.

This plasmid was successfully transferred into the mutant ∆*soxY*, thereby producing the complementation strain ∆*soxY+Y*. This strain was characterized as in the preceding experiments, concerning the degradation of sulfide and thiosulfate.

Confirming the plasmid presence:

To confirm the presence of the complementation plasmids in the respective organism, colony PCRs were performed to detect the defective gene on the genome as well as the intact gene on the plasmid.

***∆soxX+X*:** The primer pair soxXforward / soxXreverse, both primers binding to a *soxX* sequence outside of the resistance cassette, could theoretically be used to detect the intact gene as well as the inactivated gene. As the wild type DNA fragment was only 400 bp in size, it was preferentially amplificated, in contrast to the 2,5 kb mutant DNA fragment. The

PCR to detect the defective *soxX* was performed with the primer pair soxXreverse / Km1 (the latter only binding inside of the kanamycin cassette) and resulted in a 900 bp DNA fragment.

Δ*soxY*+*Y*: The PCR with the primer pair Yforward / Yreverse was used to discriminate between wild type *soxY* (1,5 kb) and mutant *soxY* (1 kb), both primers binding outside of the deletion. The second PCR applied the primers Yforward and Y2rev1, the latter binding inside of the deletion. Only with the wild type gene as template a 900 bp DNA fragment could be amplified.

The obtained results are presented in figure C44.

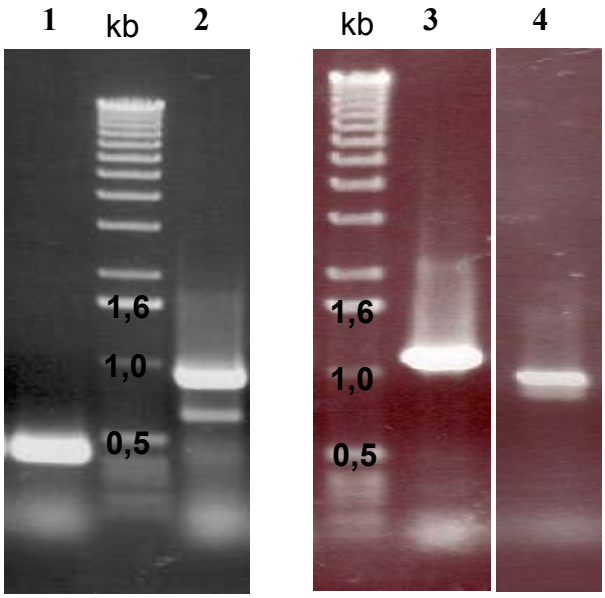

Figure C44: Colony PCR to detect (i) the plasmid-based intact *soxX* and the genome-based inactivated *soxX* in the complementation strain Δ*soxX*+*X* and (ii) the plasmid-based intact *soxY* and the genome-based deleted *soxY* in the complementation strain Δ*soxY*+*Y*. Lanes: 1. soxXforward / soxXreverse, detection of the plasmid-based *soxX*; 2. soxXreverse / Km1, detection of the inactivated *soxX* containing the kanamycin cassette; 3. Yforward / Yreverse, detection of either wild type or deleted *soxY*; 4. Yforward / Y2rev1; detection of the wild type *soxY*.

The PCR confirmed the presence of the respective wild type and mutant genes in both complementation strains.

Detection of SoxA in the complementation mutant Δ*soxX*+*X*:

A second possibility to confirm the presence of the plasmid pΔ*soxX*+*X* in the corresponding complementation mutant was the renewed production of SoxA, that was missing in the mutant Δ*soxX* because of polar effects. The detection of SoxA could also confirm the functionality of the putative promoter. Therefore crude extract of the complementation strain was examined concerning its SoxA content. The result, in comparison with wild type and Δ*soxX*, is shown in figure C45.

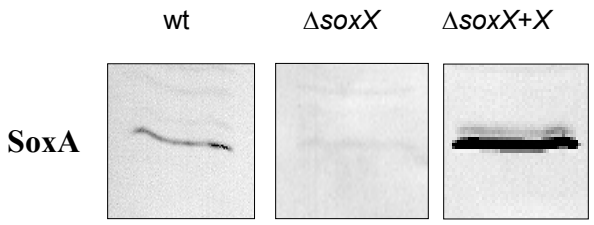

Figure C45: Detection of SoxA in crude extract of *A. vinosum* wild type (DSM 180), the mutant strain Δ*soxX* and the complementation strain Δ*soxX*+*X*. Protein amount in each lane: 186 µg.

The protein SoxA, present in the wild type and missing in the mutant, was again produced in the complementation strain. So the protein SoxX encoded upstream should also be once more present in the organism. The production of the protein from a plasmid-based *soxA* gene confirmed the actual existence of the postulated promoter upstream of *soxX*. Comparable protein concentrations have been used for SDS-PAGE, so the Western blot signal should be in accordance with the actual amount of SoxA present in the culture. Bearing this in mind, the expression of the plasmid-based *soxA* was much stronger than in the wild type or in the *soxB* mutant (for the latter see figure C29). This was potentially due to a regulative effect based on the gene sequence surrounding *soxA*. As only a small part of this surrounding sequence was transferred to the plasmid, the structures necessary for a correct regulation of *soxA* gene expression could have been destroyed or left behind. This led to the observed overexpression of *soxA*.

C.4.5 Phenotypic characterisation of the complementation strains Δ*soxX*+*X* and Δ*soxY*+*Y*

C.4.5.1 Complementation strain Δ*soxX*+*X*

The degradation of the reduced sulfur compounds thiosulfate and sulfide was determined in the complementation strain Δ*soxX*+*X*. By reintroduction of the inactivated gene and its surrounding in the mutant strain Δ*soxX* a neutralisation of the mutant phenotype was aimed for.

Thiosulfate oxidation:

The mutant strain Δ*soxX* exhibited a complete inhibition of thiosulfate oxidation to sulfate. Thiosulfate degradation in the complementation strain Δ*soxX*+*X* is shown in figure C46, together with wild type and mutant strain for comparison.

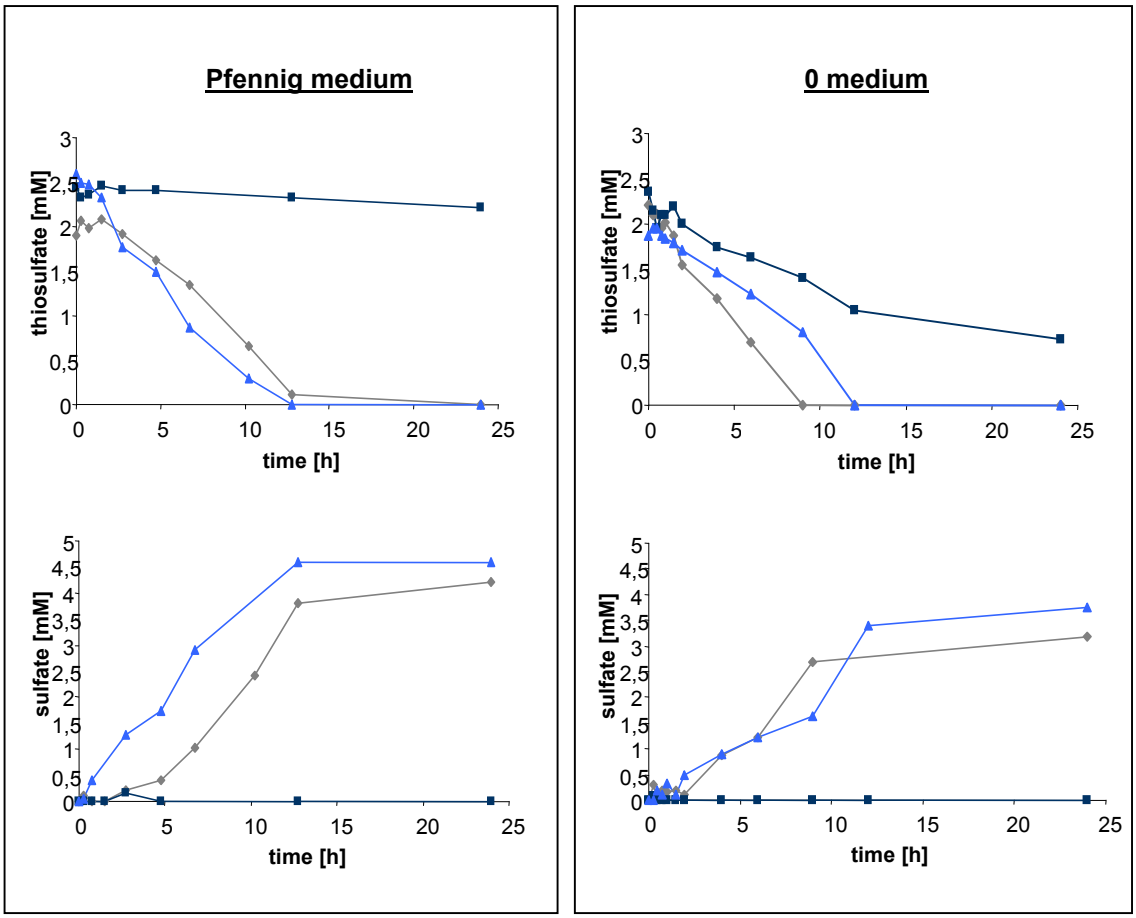

Figure C46: Thiosulfate oxidation in the *A. vinosum* complementation strain Δ*soxX*+*X* (light blue), compared to the corresponding wild type (grey) and the mutant strain Δ*soxX* (dark blue), cultivated on Pfennig and 0 medium, respectively. Presented are thiosulfate concentration and sulfate produced from thiosulfate. Protein concentrations: wild type-Pfennig: 160 µg/ml, Δ*soxX*-Pfennig: 130 µg/ml, Δ*soxX*+*X*: 155 µg/ml, wild type-0 medium: 61 µg/ml , Δ*soxX*-0 medium: 51µg/ml , Δ*soxX*+*X*-0 medium: 61 µg/ml [Lowry→ Pfennig, Bradford→ 0].

The results demonstrated the successful complementation of the Δ*soxX* phenotype by the corresponding intact genes that are expressed in trans. Thiosulfate oxidation was returned to wild type level in both approaches, the results confirmed by the renewed sulfate production. Therefore, the observed mutant phenotype could be traced back to the few plasmid-bourne genes, that have been reintroduced in the organism. In all probability this implies only the genes *soxX* and *soxA*, as the inactivation of the following two open reading frames ORF9 and *rhd* had absolutely no effect on thiosulfate oxidation. On the plasmid only fragments of *soxB* and ORFb were present, therefore being without influence.

As has been observed in the mutant strain, tetrathionate formation in the complementation Δ*soxX+X* was unaffected by the *sox* gene expression in trans and still took place on wild type level when cultivated on 0 medium. No tetrathionate was produced on Pfennig medium. The results are depicted in Figure C47.

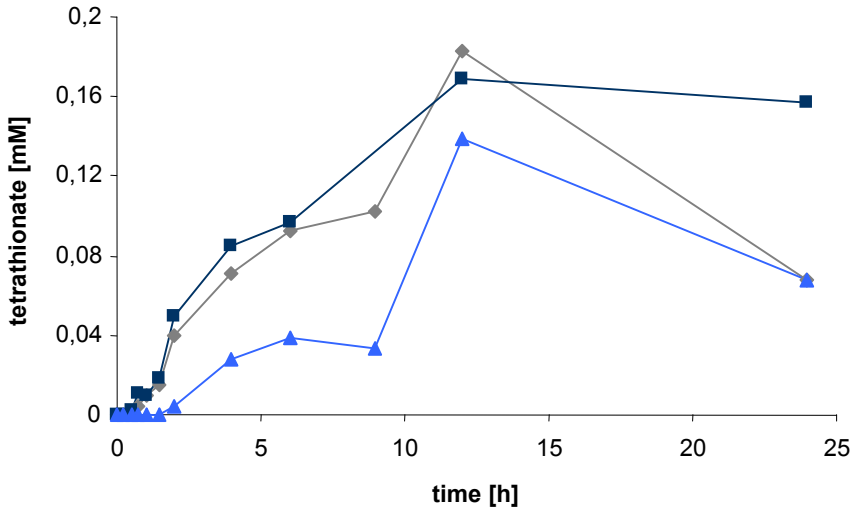

Figure C47: Tetrathionate formation on 2 mM thiosulfate in the complementation strain Δ*soxX+X* (light blue) and corresponding wild type (grey) and mutant Δ*soxX* (dark blue), cultivated on 0 medium. Protein concentration: wild type: 61 µg/ml , Δ*soxX*: 51µg/ml , Δ*soxX+X*: 61 µg/ml [Bradford].

Sulfide oxidation:

To check, whether the *sox* gene expression in trans had any effect on sulfide oxidation in the complementation, even though it was not impaired in the mutant, the culture was subjected to sulfide. The obtained results for sulfide oxidation, sulfur formation / degradation and sulfate formation in the complementation strain Δ*soxX+X* and the mutant strain Δ*soxX* are presented in Figure C48.

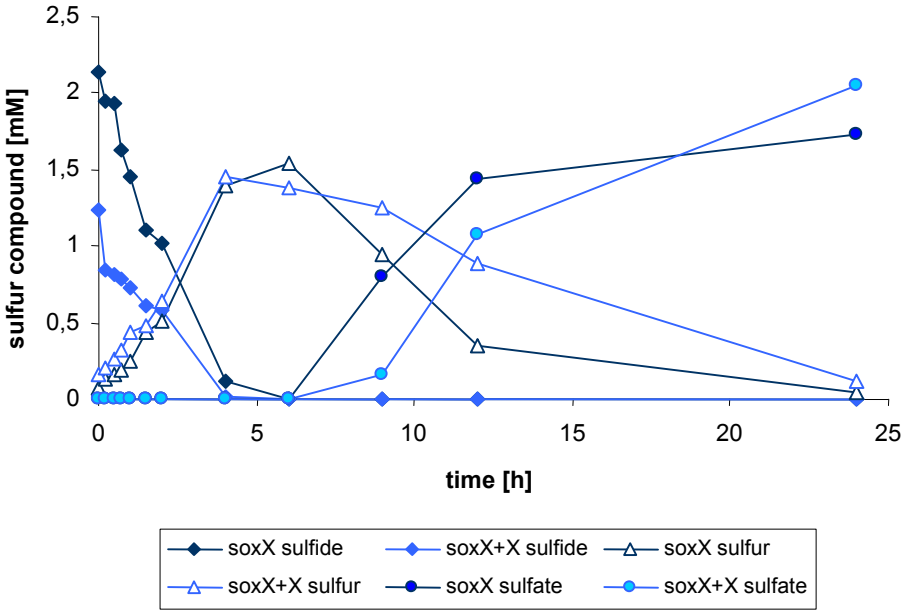

Figure C48: Sulfide oxidation and development of sulfur and sulfate in the complementation strain Δ*soxX+X* and the corresponding mutant Δ*soxX*, cultivated on 0 medium (as a representative for the comparable results obtained on Pfennig medium). Protein concentrations: wild type: 61 µg/ml , Δ*soxX*: 51µg/ml , Δ*soxX+X*: 61 µg/ml [Bradford].

Complementation strain Δ*soxX+X* and mutant strain Δ*soxX* are absolutely comparable concerning sulfide oxidation, sulfur formation and degradation and sulfate production. Therefore, the expression of *soxXA* in trans re-established the pathway from thiosulfate to sulfate, but had no effect on the oxidation of other sulfur compounds, that were not impaired in the first place.

C.4.5.2 Complementation strain Δ*soxY+Y*

The mutant Δ*soxY* was not only impaired in thiosulfate oxidation but also exhibited a problem in sulfite oxidation, which was only observed for this *sox* mutant. Sulfide oxidation, however, remained unaffected. Therefore, the aim of the complementation was to re-establish thiosulfate oxidation and remove the delay observed for sulfite oxidation.

Thiosulfate oxidation:

For this experiment solely thiosulfate was added to the culture, without the preceding addition of sulfide. The results are presented in Figure C49, together with the corresponding wild type and Δ*soxY*. In the latter approaches thiosulfate was added after preceding sulfide degradation.

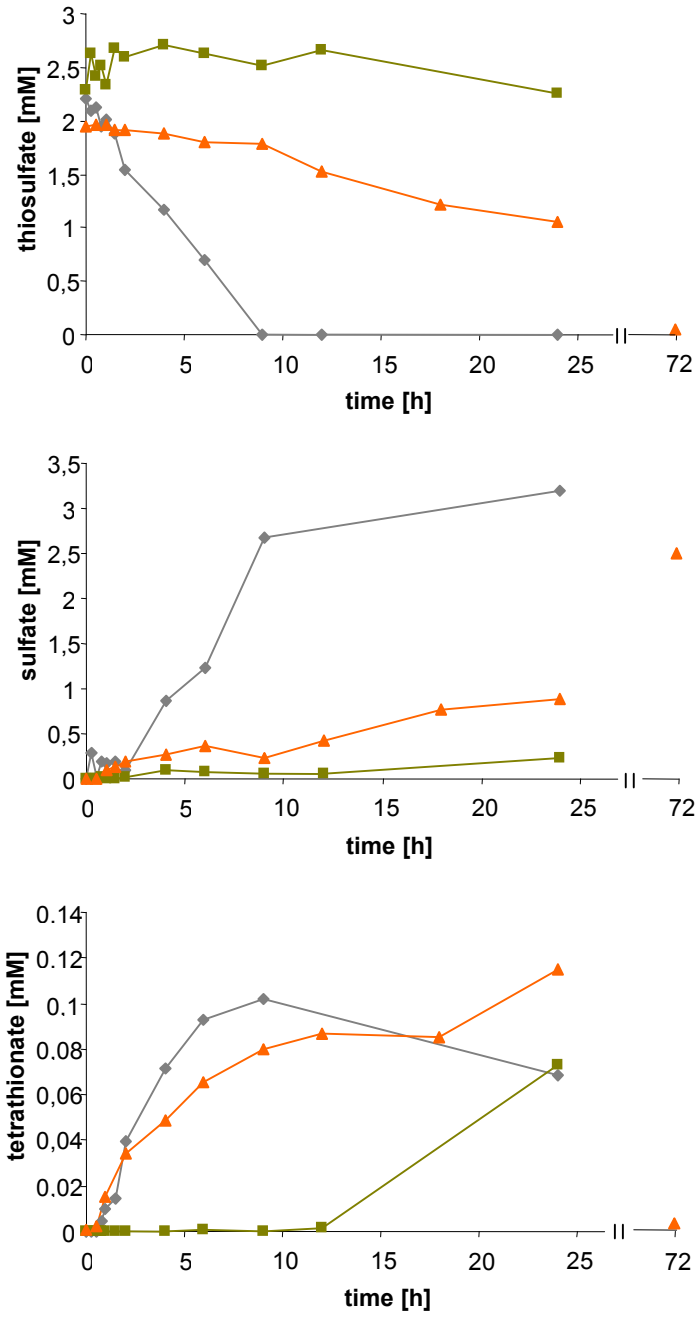

Figure C49: Thiosulfate oxidation in the *A. vinosum* complementation strain Δ*soxY*+*Y* (orange), compared to the corresponding wild type (grey) and mutant Δ*soxY* (green), cultivated solely on 0 medium. Presented are thiosulfate concentration, sulfate produced from thiosulfate and tetrathionate formation. Protein concentrations: wild type: 61 µg/ml, Δ*soxY*: 58 µg/ml, Δ*soxY*+*Y*: 68 µg/ml [Bradford].

In contrast to wild type and mutant, the complementation was cultivated for 72 h. In the wild type the complete amount of thiosulfate was oxidised after approximately 25 h. The mutant exhibited no oxidation of thiosulfate to sulfate, even over a longer period of time (duration: 80 h, data not shown). The difference in thiosulfate oxidation between the mutant and the complementation strain was very small, but detectable. After 72 hours the complete amount of thiosulfate was degraded in Δ*soxY+Y*. Significant amounts of sulfate were also detectable, thereby confirming the renewed possibility of thiosulfate oxidation to sulfate, even if at a level below the wild type. Tetrathionate was produced from thiosulfate at wild type level. This was in all probability due to thiosulfate being the only sulfur substrate in the experiment, with no residual sulfite still present from sulfide oxidation. Therefore, the thiosulfate:acceptor oxidoreductase was not inhibited. The complementation confirmed the importance of SoxY for the oxidation of thiosulfate to sulfate.

Sulfite oxidation:

To determine whether the reintroduction of the deleted gene had an effect on the delayed sulfite oxidation, the degradation of 5 mM externally added sulfite was examined. The results for the wild type and the mutant Δ*soxY* were thankfully provided by Bettina Franz. They are presented together with the results obtained for the complementation strain Δ*soxY+Y* in Figure C50.

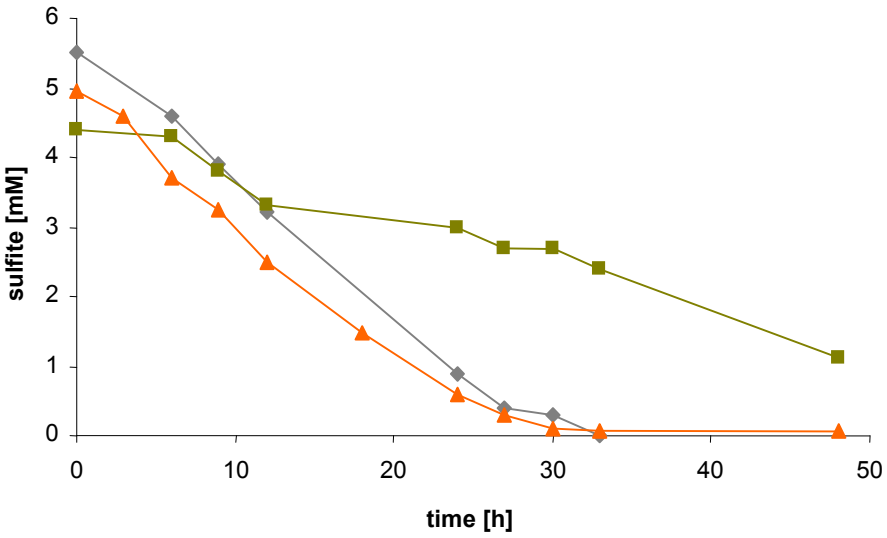

Figure C50: Sulfite oxidation in the complementation strain Δ*soxY+Y* (orange), compared to the corresponding wild type (grey) and the mutant Δ*soxY* (green), cultivated on 0 medium. Protein concentrations: wild type: 79 µg/ml, Δ*soxY*: 58 µg/ml, Δ*soxY+Y*: 59 µg/ml [Bradford].

The wild type readily oxidized the externally added sulfite in approximately 30 hours time. In the mutant strain Δ*soxY* barely half of the sulfite was degraded by this time. The impairment in the oxidation of internal sulfite had already been demonstrated for Δ*soxY* (see Figure C41). The results obtained with externally added sulfite supplied further proof for this deficiency. After the reintroduction of *soxYZ* sulfite oxidation in Δ*soxY+Y* was reverted back to wild type level. The re-introduction of the inactivated gene *soxY*, together with *soxZ*, resulted in a complete removal of the mutant phenotype concerning sulfite oxidation

Sulfide oxidation:

None of the *sox* mutants or the complementation strain Δ*soxX+X* exhibited impairments concerning the oxidation of sulfide. The complementation strain Δ*soxY+Y* was also tested regarding its sulfide oxidation capacity. The obtained results (Figure C51) are shown in comparison with the mutant strain Δ*soxY*. The latter was unaffected in sulfide oxidation, but exhibited a delay in sulfur oxidation, in all probability due to the delay in sulfite oxidation..

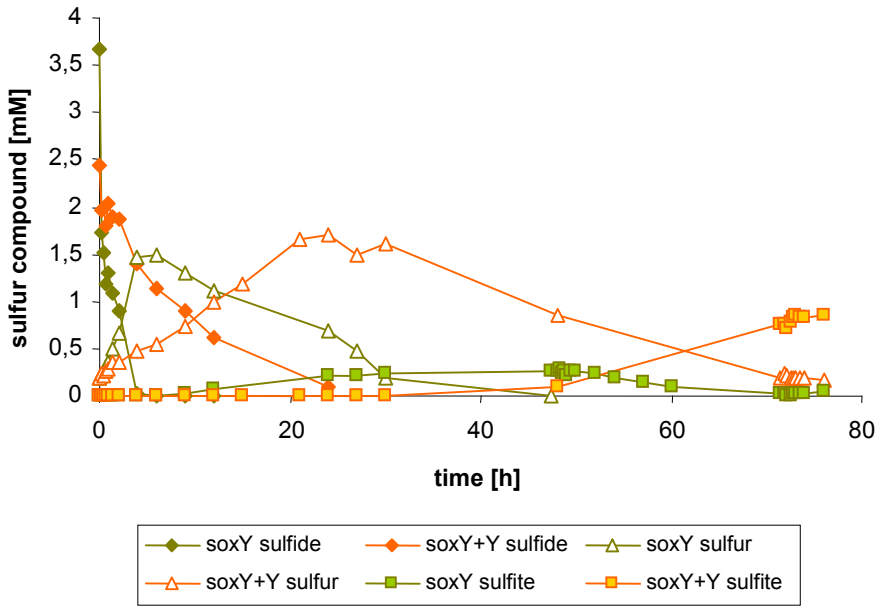

Figure C51: Sulfide oxidation and development of sulfur and sulfite in the complementation strain Δ*soxY+Y* and the corresponding mutant Δ*soxY*, cultivated on 0 medium. Protein concentrations: Δ*soxY*: 58 µg/ml, Δ*soxY+Y*: 95 µg/ml [Bradford].

While the mutant Δ*soxY* was already delayed in sulfur and sulfite oxidation, the situation has worsened significantly by the reintroduction of *soxYZ* in the complementation strain Δ*soxY+Y*. Sulfide oxidation, being at wild type level in the mutant, was decreased about four times in Δ*soxY+Y*. The sulfur formation rate was not affected in the mutant, but was significantly decreased in the complemented strain. The following steps of sulfur degradation and sulfite oxidation, already impaired in the mutant, were also even more slowed. Why the reintroduction of a deleted gene resulted in the appearance of a phenotype observed neither in the wild type nor in the corresponding mutant (or in any of the other mutants) so far remains inexplicable.

C.4.6 Oxidation rates

For a better comparison of the thiosulfate oxidation and sulfate formation in the different wild types, mutants and complementation strains, the respective oxidation rates, obtained using 0 medium, were determined. The results are summarised in Table C5.

***A. vinosum* strain**	**thiosulfate oxidation [µmol/h*mg]**	**sulfate formation [µmol/h*mg]**
DSM180	4,034	2,235
Δ*soxX*	1,367	-0,224
Δ*soxB*	0,953	-0,132
Δ*soxBX*	1,403	-0,012
Δ*soxX+X*	2,656	2,715
185SM50	5,446	1,328
ΔORF9/*rhd*	4,853	1,194
Rif50	2,605	2,259
Δ*soxY*	0,026	0,164
Δ*soxY+Y*	0,391	0,169

Table C5: Oxidation and formation rates for thiosulfate and sulfate in the different wild types, mutants and complemented mutants, cultivated on 0 medium.

The rates obtained for the oxidation of thiosulfate and the formation of sulfate essentially confirm the preceding results, e.g. the absolute necessity of the gene products of *soxBXA* and *soxY*(*Z*) for the oxidation of thiosulfate to sulfate.

D: Discussion

In the present study, the second system for thiosulfate oxidation in *A. vinosum* could be clearly identified. A thiosulfate:acceptor oxidoreductase is responsible for the oxidation to tetrathionate (Sperling, 2001), while a *sox* gene encoded multienzyme complex is essential for the oxidation to sulfate. The sequencing of the second of two independent parts of genomic DNA of *A. vinosum* revealed the presence of 15 open reading frames altogether. Seven of these showed similarity to open reading frames found in *sox* gene clusters of different organisms. Five of the genes (*soxBXA* and *soxYZ*) encode proteins thought to be essential for a functioning Sox system (Friedrich *et al.*, 2000): SoxB, SoxXA and SoxYZ. These proteins were purified from thiosulfate-grown *A. vinosum* and appear to be thiosulfate-inducible above a low constitutive level. Inactivation of *sox* genes revealed the absolute necessity of Sox proteins for thiosulfate oxidation to sulfate. Furthermore, the deletion of *soxY* additionally resulted in a delayed sulfite oxidation, thereby broadening the Sox protein substrate spectrum. Complementations of Δ*soxX* and Δ*soxY* were more or less successful, especially the latter yielding some unexpected results.

D.1 Sequence analysis

In *P. pantotrophus* and most of the other examined Sox-system-containing organisms the *sox* genes are arranged in one single cluster (Friedrich *et al.*, 2005). This situation enables a joint expression of the genes *soxXYZABCD* (Friedrich *et al.*, 2001), all of which encode proteins essential for Sox-dependent thiosulfate oxidation in *P. pantotrophus*. The situation in *A. vinosum* is in stark contrast to that. The identified *sox* genes *soxBXA* and *soxYZ* are located on two independent sites on the genome, thereby excluding an efficient co-transcription. If a functional enzyme system consists of several different proteins, as is the case for the Sox complex, it seems favourable to combine all necessary parts in one large polycistronic transcript. The involvement of a specific regulatory protein could ensure a joint transcription, combining independent sites to form one larger regulon. With SoxR and SoxS in *P. pantotrophus*, proteins with influence on the *sox* gene expression have already been identified (Rother *et al.*, 2005). But so far no proteins with a potential for protein-DNA

interaction have been identified in the vicinity of the *sox* genes in *A. vinosum*. Another interesting candidate for the regulation of at least part of the *sox* gene expression appears to be the RubisCO-like protein (RLP) identified in *C. tepidum* (Hanson and Tabita, 2001). The corresponding gene sequence was identified by sequencing of the complete genome. A highly similar gene was also identified in a thiosulfate-oxidizing strain of *C. limicola*. The encoded protein RLP is potentially not active as a ribulose-1,5-bisphosphate carboxylase / oxygenase, as several necessary active sites are missing. The disruption of this gene in *C. tepidum* resulted in an accumulation of stress response proteins, but more interestingly in an accumulation of higher levels of sulfur globules compared to the wild type. The latter phenotype could be rescued by the addition of cysteine to the culture. Sulfur globules are obligate intermediates of thiosulfate oxidation in *C. tepidum*. Therefore, the question is raised which step in sulfur compound metabolism is influenced by the lack of RLP. Further examination of the same RLP mutant revealed a defect in thiosulfate oxidation, but not in sulfide oxidation (Hanson and Tabita, 2003). This effect on thiosulfate oxidation was linked to the apparent lack of SoxY, though SoxA and SoxB were still present in the preparation. Therefore, RLP seems to have an effect on the presence of SoxY. In *A. vinosum* a gene encoding for a RubisCO-like protein has also been identified. An influence on *soxY* expression by RLP would potentially be much easier to achieve than in *C. tepidum*, as in the latter organism the *soxY* gene is the third in a thirteen gene operon. With the genes *soxYZ* separated from the other *sox* genes, an independent regulation would be possible. This independency raises the question, why an independent production of SoxYZ could be necessary, if the protein only functions as part of a larger multienzyme complex. A possible answer was given by the examination of the Δ*soxY* mutant and is discussed below.

The Sox multienzyme complex, based upon the model developed for *P. pantotrophus*, needs at least four different proteins for functionality. Apart from the three proteins SoxXA, SoxB and SoxYZ, also encoded in *A. vinosum*, the protein SoxCD plays an important role, as it oxidizes the bound sulfane sulfur of thiosulfate, that is later hydrolytically cleaved off by SoxB. Not all Sox-containing organisms encode SoxCD in the cluster, *A. vinosum* being one of them. This finding seems to be in accordance with metabolic properties and allows the separation into two groups. The genes *soxCD* have been detected in chemotrophic bacteria like *P. pantotrophus* (Wodara *et al.*, 1997), but also in phototrophic thiosulfate oxidizers like *Rhodovulum sulfidophilum* (Appia-Ayme *et al.*, 2001). The completely sequenced green sulfur bacterium *Chlorobaculum tepidum*, however, shows no sign of *soxCD* whatsoever

(Eisen *et al.*, 2002). The difference between the two groups of organisms, with and without SoxCD, seems to be the presence of elemental sulfur as a metabolic intermediate during the Sox-dependent oxidation of thiosulfate to sulfate. *P. pantotrophus*, which degrades thiosulfate without the appearance of free intermediates, contains SoxCD, which *in vitro* enhances the electron yield during thiosulfate oxidation from 2 to 8 electrons. In *C. tepidum*, which lacks the genes coding for SoxCD, sulfur globules are produced as an obligate intermediate during thiosulfate oxidation. *A. vinosum* also produces sulfur globules as an obligate intermediate during thiosulfate oxidation. In *A. vinosum* neither *soxCD* nor the corresponding protein could be detected (Kappler, 1999). This similarity between *C. tepidum* and *A. vinosum* is underlined by the closer resemblance of the genes *soxY* and *soxZ* in these organisms, compared to the homology to the corresponding proteins in *P. pantotrophus*. The consequence of a missing SoxCD protein is the necessity to modify the model for Sox-dependent thiosulfate oxidation so far based on the situation found in *P. pantotrophus* (Friedrich *et al.*, 2001). In organisms like *C. tepidum* and *A. vinosum* another way must be postulated to further degrade the sulfane sulfur still bound to the substrate binding protein SoxYZ. One of the first steps in *A. vinosum* must be a transfer of the sulfane sulfur to a growing sulfur globule, as the separate fate of the two sulfur atoms from thiosulfate has long been established (Trüper and Pfennig, 1966; Smith and Lascelles, 1966). For this transfer step the putative rhodanese, possibly acting as a sulfur transferase and encoded in the first set of *sox* genes, appeared to be a likely candidate.

Apart from the four major proteins SoxXA, SoxB, SoxCD and SoxYZ, other Sox proteins are also encoded in most of the examined *sox* gene clusters. The genes *soxEF*, situated directly downstream of *soxD* in *P. pantotrophus*, encode a c-type cytochrome (SoxE) and a flavoprotein (SoxF) (Wodara *et al.*, 1997). Mutagenesis of *soxF* resulted in a slight inhibition of sulfide oxidation *in vitro*. For thiosulfate oxidation, however, the protein was not required (Rother *et al.*, 2001). SoxF was shown to exhibit sulfide dehydrogenase activity *in vitro* (Quentmeier *et al.*, 2004). More recent studies, however, postulated an activating function of SoxF on the Sox system, when it was reconstituted with a SoxYZ protein separately inactivated by reduction. Therefore, SoxF potentially performs a redox-balancing function (Friedrich *et al.*, 2005). The genes *soxEF* in *P. pantotrophus* exhibit homology to the genes *fccAB* in *A. vinosum*. The latter encode flavocytochrome c_{552}, a protein that also exhibited sulfide dehydrogenase activity *in vitro*. The protein is located in the periplasm and contains two subunits: a cytochrome c (FccA) and a flavoprotein (FccB). The protein was originally thought to be involved in the oxidation of sulfide to intracellular sulfur in *A. vinosum*.

Disruption of *fccAB*, however, had no effect on the sulfide oxidation capability of the organism (Reinartz *et al.*, 1998). Therefore, a role in the Sox complex could be another possible field of action. The inactivation of FccAB in *A. vinosum* did not result in a complete inhibition of thiosulfate oxidation. Nevertheless, a decrease in the oxidation rates concerning thiosulfate (about 40 %) and sulfite (about 35 %) has been observed (Reinartz, 1997). Therefore, the involvement of FccAB in the oxidation of these two reduced sulfur compounds seems possible. The effect on sulfite oxidation is of special interest, regarding the phenotype of the mutant Δ*soxY*, that also exhibits a delay in sulfite oxidation.

In addition to the identified *sox* genes, two more open reading frames were identified, ORF9 and *rhd*, that are potentially involved in the Sox system of *A. vinosum*. A homologue to ORF9 is found in the *C. tepidum sox* gene cluster, situated between *soxA* and *soxB*. A BLAST search revealed no obvious function for either of the proteins. But the central position of the genes makes an involvement of the putative proteins in the Sox complex of the respective organism very likely. The *rhd* gene of *A. vinosum*, downstream of ORF9, shows similarity to a thiosulfate sulfurtransferase in *A. aeolicus*. The latter open reading frame is located in the organisms *sox* gene cluster, downstream of *soxB* and separated from it by two open reading frames without obvious connection to thiosulfate oxidation. As these homologues are both positioned in close vicinity to essential proteins of the Sox system, an involvement could be postulated for both putative sulfurtransferases. For *A. vinosum* a sulfurtransferase activity of some kind is necessary to transfer the sulfane sulfur of thiosulfate to the sulfur globules. But the ΔORF9/*rhd* mutant in *A. vinosum* exhibited no phenotype to clarify the function of the proteins encoded by the respective open reading frames.

D.2 Protein purification

Regarding the expression experiments, the *sox* genes could be well expressed in *E. coli*, but with a question mark concerning their functionality. In all probability none of the signal peptides, neither for Sec- nor Tat-dependent transport, were cleaved off, or the difference was not discernable on SDS-PAGE. Potentially SoxY was partly processed, as two bands appeared that could represent the protein with and without signal peptide. The insertion of heme groups into SoxXA was quite unlikely, regarding the *E. coli* strain used for production. For antibody testing purposes, however, the produced proteins were sufficient, even if they did not deliver the unequivocal results that we aimed for. The examination of recombinant

proteins produced in *E. coli* always raises the question as to how transferable the gained data are to the original organism. The examination of the proteins in question in the original organism must be the preferred option. Therefore, the Sox proteins were purified directly from *A. vinosum* rather than from *E. coli*.

Three Sox proteins were purified from *A. vinosum*: SoxXA, SoxB and SoxYZ. The localisation of the proteins was postulated, on the basis of sequence analysis, to be periplasmic. The signal peptide for Tat-dependent transport in SoxY hints at the transport of SoxY in its already folded form. Without any potential cofactor binding site, the co-transported element would be the second subunit SoxZ, leading to a completely periplasmic Sox complex, including the potentially involved protein FccAB (= SoxEF), that is also located in the periplasm. *A. vinosum* cell material was disrupted and separated to differentiate between the soluble and the membrane fraction. In the soluble fraction both cytoplasmic and periplasmic proteins are found. The antiserum against SoxA provided the best results in *A. vinosum* cell material and was therefore used for the first experiments concerning localisation and inducibility. The major part of the protein SoxA was found in the soluble fraction of thiosulfate-grown cell material. Together with the presence of a signal peptide and the putative c-type cytochrome nature of SoxA, a periplasmic localisation appears to be very likely, as the Sox multienzyme complex usually is assumed to be soluble in the periplasm. The Sox system in *S. novella* was thought to be anchored to the periplasmic side of the cytoplasmic membrane (Kappler *et al.*, 2001), but this would seemingly be the only membrane associated Sox system so far examined. A detection of a faint SoxA signal in the membrane could be due to an incomplete separation of the two fractions. However, also a weak association with the membrane is possible, as the electrons stored in SoxXA, by the attachment of thiosulfate to SoxYZ, need to be further transferred to eventually yield a membrane potential usable for ATP synthesis.

The comparison of SoxA detection in malate-grown and thiosulfate-grown *A. vinosum* cell material indicated the induction of the *sox* gene expression by thiosulfate and/or sulfide. Both reduced sulfur compounds were present in the applied thiosulfate medium. The importance of the two substrates depends on the substrate spectrum used by the Sox multienzyme complex. Taking the much higher concentration of thiosulfate than of sulfide in the medium into account, the former was probably responsible for the observed induction. In *P. pantotrophus* the thiosulfate-dependent induction of gene expression has been demonstrated for *soxXYZABCD* and *soxFGH* (Friedrich *et al.*, 2001). The expression is mediated by the

proteins SoxRS (Rother *et al.*, 2005). In *S. novella* the expression of *soxXA* (Kappler *et al.*, 2000; Kappler *et al.*, 2004) and *soxC* (Kappler *et al.*, 2001) was examined. The production of SoxXA appears to be inducible by thiosulfate. SoxC, however, could be detected in extracts of *S. novella* independent of the presence or absence of thiosulfate. In *A. vinosum* statements can only be made for the expression of *soxA*. As in *P. pantotrophus* and *S. novella* the production of SoxA (in all probability together with SoxX) is inducible, the inducer potentially being thiosulfate. Apart from the induction by the putative substrate, a low but detectable constitutive gene expression was observed. As is the case for the *lac* operon in *E. coli* , a low level of enzyme always present in the cell ensures the direct degradation of the substrate as soon as it is available for the organism. With the thiosulfate:acceptor oxidoreductase as a completely constitutive enzyme (Sperling, 2001), its activity dependent of the medium pH, a second alternative for thiosulfate degradation is present in *A. vinosum*, until the induction of *sox* gene expression was has taken place.

For SoxB and for SoxYZ the situation concerning inducibility remains more or less speculative. The gene *soxB* is already part of a transcription unit different from *soxXA*. Nevertheless, the close neighbourhood still makes a comparable regulation very likely. Concerning the independent position of *soxYZ* on the genome, the special nature of the Δ*soxY* mutant (delay in sulfite oxidation) and potentially the influence of RLP, the expression of these genes could be regulated in a different way. With SoxYZ on one hand as a part of the Sox multienzyme system and on the other hand as part of a putative sulfite oxidizing enzyme, a regulative mechanism must be present to ensure the production of the right amount of protein at the appropriate time for the appropriate enzyme system.

As the Sox system was mainly produced with thiosulfate present in the medium, the respective proteins were purified from thiosulfate-grown *A. vinosum* cell material. In *P. pantotrophus* the Sox proteins SoxXA, SoxB, SoxYZ and SoxCD were all purified from the ammonium sulfate fraction between 44 and 65 % saturation (Friedrich *et al.*, 2000). This is essentially in accordance with the observation, that in *A. vinosum* all Sox proteins could be isolated from the supernatant of a solution with 40 % ammonium sulfate saturation. The subsequent purification strategies, however, applied for several organisms (Friedrich *et al.*, 2000; Appia-Ayme *et al.*, 2001; Kappler *et al.*, 2004), sometimes differ quite distinctly from the strategies applied for *A. vinosum*. Therefore, the optimal purification strategy appeared to be organism-specific.

The protein SoxXA was purified as a heterodimer from *A. vinosum*. The purified protein SoxXA was further analysed concerning its heme content. Regarding the UV-visible spectrum obtained for the purified SoxXA, it exhibits a typical c-type cytochrome spectrum. However, not an air-oxidized spectrum was observed, as in the other SoxXA proteins so far purified (Friedrich *et al.*, 2000; Cheesman *et al.*, 2001; Kappler *et al.*, 2004), but the reduced spectrum. With an α peak at 550 nm, SoxXA from *A. vinosum* is a cytochrome c_{550}. SoxXA from *P. pantotrophus* exhibited an α peak at 552,5 nm (Friedrich *et al.*, 2000) and SoxXA from *R. sulfidophilum* at 551 nm (Appia-Ayme *et al.*, 2001), as was the case for SoxXA from *S. novella* (Kappler *et al.*, 2004). All measurements confirmed the presence of a c-type cytochrome. The occurrence of a reduced c-type cytochrome during and after purification was quite surprising. With all procedures taking place under oxic conditions, the heme was expected to be air-oxidised. But for so far unknown reasons the reduced form of *A. vinosum* SoxXA seems to be unusually stable. The heme staining of SoxXA after SDS-PAGE confirmed the presence of at least one heme group per subunit, as was predicted by sequence analysis. According to the sequence alignment *A. vinosum* belongs to the group of organisms that contain a monoheme SoxA. While SoxX always contains just the one heme group, SoxA can differ in its heme content, dependent on the organism it originates from. Organisms like *P. pantotrophus* and *R. sulfidophilum* contain a diheme SoxA protein (Friedrich *et al.*, 2000; Appia-Ayme *et al.*, 2001), while organisms like *S. novella* (Kappler *et al.*, 2004) and *A. vinosum* contain just a monoheme SoxA. In case of a monoheme protein, the N-terminal heme binding motif has been destroyed, leaving just the C-terminal motif behind. The pyridine spectrum analysis was difficult to analyse concerning the actual quantity of the heme groups. For the final analyses several SoxXA-containing gel filtration fractions had been combined and concentrated, regardless of the additional contaminating proteins reintroduced into the sample, because of the low amount of SoxXA. The analysis of the hemochrome and hemichrome spectra revealed the qualitative presence of heme c in the sample, which was in accordance with the preceding results. With contaminating proteins artificially raising the protein concentration, the determined heme content was below the expected amount.

The purification aproach for SoxB led to a fairly homogenous protein sample, with only one additional band visible in the Coomassie-stained gel. However, the SoxB purification provided some difficulties, based upon the lack in accuracy of the applied antiserum. SoxB purifies from *A. vinosum* as a monomer with a molecular weight of approximately 60 kDa according to the SDS-PAGE. A second so far unidentified protein with exactly the same size

was also detected by the antiserum against SoxB and purified from *A. vinosum*, which raised the need for MALDI-TOF analysis as an additional proof for protein identity. This identification was a complete success. The analysis additionally confirmed the postulated signal peptide cleavage position for SoxB, confirming the postulated location in the periplasm. The Tat-dependent transport implicates the presence of a cofactor, corresponding to SoxB from *P. pantotrophus*, that contains two manganese atoms per monomer (Friedrich *et al.*, 2000). The observed difference between postulated molecular weight (62 kDa), molecular weight determined by SDS-PAGE (~60 kDa) and molecular weight determined by gelfiltration (41 kDa) indicates the possibility of interaction between protein and column material, that leads to a delayed elution not in accordance with the actual molecular weight. A comparable difference in molecular weight determination of SoxB was observed in *C. tepidum* (Hanson and Tabita, 2003). While the protein had a predicted size of 64 kDa, it consistently migrated at approximately 48 kDa in a two-dimensional SDS-PAGE. However, as the *A. vinosum* SoxB was found at the correct size in SDS-PAGE, two independent phenomena are responsible for these observations.

The purification of SoxYZ was successful, but could nevertheless be optimised, as several proteins apart from SoxYZ were still present in the preparation. Perhaps the reintroduction of the omitted anionic exchange chromatography would already lead to desired results. According to the molecular weight determined by gel filtration, SoxYZ is purified from *A. vinosum* as a heterodimer. In *P. pantotrophus* different subunit compositions were demonstrated for SoxY and SoxZ (Quentmeier *et al.*, 2003). The heterodimer SoxYZ was found either in associated form or covalently linked by a disulfide bond. The homodimers SoxYY and SoxZZ also appeared in the covalently linked state. Interaction with SoxB converted the covalently linked heterodimer SoxYZ to the associated form, which in turn aggregated to a heterotetramer $Sox(YZ)_2$. If these kinds of interaction also appear in *A. vinosum* was not distinguishable from the gained results. In *P. pantotrophus* SoxY has been demonstrated to be the substrate binding molecule of the Sox multienzyme complex (Quentmeier and Friedrich, 2001). By comparison with sequence date from other organisms a new consensus sequence has been identified, including the cysteine residue involved in substrate binding at the SoxY C-terminus (Friedrich *et al.*, 2001). The deduced protein sequence of *A. vinosum* SoxY is in accordance with this consensus sequence. Therefore, in all probability SoxY is the substrate binding molecule of the *A. vinosum* Sox complex. To further verify the identity of SoxYZ, MALDI-TOF analysis was again performed. The analysis

revealed fragments of both SoxY and SoxZ. Because of their similar molecular weight, the discrimination on the gel was difficult and in all probability resulted in a mixture to be analysed. Four peptide fragments altogether could be assigned to either SoxY (3 fragments) or SoxZ (1 fragment). As one of the observed MALDI-TOF peptides actually represented the C-terminus of SoxY potentially with a covalently bound thiosulfate, this would be a first hint to the actual presence of the substrate binding site. Further conclusive proof is, however, needed.

The assay to detect Sox system activity was based upon the thiosulfate-dependent reduction of horse heart cytochrome c. The supernatant after ammonium sulfate precipitation contained all the Sox proteins (SoxXA, SoxB and SoxYZ) and exhibited a reduction of cytochrome c in the assay significantly above blank level, with an activity of 1,34 mU/mg. Taking the inability of the thiosulfate:acceptor oxidoreductase to act with horse heart cytochrome c as electron acceptor into consideration, this allows several conclusions: (i) Thiosulfate oxidation is catalysed by proteins in the supernatant after precipitation, (ii) these proteins are in all probability Sox proteins and (iii) thiosulfate is an *in vitro* substrate of the Sox complex. In *P. pantotrophus* a cytochrome c reduction rate of 8,9 mU/mg with thiosulfate as substrate was recorded in cell extract (Wodara *et al.*, 1997). In comparison, the activity observed in *A. vinosum* appears to be quite low. However, it must be taken into account, that the Sox system of *P. pantotrophus* yields 8 electrons, due to the complete degradation of thiosulfate to sulfate, while without SoxCD the electron yield goes down to 2 electrons, the amount supposedly obtained by the Sox system in *A. vinosum*. The proteins needed for further oxidation of the sulfane sulfur have potentially been separated from the Sox proteins in the ammonium sulfate precipitation.

The reconstituted Sox system in *P. pantotrophus* exhibited a cytochrome c reduction rate of 6,51 mU, with 0,5 nmol/ml of SoxB SoxYZ, SoxXA and SoxCD, respectively, in the preparation (Friedrich *et al.*, 2000). The first approach for a reconstitution of the *A. vinosum* Sox system contained less protein, but exhibited an activity even higher than the ammonium sulfate supernatant (1,84 mU/mg). This is quite surprising, when looking at the other two preparations, that both contain more protein, but exhibit far less activity. Therefore, the reconstitution of the Sox system from the purified proteins SoxXA, SoxB and SoxYZ exhibited mixed results. The lack of activity in the reconstituted system could be due to several factors. The first assumption is, that some essential part of the multienzyme system is missing, when only the three proteins are put together. This unknown protein would potentially still be present in the supernatant after precipitation. Considering the missing

SoxCD, a complete thiosulfate oxidation to sulfate by these proteins is in all probability not possible, but at least the substrate binding and the cleaving off of the sulfone sulfur should be possible, resulting in a release of electrons. However, without the release of the sulfane sulfur, the substrate molecule is blocked for further reaction cycles. The next assumption would be that all necessary parts are present, but in the wrong proportion. Equimolar amounts of all three proteins have been applied, but without a positive result. Perhaps also the protein to substrate concentration was unsuitable. An important factor seems to be the condition of the proteins applied to the assay. When SoxYZ of *P. pantotrophus* has been inactivated by reduction before addition to the enzyme assay, no activity was measurable (Friedrich *et al.*, 2005). This consideration pushes SoxXA into focus. This protein was isolated from *A. vinosum* in its reduced form and not artificially oxidized before usage in the assay. SoxXA is thought to take over the electrons released when binding thiosulfate to SoxYZ. With SoxXA already carrying electrons, this first step could already be inhibited. In the supernatant after ammonium sulfate precipitation some unknown electron acceptor potentially takes over the electrons from SoxXA and enables the protein to be active in the Sox complex. This protein was lost during purification. A likely candidate would be the flavocytochrome c_{552} . The homologue SoxF from *P. pantotrophus* reactivated SoxYZ, that was reduced before addition to an enzyme assay. This kind of redox-balancing function could also be postulated for FccAB in *A. vinosum*. The last possibility for the lack in activity could be degradation of proteins. Even though if a protease inhibitor was added to the preparations during purification, it is not yet clear, if this strategy was completely successful.

No explanation can be given, why the reconstituted Sox system appears to be active in one preparation and nearly inactive in two others, with all preparations not too different from one another.

D.3 Analysis of *sox* gene mutants

The inactivation of *sox* genes in different organisms had different effects. The inactivation of *soxB* in *P. pantotrophus* by transposon mutagenesis resulted in a complete loss of thiosulfate oxidation ability (Chandra and Friedrich, 1986; Wodara *et al.*, 1994). A *soxA*-deficient mutant of R. *sulfidophilum* was unable to oxidize thiosulfate as well as sulfide (Appia-Ayme *et al.*, 2001). The inactivation of different *sox* genes in *A. vinosum* resulted mostly in a lack of thiosulfate oxidation, with the exceptions of ΔORF9/*rhd* and Δ*soxY*. While on Pfennig

medium no significant amount of tetrathionate was detected during thiosulfate oxidation, the experiments performed using 0 medium resulted in the formation of tetrathionate. This sulfur compound was the product of thiosulfate:acceptor oxidoreductase activity with thiosulfate as substrate. This pathway, an alternative to the oxidation to sulfate via the Sox pathway, remained essentially unaffected by *sox* gene inactivation. The ratio of sulfate and tetrathionate as products from thiosulfate oxidation is essentially pH dependent. As both experimental approaches (Pfennig and 0 medium) were subjected to pH control, the absence or presence of tetrathionate in the different media is potentially due to another unknown effect.

The insertional inactivations of *soxX* and *soxB* both had identical phenotypes. Sulfide oxidation and all subsequent oxidation steps remained unaffected (polysulfide formation, sulfur oxidation and sulfate as end product). Thiosulfate oxidation to sulfate, however, was completely inhibited. This phenotype was less detectable in thiosulfate concentration, but more clearly in the complete lack of sulfate production. While thiosulfate concentration remained unchanged in Pfennig medium, a decrease was observed on 0 medium. As oxidation to sulfate was not possible in the mutant strains, tetrathionate formation was responsible for part of the decrease. However, as the production of tetrathionate does not account for the complete amount of thiosulfate that has been degraded, trithionate formation could fill the gap and has been confirmed for *A. vinosum* (Sperling, 2001).
The joint inactivation of *soxB* and *soxX* also resulted in an inhibition of thiosulfate oxidation as observed in both of the single mutants. The observed slight delay in sulfide oxidation, polysulfide development and sulfur oxidation in Δ*soxBX* was possibly due to a cumulative effect. There may be an increase in the severity of the phenotype the more *sox* genes are inactivated in *A. vinosum*. It must also be taken into account, that the three mutants Δ*soxX*, Δ*soxB* and Δ*soxBX* were produced by insertional inactivation. The resistance cassette destroys the reading frame and contains transcriptional and translational stop signals on each end. Thereby it blocks the expression of genes in the same transcription unit as the inactivated gene. This could be demonstrated for the example of *soxA* directly downstream of *soxX*. The inactivation of the latter always resulted in the inability to detect SoxA. Additionally, in the Δ*soxBX* mutant the intergenic region between *soxB* and *soxX*, containing the putative promoters, was destroyed. Therefore, the observed phenotypes could not be followed back to one single inactivated gene, but always involved more extensive effects.

The phenotypic characterisation of the mutant strains Δ*soxX*, Δ*soxB* and Δ*soxBX* demonstrated, that the gene products of *soxB* and *soxXA* are absolutely essential for the oxidation of thiosulfate to sulfate in *A. vinosum*. This was also supported by the successfully complemented Δ*soxX* mutant. The *sox* gene expression in trans was successful, as thiosulfate oxidation to sulfate was reverted to wild type level, confirmed by the renewed production of sulfate. The production of SoxA in the complemented mutant provided additional proof for the correct expression of the plasmid-bourne genes. Furthermore, the introduction of the plasmid had no unwanted negative effects, as the precedingly unaffected sulfide oxidation pathway remained unaffected.

The open reading frames ORF9 and *rhd*, situated downstream of *soxA*, are potentially not involved in the oxidation of thiosulfate to sulfate at all, as the corresponding mutant exhibited no phenotype under the chosen conditions. There might be the possibility that the gene products are of importance, when the experimental conditions are different. In *A. vinosum* the inactivation of APS reductase, thought to be essential for sulfite oxidation, also had no effect on growth in batch culture (Dahl, 1996), but in continuous culture resulted in a considerable decrease in growth under light saturation (Sanchez *et al.*, 2001). The proteins encoded by ORF9 and *rhd* have a function, that still remains to be investigated. As no mutational experiments have been undertaken for the homologous genes in *C. tepidum* and *A. aeolicus*, no data for further speculation is available.

When compared with Δ*soxX*, Δ*soxB* and Δ*soxBX*, the mutant strain Δ*soxY* exhibited a differing phenotype. As observed for the other *sox* mutants, the oxidation of thiosulfate to sulfate was completely inhibited. In addition, the mutant exhibited a significant delay in sulfite oxidation. This is the first *A. vinosum* mutant so far, that was impaired in the degradation of this sulfur compound. Until now, an enzyme essential for sulfite oxidation in *A. vinosum* could not be identified. With the APS reductase not being essential (see above), a sulfite:acceptor oxidoreductase activity was postulated, but without any further proof on genetic or protein basis. If SoxYZ is involved in sulfite oxidation, there are several problems to consider. Because of the *in frame* deletion of *soxY*, *soxZ* is potentially still expressed. But as SoxZ contains no signal peptide of its own and SoxY is missing for joint transport, the protein potentially accumulates in the cytoplasm and is eventually degraded. The observed phenotype could be due either to the lack of just SoxY, or, more likely, to the lack of the complete substrate binding molecule SoxYZ. A second problem is the Sox system structure.

In *P. pantotrophus* SoxYZ is part of a larger multienzyme complex. As the Sox proteins in *A. vinosum* are not purified as one large Sox complex, nothing can be said about the composition of the thiosulfate oxidising structure. Regarding the independent function of SoxYZ a rigid structure composition seems rather unlikely. Sulfite oxidation involving SoxYZ appears to function without the action of the other Sox proteins, as none of the other mutants exhibited problems in sulfite oxidation. Except for the conserved cysteine SoxYZ contains no obvious active site or redox-active group. Therefore it could only act as a substrate carrier for sulfite, with the necessity of a second catalytically active enzyme for the actual oxidation to sulfate. A last problem evolves if sulfite is not added externally, but appears as an intermediate of sulfide and sulfur oxidation. Sulfite is produced in the cytoplasm from intracellular sulfur globules by the action of Dsr proteins (Pott and Dahl, 1998; Dahl *et al.*, 2005). With SoxYZ situated in the periplasm, some kind of substrate transporter must be postulated. The observed delay in sulfur degradation in Δ*soxY* is very likely due to the delayed sulfite oxidation. It is not in the interest of the organism to accumulate huge amounts of potentially harmful sulfite. Therefore, a decrease of Dsr protein activity would be a way to deal with a lowered sulfite oxidation rate. Regarding the Δ*soxY* phenotype, the independent position of *soxYZ* on the genome gains new potential. If SoxYZ is needed for a function apart from the other Sox proteins, there would be a need for independent regulation, which is made possible by gene separation.

By complementing a mutant strain, one would expect a more ore less successful return to the wild type phenotype (as observed for the complemented Δ*soxX*). The Δ*soxY+Y* mutant only partly fulfilled this expectancy. Thiosulfate was again oxidized, although at a significantly slower rate than the wild type. Externally added sulfite was oxidised again with wild type rates. Surprisingly, the complemented mutant exhibited a significant delay in sulfide oxidation. As a follow up, sulfur globule formation and degradation, as well as sulfite oxidation, was slowed. But this could possibly be only downward effects of the initially affected sulfide oxidation. Which step in the oxidation of sulfide to sulfate is ultimately impaired, is difficult to determine. Perhaps there is a complication concerning plasmid-bourne *soxYZ* and the second copy of *soxZ* still present in the genome. Possibly there is a regulatory active region upstream of *soxY*, that has not been inserted into the plasmid. Therefore, the expression of *soxYZ* from the plasmid is unregulated, leading to problems not observed in the correctly regulated wild type or the mutant without functional SoxYZ at all. At the moment a completely satisfying explanation cannot be given for this phenotype.

D.4 A model for the Sox system in *A. vinosum*

When trying to work out a model for thiosulfate oxidation to sulfate in *A. vinosum* (depicted in figure D1), it is clear that all three Sox proteins (SoxXA, SoxB and SoxYZ) must be involved. The missing SoxCD calls for a modification of the model so far postulated for *P. pantotrophus* (Friedrich *et al.*, 2001).

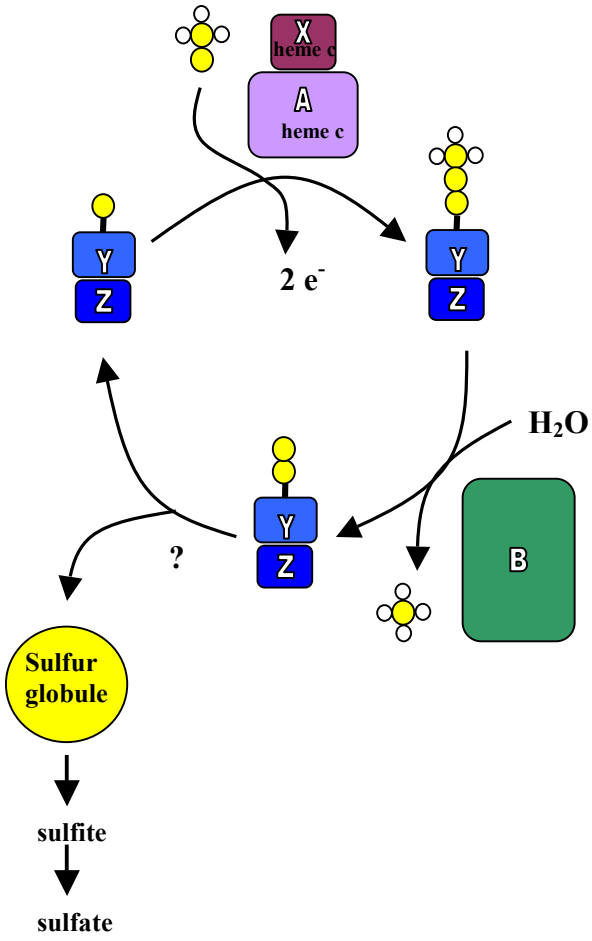

Figure D1: A model for the Sox multienzyme system in *A. vinosum*. Yellow: conserved cysteine at SoxY / sulfur, white: oxygen.

The first steps are in accordance with the *Paracoccus* model: a SoxXA-dependent binding of thiosulfate to the conserved cysteine of SoxYZ, followed by hydrolytic release of sulfate by SoxB. The remaining sulfane sulfur, however, needs to be oxidised via different pathway due to the lack of SoxCD. In *A. vinosum* as well as in *C. tepidum*, both organisms that lack

SoxCD, sulfur globules are an obligate intermediate of thiosulfate oxidation. Therefore the sulfane sulfur must be transferred to the sulfur globules in some fashion. The protein encoded by *rhd*, a sulfurtransferase, would have been a potential candidate in *A. vinosum*, but the corresponding mutant did not exhibit phenotype. Therefore, this protein remains to be identified. Once transferred to the sulfur globule, the further oxidation to sulfite is carried out by Dsr proteins. In the last oxidation step to sulfate SoxYZ must be involved. Together with other unknown components this would complete the oxidation of thiosulfate to sulfate.

Several questions still remain to be solved in the future. However, with the function of thiosulfate:acceptor oxidoreductase and the Sox multienzyme system for thiosulfate oxidation demonstrated in *A. vinosum*, further potentially undetected pathways for thiosulfate degradation are very unlikely. Additionally, new insights could be gained into the oxidation of sulfite, which was so far still unclear.

E: Summary

- Genomic sequence of *A. vinosum* was analysed, containing five open reading frames. Two open reading frames were identified as *sox* genes, *soxY* and *soxZ*. These are located on a site independent from the one containing the *sox* genes identified during the diploma thesis.

- The gene *soxY* encodes a putative periplasmic protein (12,7 kDa), *soxZ* a putative cytoplasmic protein (11,2 kDa). SoxY contains a conserved C-terminus including a cysteine residue necessary for substrate binding.

- The proteins SoxA, SoxB and SoxYZ were heterologously produced in *E. coli*. Their use for antiserum testing revealed the difference in sensitivity and background signals of the respective antisera, with SoxA antiserum delivering the best results.

- SoxA was detected in the soluble fraction of *A. vinosum* cell extract, in accordance with the postulated periplasmic location of the Sox proteins. The expression of *soxA* was inducible by thiosulfate above a constitutive level of expression. Therefore, the Sox proteins were purified from the soluble fraction of thiosulfate-grown *A. vinosum*.

- SoxXA was purified as a heterodimer. The c-type cytochrome nature of both subunits was confirmed by heme staining, additional proof provided by the recording of pyridine spectra. The spectrum recorded of SoxXA showed a reduced cytochrome c_{550}, that has not been air-oxidised.

- SoxB was purified as a monomer. To verify the identity of the purified protein MALDI-TOF analysis was applied, confirming the identity of SoxB. Additionally one peptide fragment verified the postulated signal peptide cleavage position and thereby the location of SoxB in the periplasm.

- SoxYZ was purified as a heterodimer. The identity was again verified by MALDI-TOF analysis. It confirmed the identity of SoxY and SoxZ. Additionally a first hint

was given concerning the binding of thiosulfate to the conserved cysteine residue in SoxY.

- The assay determining thiosulfate-dependent reduction of horse heart cytochrome c by the Sox proteins did not provide clear results.

- An *in frame* deletion of *soxY* in *A. vinosum* was constructed. The mutant Δ*soxY* was phenotypically characterised with the mutants constructed during the diploma thesis (Δ*soxX*, Δ*soxB*, Δ*soxBX* and ΔORF9/*rhd*).

- In the mutant strains Δ*soxX*, Δ*soxB*, Δ*soxBX* and Δ*soxY* the oxidation of thiosulfate to sulfate was completely inhibited, with tetrathionate formation unaffected. The mutants were not impaired in sulfide oxidation.

- The mutant strain Δ*soxY* exhibited an accumulation of significant amounts of sulfite during sulfide oxidation. The oxidation of externally added sulfite was also delayed.

- To complement the mutants Δ*soxX* and Δ*soxY*, plasmids were constructed that carried the intact genes and surrounding sequence containing the putative promoters. The plasmids were successfully established in the mutant strains, resulting in the complementation strains Δ*soxX*+*X* and Δ*soxY*+*Y*.

- In the strain Δ*soxX*+*X* the ability to oxidise thiosulfate to sulfate was re-established at wild type level.

- In the strain Δ*soxY*+*Y* the ability to oxidise thiosulfate to sulfate was only partly restored. The delay in sulfite oxidation, however, was completely removed. The complemented mutant exhibited a delay in sulfide oxidation and all subsequent oxidation steps.

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G: Appendix

G.1 Nucleotide Sequence

```
1  aattcctgcg cacccatccg gtcaacgcca accgcatcgc
>>..................ORFd..................>

41  cgaggcgctc ggccgggccg acgacttcgg cgcccagcag
>..................ORFd..................>

81  cgccctgaca gtctgcgctt ccagctcgca cgcgcggccc
>..................ORFd..................>

121  tgcgcgaacg ctcctacaaa cgcccggaac aggccgtcgc
>..................ORFd..................>

161  ccatttccgt gacacattgc gcgagggccg gcatcgcaat
>..................ORFd..................>

201  gccgtcgccg aacactatgg ctatgcgctg gccctgacgc
>..................ORFd..................>

241  gcgcgggtca gttcgacgcc gcccgcgccg ccctggccac
>..................ORFd..................>

281  ggccatgaaa tcgcactcca gcttgcccga gttcatcgtc
>..................ORFd..................>

321  ctggaggccc ggctcgatct cgaacagggg caggtcgagc
>..................ORFd..................>

361  gcgccgtgcg taacctggga caggccgtgg gtctgtcgcc
>..................ORFd..................>

401  gagccactgg ccgctgcgcg tggcctatgc cgaggcgttg
>..................ORFd..................>
```

```
441  atgaaagccg gccgtccggc tcaggccatc gacgagctga
>..................ORFd...................>

481  ccgccgtggc gcgactgcgc cccggcaatc ccatgttgta
>..................ORFd...................>

521  cgataagctg gagcaggcgg cctttcgcgc gggcaacaaa
>..................ORFd...................>

561  tcggcgaccc accgtttccg cgccgagaag ctctatgccg
>..................ORFd...................>

601  agggcgaccg cgagccggcg atccgtcagc tcgagatcgc
>..................ORFd...................>

641  actgcgtcag cgcgacctcc cctaccatga ggcggcgcgc
>..................ORFd...................>

681  atccaggcgc ggctcgaaac ctggaaagaa gaagaacgcg
>..................ORFd...................>

721  aagccaaacg caaagacaag agaggagata aatcatgatc
>>...>
>................ORFd................>>

761  gatgccaaac gccggacact cgtcaagggc tcgctggccg
>..................soxY...................>

801  ctggggcggt cgtcggcgcc ggactgatca cgccacgcgc
>..................soxY...................>

841  cttcgccgac tggaacgcag cggccttcca ggccaaggac
>..................soxY...................>

881  atccccacgg ccatgaccgg tctgctcggc agcgacgccg
>..................soxY...................>
```

```
921  ccgaggtcag cgaccggatc aagatcaagg ccccggacat
>..................soxY...................>


961  cgccgagaac ggtgccgtgg ttccggtcac ggtcgagacc
>..................soxY...................>


1001  gatctggagg gcgtcacttc catcagtctc atcgcggcca
>..................soxY...................>


1041  agaatcagtc gccgctgatc gcctccttcg agttcgtcga
>..................soxY...................>


1081  cccgtccgtg atccccttcg tcgccacccg catcaagatg
>..................soxY...................>


1121  gctgagaccg ccgacgtcat cgccgtggtc aaggcgggtg
>..................soxY...................>


1161  acaagctcta taagaacgcc aagagcgtca aggtcaccat
>..................soxY...................>


1201  cggcggctgc ggcggctgat cagcctctca cgcccgtacc
>......soxY.......>>


1241  caagtatcca aggctgaata ccgaacacga ggagtcgaga


1281  cgatgtccga tatcaagatc cgcgccaagc tcgagggtga
>>.................soxZ.................>


1321  cgagacgacc gtcaagtgtc tgatgagcca cccgatggag
>..................soxZ...................>


1361  accggtctgc gcaaggacag caagaccaac gaagtcatcc
>..................soxZ...................>



1401  cggcgcactt catccgggaa gtggtgtgca aggtcaaggg
>..................soxZ...................>
```

```
1441  agccgtggtg atgaagacgt cctggagcgg cggcgtctcc
>..................soxZ..................>


1481  aagaatccct atctgtcgtt caagttcaag ggcggcgccg
>..................soxZ..................>


1521  tcggcgatcc gatcgagatc gcctggaccg acaacacggg
>..................soxZ..................>


1561  cgagagccag agtgccacgg ccgagatcag cggctgacgg
>.................soxZ.................>>


1601  aacgccgagg ccccggagat ccggggcctc ggaccacgac
<


1641  tagaattcga aaccagccgg ctcgggcgca ttgcgcagcg
<..................ORFe..................<


1681  gcgccacctc gacctcgaac agcgactcgc gccgatagtc
<..................ORFe..................<


1721  gtggcgcccg acgcgggtca cgcacacaca ctccatcgcg
<..................ORFe..................<


1761  ggtgccgtgc ccaggatgca gaacagccgt ccgccgatgg
<..................ORFe..................<


1801  tcagctgctc gcgcagcatc ggcagcgcgt cctcggtcgg
<..................ORFe..................<


1841  catcgagccc ttgacggcga tcgcgtcgaa tggcgccccg
<..................ORFe..................<


1881  ctcacgggtc cggccagacc atcaccctcg cgaacctcga
<..................ORFe..................<


1921  cccagtcgaa cttgagcgct tcgagccgct cgaccgcctc
<..................ORFe..................<
```

```
1961  ggccgcctgc atcgggtcga tctccagact gatcacgcgc
<.................ORFe.................<


2001  gccccgagcc ggctcagaca ggcggcgaca tagcccgacc
<.................ORFe.................<


2041  ccgtgccgat ctccagcgcc cgatcgcccg gctggacggc
<.................ORFe.................<


2081  gagcgcctgc aacagatgac cgaccacctt gggcgcaagc
<.................ORFe.................<


2121  atgagggtgc cgttgccgtt ggggatctcg atgtcggcat
<.................ORFe.................<


2161  aggccagcgc ccgataggca tccggcacga agcgctcgcg
<.................ORFe.................<


2201  ctcgaccgtg cccatcacct ccagcacccg gtcgtcgagc
<.................ORFe.................<


2241  acgccccagg gccggatctg ctgctggatc atgttgaagc
<.................ORFe.................<


2281  gggccaactc actgctgttg tccatgaaat ggaagtaccc
<.........ORFe.........<<


2321  cgtctgaact catgtttttg gaaaagcgac cgcgtccggc
>>...........ORFf...........>


2361  gagacgcagg acgaaagatt atataatagg tgcccaacca
>.................ORFf.................>


2401  gccgaccgag tcgacggtcg gcctattcct gtcagttcag
>.................ORFf.................>


2441  tggagagtct cttcatgccc agcgccccga gcaagtcgct
>.................ORFf.................>
```

```
2481  cgaaaccttc cccaatcccc agcccgagcg cgactatacg
>..................ORFf...................>


2521  atccgcatcc gggtgcccga gttcacctgt ctctgcccca
>..................ORFf...................>


2561  agaccggcca gccggacttc gccgagctga tgctcgaata
>..................ORFf...................>


2601  cgtccccgag cagaagtgcg tcgagctgaa ggcgctcaag
>..................ORFf...................>


2641  acctatgtct ggtcctatcg tgacgagggc gccttccacg
>..................ORFf...................>


2681  aggccgtcac caaccgcatc ctcggtgatc tggtcgaggc
>..................ORFf...................>


2721  cacggcaccg cgcttcatgc gcctgaccgc cgagttcaac
>..................ORFf...................>


2761  gtgcgcggcg gtatctatac cacggtcgtc gccgagcatc
>..................ORFf...................>


2801  gcgccgctga ctggcagccg ccggtgccgg tcacgctgcc
>..................ORFf...................>


2841  gtgagcggtc tgaaccctcg gccctgcgcc gccggccggg
>.>>


2881  tcggctgacc ccgtggccaa gcgacgactc tccgcgcgcc


2921  agatcgagcg cattcaagcg atccaggaac gccgccgcga


2961  acggctcgcc gcgcggcccg aacgggcgct ggccgaactc


3001  gacgacgagc tggagccgga agaaggacga gtcgtcgtcc
```

```
3041  gacacggcgc caatctggcg gtcgaggacg cgcacggtca

3081  cttgatccac tgcctggcgc gccagaacat cgggcatgtg

3121  gtgtgcgggg accgcgtcgt ctggcagcgt ctgcccgatg

3161  gccagggtgt ggtgacagcc accctgccgc gcatcagcac

3201  cctgagccga cccgactaca gcgggcgcga caagccgctc

3241  gcggccaatc tgacacggct cgccatcctc atcgccccgg

3281  aaccggagcc gagcggttat ctgatcgata agcttggatc

3321  cggagagctc ccaacgcgtt ggatgcatag cttgagtatt

3361  ctatagtgtc acctaaatag cttggcgtaa tcatg
```

G.2 Amino acid sequence

ORFd (incomplete):

F L R T H P V N A N R I A E A L G R A D D F G A Q Q R P D S L R F Q L A R A A L R
E R S Y K R P E Q A V A H F R D T L R E G R H R N A V A E H Y G Y A L A L T R A G
Q F D A A R A A L A T A M K S H S S L P E F I V L E A R L D L E Q G Q V E R A V R
N L G Q A V G L S P S H W P L R V A Y A E A L M K A G R P A Q A I D E L T A V A R
L R P G N P M L Y D K L E Q A A F R A G N K S A T H R F R A E K L Y A E G D R E P
A I R Q L E I A L R Q R D L P Y H E A A R I Q A R L E T W K E E E R E A K R K D K R
G D K S *

SoxY:

M I D A K R R T L V K G S L A A G A V V G A G L I T P R A F A || D W N A A A F Q A
K D I P T A M T G L L G S D A A E V S D R I K I K A P D I A E N G A V V P V T V E T
D L E G V T S I S L I A A K N Q S P L I A S F E F V D P S V I P F V A T R I K M A E T
A D V I A V V K A G D K L Y K N A K S V K V T I G G C G G *

SoxZ:

M S D I K I R A K L E G D E T T V K C L M S H P M E T G L R K D S K T N E V I P A H
F I R E V V C K V K G A V V M K T S W S G G V S K N P Y L S F K F K G G A V G D P
I E I A W T D N T G E S Q S A T A E I S G *

ORFe:

M D N S S E L A R F N M I Q Q Q I R P W G V L D D R V L E V M G T V E R E R F V P
D A Y R A L A Y A D I E I P N G N G T L M L A P K V V G H L L Q A L A V Q P G D R
A L E I G T G S G Y V A A C L S R L G A R V I S L E I D P M Q A A E A V E R L E A L
K F D W V E V R E G D G L A G P V S G A P F D A I A V K G S M P T E D A L P M L R
E Q L T I G G R L F C I L G T A P A M E C V C V T R V G R H D Y R R E S L F E V E V
A P L R N A P E P A G F E F *

ORFf:

M P S A P S K S L E T F P N P Q P E R D Y T I R I R V P E F T C L C P K T G Q P D F A
E L M L E Y V P E Q K C V E L K A L K T Y V W S Y R D E G A F H E A V T N R I L G
D L V E A T A P R F M R L T A E F N V R G G I Y T T V V A E H R A A D W Q P P V P
V T L P S *

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Acknowledgements

Many thanks to...

... PD Dr. Christiane Dahl for the interesting subject, the provision of working space, the interest in my work and many helpful discussions.

... Prof. Dr. Dr. Hans G. Trüper for the readiness to give an opinion on my Ph.D. thesis.

... my co-workers in the „sulfur“ group for their support and constant readiness for discussion.

... Bettina Franz for the provision of the sulfite fermenter results.

... Dipl.Biol. Fabian Grein for the help in constructing and identifying plasmids containing the second set of *sox* genes.

... Birgitt Hüttig for the construction of the plasmid pΔ*soxX*+*X* and help whenever needed.

... all the other people in the institute for a good working atmosphere.

... the group of Cornelius Friedrich at the university in Dortmund for the provision of antisera against SoxB and SoxYZ.

... my friend Anke Gehlen for moral support that never ceased.

... my parents and family for always being there.