New Proteins Involved in Sulfur Trafficking in the Cytoplasm of *Allochromatium vinosum*[*]

Yvonne Stockdreher[‡]**, Marga Sturm**[‡1]**, Michaele Josten**[§]**, Hans-Georg Sahl**[§]**, Nadine Dobler**[‡2]**, Renate Zigann**[‡]**, and Christiane Dahl**[‡3]

From the [‡]*Institut für Mikrobiologie & Biotechnologie, Rheinische Friedrich-Wilhelms-Universität Bonn, D-53115 Bonn, Germany and* [§]*Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie, Abteilung Pharmazeutische Mikrobiologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Meckenheimer Allee 168, D-53115 Bonn, Germany*

Background: Sulfur carrier proteins Rhd_2599, TusA, and DsrE2 occur in many sulfur oxidizing prokaryotes.
Results: Rhd_2599, TusA, and possibly DsrE2 are involved in cytoplasmic sulfur trafficking during dissimilatory sulfur oxidation.
Conclusion: Sulfur transfer from persulfide intermediates to dissimilatory sulfite reductase involves Rhd_2599, TusA, and possibly DsrE2.
Significance: Proteins involved in dissimilatory sulfur oxidation have been identified.

The formation of periplasmic sulfur globules is an intermediate step during the oxidation of reduced sulfur compounds in various sulfur-oxidizing microorganisms. The mechanism of how this sulfur is activated and crosses the cytoplasmic membrane for further oxidation to sulfite by the dissimilatory reductase DsrAB is incompletely understood, but it has been well documented that the pathway involves sulfur trafficking mediated by sulfur-carrying proteins. So far sulfur transfer from DsrEFH to DsrC has been established. Persulfurated DsrC very probably serves as a direct substrate for DsrAB. Here, we introduce further important players in oxidative sulfur metabolism; the proteins Rhd_2599, TusA, and DsrE2 are strictly conserved in the Chromatiaceae, Chlorobiaceae, and Acidithiobacillaceae families of sulfur-oxidizing bacteria and are linked to genes encoding complexes involved in sulfur oxidation (Dsr or Hdr) in the latter two. Here we show via relative quantitative real-time PCR and microarray analysis an increase of mRNA levels under sulfur-oxidizing conditions for *rhd_2599, tusA,* and *dsrE2* in *Allochromatium vinosum.* Transcriptomic patterns for the three genes match those of major genes for the sulfur-oxidizing machinery rather than those involved in biosynthesis of sulfur-containing biomolecules. TusA appears to be one of the major proteins in *A. vinosum.* A *rhd_2599-tusA-dsrE2*-deficient mutant strain, although not viable in liquid culture, was clearly sulfur oxidation negative upon growth on solid media containing sulfide. Rhd_2599, TusA, and DsrE2 bind sulfur atoms via conserved cysteine residues, and experimental evidence is provided for the transfer of sulfur between these proteins as well as to DsrEFH and DsrC.

[1] Present address: University Children's Hospital, Dept. of General Pediatrics, Neonatology, and Pediatric Cardiology, Moorenstr. 5, D-40225 Düsseldorf, Germany.
[2] Present address: Autodisplay Biotech GmbH, Lifescience Center, Meringerplatz 1a, D-40225 Düsseldorf, Germany.
[3] To whom correspondence should be addressed: Institut für Mikrobiologie & Biotechnologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Meckenheimer Allee 168, D-53115 Bonn, Germany. Tel.: 49-228-732119; Fax: 49-228-747576; E-mail: ChDahl@uni-bonn.de.

Reduced inorganic sulfur compounds serve as electron donors for carbon dioxide fixation during anoxygenic photosynthesis in purple and green sulfur bacteria. In the γ-proteobacterium *Allochromatium vinosum* periplasmic globules of zero-valent sulfur are formed as obligatory intermediates during the oxidation of sulfide, polysulfides, elemental sulfur, and thiosulfate to sulfate. The degradation of these globules involves essential steps in the cytoplasm and is catalyzed by soluble as well as membrane bound proteins of the Dsr system (1, 2). Despite its central importance, activation and transport of sulfur from the periplasm into the cytoplasm is not yet fully understood. The current model involves transport of sulfur into the cytoplasm via a persulfidic carrier molecule, possibly glutathione amide persulfide (3). It is well established that the Dsr mechanism includes the transfer of sulfur atoms from DsrEFH to DsrC, which in its persulfurated state then most likely serves as the substrate for dissimilatory sulfite reductase DsrAB (4). However, DsrEFH on its own is incapable of mobilizing sulfur from persulfidic carrier molecules (4) and, therefore, needs a donor protein. Intriguingly, DsrEFH and DsrC are homologs of *Escherichia coli* TusBCD and TusE, respectively (5, 6). These proteins are involved in the sulfur relay system leading to the synthesis of 2-thiouridine in the modified wobble base 5-methyl-aminomethyl-2-thiouridine $((mnm)^5 s^2 U)$ in tRNA (7). The transferred sulfur originates from L-cysteine and is mobilized by the L-cysteine desulfurase IscS. The TusA protein is the first to accept sulfur from IscS and further transfers it to the TusD subunit of TusBCD. From here the transfer progresses to TusE and thiouridylase MnmA, which finally catalyzes 2-thiouridine formation.

A *tusA* gene also exists in *A. vinosum* opening the possibility that TusA may act as the cytoplasmic sulfur donor protein for DsrEFH in the purple sulfur bacterium. Bioinformatic and microarray analyses provided first hints that the role of TusA in

bacteria not closely related to *E. coli* may not be limited to biosynthetic processes. In the acidophilic chemolithoautotrophic γ-proteobacterium *Acidithiobacillus ferrooxidans*, a *tusA* homologous gene exhibited a very conspicuous pattern of relative mRNA levels; significantly higher levels were observed under sulfur-oxidizing than under iron-oxidizing conditions (8). In *A. ferrooxidans* the *tusA* gene (AFE_2557) is flanked in the same direction of transcription by two genes encoding a rhodanese-like protein (*rhd*, AFE_2558) and a protein of the DsrE superfamily (AFE_2556, *dsrE2*). For the *dsrE2* gene the relative mRNA level was also elevated under sulfur-oxidizing *versus* iron-oxidizing conditions. The *rhd-tusA-dsrE2* arrangement is situated immediately upstream of genes encoding the putative heterodisulfide reductase-like complex HdrC1B1A1Orf2HdrC2B2. All of these genes followed the same transcription pattern. These findings led Quatrini *et al.* (8) to the following conclusions. 1) The Hdr-like complex was predicted to be responsible for the oxidation of organic persulfides that are formed as intermediates during the oxidation of externally available elemental sulfur to sulfite. 2) The rhodanese-like Rhd proteins, TusA, and DsrE2, were proposed to be involved in the transfer of sulfur from the persulfidic intermediates toward the Hdr complex.

The *rhd-tusA-dsrE2* gene arrangement is not restricted to the genus *Acidithiobacillus* but is also present in *A. vinosum* (Alvin_2599 –2601) and in a large number of further genome-sequenced chemotrophic and phototrophic sulfur-oxidizing prokaryotes (9). In most representatives of the phototrophic green sulfur bacteria *Chlorobiaceae* the complete set *dsrE2-rhd-tusA* is situated immediately upstream of the *dsr* gene cluster. Remarkably, the whole cluster is preceded by genes encoding the SoeABC-related PSRLC3 complex for which a role in the oxidation of sulfite in the cytoplasm is very probable (10, 11). Notably, the only *Chlorobium* species with incomplete *rhd-tusA-dsrE2* clusters, *Chlorobaculum parvum* and *Chlorobium ferrooxidans*, are unable to convert accumulated sulfur globules to sulfate (12) and completely incapable of living on reduced sulfur compounds (13), respectively. Although the complete *rhd-tusA-dsrE2* cluster is present in *Chromatiaceae*, an Alvin_2599 homolog is not present in phototrophic members of the Ectothiorhodospiraceae. In some of these organisms, *tusA* and *dsrE2* are immediately linked with *hdr* genes; in others *tusA* resides close to the *dsr* and *soeABC* genes (9, 11, 14). The only phototrophic α-proteobacterium that contains *tusA-dsrE2* is *Rhodomicrobium vannielii*, and in fact this is also the only genome-sequenced phototrophic member of this class that contains genes encoding the complete Dsr pathway. In this study we set out to investigate the roles of the rhodanese-like protein encoded next to *tusA*, TusA, and DsrE2 in dissimilatory sulfur metabolism and chose *A. vinosum* as a model organism.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Table 1 lists the bacterial strains and plasmids that were used for this study. *E. coli* DH5α cells were employed for molecular cloning, whereas *E. coli* SM10 and *E. coli* S17-1 were used for conjugation with *A. vinosum*. *E. coli* BL21(DE3) was used for the overproduction of soluble proteins; for membrane-bound proteins

E. coli strains C41 and C43 were employed. All *E. coli* strains were grown on LB medium. Wild type and mutant strains of *A. vinosum* DSM 180[T] were cultivated as described before (15). For photoorganoheterotrophic growth *A. vinosum* strains were cultivated on malate (16) supplemented with trace element solution SL12 (17). When cells were grown on plates the medium was solidified with 1% (w/v) Phytagel. 0.5% (w/v) NaCl was added to support gelling. For the characterization of phenotypes, *A. vinosum* strains were grown photolithoautotrophically on Pfennig's medium (18) lacking a sulfur source. The sulfur compound of interest was added directly to the freshly inoculated medium to start the experiment. Antibiotics were used at the following concentrations: for *E. coli*, 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin; for *A. vinosum*, 10 μg ml^{-1} kanamycin, 50 μg ml^{-} rifampicin, 10 μg ml^{-1} ampicillin, and 10 μg ml^{-1} streptomycin.

RNA Preparation and cDNA Synthesis—Total RNA was extracted from *A. vinosum* wild type grown either photoorganoheterotrophically on malate (22 mM) or photolithoautotrophically on elemental sulfur (50 mM), sulfide (4 mM), or thiosulfate (5 mM) in thermostatted glass fermenters. The RNA extraction followed the protocol previously described by Weissgerber *et al.* (19). For cDNA synthesis the First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Schwerte, Germany) was used.

Analysis of Transcription by Quantitative Real-time PCR (qRT-PCR)—For qRT-PCR analysis 100 ng of RNA was used and analyzed via the QuantiTect SYBR green RT-PCR kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reactions without reverse transcriptase served as control for each sample. Alvin_0486 was used as a reference (19). The qRT-PCR conditions were as follows: 30 min at 50 °C, 15 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. This was followed by a melting curve analysis in which the temperature was increased by 1 °C s^{-1} starting at 35 °C up to 95 °C. The qRT-PCR was performed in the iCycler iQ real-time detection system (Bio-Rad). The oligonucleotides used for this experiment are listed in Table 2. Analyses of melting curves and calculation of C_t (calculated threshold) values were automatically carried out by the iCycler iQ software. C_t values for each point in time were run in triplicate. Relative expression ratios (R) were calculated by the $2^{-\Delta\Delta Ct}$ method (20) using Equations 1 and 2 with PLA referring to photolithotrophic growth and POH meaning photoorganoheterotroph growth.

$$\Delta\Delta C_t = (C_{t,target} - C_{t,Alvin\_0486})_{PLA} - (C_{t,target} - C_{t,Alvin\_0486})_{POH}$$

(Eq. 1)

$$R = 2^{-\Delta\Delta Ct}$$

(Eq. 2)

Construction of A. vinosum Mutant Strains—For in-frame deletion of the *rhd_2599-tusA-dsrE2* sequence and the single in-frame deletion of *tusA*, the plasmids pk18*mobsacB*Δ*rtd* and pk18*mobsacB*Δ*tusA* were created. PCR amplicons containing the deletions without disrupting the reading frame were obtained by gene SOEing (21) using the primers listed in Table 2. The amplicons were cloned into the respective restriction sites of pk18*mobsacB*. For replacement of the *rhd_2599-tusA-*

TABLE 1

Strains and plasmids used in this study

Strains or plasmids	Genotype or phenotype	Reference or source
E. coli strains		
DH5α	F⁻ φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1hsdR17* (rk⁻ mk ⁺) *supE44 ë⁻ thi-1 gyrA relA1*	(49)
S17-1	294 (*recA pro res mod⁺*) Tpʳ Smʳ (pRP4–2-Tc::Mu-Km::Tn7)	(50)
SM10	*Kmʳ supE44 thi-1 thr-1 recA leu B6 lacY1 ton A21* RP4–2-Tc::Mu-Km::Tn7	(50)
BL21 (DE3)	F⁻ *ompT hsdS_B (r_B⁻m_B⁻) gal dcm met*(DE3)	Novagen
C41 (DE3)	Derived from BL21 (DE3), at least one uncharacterized mutation	(51)
C43 (DE3)	Derived from C41 (DE3), at least two uncharacterized mutations	(51)
A. vinosum strains		
Rif50	Rifʳ; spontaneous rifampicin-resistant mutant of *A. vinosum* DSM 180ᵀ	(31)
rhd_2599:: ΩSm	Rifʳ; insertion of ΩSm in *rhd_2599* of Rif50	This study
Δ*tusA*	Rifʳ; Rif50 with in-frame deletion of *tusA*	This study
Δ*rtd*	Rifʳ; Rif50 with in-frame deletion of *rhd_2599*, *tusA*, and *dsrE2*	This study
Δ*rtd*::ΩKm	Rifʳ Kmʳ; Rif50 with in-frame deletion of *rhd_2599*, *tusA*, *dsrE2*, and ΩKm in center of deleted sequence	This study
Plasmids		
pHP45-Ω	Apʳ Smʳ/Spcʳ	(52)
pHP45Ω-Km	Apʳ Kmʳ	(53)
pGEM-5Zf	Apʳ; *lacZ* f1 *ori*	Promega
pGEM5-*tusA*	Apʳ; *rhd_2599-tusA-dsrE2* amplicon cloned into SalI restriction site of pGEM-5Zf	This study
pGEM5-*rhd_2599*::ΩSm	Apʳ Smʳ; SmaI fragment from pHP45-Ω ligated into Bsp68I restriction site of pGEM 5-*tusA*	This study
pK18*mobsacB*	Kmʳ, *lacZ'*, *sacB*, Mob⁺	(35)
pK18*mobsacB*Δ*tusA*	Kmʳ; 1,21 kb amplicon with deleted *tusA* cloned into XbaI restriction site of pK18*mobsacB*	This study
pK18*mobsacB*Δ*rtd*	Kmʳ; 1,21-kb amplicon with deleted *rhd_2599-tusA-dsrE2* cloned into BamHI-restriction site of pK18*mobsacB*	This study
pSUP202	Apʳ Cmʳ Tcʳ; Mob⁺ RP4 *oriT*	(50)
pSUP202-*rhd_2599*::ΩSm	SalI fragment from pGEM5-*rhd_2599*::ΩSm cloned into SalI of pSUP202	This study
pSUP301	Apʳ Kmʳ; RP4 oriT p15A *ori* Mob⁺	(50)
pSUP301Δ*rtd*	Apʳ; 1,21 kb amplicon with deleted *rhd_2599-tusA-dsrE2* cloned into HindIII-restriction site of pSUP301	This study
pSUP301Δ*rtd*::ΩKm	Apʳ Kmʳ; pHP45Ω-Km kanamycin cassette inserted into pSUP301Δ*rtd* using EcoRI restriction sites	This study
pET-15b	Apʳ, T7 promoter, His tag (N terminus)	Novagen
pET15bRhd	Apʳ, NdeI/XhoI fragment of amplified *rhd_2599* in NdeI/XhoI of pET-15b	This study
pET15bRhd-C64S	Apʳ, NdeI/BamHI fragment of amplified *rhd_2599-C64S* in NdeI/BamHI of pET-15b	This study
pET15bRhd-C74S	Apʳ, NdeI/BamHI fragment of amplified *rhd_2599-C74S* in NdeI/BamHI of pET-15b	This study
pET15bTusA	Apʳ, NdeI/BamHI fragment of amplified *tusA* in NdeI/BamHI of pET-15b	This study
pET15bTusA-C15S	Apʳ, NdeI/BamHI fragment of amplified *tusA-C15S* in NdeI/BamHI of pET-15b	This study
pET15bEcTusA	Apʳ, NdeI/BamHI fragment of amplified *E. coli tusA* in NdeI/BamHI of pET-15b	This study
pASK-IBA5plus	Apʳ, Tet promoter, Strep-Tag (N-terminal)	IBA
pIBADsrE2	Apʳ, BsaI fragment of amplified *dsrE2* in BsaI of pASK-IBA5plus	This study
pIBADsrE2-C110S	Apʳ, BsaI fragment of amplified *dsrE2-Cys110Ser* in BsaI of pASK-IBA5plus	This study
pIBADsrE2-C120S	BsaI fragment of amplified *dsrE2-C120S* in BsaI of pASK-IBA5plus	This study
pIBADsrE2-C156S	Apʳ, BsaI fragment of amplified *dsrE2-Cys156Ser* in BsaI of pASK-IBA5plus	This study
pETEFH	Apʳ, NdeI/BamHI fragment of amplified *dsrEFH* in pET-15b	(54)
pETE₇₈FH	Apʳ, NdeI/BamHI fragment of amplified *dsrE78FH* in pET-15b	(6)
pETCEX	Apʳ, NdeI/BamHI fragment of amplified *dsrC* in pET-15b	(5)
pETCEXSer-100	Apʳ, NdeI/BamHI fragment of amplified *dsrC100* in pET-15b	(5)
pETCEXSer-111	Apʳ, NdeI/BamHI fragment of amplified *dsrC111* in pET-15b	(5)

dsrE2 cluster by a kanamycin resistance interposon, plasmid pSUP301Δ*rtd*::ΩKm was generated; via gene SOEing a restriction site for *Eco*RI was created in the center of the remains of the gene cluster using pk18*mobsacB*Δ*rtd* DNA as template. Before the Ω-kanamycin cassette from pHP45Ω-Km was cloned into the EcoRI site, the amplicon itself was inserted into the HindIII restriction site of pSUP301. For the generation of pSUP202-*rhd_2599*::ΩSm the Ω-Sm/Spc cassette from pHP45-Ω was isolated by digestion with SmaI and ligated into the *Bsp*68I site in the *rhd_2599* sequence in pGEM-*rtd* (Table 1). The complete insert (*rhd_2599*::ΩSm-*tusA-dsrE2*) was then cut from the vector with SalI and cloned into the corresponding site in pSUP202. Plasmids based on pk18*mobsacB* and pSUP301 were transferred to *E. coli* S17-1, whereas pSUP202-*rhd_2599*::ΩSm was transferred to *E. coli* SM10 for conjugation with *A. vinosum* Rif50. The *E. coli* cells were mixed with stationary phase cells of *A. vinosum* in a ratio of 1:3 and incubated anaerobically on cellulose nitrate membranes (pore size 0.45 μm) on solid RCV medium (16). After 2 days the cells were washed from the filters with RCV medium. Cells were plated on RCV medium containing 1% (w/v) Phytagel for solidification. The plates were further supplemented with 0.5% (w/v) NaCl to support

the gelling process and 0.02% (w/v) Na₂S₂O₃ × 5 H₂O, 2 mM sodium acetate, and 2.6 ml feeding solution (for 100 ml: 3.1 g of NaSH × H₂O, 5.0 g of NaHCO₃) per liter for growth enhancement. The selection process for in-frame mutants was as follows. Single-crossover mutants were identified via the resistance toward kanamycin provided by the pk18*mobsacB*-based plasmids and verified via colony PCR. After growth in non-selective RCV medium for three generations, double-crossover mutants were identified by plating cells on solid medium (see above) that contained 10% (w/v) sucrose. When plasmids of the pSUP series were used, double-crossover mutants were identified by their resistance to kanamycin or streptomycin and loss of the vector-encoded ampicillin resistance. Genotypes were verified via colony PCR and Southern blotting.

Characterization of A. vinosum Mutant Strain Phenotypes—To determine the turnover of reduced sulfur compounds under photolithoautotrophic conditions *A. vinosum* wild type and mutant strains were first grown photoorganoheterotrophically in liquid culture. Then 80 ml of the cultures were harvested, washed with Pfennig's medium without sulfur compounds (2500 × *g*; 10 min; room temperature), and used to inoculate

TABLE 2

Oligonucleotides used in this study

Restriction sites and mutated nucleotides are printed in bold.

Purpose	Oligonucleotide	Sequence 5′-3′	Source or reference
Cloning of *rhd_2599*	RhdTusA_for	GGGTAGGAC**CATATG**GTGGTCAAC	This study
	Rhd_rev	GTA**CTCGAG**TCAGCCCTCGGG	This study
Cloning of *tusA* into pET-15b	TusA_NdeI	GAGGCT**CATATG**GCTGATTTCGAT	This study
	TusA_BamHI	GCCGTGC**GGATCCA**TCAGGACTT	This study
Cloning of *EctusA* into pET-15b	EcTusA_for	TGAAG**CATATG**CCCGATCTC	This study
	EcTusA_rev	CATCAG**GGATCC**TCAACCGCC	This study
Cloning of *dsrE2* into pASK-IBA5+	DE2_Strep_for	ATGGTA**GGTCTC**AGCGCCATGGAACAAAAGAAACTGGCGATC	This study
	DE2_Strep_rev	ATGGTA**GGTCTC**ATATCAGATGTAGAGACAGATGTCGCTCT	This study
Cloning of *rtd* into pET-15b	RhdTusA_for	GGGTAGGAC**CATATG**GTGGTCAAC	This study
	DE2_rev	ACGTGCGGT**CTCGAG**ACTTCAGATGTA	This study
Cys-Ser exchange	DE_C110S_for	CGAGCTCT**CA**CAGGAAGCC	This study
	DE_C110S_rev	GGCTTCCTG**TG**AGAGCTCG	This study
	DE_C156S_for	AGCGACATCT**CA**CTCTACATC	This study
	DE_C156S_rev	GATGTAGAG**TG**AGATGTCGCT	This study
	DE_C120S_rfor	TGATCGCCT**CA**CAGATGACCG	This study
	DE_C120S_rev	CGGTCATCTG**TG**AGGCGATCA	This study
	IBA5_for	TGAGCTATGAGAAAGCGCC	This study
	IBA5_rev	GGCGACACGGAAATGTTGA	This study
	TusA_C15S_for	CCTGAACT**C**CCCGCTGC	This study
	TusA_C15S_rev	GCAGCGGG**G**AGTTCAGG	This study
	Rhd_C64S_for	GTGGTCATC**T**ACTCCCGCAGC	This study
	Rhd_C64S_rev	GCTGCGGG**A**GTAGATGACCAC	This study
	Rhd_C74S_for	CAGGCTTC**C**CGCCTATCTGATGC	This study
	Rhd_C74S_rev	GCATCAGATAGGC**G**GAAGCCTG	This study
	T7Prom	TAATACGACTCACTATAGGG	This study
	T7Term	GCTAGTTATTGCTCGCGG	This study
Deletion of *tusA*	ΔTusA_for1	**GATCAAGAACTCGAC**TTCCACTTCCTGATC	This study
	ΔTusA_rev1	**GATCAGGAAGTGGAA**GTCGAGTTCTTGATC	This study
	ΔTusA_for2	CAGCGC**TCTAGA**CAGCTCAGGCGACAC	This study
	ΔTusA_rev2	TTTGGC**TCTAGA**ACGGTCATCTGACAG	This study
Deletion of *rtd*	ΔRTD_F1	CTTTGACAC**GGATCC**GATAACG	This study
	ΔRTD_R1	GAGACTTCAGATGTAGA**CGTTGACCACACTATCG**	This study
	ΔRTD_F2	CGATAGTGTGGTCAACG**TCTACATCTGAAGTCTC**	This study
	ΔRTD_R2	AAGTCGAC**GGATCC**GCGATG	This study
Interposon mutagenesis of *rtd*	RTD_Kan_F1	CAGCGC**AAGCTT**CCGCGTG	This study
	RTD_Kan_R1	CTTCAGATGTAG**GAATTC**ACCACACTATC	This study
	RTD_Kan_F2	GATAGTGTGGT**GAATTC**CTACATCTGAAG	This study
	RTD_Kan_R2	GACGCA**AAGCTT**GCACATTGG	This study
qRT-PCR	RT-UROD-for	GTACCGCGCATCGAGGATT	(19)
	RT-UROD-rev	GCATTACCGGCAGCGAGAA	(19)
	RT-Rhd-for	GTGTTGCTGGTGGACATCC	This study
	RT-Rhd-rev	GGCAGTAGATGACCACGTCG	This study
	RT-TusA-for	CGATCAAGAACTCGACGCAAGC	(19)
	RT-TusA-rev	GTTGCCGGTCTGCTTGGC	(19)
	RT-DsrE2-for	CGGTATGCAGGGCATGATGAC	(19)
	RT-DsrE2-rev	TTCGGCATATCGAAGAGGTCG	(19)

100 ml of Pfennig's medium in completely filled screw-capped bottles. Sulfide (4 mM), thiosulfate (5 mM), or sulfur (50 mM) was then added as sulfur substrates. The cultures were continuously illuminated and kept at 30 °C throughout the experiment. Elemental sulfur was determined via cyanolysis (22), and sulfate was measured by the method of Sörbo (23).

Cloning, Site-directed Mutagenesis, Overproduction, and Purification of Recombinant Proteins—For the amplification of the Alvin_2599 (*rhd_2599*), Alvin_2600 (*tusA*), and Alvin_2601 (*dsrE2*) genes, genomic DNA of *A. vinosum* served as the template. The oligonucleotides used for amplification and introduction of restriction sites for molecular cloning are listed in Table 2. After digestion with the respective restriction enzymes, the PCR products for *rhd_2599* and *tusA* were ligated into the corresponding sites of pET-15b (Novagen) resulting in amino-terminally His-tagged proteins. The *dsrE2* amplicon was ligated into pASK-IBA5plus (IBA, Göttingen, Germany) and thereby fused to an amino-terminal Strep tag. 500 ml of LB medium were inoculated with 5% (v/v) *E. coli* precultures hosting the respective plasmid and cultivated at 37 °C and 180 rpm until an A_{600} of 0.6–0.8 was reached. After induction with 0.1

mM isopropyl 1-thio-β-D-galactopyranoside (for pET-15b-derived plasmids) or 50 μg ml^{-1} anhydrotetracycline (for the pASK-IBA5plus derived plasmid), the cells were cultivated for 2 more hours under the same conditions and harvested (14,000 × *g*; 20 min; 4 °C). Pellets were resuspended in a lysis buffer specific for the respective overproduction system, disrupted via sonication, and purified according to the manufacturer's instructions. DsrE2 and its mutant variants were isolated from the membrane fraction of *E. coli*. The membranes were separated from the soluble fraction after sonication via ultracentrifugation (145,000 rpm; 2 h; 4 °C). Solubilization was carried out with 1% Triton X-100 overnight on ice under gentle stirring. After another ultracentrifugation step the proteins were isolated from the supernatant. Buffers for purification and the storage buffer contained 0.1% (v/v) Triton X-100. Point mutations were introduced into *rhd_2599*, *tusA*, and *dsrE2* via gene splicing by overlap extension (21) using standard PCR with *Pfu* polymerase (Thermo Fisher Scientific, Schwerte, Germany). Plasmids pET15bTusA, pET15Rhd, and pIBADsrE2 (Table 1), respectively, served as templates for PCR reactions using the primers listed in Table 2.

Preparation of Proteins for Electrophoretic Separation—For the electrophoretic separation of proteins by their molecular mass, the protocol by Laemmli was used. For native gel electrophoresis, 100 pmol of DsrEFH and 800 pmol of TusA from *A. vinosum* or *E. coli* were incubated as described previously (4). For the separation of native proteins, the Laemmli method was modified by preparing buffers without SDS. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol, and 10% (v/v) acetic acid. For destaining, 20% (v/v) methanol, 10% (v/v) acetic acid was used.

Thiosulfate-Cyanide Sulfurtransferase, Glutathionepersulfide: Cyanide Sulfurtransferase Activity—Thiosulfate:cyanide sulfurtransferase (rhodanese) activity was measured according to Ray *et al.* (24). The assay contained 100 mM glycine, pH 8.9, 50 mM sodium thiosulfate, 50 mM NaCN, and enzyme in a final volume of 500 μl. The reaction was started by adding NaCN, and the mixture was incubated for 1 min at 30 °C. 250 μl of 15% formaldehyde was used to stop the reaction before 750 μl of ferric nitrate reagent (25 g of Fe(NO$_3$)$_3 \times$ 9 H$_2$O and 50 ml of 65% HNO$_3$ per 375 ml) was added. Absorption was measured at 460 nm. Enzyme units are defined as the amount that catalyzed the production of 1 μmol of thiocyanate per min. As an alternative substrate, GSSH was tested in a concentration of 50 mM. GSSH was synthesized by incubating 500 mM oxidized glutathione with 500 mM sulfide for 30 min at 30 °C (25). Completion of the reaction was verified by the absence of sulfide in the product solution following the protocol for sulfide quantification published by Rethmeier *et al.* (26).

Sulfur Binding and Transfer Experiments with MALDI-TOF or N-(iodoacetyl)-N′-(5-sulfo-1-naphtyl)-ethylenediamine (1,5-IAEDANS)—Sulfur binding and transfer experiments with MALDI-TOF were performed exactly as described before (4). Under the applied experimental conditions, masses were detected for all tested proteins that agreed with the calculated masses within a range of a maximum of 3 Da. The basic principle of the identification of protein-bound persulfides by use of an 1,5-IAEDANS is documented in Zheng *et al.* (27). We applied this method based on the protocol of Thomé *et al.* (28). Proteins were always treated first with 2 mM dithiothreitol (DTT) for 30 min at room temperature to reduce thiol groups and to remove any preexisting persulfides. Excess DTT was removed by gel filtration on PD Mini-Trap columns (GE Healthcare) using 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. When DsrE2 was analyzed, the buffer in addition contained 0.1% Triton X-100. For testing the principle reactivity of the reduced proteins with 1,5-IAEDANS, the proteins were concentrated using Vivaspin 500 centrifuge concentrators (5-kDa molecular weight cutoff, Sigma) to about 800 μg ml^{-1} at this stage. A volume containing 200 pmol of protein was then brought to a volume of 20 μl, and 1,5 IAEDANS was added. Conditions and further treatment were as described below for the sulfide-treated proteins. For persulfuration of proteins before treatment with 1,5-IAEDANS, the protein solutions eluted from PD Mini-Trap columns were immediately incubated with 4 mM NaHS or 4 mM sodium thiosulfate for 1 h at 30 °C followed by dialysis against the aforementioned Tris-HCl buffers to remove any excess of the reduced sulfur compound. The protein solutions were then concentrated to 300–700 μg ml^{-1}. 100 pmol (in the case of DsrEFH) or 200 pmol (all other proteins) were incubated with 0.5 nmol (1 μl

of a 0.5 mM stock solution) of 1,5-IAEDANS in a final volume of 21 μl for 1 h in the dark at 4 °C. Unbound 1,5-IAEDANS was allowed to react with 100 nmol L-cysteine (1 μl of a 100 mM stock solution were added) for 30 min at room temperature to prevent reaction of 1,5-IAEDANS in subsequent reaction steps. The reaction mixtures were now treated with DTT (2 μl of a 200 mM stock solution, *i.e.* 200 nmol) for 30 min at room temperature to reductively release bound persulfides as 1,5-AEDANS-sulfide conjugates. The samples were then mixed with 1 μl of native loading buffer (2 M sucrose, 1% (w/v) bromphenol blue), and the complete reaction mixture was applied to a 15% Laemmli gel that was run in the dark. Note that the pH of the resolving gel was adjusted to pH 9.5 to improve separation. The gels were then analyzed under UV light for visualization of 1,5-AEDANS-labeled proteins. The same gels were later stained with Coomassie Brilliant Blue to allow comparison of protein amounts in each lane. When sulfur transfer from one protein to another was studied, 100 pmol (in case of DsrEFH) or 200 pmol (all other proteins) of persulfurated donor protein was combined with the same amount of acceptor protein in a final volume of 20 μl of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl for 1 h at 30 °C followed by the addition of 1,5 IAEDANS and further treatment as described above.

RESULTS

In A. vinosum Expression of rhd_2599, tusA, and dsrE2 Is Triggered under Sulfur Oxidizing Conditions—Recently, we assessed the genome wide transcriptional response of *A. vinosum* upon a shift from photoorganoheterotrophic to photolithoautotrophic growth on different inorganic sulfur compounds (19). In this work the cells were harvested at the time of maximum oxidation rate for the respective sulfur compound. In this experimental setting, relative mRNA levels for *tusA* and *dsrE2* increased on sulfide and thiosulfate compared with malate, whereas the relative transcription of the rhodanese gene appeared unchanged. These results were verified via relative qRT-PCR of *tusA* and *dsrE2* but not for *rhd_2599* (19).

For assessment of a potential co-transcription of the three genes, we synthesized cDNA using the DE2_rev oligonucleotide from RNA that was isolated from sulfide-grown cells. Subsequent PCR yielded amplicons for the individual genes as well as for the *rhd_2599-tusA* and the *tusA-dsrE2* combinations (Fig. 1, *A* and *B*), indicating that the three genes form a transcriptional unit. The bands obtained for *rhd_2599* and the *rhd_2599-tusA* combinations (Fig. 1*B*, *lanes 1* and *4*) appear faint compared with those for *tusA*, *dsrE2*, and the *tusA-dsrE2* combination (Fig. 1*B*, *lanes 2*, *3*, and *5*). This could be explained by two promoter sites of different strengths such that there is transcriptional linkage of *rhd_2599* and its downstream genes but transcription of *tusA/dsrE2* also occurs independently. Indeed, the promoter recognition program BPROM (29) predicts two promoter sites for the *rhd_2599-tusA-dsrE2* region; the first is located upstream of *rhd_2599*, and the second in the intergenic space between *rhd_2599* and *tusA* (Fig. 1*A*).

Our initial whole genome transcriptional profiling study on *A. vinosum* covered only a single time point during the complex process of sulfur species conversion (19). We, therefore, reevaluated mRNA levels for *tusA*, *dsrE2*, and *rhd_2599* by relative qRT-PCR at ample time points that were set such that they
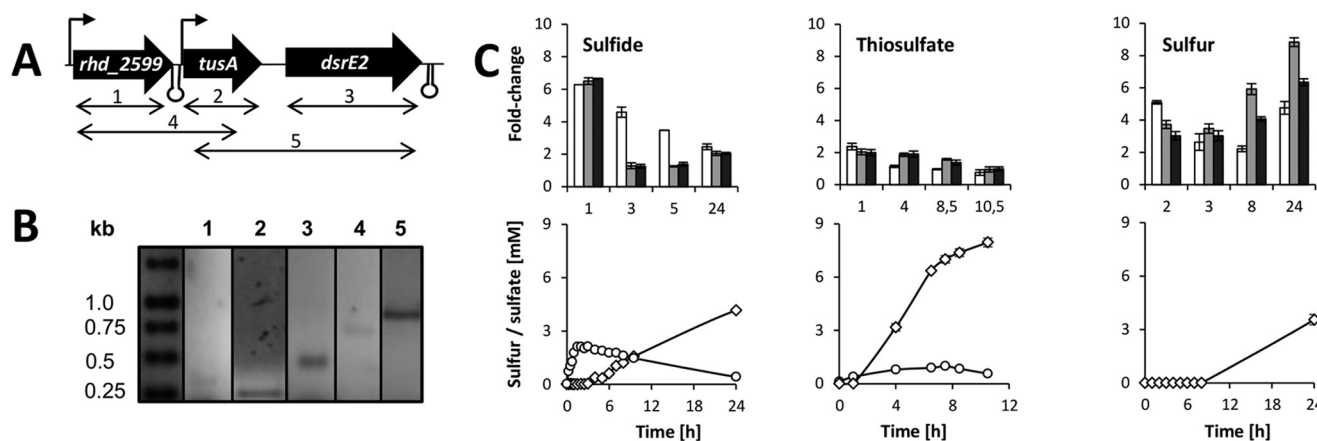
FIGURE 1. **Transcription of *rhd_2599*, *tusA*, and *dsrE2*.** *A*, genomic organization of the *rhd_2599-tusA-dsrE2* gene cluster. Promoter and terminator sites predicted by BPROM (Softberry) (29) and TransTermHP (45), respectively, are indicated. *Arrows below the genes* indicate sequences amplified in PCR reactions 1–5 intended to evaluate (co)-transcription of the genes. *B*, agarose gel of the PCR products with the following expected sizes: *1*, 330 bp; *2*, 235 bp; *3*, 495 bp; *4*, 700 bp; *5*, 860 bp. *C*, relative mRNA levels in *A. vinosum* during growth on sulfide, thiosulfate, and elemental sulfur compared with growth on malate. Results for *rhd_2599* are shown as *white bars*, those for *tusA* as *gray bars*, and *dsrE2* as *black bars*. mRNA levels for Alvin_0486 served as an endogenous reference (19). Concentrations of internal sulfur (○) and sulfate (◇) in the *A. vinosum* cultures are presented in the *lower panels*. Points represent means of three replicates. *Error bars* are too small to be visible in almost all cases.

covered the phases of maximum oxidation rates for the respective sulfur compound, the point of maximum sulfur globule accumulation, and the period of sulfur globule conversion to sulfate (Fig. 1*C*). In the phases of maximum oxidation rates, our relative qRT-PCR results for *tusA* and *dsrE2* exactly matched those of the previous study. qRT-PCR is more reliable and sensitive than genome-wide microarray analysis, and accordingly, we now also detected a significant positive transcriptional response for *rhd_2599* in the presence of sulfide, thiosulfate, and elemental sulfur (Fig. 1*C*). The overall expression patterns for *tusA* and *dsrE2* were very similar and differed by only a few percentage points with sulfide and thiosulfate as substrates. The effect exerted by thiosulfate was generally less prominent for all three genes than that observed on sulfide and elemental sulfur. Cells growing on thiosulfate contain lower concentrations of sulfur stored in intracellular sulfur globules (Fig. 1*C*, *lower panels*) opening the possibility that the increased expression of *rhd_2599*, *tusA*, and *dsrE2* is correlated with high concentrations of stored sulfur.

Transcriptome Evidence Toward the Physiological Function of Rhd, TusA, and DsrE2—In *E. coli*, TusA affects not only the biosynthesis of 2-thiourdine but also the formation of FeS-clusters and the molybdenum cofactor (30). Besides proteins of central carbon metabolism, several important enzymes of the sulfur oxidation pathway in *A. vinosum* contain FeS-clusters, including DsrAB, DsrL, and the membrane complex DsrMKJOP (1, 2, 31, 32). Another example is the iron-sulfur molybdoenzyme SoeABC, the major player in the oxidation of the DsrAB product sulfite (11). It can, therefore, not *a priori* been excluded that the close genomic association of the *rhd-tusA-dsrE2* genes with sulfur oxidation genes is related with a function of the former in biosynthesis of co-factors of the latter. If the function of *A. vinosum* TusA and also possibly Rhd and DsrE2 is indeed limited to biosynthesis, this should be reflected in similar transcription patterns of *rhd-tusA-dsrE2* and the genes encoding proteins involved in biosynthetic machineries. Therefore, we compared the expression profiles of *rhd-tusA-dsrE2* with

those of the genes involved in biosynthesis of sulfur-containing co-factors. Data were taken from Weissgerber *et al.* (19) and were complemented by the qRT-PCR results described above (Fig. 2).

A. vinosum encodes the complete Isc and the Suf systems for the biosynthesis of FeS clusters. Genes crucial for the biosynthesis of the molybdenum cofactor and thiamine were also found (Fig. 2). Transcription of *rhd-tusA-dsrE2* follows the same general pattern as that of the established sulfur-binding proteins of the Dsr pathway, DsrEFH, and DsrC as well as that of the dissimilatory sulfite reductase DsrAB itself. A very similar rise in relative mRNA levels in response to the presence of reduced sulfur compounds was seen for the genes encoding most other components of the Dsr system, genes for sulfur globule proteins, and for the cytoplasmic sulfite oxidation pathways (19). In contrast, similar patterns were not observed for any of the biosynthetic pathways (Fig. 2). Among the *isc* genes, only two (Alvin_0110 and Alvin_1420, encoding the iron-carrier IscA and the regulator IscR, respectively), responded positively under just single-growth conditions, *i.e.* in the presence of elemental sulfur. The same substrate triggered the expression of *sufEBCDA*, whereas the transcription of the cysteine desulfurase gene *sufS* remained unaltered under all tested conditions. Neither *isc* nor *suf* gene expression changed in the presence of sulfide and thiosulfate, the substrates that clearly exerted the strongest effect on *rhd-tusA-dsrE2* and the established sulfur oxidation genes (Fig. 2 and Weissgerber *et al.* (19)). The levels for mRNAs of genes for molybdenum cofactor biosynthesis were either not affected or negatively affected by reduced sulfur compounds.

It is currently unclear which proteins might be involved in the synthesis of 2-thiourdine in *A. vinosum*. In *E. coli*, a multiprotein sulfur relay system involving TusA, TusBCD, TusE, and finally MnmA is responsible for this process. *A. vinosum* harbors a *mnmA* homolog the relative mRNA levels of which do not respond to reduced sulfur compounds. Extra copies of *tusBCD* apart from *dsrEFH* are not present. The *tusA* gene is
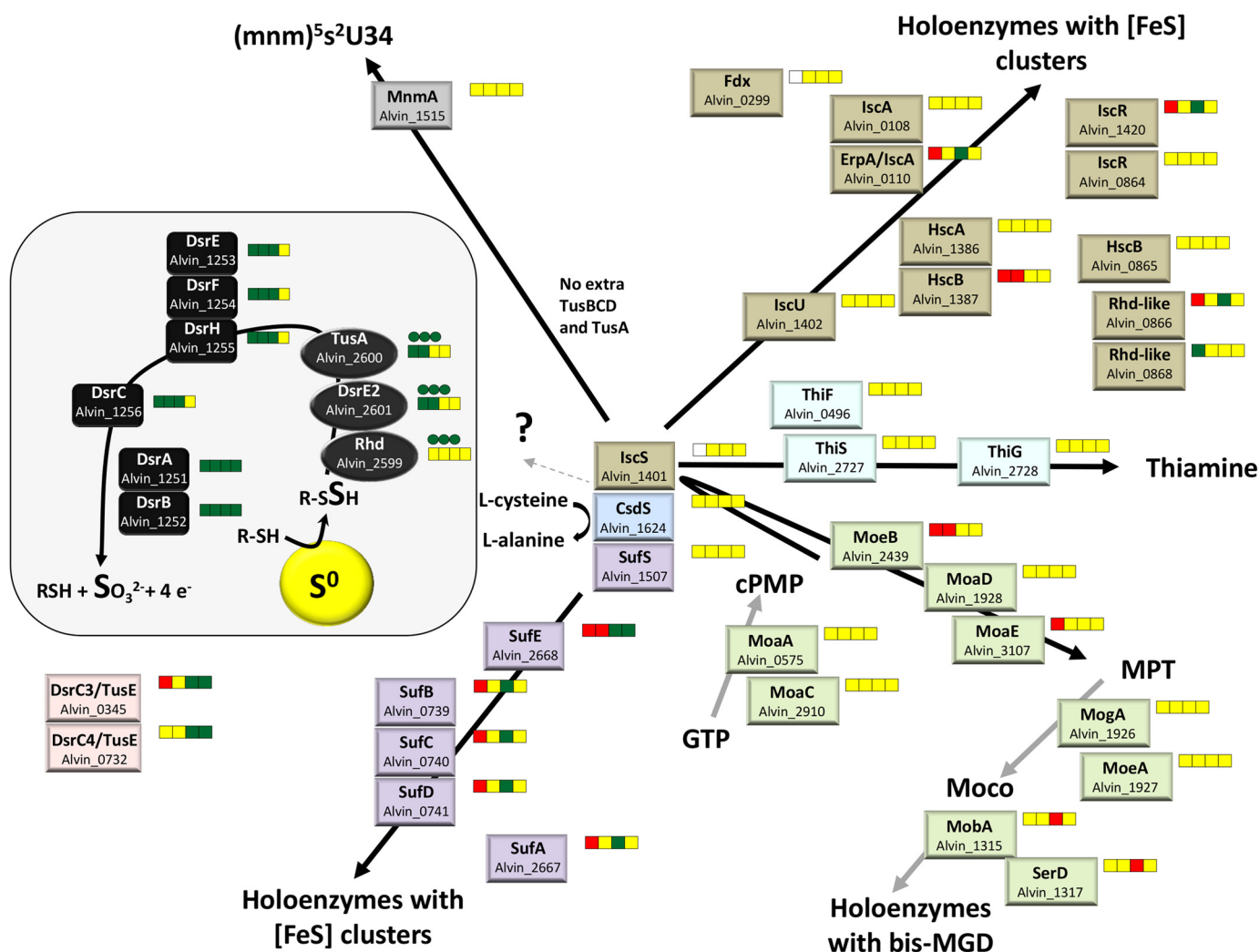
FIGURE 2. Analysis of pathways for biosynthesis of FeS clusters, the molybdenum cofactor (*Moco*), thiamine, and 2-thiouridine in *A. vinosum* as predicted from the genome sequence and the transcription patterns of the respective genes. The transcriptomic profiles are depicted next to the respective protein and are color-coded; relative -fold changes in mRNA levels >2 (*green*) were considered significantly enhanced. Relative changes <0.5 (*red*) were considered as indicating significant decreases in mRNA levels. Relative -fold changes between 0.5 and 2 (*yellow*) indicated unchanged mRNA levels. For *rhd_2599*, *tusA*, and *dsrE2*, the results obtained via qRT-PCR in this study (values taken from the experiments presented in Fig. 1) are depicted in cycles. Administered sulfur compounds from left *to right*: sulfide, thiosulfate, elemental sulfur, and sulfite. In the figure DsrC/TusE proteins without any cysteine residues are omitted. *cPMP*, cyclic pyranopterin monophosphate; *MPT*, molybdopterin; *bis-MGD*, bis-molybdopterin-guanine-dinucleotide cofactor.

also present in only a single copy. The *tusE* homolog *dsrC* is present with five genomic copies. All TusE/DsrC polypeptides functional in sulfur transfer contain a strictly conserved Cys residue (termed Cys_A) at the penultimate position in the carboxyl terminus (9) that serves as the site of sulfur binding (6). At a distance of 10 residues, DsrC proteins from sulfur oxidizers have an additional conserved cysteine (Cys_B). One *A. vinosum* DsrC homolog (Alvin_0345) has both conserved cysteine residues. This protein has recently been proposed to functionally operate as TusE (9). Neither Cys_A nor Cys_B is present in Alvin_0028 and Alvin_1508. These proteins are predicted to serve a regulatory function and have been termed regulatory sulfur-related proteins, RspA (9). Alvin_0732 contains only Cys_B that has no equivalent in *E. coli* TusE. Alvin_0732 also falls into the RspA group (6, 9). Increased mRNA levels were found on elemental sulfur and sulfite for Alvin_0732 and Alvin_0345.

Construction of rhd_2599-, tusA-, and dsrE2-deficient A. vinosum Mutants—To further assess the importance of the Rhd_2599, TusA, and DsrE2 proteins in *A. vinosum*, we set out to

generate the mutant strains *A. vinosum* Δ*tusA* and *A. vinosum* Δ*rtd*, carrying in-frame deletions of the *tusA* and the complete *rhd-tusA-dsrE2* cluster, respectively. We have applied similar methodology successfully for construction of a whole range of *A. vinosum* deletion strains (33, 34). Amplified sequences carrying the deletion without disrupting the reading frame were cloned into the mobilizable suicide vector pk18*mobsacB* (35). Single crossover mutants, which were identified via the plasmid-encoded kanamycin resistance, were grown for several generations in non-selective RCV medium before they were plated on solid RCV medium containing 10% sucrose. Sucrose induces the gene expression of *sacB* encoding levansucrase leading to the death of cells still harboring the plasmid. Although single crossover mutants were verified, the next step yielded only revertants to the wild type genotype. Usually we obtain ~50% deletion strains as would be theoretically expected. Thus this observation was a first hint toward a vital function of at least TusA for fitness of *A. vinosum*. We, therefore, changed our approach such that we now introduced a

FIGURE 3. **Different colony types of *A. vinosum* after transfer of plasmid pSUP301Δ*rtd*::ΩKm.**

positive selection marker for the gene deletion; an Ω-kanamycin cassette was introduced into the remains of the *rhd_2599-tusA-dsrE2* cluster to create *A. vinosum* Δ*rtd*::ΩKm. The plates for selection contained kanamycin. Our selection plates routinely contain 0.5% yeast extract, 22 mM malate, 0.8 mM Na$_2$S$_2$O$_3$, 2 mM sodium acetate, and 1.4 mM NaHS for growth. After 2 weeks of incubation under anoxic conditions in the light, we observed two colony types that differed with regard to diameter and color (Fig. 3). The first kind resembled typical *A. vinosum* colonies in radius, dark red color, and a shiny surface. The second kind was significantly smaller and had a non-shiny surface, and the color was a milky pink. Light microscopy showed that the milky appearance of the smaller colonies arose from massive accumulation of intercellular sulfur globules. Apparently, these cells were impaired in their ability to degrade the sulfur formed during the oxidation of sulfide and thiosulfate. A difference in the appearance of individual cells as reported for the *E. coli* TusA-deficient mutant (36) was not observed. Single colonies of both types were transferred to kanamycin/ampicillin and kanamycin-only plates to discriminate between single crossover recombinants that still contained the ampicillin resistance conferring shuttle plasmid and double crossover recombinants that had lost the vector-encoded ampicillin resistance. All of the regular colonies turned out to contain only single crossover recombinants, whereas the slowly growing cells of the smaller colonies were solely kanamycin-resistant. A considerable number of attempts to re-streak these colonies and transfer them into liquid media with and without reduced sulfur compounds remained unsuccessful. Inevitably, the cultures died after a few generations. It should be noted that we never obtained colonies of double-crossover recombinants on selection plates without sulfide and thiosulfate, indicating a requirement of *A. vinosum* for reduced sulfur in the absence of the *rhd_2599-tusA-dsrE2* cluster. Yet it was possible to create *A. vinosum rhd_2599*::ΩSm, a mutant strain with a disruption in the reading frame of *rhd_2599*. Neither during photoorganoheterotroph nor during photolithoautotroph growth on sulfide, thiosulfate, or elemental sulfur did this strain behave differently from the wild type under the conditions applied. We conclude

that Rhd_2599 can be either functionally replaced by another cytoplasmic rhodanese or the protein has a minor metabolic function. Although *tusA* and *dsrE2* are present only once, the *A. vinosum* genome encodes three more cytoplasmic rhodaneses, Alvin_0866, Alvin_0868, and Alvin_1587. Two of these genes, Alvin_0866 and Alvin_1587, exhibit elevated relative mRNA levels under sulfur-oxidizing conditions and would thus be prime candidates for replacing Rhd_2599 (19). The different phenotypes for the *rhd_2599*-deficient mutant on the one hand and the *rhd_2599-tusA-dsrE2*-lacking mutant on the other hand indicated different promoter sites for *rhd_2599* and *tusA-dsrE2* and, therefore, supported the predictions by BPROM described above.

Properties and Catalytic Activities of Rhd_2599, TusA, and DsrE2—With only one rhodanese domain and a molecular mass of 11.9 kDa, Rhd_2599 belongs in the group of single domain rhodaneses (pfam00581) together with GlpE and PspE from *E. coli*. Unlike the *E. coli* enzymes, Rhd_2599 has a second cysteine residue, Cys-74, in close proximity to the active site cysteine Cys-64. Both cysteines are strictly conserved among homologous rhodanese-like proteins of the *Chromatiaceae* and the *Ectothiorhodospiraceae*. The amino acid sequence does not contain predicted transmembrane helices. Rhd_2599 fused to an amino-terminal His tag was produced in and purified from *E. coli* BL21 (DE3). The protein eluted as a monomer from an analytical gel filtration column.

TusA (cd00291) from *A. vinosum* is a small 8.3-kDa protein. The characteristic active site motif for TusA-like proteins is Cys-19-Pro-*X*-Pro. In *E. coli* the motif contains a glutamate residue at the *X* position, and the residue contributes to the stabilization of the protein (37). In TusA from sulfur-oxidizing bacteria, glutamate is mostly replaced by a hydrophobic residue. For purification from *E. coli*, recombinant *A. vinosum* TusA was fused to a His tag at the amino terminus and isolated from the soluble fraction. From an analytical gel filtration column the protein eluted mostly as a monomer, and only a small portion eluted as a dimer. Note that *A. vinosum* TusA is not detectable at 280 nm due to the complete lack of tryptophan and tyrosine residues.

Alvin_2601 encodes a 159-amino acids, 17.5-kDa protein that is annotated as hypothetical protein of the DsrE/DsrF/DsrH superfamily (pfam13686). Two transmembrane helices are predicted that are arranged such that the carboxyl terminus is located in the cytoplasm. The *A. vinosum* protein harbors three cysteine residues located in the cytoplasmically oriented carboxyl terminus. Only Cys-120 is conserved in bacteria and archaea and corresponds to the active site cysteine of DsrE and TusD (6, 38). Recombinant DsrE2 was purified from the *E. coli* membrane fraction via affinity chromatography with an amino-terminal Strep tag. Cofactors were not identified for DsrE2 or for TusA and Rhd_2599.

All three recombinant proteins were subjected to thiosulfate: cyanide sulfurtransferase assays (24). Rhd_2599 proved to be active, mobilizing sulfur from thiosulfate and transferring it to cyanide (878 units mg^{-1}). Because thiosulfate is already metabolized in the periplasm by the Sox proteins in *A. vinosum* (39), it is an unlikely *in vivo* substrate for cytoplasmic Rhd_2599. To date, persulfidic glutathione amide (GASSH) and glutathione (GSSH) are discussed as the organic carrier molecules for sul-
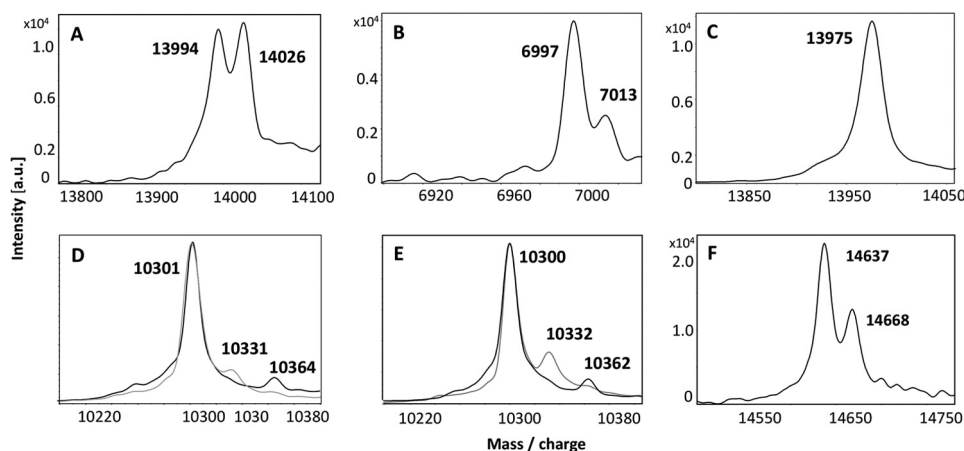
FIGURE 4. **MALDI-TOF spectra of persulfurated Rhd_2599, TusA, and DsrC proteins.** 30 μM protein solutions were incubated with a suitable sulfur substrate or sulfur-donating protein for 60 min at 30 °C. *A*, Rhd_2599 (mass 13,991 Da) after incubation with 2 mM thiosulfate. *B*, Rhd_2599 after incubation with 0.5 mM GSSH. *C*, the Rhd_2599-Cys-64Ser variant protein (mass 13,975 Da) is shown after incubation with 2 mM thiosulfate. *D*, TusA (mass 10,300 Da) after incubation with 2 mM sulfide. *E*, TusA after incubation with Rhd_2599 and thiosulfate. In *D* and *E*, two superimposed spectra obtained for two individually prepared samples are shown. *F*, persulfidic TusA was incubated with 30 μM DsrC (mass 14638 Da) as acceptor molecule. Binding of sulfur atoms is indicated by an additional mass of 32 Da. Note that in *B* the spectrum for the double-charged protein is shown. *a.u.*, absorbance units.

fane sulfur that is transported from the periplasmic sulfur globules to the cytoplasm (3, 4) and might, therefore, be substrates for rhodaneses *in vivo*. The unamidated GSSH was tested as the substrate for Rhd_2599, and indeed Rhd_2599 showed activity (25 units mg^{-1}). In contrast, TusA and DsrE2 were inactive in assays with thiosulfate and glutathione persulfide.

Rhd_2599, TusA, and DsrE2 Are Sulfur Carriers—In our previous work on DsrC and DsrEFH we proved the sulfur binding capabilities of and sulfur transfer between these proteins via mass changes observed through MALDI-TOF mass spectrometry. However, we found this method not to be applicable to proteins exceeding a molecular mass of ~18 kDa. Analysis of membrane proteins is also problematic due the detergents used during isolation and storage of the proteins. Therefore, we employed 1,5-IAEDANS as an additional or alternative method for visualization of persulfide-bound sulfur. 1,5-IAEDANS is a fluorescent reagent that can be coupled to proteins by displacement of its iodide group with the sulfur atom of a thiol group or sulfane sulfur of a persulfide. Excitation with UV light leads to the emission of light and allows the in-gel detection of proteins (27, 28). The method was validated by verification of sulfur binding of and sulfur transfer between DsrC and DsrEFH (not shown).

Above we showed that Rhd_2599 is able to mobilize sulfur from thiosulfate and GSSH, whereas TusA and DsrE2 cannot perform this reaction. We now verified the transfer of sulfane sulfur from thiosulfate and GSSH to the active site of Rhd_2599. This was done by mass spectrometry; exactly one sulfur atom (*i.e.* an additional mass of 32 Da for the single-charged and 16 Da for the double-charged protein molecules) was bound to the protein after incubation with each of the substrates (Fig. 4, *A* and *B*). When Rhd_2599 was incubated with sulfide, we found species carrying up to three sulfur atoms (not shown). We then incubated the Rhd_2599 mutant protein that carried a replacement of the probable active site cysteine Cys-64 to serine with thiosulfate. In this case a mass increase was not observed (Fig. 4*C*), clearly identifying Cys-64 as the sulfur binding residue and also showing that the second, partly conserved cysteine (Cys-

74) is not able to mobilize sulfur from thiosulfate. This result was further verified by our finding that the Rhd_2559-C74S variant protein still exhibited the 32-Da mass addition after incubation with thiosulfate (not shown). The 1,5-IAEDANS method was not applicable to Rhd_2599. Neither the wild type nor the mutant proteins reacted with the reagent in the native or in their persulfurated states, which leads us to conclude that, both Cys-64 and Cys-74 are buried inside the enzyme and not surface-exposed.

Persulfuration of TusA was verified by additional masses of 32 and 64 Da after incubation with sulfide (Fig. 4*D*). In accordance with the enzyme assays described above, GSSH (0.5 mM) was not a suitable sulfur donor for the protein (not shown). The treatment of the variant protein TusA-C15S with sulfide did not lead to a mass change (not shown). Taken together these results unambiguously demonstrate the binding of sulfane sulfur to Cys-15 of TusA.

MALDI-TOF analysis could not be applied to the membrane protein DsrE2 because the presence of Triton X-100 in the storage buffer prevented its detection. However, the 1,5-IAEDANS method clearly demonstrated persulfuration of this protein with sulfide (Fig. 5*A*); DsrE2 reacted with the fluorescent reagent, and the protein was detected under UV light. Persulfurated DsrE2 lost the fluorescence upon reduction with DTT. Proteins variants carrying C110S or C156S replacements still showed the same reaction (not shown). The C120S variant of DsrE2 could not be tested because overproduction of this mutant protein resulted in massive formation of inclusion bodies, indicating that this cysteine is important for correct folding of the protein. It is therefore not yet possible to say which of the three cysteines present in the protein is responsible for sulfur binding.

In Vitro Sulfur Transfer Reactions—Our previous experiments established Rhd_2599 as capable of mobilizing sulfane sulfur from persulfides of low molecular weight compounds like glutathione persulfide. We now set out to identify possible acceptor proteins for Rhd_2599 and first tested TusA. To this end Rhd_2599 was incubated with 2 mM thiosulfate and TusA
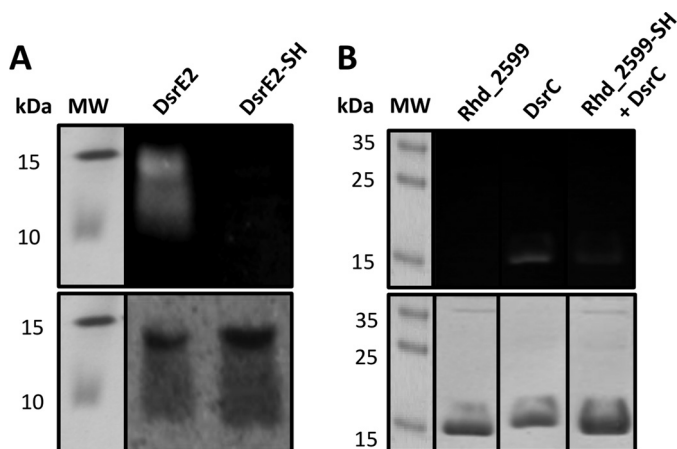
FIGURE 5. **Identification of protein persulfides using 1,5-IAEDANS.** *A,* persulfuration of DsrE2. The protein was reduced with DTT before incubation with sulfide, dialyzed, and treated with 1,5-IAEDANS and DTT as described under "Experimental Procedures." Aliquots containing 200 pmol of protein were separated via SDS-PAGE (15%). *B,* persulfuration of DsrC after incubation with persulfidic Rhd_2599. 200 pmol of DsrC were added to 200 pmol of Rhd_2599, which had before been incubated with thiosulfate. After treatment with fluorescent 1,5-IAEDANS, the protein solution was analyzed via SDS-PAGE (15%). The *upper images* show the gels under UV light, and the *lower images* show the gels after staining with Coomassie. The molecular mass (*MW*) of the marker proteins is given in kDa.

for 1 h. As a control TusA was incubated with thiosulfate under the same conditions as in the absence of Rhd_2599. TusA clearly remained unchanged in the control, whereas additional masses of 32 and 64 Da were observed when Rhd_2599 was present in the reaction mixtures (Fig. 4*E*). We then tested whether Rhd_2599 can also transfer sulfur to DsrE2 and possibly also directly to the sulfur-binding proteins of the Dsr system, DsrEFH and DsrC. The transfer to DsrE2 was assessed with 1,5-IAEDANS yielding a negative result. Rhd_2599 also proved unable to transfer sulfur to DsrEFH, which was verified with both mass spectrometry and the fluorescence method. A different result was obtained when DsrC served as the acceptor (Fig. 5*B*). When persulfurated Rhd_2559 was incubated with DsrC and 1,5-IAEDANS treatment was performed afterward, the fluorescence associated with DsrC was found to be noticeably decreased as compared with the control containing only DsrC. Please note that Rhd_2599 does not react with 1,5-IAEDANS (see above) and is, therefore, not visible under UV light (*upper panel* of Fig. 5*B*). DsrC and Rhd_2599 have similar molecular masses, 14.6 and 13.9 kDa, respectively, leading to similar migration patterns in the gel (Fig. 5*B*, *lower panel*).

We now evaluated the sulfur transfer capabilities of TusA. Although the reaction with DsrE2 was negative, we found Rhd_2599 to be persulfurated upon incubation with persulfidic TusA (not shown). The reaction between TusA and DsrEFH was also tested (Fig. 6). It should be pointed out that DsrEFH migrated mainly in one band around 38 kDa in non-reducing SDS-PAGE, *i.e.* the three subunits were not separated from each other. Only small portions ran in the monomeric state. After incubation with persulfidic TusA, the fluorescence of DsrEFH was significantly reduced compared to the control sample, whereas that of TusA became more prominent again, thereby illustrating the transfer of sulfane sulfur from TusA to DsrEFH *in vitro*. Furthermore, the sulfur transfer was reversible

(Fig. 6*B*); after TusA was incubated with persulfurated DsrEFH, the formation of a persulfide was detected in TusA. Although the fluorescence of TusA was reduced relative to the untreated sample, the fluorescence of DsrEFH was almost completely restored to the state before it had been incubated with sulfide. As a control, persulfurated DsrEFH was also incubated with the mutant TusA protein lacking Cys-15. In this case, the fluorescence of DsrEFH remained unchanged (not shown).

In a further experiment persulfidic TusA was mixed with DsrC resulting in one sulfur atom bound to DsrC (Fig. 4*F*). Sulfur was clearly transferred to Cys_A (Cys-111 in *A. vinosum* DsrC) because the reaction did not occur with a mutant protein that contains only Cys_B (Cys-100 in *A. vinosum* DsrC) (not shown). A transfer of sulfur atoms from DsrC to TusA was not detected. Although DsrE2 was incubated with persulfidic TusA, Rhd_2599, DsrEFH, and DsrC, we did not detect persulfide formation for DsrE2 in any case. At this point it is not possible to say whether DsrE2 does not accept sulfane sulfur from any of these donor proteins or the failure to document sulfur transfer is due to the use of Triton X-100. The detergent is also the reason why DsrE2 could not be tested as the sulfur donor for other proteins. Dialysis of DsrE2 after incubation with sulfide in a buffer containing 0.1% Triton X-100 failed to remove excess sulfide.

Interaction of DsrEFH and TusA—In *E. coli*, an interaction between TusBCD and TusA was not detected, although sulfur transfer from TusA to TusBCD was evident (7). To evaluate a possible interaction between *A. vinosum* TusA and DsrEFH, 100 pmol of DsrEFH was incubated with 800 pmol of TusA for 30 min at 30 °C. Subsequently, the protein mix was applied to a native PAGE. Indeed, the migrating behavior of DsrEFH and TusA changed when incubated together. Two extra bands appeared (Fig. 6*C*). The presence of DsrE-Cys-78 and TusA-Cys-15 proved to be crucial for this reaction as these additional bands were missing when mutant proteins were used. Interestingly, the use of TusA from *E. coli* did not result in altered migration patterns. *E. coli* TusA did not interact with DsrEFH. The native gel also revealed that TusA forms dimers via formation of intermolecular disulfide bonds. For the mutated protein, only the monomeric form was observed. Together, these data strongly resemble the results found for the interaction of DsrEFH with DsrC (4–6).

DISCUSSION

In this work we combined several independent lines of evidence that all strongly indicate a function of the *rhd_2599-tusA-dsrE2*-encoded proteins in oxidative dissimilatory sulfur metabolism in *A. vinosum* and probably also in other sulfur oxidizing phototrophs.

The frequent occurrence of the *rhd-tusA-dsrE2* genes in phototrophic sulfur-oxidizing bacteria as well as their conspicuous co-localization with genes encoding established (Dsr) or very probable (Hdr) major components of dissimilatory sulfur oxidation pathways point at the general and major importance of the encoded proteins for the oxidation of reduced sulfur compounds. Transcriptomic patterns for *rhd_2599*, *tusA*, and *dsrE2* match those of major genes of the sulfur-oxidizing system (here the *dsr* genes) rather than those of genes encoding
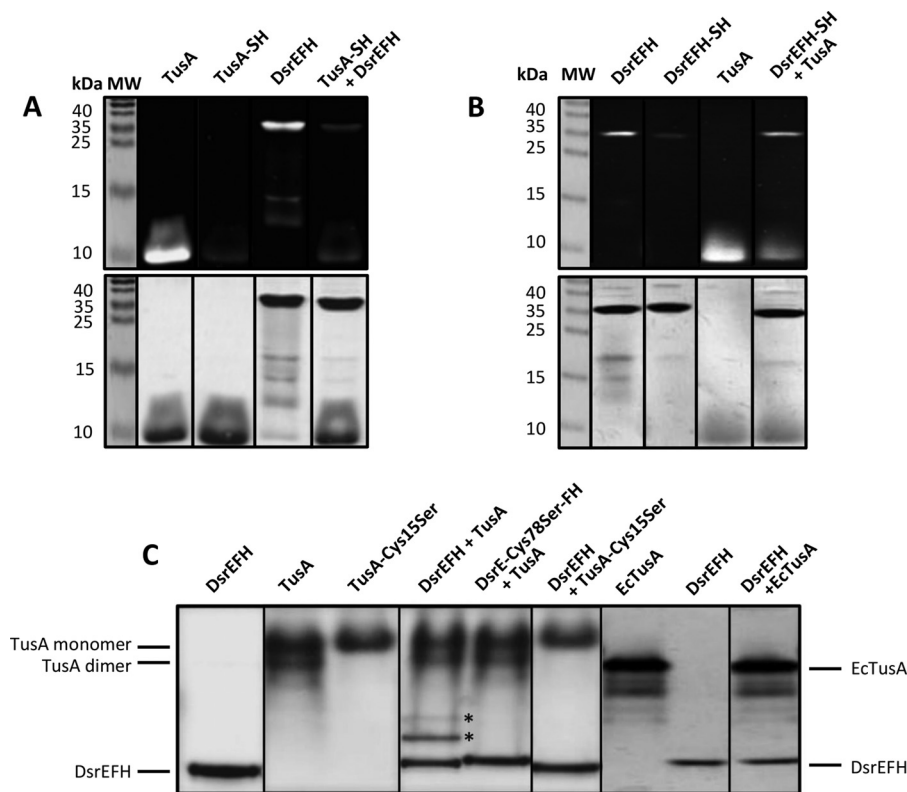
FIGURE 6. Sulfur transfer between and interaction of TusA and DsrEFH. In *panel A* the capacity of TusA for sulfur transfer to DsrEFH was analyzed by the 1,5-IAEDANS method. In *panel B* the results for the reverse reaction, *i.e.* sulfur transfer from persulfurated DsrEFH to TusA, are presented. In both cases (*A* and *B*), the respective persulfurated donor protein was incubated with the acceptor protein for 60 min at 30 °C before the reaction mixtures were treated with 1,5-IAEDANS and DTT as described under "Experimental Procedures." Samples were then electrophoresed on non-reducing 15% SDS-polyacrylamide gels. Gels were analyzed under UV-light (*upper gels* of *panel A* and *B*) and subsequently stained with Coomassie Brilliant Blue (*lower gels* of *panels A* and *B*). *Part C* shows the interaction of DsrEFH and TusA from *A. vinosum* (*TusA*) and *E. coli* (*EcTusA*), respectively. For the interaction experiments, 800 pmol of TusA/EcTusA were incubated with 100 pmol of DsrEFH (wild type and mutant proteins). Samples were then analyzed via native SDS-PAGE (7.5%). Bands arising from interaction complexes are marked with *stars*. Molecular mass (*MW*) of marker proteins is given in kDa.

components of machineries for biosynthesis of sulfur-containing biomolecules like iron-sulfur clusters, thionucleosides, thiamine, or molybdopterin (Fig. 2). Further hints toward the function of TusA come from proteomic data (40). TusA is one of the major proteins in *A. vinosum* cells grown on reduced sulfur compounds. Its abundance is comparable with that for proteins involved in central carbon and energy metabolism as well as in photosynthesis. TusA even outnumbers the most prominent proteins of the Dsr system, DsrC, and DsrAB (40). Taken together, these observations strongly support the idea that *A. vinosum* TusA is not restricted to biosynthesis of sulfur-containing co-factors.

However, TusA cannot be exclusively dedicated to sulfur oxidation, which is indicated by the strongly decreased vitality on solid media and the incapability to grow in liquid media of the *rhd_2599-tusA-dsrE2*-deficient *A. vinosum* mutant strain even in the presence of the organic substrate malate. Concerning a possible important function at least for TusA in 2-thiouridine biosynthesis, it should be emphasized that 5-methyl-2-thiouridine tRNA derivatives are universally present (41); however, the Tus proteins are not conserved in all domains of life (7). TusB, TusC, TusD, and TusE are not ubiquitous even in bacteria. *A. vinosum* harbors no extra copies for *tusA* or *tusBCD* apart from Alvin_2600 and *dsrEFH*, respectively. The *tusE* homolog *dsrC* is present with five genomic copies. This implies that

either the role of TusA and TusBCD is exerted by completely different sulfur transferases or 2-thiouridine is formed via IscS and MnmA alone as has originally been proposed for *E. coli* (42). A role of TusA in molybdopterin biosynthesis and the impact of the protein on the iron-sulfur cluster formation as described for *E. coli* (30) is also possible in *A. vinosum* and would result in a much stronger phenotype than that described for the *E. coli tusA* mutant (43). A balanced synthesis of iron-sulfur clusters would be expected to be of essential importance for a photolithotrophic organism. The biosynthesis of a high potential iron-sulfur protein, the primary electron donor to the reaction center in *A. vinosum*, may serve as an example.

As demonstrated via the bandshift assays with DsrEFH, *A. vinosum* TusA and *E. coli* TusA behave differently *in vitro*. The incubation of DsrEFH with AvTusA led to altered migration patterns in native PAGE gels, whereas EcTusA had no effect. Because we also showed the dependence of the interaction of the cysteine residues DsrE-Cys-78 and AvTusA-Cys-15, it is tempting to conclude that the entire Cys-Pro-*X*-Pro sequence and not only the conserved sulfur binding cysteine residue is essential for interaction with other proteins. With a few exceptions, TusA proteins encoded next to *rhd_2599* and *dsrE2* homologs harbor a hydrophobic non-aromatic amino acid at the *X* position in the motif. Within *Chromatiaceae*, the *E. coli* motif Cys-Pro-Glu-Pro is only found in *Rheinheimera*
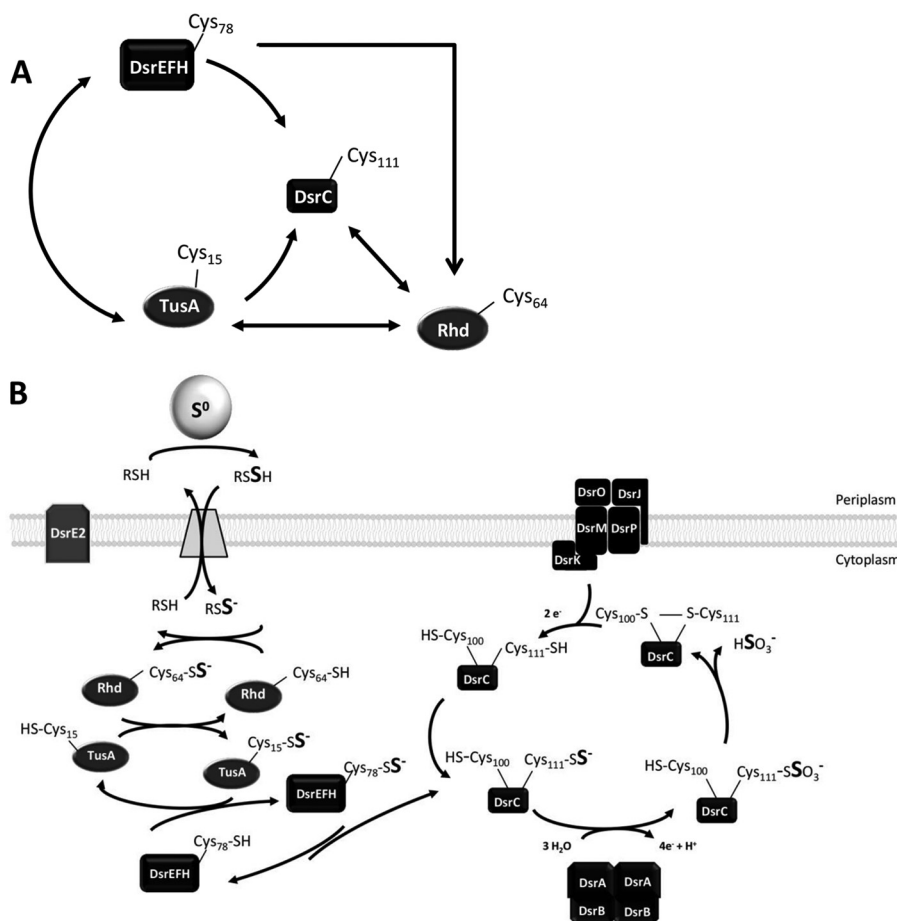
FIGURE 7. **Cytoplasmic sulfur trafficking during sulfur globule oxidation in *A. vinosum*.** *A*, summary of sulfur transfer reactions detected *in vitro*. The cysteines responsible for sulfur binding are depicted. *B*, model of sulfur transfer reactions occurring *in vivo*. Rhd_2599 and TusA are introduced as sulfur-donating proteins for the Dsr system. Thiol groups and persulfides are shown in the ionized or protonated state according to their supposed pK_a values of around 8.5 (46) and 6.2 (47), respectively. Because persulfides are 1–2 pK_a units more acidic than their thiol equivalents, we calculated the pK_a of the assumed carrier molecule glutathione amide persulfide on the basis of the pK_a for glutathione (48) to be ~7.2. DsrC-Cys-111 and Cys-100 correspond to Cys$_A$ and Cys$_B$, respectively (9). See "Discussion" for detailed explanation.

strains, which lack both sulfur oxidizing systems and DsrE2. The two TusA homologs of *E. coli*, YeeD and YedF, both harbor hydrophobic aromatic amino acids in the motif: Cys-Pro-Phe-Pro and Cys-Pro-Tyr-Pro, respectively. Neither YeeD nor YedF were able to complement a *tusA*-deficient *E. coli* mutant (30), which further points at the non-conserved amino acid in the motif as the decisive element for protein-protein interaction.

The successful overproduction and purification of Rhd_2599, TusA, and DsrE2 allowed characterization of the proteins as sulfur carriers and sulfur transferases. A schematic summary of the sulfur transfer reactions detected *in vitro* is given in Fig. 7*A*. Rhd_2599 was the only enzyme showing thiosulfate and GSSH: cyanide sulfurtransferase activity. Thus, cytoplasmic sulfur trafficking in the course of dissimilatory sulfur oxidation in *A. vinosum* most probably starts with mobilization of sulfane sulfur from the low molecular weight persulfide serving as a carrier for sulfur from the periplasmic sulfur globules to the cytoplasm by Rhd_2599. Persulfidic proteins also serve as substrates for this enzyme; Rhd_2599 accepted sulfane sulfur from all protein persulfides tested in this study. More importantly, the rhodanese served as an efficient sulfur donor for TusA and DsrC *in vitro*. However, a direct sulfur transfer from Rhd_2599 to DsrC *in vivo* is unlikely because the presence of the sul-

furtransferase DsrEFH is essential for the degradation of sulfur globules in *A. vinosum* (4). Thus, sulfur transfer from Rhd_2599 to TusA is strongly indicated as the relevant reaction *in vivo*. TusA was the only protein that was capable of transferring sulfur to DsrEFH *in vitro*. Taken together with the tight *in vitro* interaction of the TusA and DsrEFH proteins (Fig. 6), a direct sulfur transfer from TusA to DsrEFH is also the most likely scenario *in vivo*.

From DsrEFH, sulfur is further transferred to DsrC (4). The proposed sulfur relay system involving transfer of sulfur atoms from TusA via DsrEFH to DsrC (Fig. 7*B*) agrees with that derived for the related proteins from *E. coli*, TusA, TusBCD, and TusE (7). DsrC in its persulfidic state appears to be very stable as we did not detect any reverse transfer reaction from DsrC to TusA or DsrEFH. Persulfurated DsrC very probably serves as a direct substrate for DsrAB *in vivo* (4, 44), and it is, therefore, feasible that DsrC acts as a sulfur trap. This would ensure a constant flow of sulfur atoms necessary for a high turnover rate of DsrAB.

REFERENCES

1. Pott, A. S., and Dahl, C. (1998) Sirohaem-sulfite reductase and other proteins encoded in the *dsr* locus of *Chromatium vinosum* are involved in the

oxidation of intracellular sulfur. *Microbiology* **144,** 1881–1894

2. Dahl, C., Engels, S., Pott-Sperling, A. S., Schulte, A., Sander, J., Lübbe, Y., Deuster, O., and Brune, D. C. (2005) Novel genes of the *dsr* gene cluster and evidence for close interaction of Dsr proteins during sulfur oxidation in the phototrophic sulfur bacterium *Allochromatium vinosum*. *J. Bacteriol.* **187,** 1392–1404

3. Frigaard, N.-U., and Dahl, C. (2009) Sulfur metabolism in phototrophic sulfur bacteria. *Adv. Microb. Physiol.* **54,** 103–200

4. Stockdreher, Y., Venceslau, S. S., Josten, M., Sahl, H. G., Pereira, I. A., and Dahl, C. (2012) Cytoplasmic sulfurtransferases in the purple sulfur bacterium *Allochromatium vinosum*: evidence for sulfur transfer from DsrEFH to DsrC. *PLoS ONE* **7,** e40785

5. Cort, J. R., Selan, U., Schulte, A., Grimm, F., Kennedy, M. A., and Dahl, C. (2008) *Allochromatium vinosum* DsrC: solution-state NMR structure, redox properties, and interaction with DsrEFH, a protein essential for purple sulfur bacterial sulfur oxidation. *J. Mol. Biol.* **382,** 692–707

6. Dahl, C., Schulte, A., Stockdreher, Y., Hong, C., Grimm, F., Sander, J., Kim, R., Kim, S.-H., and Shin, D. H. (2008) Structural and molecular genetic insight into a wide-spread bacterial sulfur oxidation pathway. *J. Mol. Biol.* **384,** 1287–1300

7. Ikeuchi, Y., Shigi, N., Kato, J., Nishimura, A., and Suzuki, T. (2006) Mechanistic insights into sulfur relay by multiple sulfur mediators involved in thiouridine biosynthesis at tRNA wobble positions. *Mol. Cell* **21,** 97–108

8. Quatrini, R., Appia-Ayme, C., Denis, Y., Jedlicki, E., Holmes, D. S., and Bonnefoy, V. (2009) Extending the models for iron and sulfur oxidation in the extreme acidophile *Acidithiobacillus ferrooxidans*. *BMC Genomics* **10,** 394

9. Venceslau, S. S., Stockdreher, Y., Dahl, C., and Pereira, I. A. C. (2014) The "bacterial heterodisulfide" DsrC is a key protein in dissimilatory sulfur metabolism. *Biochim. Biophys. Acta* 10.1016/j.bbabio.2014.03.007

10. Frigaard, N.-U., and Bryant, D. A. (2008) Genomic insights into the sulfur metabolism of phototrophic green sulfur bacteria. In (Hell, R., Dahl, C., Knaff, D. B., and Leustek, T., eds) pp. 337–355, *Sulfur metabolism in phototrophic organisms*, Springer, Dordrecht, The Netherlands

11. Dahl, C., Franz, B., Hensen, D., Kesselheim, A., and Zigann, R. (2013) Sulfite oxidation in the purple sulfur bacterium *Allochromatium vinosum*: identification of SoeABC as a major player and relevance of SoxYZ in the process. *Microbiology* **159,** 2626–2638

12. Kelly, D. P. (2008) Stable sulfur isotope fractionation by the green bacterium *Chlorobaculum parvum* during photolithoautotrophic growth on sulfide. *Pol. J. Microbiol.* **57,** 275–279

13. Heising, S., Richter, L., Ludwig, W., and Schink, B. (1999) *Chlorobium ferrooxidans* sp. nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "*Geospirillum*" sp. strain. *Arch. Microbiol.* **172,** 116–124

14. Dahl, C. (2008) Inorganic sulfur compounds as electron donors in purple sulfur bacteria. In *Sulfur in Phototrophic Organisms* (Hell, R., Dahl, C., Knaff, D. B., and Leustek, T., eds) pp. 289–317, Springer, Dordrecht, The Netherlands

15. Pattaragulwanit, K., and Dahl, C. (1995) Development of a genetic system for a purple sulfur bacterium: conjugative plasmid transfer in *Chromatium vinosum*. *Arch. Microbiol.* **164,** 217–222

16. Weaver, P. F., Wall, J. D., and Gest, H. (1975) Characterization of *Rhodopseudomonas capsulata*. *Arch. Microbiol.* **105,** 207–216

17. Overmann, J., Fischer, U., and Pfennig, N. (1992) A new purple sulfur bacterium from saline littoral sediments, *Thiorhodovibrio winogradskyi* gen. nov. and sp. nov. *Arch. Microbiol.* **157,** 329–335

18. Pfennig, N., and Trüper, H. G. (1992) The family Chromatiaceae. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications* (Balows, A., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K.-H., eds) pp. 3200–3221, Springer-Verlag, New York

19. Weissgerber, T., Dobler, N., Polen, T., Latus, J., Stockdreher, Y., and Dahl, C. (2013) Genome-wide transcriptional profiling of the purple sulfur bacterium *Allochromatium vinosum* DSM 180^T during growth on different reduced sulfur compounds. *J. Bacteriol.* **195,** 4231–4245

20. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* **25,** 402–408

21. Horton, R. M. (1995) PCR mediated recombination and mutagenesis: SOEing together tailor-made genes. *Mol. Biotechnol.* **3,** 93–99

22. Kelly, D. P., Chambers, L. A., and Trudinger, P. A. (1969) Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. *Anal. Chem.* **41,** 898–901

23. Sörbo, B. (1987) Sulfate: turbidometric and nephelometric methods. *Methods Enzymol.* **143,** 3–6

24. Ray, W. K., Zeng, G., Potters, M. B., Mansuri, A. M., and Larson, T. J. (2000) Characterization of a 12-kilodalton rhodanese encoded by *glpE* of *Escherichia coli* and its interaction with thioredoxin. *J. Bacteriol.* **182,** 2277–2284

25. Rohwerder, T., and Sand, W. (2003) The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp. *Microbiology* **149,** 1699–1710

26. Rethmeier, J., Rabenstein, A., Langer, M., and Fischer, U. (1997) Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high- performance liquid chromatography methods. *J. Chromatogr. A* **760,** 295–302

27. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry* **33,** 4714–4720

28. Thomé, R., Gust, A., Toci, R., Mendel, R., Bittner, F., Magalon, A., and Walburger, A. (2012) A sulfurtransferase is essential for activity of formate dehydrogenases in *Escherichia coli*. *J. Biol. Chem.* **287,** 4671–4678

29. Solovyev, V., and Salamov, A. (2010) Automatic annotation of microbial genomes and metagenomic sequences. In *Metagenomics and Its Applications in Agriculture, Biomedicine, and Environmental Studies* (Li, R. W., ed.) pp. 71–78, Nova Science Publishers, Hauppage, NY

30. Dahl, J. U., Radon, C., Bühning, M., Nimtz, M., Leichert, L. I., Denis, Y., Jourlin-Castelli, C., Iobbi-Nivol, C., Méjean, V., and Leimkühler, S. (2013) The sulfur carrier protein TusA has a pleiotropic role in *Escherichia coli* that also affects molybdenum cofactor biosynthesis. *J. Biol. Chem.* **288,** 5426–5442

31. Lübbe, Y. J., Youn, H.-S., Timkovich, R., and Dahl, C. (2006) Siro(haem)amide in *Allochromatium vinosum* and relevance of DsrL and DsrN, a homolog of cobyrinic acid *a,c* diamide synthase for sulphur oxidation. *FEMS Microbiol. Lett.* **261,** 194–202

32. Grein, F., Pereira, I. A., and Dahl, C. (2010) The *Allochromatium vinsosum* DsrMKJOP transmembrane complex: biochemical characterization of individual components aids understanding of complex function *in vivo*. *J. Bacteriol.* **192,** 6369–6377

33. Grimm, F., Cort, J. R., and Dahl, C. (2010) DsrR, a novel IscA-like protein lacking iron- and FeS-binding function involved in the regulation of sulfur oxidation in *Allochromatium vinosum*. *J. Bacteriol.* **192,** 1652–1661

34. Sander, J., Engels-Schwarzlose, S., and Dahl, C. (2006) Importance of the DsrMKJOP complex for sulfur oxidation in *Allochromatium vinosum* and phylogenetic analysis of related complexes in other prokaryotes. *Arch. Microbiol.* **186,** 357–366

35. Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145,** 69–73

36. Yamashino, T., Isomura, M., Ueguchi, C., and Mizuno, T. (1998) The *yhhP* gene encoding a small ubiquitous protein is fundamental for normal cell growth of *Escherichia coli*. *J. Bacteriol.* **180,** 2257–2261

37. Katoh, E., Hatta, T., Shindo, H., Ishii, Y., Yamada, H., Mizuno, T., and Yamazaki, T. (2000) High precision NMR structure of YhhP, a novel *Escherichia coli* protein implicated in cell division. *J. Mol. Biol.* **304,** 219–229

38. Numata, T., Fukai, S., Ikeuchi, Y., Suzuki, T., and Nureki, O. (2006) Structural basis for sulfur relay to RNA mediated by heterohexameric TusBCD complex. *Structure* **14,** 357–366

39. Hensen, D., Sperling, D., Trüper, H. G., Brune, D. C., and Dahl, C. (2006) Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*. *Mol. Microbiol.* **62,** 794–810

40. Weissgerber, T., Sylvester, M., Kröninger, L., and Dahl, C. (2014) A comparative quantitative proteome study identifies new proteins relevant for

sulfur oxidation in the purple sulfur bacterium *Allochromatium vinosum. Appl. Environ. Microbiol.* **80,** 2279–2292

41. Suzuki, T. (2005) Biosynthesis and function of tRNA wobble modifications. In *Topics in current genetics* (Grosjean, H., ed.) pp. 24–69, Springer-Verlag, New York

42. Kambampati, R., and Lauhon, C. T. (2003) MnmA and IscS are required for *in vitro* 2-thiouridine biosynthesis in *Escherichia coli. Biochemistry* **42,** 1109–1117

43. Ishii, Y., Yamada, H., Yamashino, T., Ohashi, K., Katoh, E., Shindo, H., Yamazaki, T., and Mizuno, T. (2000) Deletion of the *yhhP* gene results in filamentous cell morphology in *Escherichia coli. Biosci. Biotechnol. Biochem.* **64,** 799–807

44. Oliveira, T. F., Vonrhein, C., Matia, P. M., Venceslau, S. S., Pereira, I. A. C., and Archer, M. (2008) The crystal structure of *Desulfovibrio vulgaris* dissimilatory sulfite reductase bound to DsrC provides novel insights into the mechanism of sulfite respiration. *J. Biol. Chem* **283,** 34141–34149

45. Kingsford, C. L., Ayanbule, K., and Salzberg, S. L. (2007) Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. *Genome Biology* **8,** R22

46. Riederer, B. M. (2009) Oxidation proteomics: the role of thiol modifications. *Curr. Proteomics* **6,** 51–62

47. Everett, S. A., Folkes, L. K., Wardman, P., and Asmus, K. D. (1994) Free radical repair by a novel perthiol: reversible hydrogen-transfer and perthiyl radical formation. *Free Radic. Res.* **20,** 387–400

48. Tajc, S. G., Tolbert, B. S., Basavappa, R., and Miller, B. L. (2004) Direct determination of thiol pK_a by isothermal titration microcalorimetry. *J. Am. Chem. Soc.* **126,** 10508–10509

49. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166,** 557–580

50. Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1,** 784–791

51. Miroux, B., and Walker, J. E. (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260,** 289–298

52. Prentki, P., and Krisch, H. M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29,** 303–313

53. Fellay, R., Frey, J., and Krisch, H. (1987) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vivo* insertional mutagenesis of Gram-negative bacteria. *Gene* **52,** 147–154

54. Dahl, C., Schulte, A., and Shin, D. H. (2007) Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of DsrEFH from *Allochromatium vinosum. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **63,** 890–892