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ORIGINAL PAPER

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The role of the sulfur globule proteins of *Allochromatium vinosum*: mutagenesis of the sulfur globule protein genes and expression studies by real-time RT-PCR

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Abstract During oxidation of reduced sulfur compounds, the purple sulfur bacterium *Allochromatium vinosum* stores sulfur in the periplasm in the form of intracellular sulfur globules. The sulfur in the globules is enclosed by a protein envelope that consists of the homologous 10.5-kDa proteins SgpA and SgpB and the smaller 8.5-kDa SgpC. Reporter gene fusions of *sgpA* and alkaline phosphatase showed the constitutive expression of sgpA in *A. vinosum* and yielded additional evidence for the periplasmic localization of the sulfur globules. Expression analysis of the wild-type *sgp* genes by quantitative RT-PCR using the LightCycler system showed the constitutive expression of all three *sgp* genes. The expression of *sgpB* and *sgpC* is significantly enhanced under photolithotrophic conditions. Interestingly, *sgpB* is expressed ten times less than *sgpA* and *sgpC* implying that SgpA and SgpC are the "main proteins" of the sulfur globule envelope. Mutants with inactivated *sgpA* or *sgpB* did not show any differences in comparison with the wild-type, i.e., the encoded proteins can replace each other, whereas inactivation of *sgpC* leads to the formation of considerably smaller sulfur globules. This indicates a role of SgpC for globule expansion. A *sgpBC* double mutant was unable to grow on sulfide and could not form sulfur globules, showing that the protein envelope is indispensible for the formation and deposition of intracellular sulfur.

Keywords *Allochromatium vinosum* · Sulfur globule proteins · Sulfide oxidation · Sulfur globules · Phototrophic sulfur bacteria

Abbreviation Sgp Sulfur globule protein · *sgp* Sulfur globule protein gene

Introduction

A typical feature of the anoxygenic phototrophic sulfur bacteria is their ability to form sulfur globules as an intermediate during the oxidation of reduced sulfur compounds such as sulfide or thiosulfate (Schlegel 1963; Trüper and Pfennig 1966; Trüper 1981). The site of sulfur globule deposition differs in the different taxonomic groups of phototrophic sulfur bacteria. While purple sulfur bacteria of the family *Chromatiaceae* store the globules inside the cell (in the molecular structure of sulfur chains, Prange et al. 2002), they are deposited outside the cells in green sulfur bacteria (*Chlorobiaceae*) and in purple sulfur bacteria of the family *Ectothiorhodospiraceae* (Trüper 1981; Brune 1989). The sulfur globules in the *Chromatiaceae* are enclosed by a protein envelope, a feature shared by most if not all of the chemotrophic sulfur-oxidizing bacteria that form intracellular sulfur globules (Schmidt et al. 1971; Nicolson and Schmidt 1971; Strohl et al. 1981, 1982; Brune 1995; Pattaragulwanit et al. 1998; Dahl 1999).

In *Allochromatium* (formerly *Chromatium*) *vinosum* (Imhoff et al. 1998), this envelope consists of three different hydrophobic sulfur globule proteins (Sgps) of 10.5 kDa, 10.6 kDa (SgpA and SgpB) and 8.5 kDa (SgpC), while

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that of the related *Thiocapsa roseopersicina* contains only two proteins of 10.7 and 8.7 kDa (Brune 1995; Pattaragulwanit et al. 1998). Protein and nucleotide sequencing showed that the two larger proteins of *A. vinosum* are homologous to each other and to the larger protein of *T. roseopersicina* (Brune 1995; Pattaragulwanit et al. 1998). The sequences of the *sgp* genes were published by Pattaragulwanit et al. (1998). The smaller sulfur globule proteins in *A. vinosum* and *T. roseopersicina* are also homologous, indicating that these proteins are highly conserved between different species of the family *Chromatiaceae*. Interestingly, they share significant similarity with proteins such as cytoskeletal keratin and plant cell wall protein, suggesting that they are structural proteins rather than enzymes involved in sulfur metabolism (Brune 1995). Topologically, the sulfur globule proteins and the sulfur globules of *A. vinosum* and probably in other *Chromatiaceae* are located extracytoplasmically, in the periplasm (Pattaragulwanit et al. 1998).

In bacteria forming extracellular sulfur globules, Sgp proteins do not appear to be present (Then 1984; Pattaragulwanit et al. 1998). Consistent with this observation, the complete genome sequence of the green sulfur bacterium *Chlorobium tepidum* (Eisen et al. 2002) does not encode homologs of the purple bacterial sulfur globule protein genes. X-ray absorption near-edge structure (XANES) spectroscopy measurements show that the sulfur speciation in sulfur globules of anoxygenic phototrophic bacteria is nearly identical irrespective whether it is accumulated in globules inside or outside the cells (Prange et al. 1999, 2002). It therefore appears that the Sgp proteins themselves are not responsible for keeping the sulfur in a certain chemical structure. A direct/covalent attachment of chains of stored sulfur to the proteins enclosing the globules is unlikely as none of the Sgp proteins sequenced so far contains cysteine residues (Brune 1995; Pattaragulwanit et al. 1998).

Very little is known about the function of the sulfur globule proteins and their genetic regulation. It has been postulated that the protein envelope serves as a barrier to separate the sulfur from other cellular constituents (Shively et al. 1989) and/or that it provides binding sites for sulfur-metabolizing enzymes (Schmidt et al. 1971). Furthermore, it was speculated that *A. vinosum* mutants lacking *sgp* genes might deposit sulfur extracellulary, as in the case of the *Ectothiorhodospiraceae* (Brune 1995). In order to gain more insight into the function of the Sgp proteins in *A. vinosum*, we followed up the work of Pattaragulwanit et al. (1998), who showed by insertional inactivation of *sgpA* that the presence of SgpB is sufficient to form intact sulfur globules and that the mutant's ability to grow photoautotrophically on sulfide-containing medium was not affected. It was the goal of this study to investigate the expression of the *sgp* genes by quantitative RT-PCR, to inactivate the genes for the three different *sgp* genes separately and also simultaneously in *A. vinosum* and to study the phenotypic effects.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains used in this study are listed in Table 1. *A. vinosum* was grown (pH 6.8–7.0) and harvested as described earlier (Pattaragulwanit and Dahl 1995; Dahl 1996): photolithoautotrophically in sulfide-containing medium (Pfennig's medium; Pfennig and Trüper 1992) and photoorganoheterotrophically on malate (RCV medium; Weaver et al. 1975). In the case of *A. vinosum* strain DSMZ 185 and derivatives, 1% (w/v) sodium chloride was added. *Escherichia coli* strains were cultured in LB medium (Sambrook et al. 1989). IPTG and X-Gal were included in solid media to identify recombinant plasmids containing inserts in the α portion of *lacZ*. Antibiotics were used in the following concentrations (in $\mu g\,ml^{-1}$): for *E. coli*, ampicillin 100; erythromycin 50; gentamycin 25; kanamycin, 50; for *A. vinosum*, ampicillin, 20; erythromycin 10; gentamycin 5; kanamycin 10; streptomycin 50.

Recombinant DNA techniques

Standard methods were used for molecular techniques (Sambrook et al. 1989; Ausubel et al. 1994–2000). Cloning experiments were carried out in *E. coli* DH5α. Chromosomal DNA of *A. vinosum* was obtained by Sarkosyl lysis (Bazaral and Helinski 1968) and purified by phenol/chloroform extraction and dialysis against water. Southern hybridizations were performed overnight at 60–68°C as described previously (Dahl et al. 1994). PCR with *Taq*-DNA polymerase was done as described by Dahl (1996), and PCR with mutagen primers using *Pfu*-DNA polymerase to insert restriction sites according to the protocol supplied by Stragene. Inserted restriction sites were confirmed by sequencing using the Silver Sequence System (Promega) on the basis of thermal cycling sequencing (Murray 1989; Carothers et al. 1989). DNA probes for Southern hybridizations were digoxygenin-labeled by PCR (Seibl et al. 1990).

Construction and analysis of *A. vinosumsgp* mutants

The plasmids used for the construction of *A. vinosum* strains with mutations in the *sgp* genes are listed and described in Table 1. Mobilizable plasmids were transferred by conjugation from *E. coli* SM10 to *A. vinosum* SM50, *A. vinosum* 185SM50 or to *sgp*-deficient mutants, respectively (Pattaragulwanit and Dahl 1995) (Table 1). Transformants were selected on RCV plates containing the appropriate antibiotics under anoxic conditions in the light. Approximately 90% of the *sgp* recombinants analyzed had lost the vector-encoded antibiotic resistance, indicating that a double cross-over recombination had occurred. In the case of the *sgpA-phoA* fusion mutant of *A. vinosum* SM50, a single cross-over recombination mutant was se-

Table 1 Bacterial strains and plasmids used in this study. *DSMZ* Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig

Bacterial strains	Genotype or phenotype	Source or reference
Escherichia coli strains		
DH5α	*sup*E44 Δ*lac*U169 *hsd*17 (ϕ*lac*Z ΔM15) *rec*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan (1983)
SM10	Km^r*recA*: :S49-20 *thi*-1 *thr*-1 Leu su$_{III}$ RP4-2-TC: :Mu-Km: :Tn7 in chromosome	Simon et al. (1983)
Allochromatium vinosum strains		
DSMZ 180^T	Wild-type, type strain	DSMZ
DSMZ 185	Wild-type	DSMZ
SM50	Sm^r; spontaneous streptomycin-resistant mutant of *A. vinosum* DSMZ 180^T	Dahl (1996)
185SM50	Sm^r; spontaneous streptomycin-resistant mutant of *A. vinosum* DSMZ 185	This work
PR1	Sm^r Km^r Ap^r, *sgpA*: :pBN27	This work
K11	Sm^r Km^r; *sgpA*: :ΩKm (in *A. vinosum* SM50)	Pattaragulwanit et al. (1998)
B11	Sm^r Km^r; *sgpB*: :ΩKm (in *A. vinosum* SM50)	This work
C11	Sm^r Km^r; *sgpC*: :ΩKm (in *A. vinosum* SM50)	This work
BC11	Sm^r Km^r Gm^r, *sgpB*: :ΩGm *sgpC*: :ΩKm (in *A. vinosum* SM50)	This work
A1185	Sm^r Em^r; *sgpA*: :Em (in *A. vinosum* 185SM50)	This work
B1185Km	Sm^r Km^r; *sgpB*: :ΩKm (in *A. vinosum* 185SM50)	This work
B1185Gm	Sm^r Gm^r; *sgpB*: :ΩGm (in *A. vinosum* 185SM50)	This work
C1185Km	Sm^r Km^r; *sgpC*: :ΩKm (in *A. vinosum* 185SM50)	This work
C1185Gm	Sm^r Gm^r; *sgpC*: :ΩGm (in *A. vinosum* 185SM50)	This work
BC1185	Sm^r Km^r Gm^r; *sgpB*: :ΩGm *sgpC*: :ΩKm (in *A. vinosum* 185SM50)	This work
Plasmids	**Genetic markers or description of construction**	**Source or reference**
p134	Ap^r 1.4-kb *Sal*I fragment with *sgpA* in *Sma*I of pSUP301	Pattaragulwanit et al. (1998)
p13B6	Ap^r Km^r-cartridge (*Eco*RI) from pHP45Ω-Km replaces 681-bp *Bss*HII fragment of p134	Pattaragulwanit et al. (1998)
p335	Ap^r 2.6-kb *Sal*I fragment with *sgpC* in *Sma*I of pSUP301	Pattaragulwanit (1998)
pBlueskript II SK	Ap^r*lacZ*′	Stratagene
pBBR1MCS-5	Gm^r Mob$^+$	Kovach et al. (1995)
pETP14	Ap^r 1.4-kb *Kpn*I *phoA* fragment of pSWFII in *Kpn*I of pETP1	Pattaragulwanit et al. (1998)
pGEM3-Zf(+)	Ap^r*lacZ*′	Promega
pGEM-T	Ap^r*lacZ*′	Promega
pGTV21	Ap^r*lacZ*′ 0.5-kb PCR product with signal encoding sequnce of *sgpB* in pGEMT-T	Pattaragulwanit (1998)
pHP45Ω	Ap^r Spc^r Sm^r	Fellay et al. (1987)
pHXE39	Ap^r 1.1-kb *Hind*III/*Xho*I fragment with *sgpB* in pET22b(+)	Pattaragulwanit et al. (1998)
pS3	Cm^r 2.6-kb *Sal*I fragment with *sgpC*in *Sal*I of pHSG575	Pattaragulwanit et al. (1998)
pSUP202	Ap^r Cm^r Tc^r, Mob$^+$	Simon et al. (1983)
pSUP301	Ap^r Km^r, RP4 *oriT* p15A *ori* Mob$^+$	Simon et al. (1983)
pSWF II	Ap^r*phoA*	Ehrmann et al. (1990)
pUC19Ery	Ap^r Em^r*lacZ*′	Leenhouts et al. (1990)
pAR28	Ap^r 1.1-kb mutagen PCR product (insertion of *Bam*HI-restriction site) (Primers: MutA: CCTGGTG*GGATCC*GGGCTG; T7T: GCTAGTTATTGCTCAAGCGG) with a part of *sgpB* (amplified from pHXE39) cloned in *Hinc*II of pGEM-3Zf(+) (Primers: MutA and T7T)	This work
pAR75	Ap^r 0.5-kb *Kpn*I/*Bam*HI fragment with the signal encoding sequence of *sgpB*of pMES21F and *phoA* (*Bam*HI) of pSWFII in *Kpn*I/*Bam*HI of pAR28	This work
pBN2	Ap^r Mob$^+$ 1.5-kb *Nar*I/*Xho*I fragment with the signal encoding sequence of *sgpA* of pETP14 cloned in p134	This work
pBN27	Ap^r Km^r Mob$^+$ Ω-Km-cartridge from pHP45Ω-Km in *Eco*RV of pBN2	This work
pBSEry	Ap^r Em^r 1.1-kb *Eco*RI/*Hin*dIII fragment of pUC19Ery in *Eco*RI/*Hin*dIII of pBlueskript	This work
pGEM-Gm	Ap^r Gm^r 0.87-kb PCR product with Gm^r from pBBR1MCS-5 in pGEM-T	This work
pHP45-Em	Ap^r Em^r 1.1-kb *Sph*I/*Spe*I fragment of pBSEry in *Sph*I/*Spe*I of pHP45Ω	This work
pHP45Ω-Gm1	Ap^r Gm^r 0,98 kb *Sph*I/*Spe*I fragment of pGEM-Gm in *Bss*HII/*Sph*I of pHP45Ω	This work
pLIP27-Gm	Ap^r Gm^r Mob$^+$ 1.5-kb *Sma*I fragment of pHP45Ω-Gm1 in *Nco*I of p335	This work
pLIP27-Km	Ap^r Km^r Mob$^+$ 2.2-kb *Hin*dIII fragment of pHP45Ω-Km in *Nco*I of p335	This work
pMAR27	Ap^r Mob$^+$Em^r-cartridge (*Sma*I) of pHP45Ω-Em in *Sma*I of p13B6	This work
pMED2	Ap^r Gm^r-cartridge (*Bam*HI) of pHP45Ω-Gm1 in *Bam*HI of pAR75	This work
pMES21F	Ap^r 0.37-kb *Hin*dIII/SalI fragment of pGTV21 in *Hin*dIII/*Sal*I of pMES23	This work
pMES23	Ap^r 0.28-kb mutagen PCR product (insertion of *Bam*HI-restriction site) (Primer: T7P: TAATACGACTCACTATAGGG; MutB: CAGCCC*GGATCC*CACCAGG) with the signal encoding sequence of *sgpB* cloned in *Eco*RV of pBlueskript II SK	This work
pMED27	Ap^r Gm^r Cm^r Mob$^+$ 2.9-kb *Xho*I fragment of pMED2 in *Sal*I of pSUP202	This work
pWIN2	Ap^r Km^r Ω-Km-cartridge of pHP45Ω-Km in *Bam*HI of pAR75	This work
pWIN27	Ap^r Km^r Cm^r Mob$^+$ 3.7-kb *Xho*I fragment of pWIN2 in *Sal*I of pSUP202	This work

lected. The genotypes of the *A. vinosum* recombinants used in this study were confirmed by PCR and Southern hybridization.

Extraction of sulfur globule proteins and analysis by HPLC

Proteins from sulfur globules of *A. vinosum* mutants were extracted with 50% aqueous acetonitrile containing 1% trifluoroacetic acid and 10 mM dithiothreitol and were dried in a vacuum centrifuge as described previously (Schmidt et al. 1971; Brune 1995). The extracts were redissolved in a small volume of 50% acetonitrile/0.2% trifluoroacetic acid. Aliquots of 200 µl were analyzed by reversed-phase HPLC on a NUCLEOSIL CC125/4, 300-5C4 column (Macherey–Nagel). Proteins were separated using a gradient of 30–60% acetonitrile in water with 10 mM trifluoroacetic acid. The solvent flow rate was 0.8 ml min^{-1}.

Growth experiments and HPLC analysis of sulfur compounds

To determine the turnover of reduced sulfur compounds by *A. vinosum* wild-type and mutants, batch culture experiments were carried out in a thermostatted glass fermenter with a culture volume of 1.6 l at 30°C under continuous illumination of 3,000 lux and with a nitrogen gas atmosphere. The pH was kept at 7.0 with the aid of a pH-stat. *A. vinosum* was grown photoorganoheterotrophically on malate and fed at the beginning of the exponential growth phase with 4 mM sodium sulfide solution (prepared according to Siefert and Pfennig (1984)) or sodium thiosulfate solution to induce sulfur globule formation. The protein content of the cultures was comparable in all cases and was determined using the Bradford assay (Bradford 1976). Sulfur compounds (sulfide, elemental sulfur (after extraction with chloroform), thiosulfate, sulfate) were determined by HPLC (Thermo Separation Products TSP) using the methods of Rethmeier et al. (1997).

Electron microscopy

Freshly grown cells of *A. vinosum* SM50 and *A. vinosum* C11 were harvested and resuspended in buffer solution, chemically fixed and embedded in Epon following the procedures described in Glauert and Lewis (1998). Samples were fixed in 2.5% glutaraldehyde and 0.5% OsO_4. Ultrathin sectioning was done by means of a Reichert-Jung Ultracut E ultramicrotome. The sections were put on carbon-coated copper grids and inspected in a Philips CM12 transmission electron microscope at a primary magnification of 21,000*x*. Micrographs were recorded on Agfa film material (7×10 cm). Isolated sulfur globules were suspended in water, adsorbed to the grids and negatively stained with 2% unbuffered uranyl acetate. The diameter of the globules was determined on micrographs recorded at a magnification of 13,500*x*. The magnification was calibrated and had an absolute error of less than ±5%.

Assessing the amount of sulfur globules in cells from ultrathin sections

Assuming that N particles (e.g., sulfur globules) of spherical shape and diameter D_P are statistically equally distributed in a cylindrical cell of thickness D_C, a mass or volume fraction of $N(d/D_C)$ particles will be found in ultrathin sections of thickness d (as used for electron microscopy) on average. Sections are perpendicular to the longitudinal axis of the cylinder and cover the cell in the x,y-direction completely. In cases in which the cell shape is such that the volumes extracted by sectioning show a distribution, e.g., with a spherical cell, the average of a number of sections was taken. If instead of determining the fractional volumes of the particles in the sections the number of visible particle traces is counted, a particle of diameter D_P is found as decribed by $(D_P + d)/d = D_P/d + 1$ out of D_C/d sections (a particle of the size D_P=d is usually contained in two sections because only in a single out of an infinite number of situations is the particle completely positioned inside one section). Therefore, the average number N_S of particle traces per section is given as:

$$N_S = \frac{N(d/D_C)(D_P + d)}{d} = \frac{N(D_P + d)}{D_C}. \quad (1)$$

Comparing two sets (A and B) of cells of the same size D_C and with the same number of particles N per cell but with different particle diameters D_{PA} and D_{PB} (D_{PA}>D_{PB}), then according to Eq. 2 the number of particle traces in sections of cell type A is different by a factor >1:

$$\frac{N_{SA}}{N_{SB}} = \frac{(D_{PA} + d)}{(D_{PB} + d)}. \quad (2)$$

This fact has to be accounted for with quantitative comparisons of sulfur globules in ultrathin sections of *Allochromatium* wild-type and mutant cells.

Alkaline phosphatase assay

Alkaline phosphatase activity was measured photometrically by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate according to Brickman and Beckwith (1975). Cells (100 µl sample) were mixed with 900 µl of 1 M Tris–HCl (pH 8.0) and incubated with 30 µl chloroform plus 20 µl 0.1% SDS before the reaction was started by adding *p*-nitrophenyl phosphate to a final concentration of 1.8 mM. The reaction was stopped after 2 min by adding 200 µl of 1 M dipotassium hydrogen phosphate solution. After centrifugation at 9,500×*g* for 2 min, the absorbance of the supernatant at 420 nm was determined.

RNA isolation and expression studies by real-time RT-PCR

Total RNA from *A. vinosum* SM50 and *A. vinosum* transformants was isolated following the protocol for rapid RNA isolation from gram-negative bacteria described by Ausubel et al. (1994–2000). The RNA was further purified using the RNAeasy Total RNA kit (Qiagen). After adding 1 U RNasin ribonuclease inhibitor (Promega) μl^{-1}, RNA was stored at –70°C. RNA was measured photometrically with a Lambda11 UV/VIS spectrophotometer (Perkin–Elmer) according to Sambrook et al. (1989). Total RNA (0.8 µg) was used for real-time RT-PCR analyses with a LightCycler instrument (Roche Diagnostics) using the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. A 114-bp fragment of *sgpA* was amplified by use of the forward primer CV1-RNAF (5′-TGGGACAACATGGGTGA-3′) and the reverse primer CV1-RNAR (5′-CGTAACCATAGCCATTG-3′) (annealing temperature: 49°C), a 168-bp fragment of *sgpB* was amplified by use of the forward primer CV2-1F (5′-AATGACATGTTCGGCGA-3′) and the reverse primer CV2-4R (5′-ATACGCGCCGTAGGGAG-3′) (annealing temperature: 52°C) and a 124-bp fragment of *sgpC* was amplified by use of the forward primer CV3RNAF (5′-CTGGAATCCCTTCGGCT-3′) and the reverse primer CV3RNAR (5′-CGCCGTAGAGTCCGTAG-3′) (annealing temperature: 52°C). The RT-PCR conditions were: 50°C for 20 min (reverse transcription), 95°C for 15 min (activation of the polymerase), followed by 45 cycles of 95°C for 15 s, 49–52°C (primer annealing temperatures, see above) for 20 s and 72°C for 20 s. This RT-PCR quantification program was followed by a melting curve program consisting of a melting and continuous measuring step at 0.1°C s^{-1} up to 95°C to detect the melting points of every PCR product for each sample. Chromosmal DNA (1.7–500 ng) of *A. vinosum* SM50 was used under the same RT-PCR conditions to obtain standard curves for calibration. Using the DNA standards, the LightCycler automatically quantified cDNA amounts of the samples of interest.

Chemicals

All chemicals were of analytical grade or corresponding purity from Merck or Sigma-Aldrich.

Results and discussion

Reporter gene fusion of *sgpA* and alkaline phosphatase

By conjugation of pBN27 (containing a *phoA* reporter gene fusion to the sequence encoding the proposed signal peptide of *sgpA*) in *A. vinosum* SM50 the single cross-over mutant *A. vinosum* PR1 (Km^r, Ap^r) was obtained (Table 1). As expected for a single cross-over mutant containing the altered and the unchanged gene simultaneously, growth and oxidation of sulfide were similar to the wild-type. PhoA-activity of *A. vinosum* PR1 was 240 times higher than in the wild-type. Furthermore, PhoA-activity in *A. vinosum* PR1 was constitutively present irrespective of the growth conditions. The expression of PhoA in *A. vinosum* is also evidence for the periplasmic localization of SgpA because PhoA only shows activity when transported into the periplasm (Michaelis et al. 1983; Derman et al. 1993). This finding confirms the results of Pattaragulwanit et al. (1998), who investigated a *sgpA-phoA* reporter gene fusion construct in *E. coli*.

Expression of *sgp* genes analyzed by real-time RT-PCR

Semi-quantitative RT-PCR of RNA isolated from cells grown photoorganoheterotrophically with and without sulfide-induction was carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) to study induction of *sgp* transcription by the presence of sulfide. Specifity of the PCR reactions was confirmed by melting-curve analyses and by agarose gel electrophoresis of the PCR products. Different chromosomal *A. vinosum* DNA standards of known concentrations were used for (semi-) quantification. Real-time RT-PCR expression analyses of *sgpA*, *sgpB* and *sgpC* clearly showed that all three *sgp* genes are expressed constitutively under photolithotrophic and photoorganotrophic conditions. Expression of *sgpA* was comparable when cells were grown with or without sulfide, respectively, and is therefore not induced or regulated by sulfide addition. Expression of *sgpB* and *sgpC*, however, was significantly enhanced when cells were grown on sulfide. Expression of these genes was three to four times higher under photolithoautotrophic conditions than under photoorganoheterotrophic conditions. Interestingly however, when comparing absolute values of cDNA amounts as calculated by the LightCycler software, it could be clearly shown that expression of *sgpB*, although clearly induced by sulfide, was ten times less than that of *sgpA* and *sgpC* under photolithotrophic and photoorganoheterotrophic conditions. This clearly shows that SgpA and SgpC are the "main proteins". This finding correlates to the observation that SgpA and not SgpB from *A. vinosum* is the protein more closely related to the single large 10.6-kDa sulfur globule protein of *T. roseopersicina* (Brune 1995; Prange and Dahl, unpublished results).

Insertional inactivation of the sulfur globule protein genes in *A. vinosum*

All three *sgp* genes of *A. vinosum* are preceded by putative promotor sequences and followed by probable transcription terminators, i.e., each single gene forms a separate transcriptional unit (Pattaragulwanit et al. 1998). Downstream polar effects of the Ω cartridges (Table 1) used for *sgp* inactivation should therefore be minimal. Mutations in the *sgp* genes of *A. vinosum* SM50 and *A. vi-*

nosum 185SM50 (Table 1) were constructed by insertion of polar antibiotic Ω interposons into the *sgp* genes of the wild-type genomes via conjugation (Pattaragulwanit and Dahl 1995). Integration of these interposons leads to premature termination of transcription of both the affected genes and genes located downstream in the same transcriptional unit. To develop the work of Pattaragulwanit et al. (1998), who inactivated *sgpA* in *A. vinosum* SM50 using a kanamycin cartridge, resulting in mutant *A. vinosum* K11, the mutants *A. vinosum* B11 and C11 with inactivated *sgpB* and *sgpC*, respectively, were constructed. Furthermore, we were able to inactivate *sgpC* by introduction of a gentamycin Ω cartridge into mutant *A. vinosum* B11, resulting in mutant *A. vinosum* BC11 with inactivated *sgpB* and *spgC*. We did not succeed in constructing a mutant with inactivated *sgpA* and *sgpC* or with inactivated *sgpA* and *sgpB* or a mutant with three inactivated *sgp* genes, although different antibiotic catridges (kanamycin, erythromycin, gentamycin) and combinations thereof were tested. To verify our results with *A. vinosum* SM50, the *sgp* genes were inactivated in another strain of *A. vinosum*, *A. vinosum* DSMZ 185, using the same antibiotic cartridges. All "single" *sgp* genes could be inactivated: *sgpA* (Em), *sgpB* (Km or Gm), *sgpC* (Km or Gm) (Table 1). It was also possible to inactivate *sgpB* and *sgpC* simultaneously, resulting in mutant *A. vinosum* BC1185. However, just as in the case of the strains derived from *A. vinosum* DSMZ 180 (see above), it was not possible to inactive all three *sgp* genes or *sgpA* and *sgpB* or *sgpA* and *sgpC* in one mutant.

Furthermore, proteins from the sulfur globules of the *A. vinosum* mutants lacking one Sgp were analyzed by HPLC. In all cases the expected results were obtained. As an example, the HPLC chromatograms of the sulfur globule proteins (qualitative assessment) of the *sgpC* mutant *A. vinosum* C11 and the wild-type *A. vinosum* SM50 are shown (Fig. 1). According to the results of Brune (1995), for the wild-type three major peaks, at 11.4, 13.2, and 18.5 min, could be assigned to the three sulfur globule proteins SgpA, SgpB, and SgpC. In contrast, the chromatogram of the Sgp proteins isolated from *A. vinosum* C11 showed peaks at 11.7 min (SgpA) and 13.5 min (SgpB), but the SgpC peak was clearly lacking. The sulfur speciation of the sulfur globules of those mutants that were still able to form sulfur globules was investigated by X-ray absorption near edge structure (XANES) spectroscopy: as in the wild-type, the globules consisted of sulfur chains as the main form of sulfur (data not shown; details to method and sulfur speciation in *A. vinosum*, Prange et al. 1999, 2002).

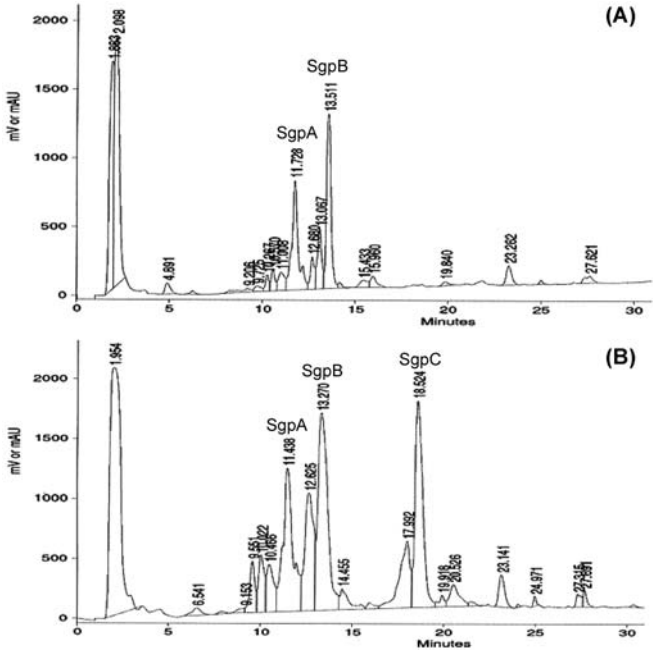

Fig. 1 HLPC chromatograms (qualitative assessment) of the sulfur globule proteins from **a** *Allochromatium vinosum* C11 (*sgpC*-deficient mutant) and **b** *A. vinosum* SM50 (wild-type)

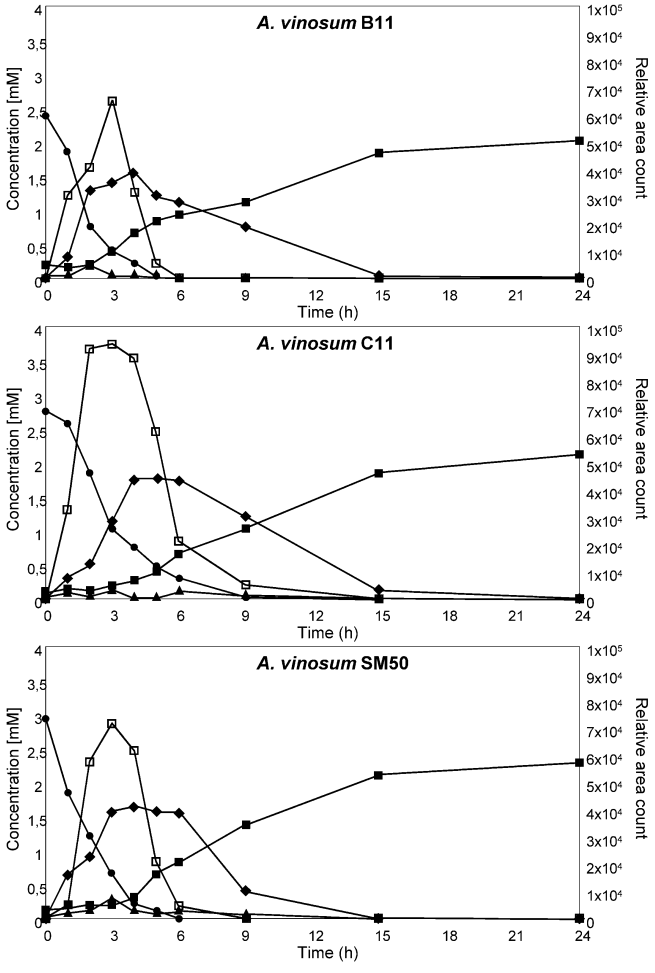

Fig. 2 Turnover of sulfur compounds by **a** *A. vinosum* B11 (*sgpB*-deficient mutant) and **b** *A. vinosum* C11 (*sgpC*-deficient mutant) in comparison with **c** *A. vinosum* SM50 (wild-type) during photolithoautotrophic growth on sulfide. Protein content at the onset and end of the experiments: 60–90 µg/ml. *Filled circles* sulfide (mM), *filled diamonds* sulfur (mM), *open squares* polysulfide (relative area count), *filled squares* sulfate (mM), *filled triangles* thiosulfate (mM)

Growth experiments with *sgp*-deficient mutants

A. vinosum B11 clearly lacks SgpB, but its ability to grow photoautotrophically on sulfide-containing media and to store sulfur as intracellular sulfur globules was not affected. Similiar to the wild-type, the mutant oxidized sulfide first to polysulfide and then to "zero-valent" polysulfane sulfur stored in sulfur globules, which was finally oxidized to sulfate (Fig. 2). Lack of SgpB influenced neither the size nor the quantity of sulfur gobules. This result is similar to the finding of Pattaragulwanit et al. (1998), who showed that the lack of SgpA in mutant *A. vinosum* K11 did not affect either sulfide-oxidizing ability or sulfur globule formation. Consistent with these observations, *T. roseopersicina* contains only one corresponding sulfur globule protein that is homologous to both SgpA and SgpB from *A. vinosum* (Brune 1995). Therefore, we conclude that the homologous proteins SgpA and SgpB can replace each other in the presence of SgpC.

In mutant *A. vinosum* C11, which lacks SgpC, the ability to oxidize sulfide or thiosulfate via intracellar sulfur to sulfate was also not affected. Sulfide and thiosulfate were depleted from the cultures with a rate similar to that of the wild-type (Figs. 2, 3). It should also be stressed that, in contrast to the oxidation of sulfide, neither the wild-type nor the mutant formed polysulfides as intermediates of thiosulfate oxidation. A significant difference between the wild-type and mutant B11 on the one hand and mutant C11 on the other became obvious upon electron microscopy of thin sections of embedded cells (Fig. 4) and of isolated sulfur globules (Fig. 5). The globules of the *sgpC* mutant strain C11 were ≈50% smaller in diameter on average than those of the wild-type (Figs. 4, 5). As evident from Figs. 2, 3, the maximum concentration of stored sulfur was the same for the wild-type and mutant B11, both possessing globules of regular size, and for C11, containing much smaller sulfur globules. Since a smaller diame-

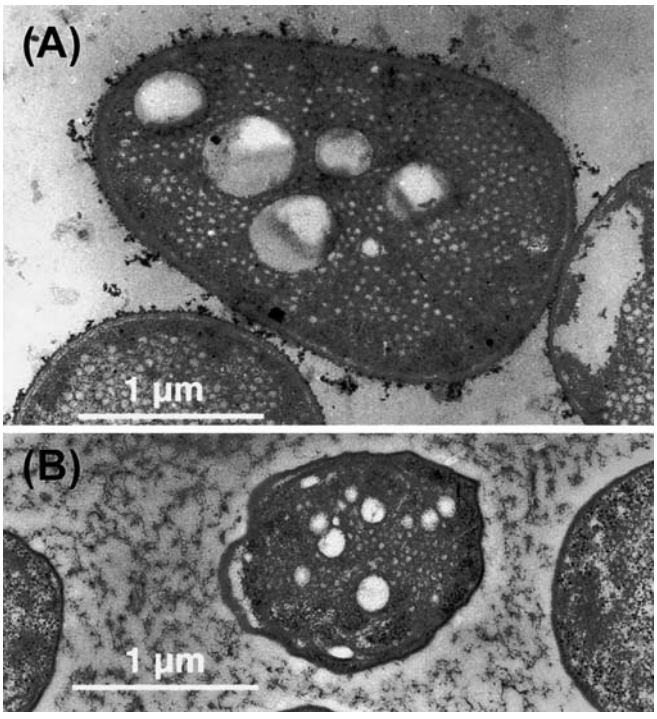

Fig. 4 Electron micrographs of embedded and thin sectioned cells of **a** *A. vinosum* DSMZ 180 (wild-type) and **b** *A. vinosum* C11 (*sgpC*-deficient mutant). Intracellular sulfur globules are identified as bright, almost circular areas

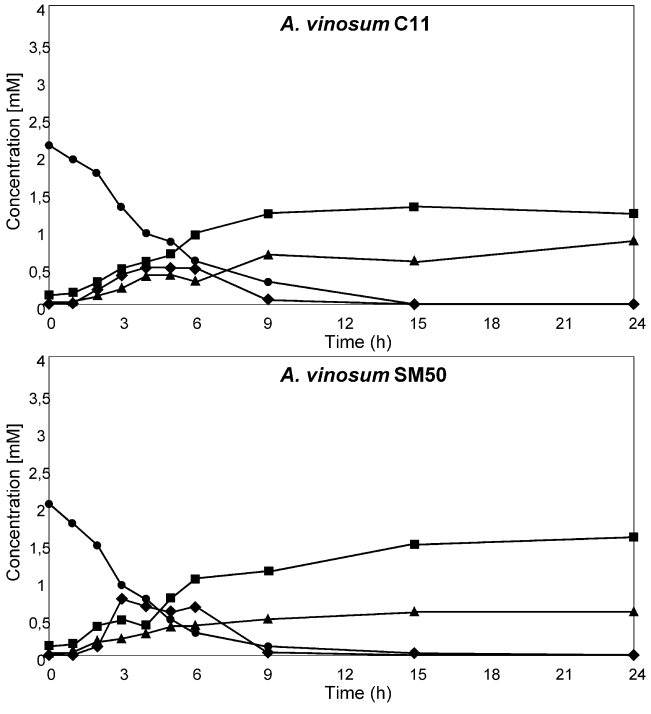

Fig. 3 Turnover of sulfur compounds by **a** *A. vinosum* C11 (*sgpC*-deficient mutant) in comparison with **b** *A. vinosum* SM50 (wild-type) during photolithoautotrophic growth on thiosulfate. Protein content at the onset and end of the experiments: 50–70 µg/ml. *Filled circles* thiosulfate (mM), *filled diamonds* sulfur (mM), *filled squares* sulfate (mM), *filled triangles* tetrathionate (mM)

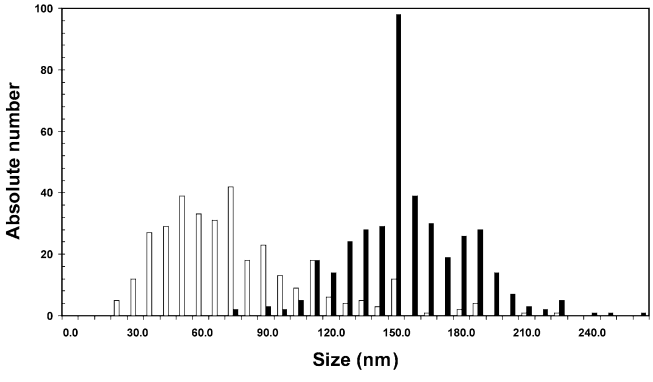

Fig. 5 Size distribution of isolated sulfur globules of *A. vinosum* C11 (*sgpC*-deficient mutant) (*white bars*) and of *A. vinosum* SM50 (wild-type) (*black bars*) grown photolithoautotrophically on sulfide. The globules were inspected by electron microscopy and their diameters determined from electron micrographs. Statistical values: *A. vinosum* SM50: *n*=399, diameter 155±27 nm; *A. vinosum* C11: *n*=338, diameter 76±35 nm

ter of a spherical body means a significant decrease of its volume, we must expect that mutant C11 compensates for the loss of material per particle by a higher number of sulfur globules per cell. This appeared indeed to be the case.

If we take the size distribution of isolated sulfur globules in Fig. 5 as representative for the globules in the cells and assume that the globules are ideal spheres (which is an approximation according to Fig. 4), the distribution of the volumes of wild-type and mutant globules can be assessed. The corresponding average values were 2.14×10^6 nm^3 (wild-type) and 0.41×10^6 nm^3 (mutant), respectively. This means that the "characteristic" wild-type globule contains about five times more sulfur than the average globule from C11 cells. By contrast, microscopy of the thin sections (number of sections analyzed, n=122) showed 2.4 times more sulfur globules in mutant cells than in wild-type cells (n=57). In addition we have to take into account that particles of 155 nm in diameter on average are detected with a higher probability in ultrathin sections of about 50-nm thickness than the smaller globules from the mutant strain. According to Eq. 2 (see Materials and methods), the number of sulfur globules in C11 cells must be corrected by factor of about 1.6, i.e. (155+50)/(76+50), resulting in an apparently four times higher content of sulfur globules in mutant cells. This assessment is in reasonable agreement with the expected value of five and suggests an approximately balanced mass of total sulfur between the wild-type and mutant strains.

In summary, our results with the *sgpC* mutant *A. vinosum* C11 suggest a role for SgpC during expansion of the sulfur globules. Gas vesicle membranes of halobacteria and cyanobacteria consist, similar to the sulfur globule envelope, of different proteins. The gas vesicle membrane of *Halobacterium salinarum* PHH1, for example, is formed by the main proteins GvpA and GvpC and other proteins (Offner et al. 1998; Offner et al. 2000). Whereas GvpA is the main protein of the gas vesicle membrane, GvpC is responsible for the enlargement and reinforcement of the membrane (Walsby 1994; Kinsman et al. 1995; Offner et al. 1998). Probably, the "small" sulfur globule protein SgpC has a function comparable to GvpC in the case of the gas vesicle membrane. As suggested for the role of GvpC during the formation of gas vesicle membranes (Walsby 1994), SgpC might be fitted between the "big" sulfur globule proteins during enlargement of the sulfur globule protein envelope.

The "double mutant" *A. vinosum* BC11, which lacks SgpB and SgpC, could not grow on sulfide or on thiosulfate and was not able to form sulfur globules. Therefore, we conclude that at least two Sgps (one of the proteins SgpA and SgpB and the "small" protein SgpC) are necessary for intact sulfur globule formation. Sulfide and also thiosulfate oxidation are not possible without sulfur globule formation, which supports earlier findings of Pott and Dahl (1998) that sulfur is an obligate intermediate of thiosulfate oxidation.

Whereas inactivation of *sgpA* and *sgpB* in "single mutants" (*A. vinosum* strains: A1185, B1185Km, B1185Gm, s. Table 1) did not lead to any differences in comparison with the wild-type, inactivation of *sgpC* in the "single mutants" *A. vinosum* C1185Km and *A. vinosum* C1185Gm (Table 1), respectively, led to significantly smaller sulfur globules. Likewise, as in the case of *A. vinosum* BC11 (see above), the "double mutant" *A. vinosum* BC1185 was only able to grow on malate and was not able to grow on sulfide or thiosulfate. An extracellular deposition of sulfur by *A. vinosum* mutants lacking *sgp* genes as speculated by Brune (1995) was not observed.

With respect to the function of SgpA and SgpB, we can conservatively state that these proteins can substitue for each other in the presence of SgpC. However, in its absence this appears not to be true. A mutant possessing SgpA and SgpB but lacking SgpC can grow on sulfide and thiosulfate (albeit with smaller sulfur globules). If SgpA and SgpB were fully competent to replace each other, growth and sulfur globule formation should also be possible for mutants possessing solely SgpA or SgpB. However, this is clearly not the case: while mutants with only SgpA are viable under photoorganotraophic conditions, they are not able to grow in the presence of sulfide or thiosulfate, and mutants with only SgpB are apparently not even viable under photoorganotrophic conditions.

So far, we have no experimentally proven explanation for our finding that the construction of *A. vinosum* mutants lacking SgpA and SgpC or all three Sgp proteins is not possible. A manifest conclusion would be that the presence of a basic level of sulfur globule proteins is obligatory for cell survival even under conditions that do not allow or lead to sulfur globule formation, i.e., during photoorganotrophic growth. This may be related to the probable function of the Sgp proteins as "pure" structural proteins. Calculations show that the sulfur globule proteins account for up to 3.5% of the total cellular protein (Mas and van Gemerden 1987; Brune 1995). Furthermore, Fourier-transform-infrared (FTIR) spectroscopy indicated that the sulfur globule proteins most probably consist of a β-sheet structure (Engelhardt, Prange, Trüper, Dahl, unpublished data). It is well known that the photosynthetic membranes do not contain β-sheet structures. Therefore, the indication for β-sheet structures must be correlated to the sulfur globule proteins, corroborating previous findings and suggestions that they are structural proteins (Brune 1995; Pattaragulwanit et al. 1998). In summary, our results indicate limits on the extents to which SgpA and SgpB can substitute for one another, and suggest that more remains to be learned about the functions of these proteins.

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