Insertional gene inactivation in a phototrophic sulphur bacterium: APS-reductase-deficient mutants of *Chromatiurn vinosum*

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In purple sulphur bacteria of the family *Chromatiaceae* sulphite oxidation via intermediary formation of adenylylsulphate is an enzymologically well characterized process. In contrast, the role of an alternative direct oxidation pathway via the enzyme sulphite:acceptor oxidoreductase has not been resolved. This paper reports the cloning of the genes encoding the adenylylsulphate-forming enzyme adenosine-5'-phosphosulphate (APS) reductase from *Chromatiurn vinosum* strain D (DSM 1807), a representative of the purple sulphur bacteria, and the construction of mutations in these genes by insertion of a kanamycin Ω cartridge. The mutated genes were transferred to *C. vinosum* on suicide vectors of the pSUP series by conjugation and delivered to the chromosome by double homologous recombination. Southern hybridization and PCR analyses of the recombinants obtained verified the first insertional gene inactivation in purple sulphur bacteria. Enzymological studies demonstrated the absence of APS reductase from the mutants. Further phenotypic characterization showed no significant effect of APS reductase deficiency on the sulphite-oxidizing ability of the cells under photolithoautotrophic growth conditions. In the wild-type as well as in mutant strains, tungstate, the specific antagonist of molybdate, led to the intermediary accumulation of sulphite in the medium during sulphide oxidation and strongly inhibited growth with sulphite as photosynthetic electron donor; this indicates that a molybdoenzyme, probably sulphite:acceptor oxidoreductase, is the main sulphite-oxidizing enzyme in *C. vinosum*. Specific inactivation of selected genes as developed for *C. vinosum* in this study provides a powerful genetic tool for further analysis of sulphur metabolism and other metabolic pathways in phototrophic sulphur bacteria.

**Keywords**: *Chromatiurn vinosum*, adenosine-5'-phosphosulphate reductase, sulphite oxidation, interposon mutagenesis, molybdenum

**INTRODUCTION**

Anaerobic phototrophic purple sulphur bacteria of the family *Chromatiaceae* are able to derive energy from light by using reduced sulphur compounds as photosynthetic electron donors. The pathway of reduced sulphur oxidation in these organisms includes three essential steps: (1) formation of elemental sulphur, which appears as globules inside the bacteria during oxidation of sulphide or thiosulphate; (2) oxidation of sulphide or elemental sulphur to sulphite; and (3) formation of sulphate as the final product (Trüper & Fischer, 1982; Trüper, 1984; Fischer, 1989; Dahl & Trüper, 1994). Thus, sulphite is a central intermediate in the metabolism of reduced sulphur compounds in purple sulphur bacteria and accordingly the oxidation of this compound has received focal attention over the past decades. However, the sulphite oxidation pathways in *Chromatiaceae* are still a matter of debate.

It is well established that the enzyme adenylylsulphate (adenosine-5'-phosphosulphate, APS) reductase (EC
1.8.99.2) participates in the oxidation of sulphite and catalyses its reaction with AMP to form APS (Trüper & Rogers, 1971; Trüper & Fischer, 1982). Whereas APS reductases from dissimilatory sulphate-reducing prokaryotes and some *Thiobacillus* species are soluble and reside in the cytoplasm (Fauque et al., 1991; Takakuwa, 1992), the enzyme from purple sulphur bacteria appears to be associated with the membrane fraction and is firmly membrane-bound in *Chromatium vinosum* (Schwenn & Biere, 1979; Trüper & Fischer, 1982). Sulphite is liberated from APS by the enzymes ADP sulphurylase and/or ATP sulphurylase, which replace the sulphate moiety by inorganic phosphate or pyrophosphate, thus producing ADP or ATP, respectively. This step allows the conservation of energy via substrate-level phosphorylation.

An enzyme postulated to bypass the formation of APS in phototrophic sulphur bacteria is sulphite: acceptor oxidoreductase (Trüper & Fischer, 1982; Trüper, 1984; Fischer, 1989). Analogous to sulphite: cytochrome *c* oxidoreductase from *thiobacilli*, which has been shown to contain molybdopterin and haem *c* (Toghrul & Southern, 1983), this enzyme may catalyse the direct oxidation of sulphite to sulphate. However, since the enzyme has never been purified to homogeneity from a purple sulphur bacterium and enzyme assays with crude extracts are strongly influenced by nonenzymic artefacts (Dahl & Trüper, 1989; Brune, 1995), it is still questionable whether this alternative sulphite oxidation pathway really exists in *Chromatiaceae*.

Since the biochemical methods used in all previous studies failed to prove the existence of sulphite: acceptor oxidoreductase in *Chromatiaceae* I took advantage of the recently implemented system for DNA transfer in *C. vinosum* strain D (DSM 180T) (Pattaragulwanit & Dahl, 1995) and developed a strategy for insertional inactivation of the genes for APS reductase in this organism.

**METHODS**

**Bacterial strains, plasmids, media and growth conditions.** The strains and plasmids used in this study are listed in Table I. *Escherichia coli* strains were cultured either in LB medium (Sambrook *et al.*, 1989) or on AB3 plates (17.5 g antibiotic medium no. 3 (Difco) per litre, 15%, w/v, agar). IPTG and X-Gal were included in solid media to identify recombinant plasmids containing inserts in the *α* portion of lacZ. The medium for photoorganoheterotrophic growth (medium MYE) of *C. vinosum* contained (per litre): 3 g dl-malic acid, 1.2 g NH₄Cl, 0.2 g MgSO₄, 7H₂O, 0.07 g CaCl₂, 2H₂O, 9 mM potassium phosphate buffer, 0.5 g yeast extract, 1 ml trace element solution SL 12 (Pfenning & Trüper, 1992), pH adjusted to 7.0. For tests on utilization of sulphate as the sole source of sulphur, the medium was modified by omission of yeast extract and addition of 1 ml 0.2% vitamin B₆ and 1 ml 1% (w/v) sodium ascorbate from sterile-filtered stock solutions (medium M). Precultures were grown on MYE and washed with medium M before inoculation to remove compounds containing reduced sulphur. Photolithothiotrophic liquid cultures of *C. vinosum* were cultivated on PF medium (medium 2 of Pfenning & Trüper, 1992). When required, sodium thiosulphate (medium PT) or sodium sulphite (medium PS), which were added from sterile-filtered stock solutions immediately before inoculation. Cells were harvested in the exponential phase of growth according to Dahl & Trüper (1994). For growth of *C. vinosum* on solid media, agar was replaced by 1% (w/v) phytogel (P-8169, Sigma), which allows incubation in the light for several weeks without desiccation of the plates. Solidification with phytogel requires the addition of 0.5% NaCl to the medium. Antibiotics were used at the following concentrations (µg ml⁻¹): for *E. coli*, ampicillin, 100; kanamycin, 50; tetracycline, 10, chloramphenicol, 50; for *C. vinosum*, kanamycin, 25; ampicillin, 20, streptomycin, 50.

**Recombinant DNA techniques.** All general cloning techniques were performed according to standard methods (Sambrook *et al.*, 1989). Chromosomal DNA of *C. vinosum* strains was obtained by Sarkosyl lysis (Bazaral & Helinski, 1968) and purified by CsCl density-gradient centrifugation. Bacterial matings were performed according to Pattaragulwanit & Dahl (1995). Hybridizations were performed overnight at 55 °C for heterologous probes and at 68 °C for homologous probes as described earlier (Dahl *et al.*, 1994).

**Construction of DNA libraries.** *C. vinosum* DSM 180 libraries were constructed from size-fractionated HindIII/Xhol fragments of 3–4 kb which were ligated into pSUP301 digested with the respective enzymes, or into pSUP202 digested with Sall and HindIII. *E. coli* clones carrying recombinant plasmids were identified by loss of kanamycin and tetracycline resistance, respectively.

**PCR.** Oligonucleotide primers were dissolved at a concentration of 50 pmol µl⁻¹. For DNA amplification, 2 µl of each primer, 500 ng chromosomal template DNA, 2.5 U Taq DNA polymerase, 5 µl Taq DNA polymerase buffer (200 mM Tris/HCl, pH 8.4, 500 mM KCl), 2 µl 50 mM MgCl₂ and 1 µl deoxy-nucleotide triphosphates (10 mM each dTTP, dCTP, dGTP and dATP) were combined in a final reaction volume of 50 µl. After an initial denaturation step at 95 °C for 3 min amplification was carried out for 34 cycles, with each cycle consisting of 30 s at the annealing temperature, 2 min at 72 °C, and 30 s at 95 °C, followed by annealing for 30 s and incubation at 72 °C for 5 min. PCR reactions with primers CWECYC [5'-TGCTGGGA(G/A)TG(T/C)TACTC(C/G)TG-3'] and DLILLG [5'-CCGATGATG(C/G)AGGAT(G/A)TC-3'], which correspond to nucleotide positions 211–230 and 611–630 of the *Archaeoglobus fulgidus* APS reductase gene locus (Speich *et al.*, 1994) were annealed at 60 °C, while for reactions with primers CWECYS and KM1 [5'-TTGATCCCAGGTGC(A/G)AC-3'] an annealing temperature of 50 °C was chosen. The primer between corresponds to positions 1499–1515 of the *Trs apb* gene locus published by Beck *et al.* (1982). DNA probes for Southern blot experiments and screening of libraries by colony hybridization were digoxigenin-labelled by PCR following the protocol of Boehringer. PCR products were purified by gel electrophoresis and electroelution prior to cloning and hybridization steps.

**Enzyme assays.** Crude extracts were prepared and APS reductase was measured by the adenyllylsulphate formation assay as described by Dahl & Trüper (1994). The APS formation reaction contained 0.4 mM AMP, 0.5 mM potassium ferriyanide, 4 mM sodium sulphite, 7.5 mM EDTA and 50 mM Tris/HCl, pH 8.0. Sulphite: acceptor oxidoreductase was assayed in the same reaction mixtures without AMP. Ferriyanide reduction was followed at 420 nm (ε₅₉₀ = 1.09 × 10⁻⁴ cm⁻¹). One unit was defined as 1 pmol sulphate oxidized min⁻¹. Single colonies were screened for presence of APS reductase as follows. Cells from a single colony were suspended in 25 µl TES (0.1 M NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 8.0), followed by addition of 20 µl lysozyme solution (20 mg ml⁻¹) and incubation at 37 °C for 20 min. Cells were lysed by adding 5 µl 10% (w/v)
Inactivation of APS reductase genes in *Chromatium vinosum*

**Table 1.** Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>DH5α</td>
<td>F−, ∆80lacZΔM15, ∆lacZYA-argF/(U169 recA1 endA1 hsdR17 (rK mK) supE44 thi-1 gyrA relA1)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>SM10</td>
<td>Km', supE44 thi-1 thr-1 recA leuB6 lacY1 tonA21 R4-2-Tc::Mu- Km': Tn7 in chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Chromatium vinosum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM50</td>
<td>Sm', spontaneous streptomycin-resistant mutant of <em>C. vinosum</em></td>
<td>This work</td>
</tr>
<tr>
<td>D3</td>
<td>Sm', Km', aprB::KmΩ (orientation A)</td>
<td>This work</td>
</tr>
<tr>
<td>Ap4</td>
<td>Sm', Km', Ap', aprB::pNTS35</td>
<td>This work</td>
</tr>
<tr>
<td>25:26</td>
<td>Sm', Km', aprB::KmΩ (orientation B)</td>
<td>This work</td>
</tr>
<tr>
<td>28:2</td>
<td>Sm', Km', aprA::KmΩ (orientation A)</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pSUP202</td>
<td>Ap', Cm', Tc', Mob+</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pSUP301</td>
<td>Ap', Km', Mob+</td>
<td>Simon et al. (1983)</td>
</tr>
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<td>3.6 kb HindIII/XbaI fragment in pSUP301</td>
<td>This work</td>
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<td>pNTS35</td>
<td>Km' cartridge (EcoRI) from pPH450 in PseII of pNTS30</td>
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</tr>
<tr>
<td>pNTS38</td>
<td>Km' cartridge (HindIII) from pPH450 in PseII of pNTS30</td>
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<td>pNTS25</td>
<td>5.9 kb HindIII/XhoI fragment from pNTS38 in pSUP202</td>
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<td>pNTS28</td>
<td>Km' cartridge (EcoRI) from pPH450 in SalI of pNTS20</td>
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<tr>
<td>pHP450</td>
<td>Ap', Km'</td>
<td>Fellay et al. (1987)</td>
</tr>
</tbody>
</table>

lauroylsarcosine. In a final volume of 100 μl, AMP, sulphite, EDTA and ferricyanide were added to the final concentrations given above. After incubation for 15 min at room temperature and centrifugation at 15000 g for 5 min in a microcentrifuge the supernatant was inspected for the presence of APS by high-performance thin-layer chromatography (Cooper & Trüper, 1979). Protein contents of cell extracts were measured by the Lowry method.

**Turnover of reduced sulphur compounds.** Batch culture experiments concerned with the turnover of reduced sulphur compounds were performed under photolithoautotrophic conditions in a thermostatted glass fermenter with a culture volume of 1.6 l at 30 °C under continuous illumination of 3000 lx and with N₂ as the gas atmosphere. The pH was kept at 7.0 with the aid of a pH-stat. When calculating specific sulphite oxidation rates, a chemical oxidation rate of 0.36 pmol min⁻¹ ml⁻¹ was determined in fed-batch cultures in completely filled 1-litre screw-capped bottles. Thiosulphate was determined via cyanolysis (modified after Urban, 1961) in the following assay system: a 650 μl sample with up to 0.25 nmol thiosulphate was mixed with 200 μl 0.2 M sodium acetate, pH 4.8, 50 μl 40 mM CuCl₂ and 50 μl Fe(NO₃)₃ reagent [30 g Fe(NO₃)₃, 9H₂O and 40 ml 55% (v/v) HNO₃ made up to 100 ml with distilled water]. Tetrathionate was determined as follows (modified after Kelly, 1969): a sample of 850 μl containing up to 160 nmol tetrathionate with 100 μl 1 M Tris/acetate, pH 8.7, and 200 μl NaCN was incubated at 30 °C for 30 min followed by the addition of 100 μl Fe(NO₃)₃ reagent. In both cases the absorbance was measured at 460 nm against a reagent blank. The assays were calibrated with NaSCN. Sulphur compounds were determined in 1 ml reaction volumes according to the following methods described in the literature: sulphide (Trüper & Schlegel, 1964); elemental sulphur (Schedel & Trüper, 1980); sulphate (Sörbo, 1987). Cell pellets for determination of elemental sulphur were obtained by centrifugation of 100 μl culture aliquots for 20 min at 4 °C and 18000 g in a microcentrifuge. Thiosulphate was determined via cyanolysis (modified after Urban, 1961) in the following assay system: a 650 μl sample with up to 0.25 nmol thiosulphate was mixed with 200 μl 0.2 M sodium acetate, pH 4.8, 50 μl 40 mM CuCl₂ and 50 μl Fe(NO₃)₃ reagent [30 g Fe(NO₃)₃, 9H₂O and 40 ml 55% (v/v) HNO₃ made up to 100 ml with distilled water]. Tetrathionate was determined as follows (modified after Kelly, 1969): a sample of 850 μl containing up to 160 nmol tetrathionate with 100 μl 1 M Tris/acetate, pH 8.7, and 200 μl NaCN was incubated at 30 °C for 30 min followed by the addition of 100 μl Fe(NO₃)₃ reagent. In both cases the absorbance was measured at 460 nm against a reagent blank. The assays were calibrated with NaSCN. Sulphur was measured by the fuchsin method (modified after Leinweber & Monty, 1987), as follows. A 700 μl sample with up to 40 nmol sulphite was incubated with 9.2 ml 2% zinc acetate and 0.1 ml 0.04% fuchsin (in 10%, v/v, H₂SO₄) for 10 min at room temperature followed by the addition of 10 μl 35% (v/v) formaldehyde and a second incubation for 10 min. The absorbance was measured at 570 nm against a reagent blank. Since sulphone interferes with this assay, sulphite was only determined in the absence of sulphide. The protein content of the cultures was determined as described earlier (Dahl & Trüper, 1989).
RESULTS AND DISCUSSION
Cloning the DNA region encoding APS reductase

As a key enzyme in dissimilatory sulphur metabolism APS reductase has not only been found in phototrophic sulphur-oxidizing bacteria but also in chemotrophic sulphur oxidizers and dissimilatory sulphate-reducing prokaryotes of different phylogenetic positions (Trüper & Fischer, 1982; Fauque et al., 1991; Takakuwa, 1992; Speich et al., 1994). All APS reductases studied so far have a similar structure and bind iron–sulphur clusters and FAD. The genes for the iron–sulphur protein (aprB) and the flavoprotein (aprA) subunits of the enzymes from both the archaeal sulphate reducer Archaeoglobus fulgidus (Speich et al., 1994) and the purple sulphur bacterium Thiocapsa roseopersicina (I. Faath & H. G. Trüper, personal communication) are arranged in the order aprBA. In particular, they show two regions of extensive amino acid sequence similarities: the amino acid sequence stretch CWECYSC, which is located in the β-subunit and involved in [Fe₃S₄] cluster binding, and the sequence motif DILIIG, which is found close to the amino-terminus of AprA and constitutes part of the FAD-binding site (Speich et al., 1994). On the basis of codon usage data from existing Chromatium vinosum D (DSM 180) genes (Viale et al., 1989) two oligonucleotide primers (given in Methods) were deduced from these sequence identities and a DNA fragment of the expected size of 400 bp was obtained by PCR with C. vinosum DNA as the template. The 400 bp amplicon hybridized with the corresponding DNA fragment from T. roseopersicina. In Southern blot hybridizations the 400 bp gene probes from both T. roseopersicina and C. vinosum hybridized to identical restriction fragments of C. vinosum DNA, further confirming the identity of the amplicon. Since both probes gave only one band in most restriction digests it is concluded that the apr genes are single-copy genes in this organism. The amplified C. vinosum DNA fragment was used as a probe to identify several positive clones in libraries of 3–4 kb HindIII/XhoI fragments in the vectors pSUP202 and pSUP301 (Simon et al., 1983). Positive plasmids were designated pNTS20 and pNTS30, respectively. A partial restriction map of the cloned 3.6 kb DNA fragment and neighbouring DNA regions is shown in Fig. 1. The aprBA genes were located on this map by a combination of nucleotide sequencing (W. Hipp & H. G. Trüper, personal communication), restriction analysis and Southern hybridization.

Insertional inactivation of the aprBA locus in C. vinosum

In order to confirm the coding nature of the cloned fragment and to investigate the consequences of an APS reductase deficiency on the sulphite-oxidizing capabilities of C. vinosum, strains with defined mutations in the putative coding region were constructed. For this purpose, a kanamycin Ω interposon (Frey & Krisch, 1985; Fellay et al., 1987) was inserted into aprB. Integration of this widely used antibiotic cartridge leads to premature termination of transcription of the affected gene and genes which might be located downstream in the same transcription unit. Since plasmid vectors suitable for delivery of such constructs to the chromosome of phototrophic sulphur bacteria have not been reported, the mobilizable suicide vehicles pSUP301 and pSUP202 were chosen; these are generally unable to replicate in strains outside the enterobacterial group (Simon et al., 1983) and have been successfully applied for anoxygenic phototrophic bacteria of the family Rhodospirillaceae (Donohue & Kaplan, 1991). The two plasmids carry the origins of replication from p15A and CoIE1, respectively (Simon et al., 1983, 1986), enabling us to test the suitability of suicide vectors based on two different replicons in C. vinosum.

For construction of a mutated fragment in pSUP301, plasmid pNTS30 was used to introduce the 2.3 kb EcoRI kanamycin Ω interposon from plasmid pH45Ω (Fellay et al., 1987), which had been blunt-ended by treatment with Klenow polymerase, into a single PvuII site located about 300 bp 3' to the start of aprB. The kanamycin resistance gene was determined by restriction mapping to lie in such an orientation that it was transcribed in the same direction as aprB. For generation of a mutated fragment in pSUP202 the kanamycin resistance gene was isolated by digestion with HindIII, blunt-ended and first ligated into the PvuII site of pNTS30, resulting in pNTS38. The kanamycin cartridge was determined to lie in the opposite direction as compared to pNTS35. The mutated 5.9 kb XhoI/HindIII DNA fragment from pNTS38 was cloned between the HindIII and SalI sites of pSUP202, resulting in pNTS25. The kanamycin interposon was also introduced downstream of aprB into aprA by cloning the blunt-ended 2.3 kb EcoRI fragment from pH45Ω into the blunt-ended SalI site of pNTS20, which is 750 bp away from the PvuII site, resulting in pNTS28. pNTS35, pNTS25 and pNTS28 were transferred to C. vinosum SM50 by conjugation from the E. coli donor strain SM10. Resistance to streptomycin and kanamycin was selected for on plates with malate as carbon source and photosynthetic electron donor because APS-reductase-deficient mutants might have been severely impaired in oxidative sulphur metabolism. Independent of the replicon of the delivery plasmid, kanamycin-resistant C. vinosum recombinants were obtained with frequencies of

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Fig. 1. Restriction map of the apr locus in C. vinosum. E, EcoRI; H, HindIII; S, SalI; Ss, SstI; X, XhoI.
Inactivation of APS reductase genes in *Chromatium vinosum*

(a) Schematic representation of the *aprBA* gene region in the chromosome of *C. vinosum* SM50, the derived single-crossover mutant Ap4, and the derived APS-reductase-deficient double-crossover mutants D3 and 25:26, in which the *aprB* gene is interrupted by a kanamycin resistance cartridge. E, EcoRI; S, SalI. (b) The upper part shows Southern blot analyses of total DNA from *C. vinosum* SM50 (wild-type), the single-crossover mutant Ap4 and the double-crossover mutants D3 and 25:26. Total DNA was restricted with EcoRI or SalI and hybridized with the *aprBA* probe indicated in (a). Sizes of DNA length standards are indicated. The lower part shows a thin-layer chromatogram of APS reductase reactions performed with single colonies of the wild-type and mutants Ap4 and D3 as described in Methods. The small spot found for strain D3 slightly above the heavy APS spots detected for the wild-type and mutant Ap4 was proven by an enzyme-coupled assay (Dahl et al., 1994) not to contain any APS.

Fig. 2. (a) Schematic representation of the *aprBA* gene region in the chromosome of *C. vinosum* SM50, the derived single-crossover mutant Ap4, and the derived APS-reductase-deficient double-crossover mutants D3 and 25:26, in which the *aprB* gene is interrupted by a kanamycin resistance cartridge. E, EcoRI; S, SalI. (b) The upper part shows Southern blot analyses of total DNA from *C. vinosum* SM50 (wild-type), the single-crossover mutant Ap4 and the double-crossover mutants D3 and 25:26. Total DNA was restricted with EcoRI or SalI and hybridized with the *aprBA* probe indicated in (a). Sizes of DNA length standards are indicated. The lower part shows a thin-layer chromatogram of APS reductase reactions performed with single colonies of the wild-type and mutants Ap4 and D3 as described in Methods. The small spot found for strain D3 slightly above the heavy APS spots detected for the wild-type and mutant Ap4 was proven by an enzyme-coupled assay (Dahl et al., 1994) not to contain any APS.

up to $10^{-4}$ per recipient cell. No transconjugants were obtained on selective plates using the pSUP301 or pSUP202 plasmids alone, which carry resistance genes but no *C. vinosum* DNA. It can therefore be concluded that integration of foreign DNA into the genome of *C. vinosum* requires the presence of homologous DNA and that conjugation with the vectors used is possible only by homologous recombination. Approximately 95% of the recombinants analysed were Km$^\text{r}$ and Ap$^\text{r}$, indicating that they had lost the vector-encoded ampicillin resistance and that a double-crossover recombination had occurred. In *Rhodospirillaceae* double-crossover rates generally amount to only 1–10% (Donohue & Kaplan, 1991), while rates comparable to those observed for *C. vinosum* have been found for single-celled cyanobacteria (Bockholt et al., 1991). The high double-crossover rate is indicative of a very active recombination system in *C. vinosum*.

Genotypic characterization of mutant strains

Several independent colonies obtained after conjugation with pNTS35 or pNTS25 were analysed by Southern blotting. Total DNA from the wild-type and transformants was digested with different enzymes and probed with the 400 bp probe spanning most of *aprB* and approximately the first 60 bp of *aprA*. As expected, the probe fragment hybridized to an 80 kb EcoRI band in the wild-type (Fig. 2). In DNA of the mutant strain D3, which arose from double-crossover with pNTS35, the predicted two EcoRI fragments of 3·3 kb and 4·7 kb were detected, while in the Km$^\text{r}$ Ap$^\text{r}$ mutant Ap4 the EcoRI bands shifted to 3·3 kb and 16·5 kb, clearly identifying this strain as a single-crossover mutant in which pNTS35 is integrated in the chromosome and both interrupted and wild-type *aprB* are present (Fig. 2). Analysis of HindIII- and PstII-digested DNA confirmed these results (not shown). Results with SalI-digested DNA from strain D3 and strain 25:26, the latter obtained after recombination with pNTS25, showed that the kanamycin resistance cartridge is oriented in the opposite direction in these two strains as expected from the insertion of the kanamycin cassette in the original plasmid vectors (Fig. 2). Bands of 12·9 kb and 8·6 kb, which would have corresponded in size to the autonomously replicating shuttle vectors pNTS25 and pNTS35, respectively, were not detected in EcoRI digests. While the expected fragments were found...
Table 2. Phenotypic characterization of C. vinosum SM50 (wild-type) and the APS-reductase-deficient mutant strain D3

<table>
<thead>
<tr>
<th>Property</th>
<th>Wild-type</th>
<th>Apr⁻ mutant D3</th>
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<tbody>
<tr>
<td>Growth rate on malate and sulphate (h⁻¹)*</td>
<td>0.072</td>
<td>0.075</td>
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<tr>
<td>Specific oxidation rates</td>
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<tr>
<td>[nmol min⁻¹ (mg protein)⁻¹]*</td>
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<td>Sulphide (3 mM)†</td>
<td>114.6</td>
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<td>Intracellular sulphur (2 mM)†</td>
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<td>Thiosulphate (1.5 mM)†</td>
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<td>Sulphite (1.5 mM)†</td>
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<td>Molar growth yields (g protein mol⁻¹)‡</td>
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<td>Sulphite</td>
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<td>Specific activity APS reductase/</td>
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<td>sulphite:acceptor oxidoreductase§</td>
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<td>[mU (mg protein)⁻¹] in cells grown on</td>
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<td>Sulphide</td>
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<tr>
<td>Sulphide + 2 mM tungstate</td>
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<td>Malate + sulphate</td>
<td>8/2.2</td>
<td>0/2.2</td>
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* Means of two independent measurements.
† The initial substrate concentration is given in parentheses.
‡ Means of six independent measurements.
§ Due to nonenzymic artefacts, specific activities of sulphite:acceptor oxidoreductase are only estimates; ND, not determined.

in mutant strains when using the 2.3 kb Km⁺ fragment as a probe, no bands were detected in the wild-type with this probe (not shown). In summary, the Southern blotting analyses clearly confirm that the kanamycin Ω cartridge is correctly integrated in the chromosome of C. vinosum, that conjugation and subsequent selection did not result in spontaneous resistance and that the pSUP301 and pSUP202 vectors are not replicated in C. vinosum.

Since we could prove the absence of the shuttle vectors from C. vinosum recombinants it was possible to distinguish mutant and parent strains by PCR analysis. In addition to the CWECYSC primer, the KM1 primer was designed from a sequence located within the Km⁺ gene. Using these primers, no amplicon was generated from the wild-type, while 1.2 kb and 1.9 kb fragments were amplified from mutants D3 and 28:2, respectively, proving that the Km⁺ gene was inserted in the aprA gene in mutant 28:2, as would have been expected after recombination with plasmid pNTS28 (not shown).

Biochemical and physiological characterization of mutant strains

Single colonies of the different double- and single-crossover C. vinosum mutant strains were tested for the presence of APS reductase activity. Fig. 2(b) shows representative results obtained with the wild-type strain and the double- and single-crossover mutants D3 and Ap4, respectively. Thin-layer chromatography clearly showed that double-crossover mutants with insertions in aprB or aprA were not able to form APS from sulphite and AMP, while the wild-type and single-crossover mutants with the genotype of Ap4 contained APS reductase activity. These experiments demonstrate that interrupting the aprB gene in fact inactivated it. In mutant Ap4 the intact copy of the APS reductase genes is transcribed either from its own or from a plasmid-borne promoter.

In the assimilatory sulphate reduction pathway sulphite is not formed via APS as it is in dissimilatory sulphate reducers (which perform reactions exactly opposite to those found in the photosynthetic sulphur oxidizer C. vinosum) but in an equivalent reaction via 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The reduction of PAPS is catalysed by the enzyme PAPS reductase, which does not exhibit any homologies to APS reductase (Krone et al., 1991; Gisselmann et al., 1992; Niehaus et al., 1992; Speich et al., 1994). As shown above, the product of the genes inactivated in the representative mutant strain C. vinosum D3 was identified as an APS reductase by Southern hybridizations and enzyme assays. To fully exclude the possibility that the aprBA-encoded enzyme has a function equivalent to PAPS reductase in assimilatory sulphate reduction in C. vinosum, growth of mutant D3 on sulphite as the sole source of sulphur was tested. After ten passages on a medium free of reduced sulphur compounds, growth rates of the wild-type and the mutant strain did not differ significantly (Table 2). This clearly shows that the product
Inactivation of APS reductase genes in *Chromatium vinosum*

In order to learn more about the physiological effects of APS reductase deficiency in phototrophic sulphur bacteria, oxidation of various reduced sulphur compounds by the mutant strain D3 and the wild-type strain SM50 was compared. Like the wild-type, the APS-reductase-negative mutant was able to oxidize sulphide, thiosulphate and sulphite under photolithoautotrophic conditions. Neither the specific oxidation rates nor the oxidation patterns and the end products differed significantly for these two strains (Fig. 3, Table 2).

As expected for a classical purple sulphur bacterium (Brune, 1989), sulphide was in a biphasic process first oxidized to intracellular sulphur which in both the wild-type and the mutant strain began to be oxidized to sulphate with rates of 26–27 nmol min⁻¹ (mg protein)⁻¹ after sulphide was depleted (Fig. 3a, b, Table 2). One might have predicted that a defect in APS reductase would abolish oxidation of sulphite, which is formed as an intermediate during sulphur oxidation to sulphate, or at least lower the rate of sulphite oxidation, thus leading to an accumulation of this sulphur compound in the medium. In fact, during growth of *C. vinosum* D3 on sulphide a fuchsin-reactive compound, probably sulphite, appeared in the medium after sulphide was depleted and disappeared again 5–10 h later. However, the concentration reached a maximum of only 22 μM (Fig. 3b). Furthermore, the same concentration was found for the wild-type, indicating that the observed effect was not specific for APS reductase deficiency (Fig. 3a). As expected for *C. vinosum* at neutral to slightly acidic pH (Smith, 1966), both wild-type and mutant strain oxidized thiosulphate partly to sulphate with an intermediary formation of intracellular sulphur and partly to tetrathionate, which was not further metabolized (Fig. 3c, d). *C. vinosum* is one of the few phototrophic sulphur bacteria able to grow on externally supplied sulphite. This ability was not affected in the APS reductase negative mutant (Fig. 4). Under phototrophic conditions cell yields were close to 33 g protein (mol sulphide)⁻¹ and 7 g (mol sulphite)⁻¹ in fed-batch cultures irrespective of the presence of APS reductase in the cells (Table 2). Comparable results were reported by Smith & Lascelles (1966), who also pointed out that the observed growth yields are proportional to the number of electrons released during oxidation of sulphide and sulphite to sulphate (8 and 2 e⁻, respectively).
No significant differences were observed between wild-type and mutant strains in their growth rates on the reduced sulphur compounds tested (Figs 3 and 4).

The experiments described clearly show that replacing the wild-type APS reductase gene of *C. vinosum* with the inactivated gene does not exert any significant effect on the turnover of reduced sulphur compounds under photolithoautotrophic growth conditions. It is therefore concluded that APS reductase is not obligatory for photolithoautotrophic growth on reduced sulphur compounds in this organism and that *C. vinosum* contains an alternative sulphite-oxidizing enzyme, probably sulphite:acceptor oxidoreductase. These results are confirmed by work of Ulbricht (1984, cited in Neutzling et al., 1985), who postulated the presence of sulphite:acceptor oxidoreductase on the basis of protein-chemical data. The conclusions of the present study are furthermore supported by evidence of Fry et al. (1985), who examined sulphur isotope fractionation during sulphite photo-oxidation by *C. vinosum* and found that an inverse isotope effect changed to a normal isotope effect after 20% of the sulphite was oxidized, leading to the speculation that this change might have been due to competition of two different enzymes for the substrate sulphite.

To obtain additional experimental evidence for the presence of sulphite:acceptor oxidoreductase in *C. vinosum*, mutant and wild-type strain were grown with different photosynthetic electron donors and the specific activities of APS reductase and sulphite:acceptor oxidoreductase were determined. As expected, APS reductase was only present in the wild-type (Table 2), exhibiting specific activities comparable to those reported by Schwenn & Bire (1979). A sulphite-dependent ferricyanide reduction in the absence of AMP was observed in all cell extracts tested (Table 2), indicating the presence of sulphite:acceptor oxidoreductase. However, due to non-enzymic artefacts, specific activities could only be estimated. The maximum estimated specific activity amounted to 7-3 mM (mg protein)^{-1} for sulphite-grown cells. Purification and further characterization of sulphite:acceptor oxidoreductase will have to await the development of a more reliable test system. An induction of sulphite:acceptor oxidoreductase in the absence of APS reductase was not observed.

To further test the hypothesis that the potential molybdenum-enzyme sulphite:acceptor oxidoreductase plays an important role in sulphite oxidation in *C. vinosum*, the effect of tungsten, a competitive inhibitor of molybdenum (Giordano et al., 1980), on the sulphite-oxidizing capability of the wild-type and the mutant strain was tested. As shown in Fig. 4 both the wild-type and the APS reductase-deficient mutant showed severely impaired growth on and oxidation of sulphite in the presence of tungsten. Cultures of the wild-type and the mutant containing 0-1 mg protein ml^{-1} excreted a maximum of 1-1 mM sulphite when fed with 1-5 mM Na_2S in tungsten medium (not shown), coinciding with a reduced sulphite:acceptor oxidoreductase activity in strain D3 (Table 2). These findings lend strong support to the assumption that the *C. vinosum* sulphite-oxidizing enzyme, like other bacterial sulphite:acceptor oxidoreductases (Togbrol & Southerland, 1983) contains a molybdenum cofactor. This enzyme cannot be substituted for by APS reductase, since the effect of tungsten on the sulphite-oxidizing capabilities of the wild-type and the mutant are indiscernible. The contribution of APS reductase to photolithotrophic sulphite oxidation in *C. vinosum* is so small that it cannot be detected with the methods applied.

However, it seems unlikely that *C. vinosum* does not benefit from the presence of APS reductase. This enzyme, like sulphite:acceptor oxidoreductase, is constitutive in *C. vinosum* since it is still detectable after prolonged growth in the absence of reduced sulphur compounds (Table 2). The APS reductase pathway allows additional energy conservation and may therefore be of significant importance under energy-limiting conditions. This might be especially true for chemolithotrophic growth, when in the absence of light the sole source of energy is the oxidation of reduced sulphur compounds. It has been shown that under such conditions the specific activity of the enzymes participating in sulphite oxidation increases substantially in *T. roseopersicina* (Ivanovska & Petushkova, 1976; Petushkova & Ivanovski, 1976; Dahl & Trüper, 1989). Work on the co-ordination of the two sulphite oxidation pathways under chemolithotrophic conditions is under way to acquire a better understanding of their *in vivo* function.

**Conclusions**

Sulphite oxidation is central to the sulphur metabolism of phototrophic sulphur bacteria and complicated by alternative routes. Insertional inactivation of the genes encoding APS reductase in *Chromatium vinosum* allowed important insights into the metabolism of this central
intermediate and provided strong evidence for the existence of the molybdoenzyme sulphite:acceptor oxidoreductase in *C. vinosum*. Sulphite:acceptor oxidoreductase appears to be the main sulphite-oxidizing enzyme in *C. vinosum*, whether sulphite is supplied externally or whether it is oxidized as an intermediate in sulphur metabolism. Furthermore, the demonstration in this paper that allelic replacement is possible in *C. vinosum* and possibly other members of the *Chromatiaceae* reveals the potential of this invaluable genetic tool for the investigation of sulphur metabolism and other metabolic pathways in these fascinating organisms.

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