Dissimilatory Sulfite Reductase (Desulfoviridin) of the Taurine-Degrading, Non-Sulfate-Reducing Bacterium *Bilophila wadsworthia* RZATAU Contains a Fused DsrB-DsrD Subunit

HEIKE LAUE,[1]* MICHAEL FRIEDRICH,[2] JÜRGEN RUFF,[1] AND ALASDAIR M. COOK[1]

Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz,[1] and Max-Planck-Institut für Terrestrische Mikrobiologie, D-35043 Marburg,[2] Germany

A dissimilatory sulfite reductase (DSR) was purified from the anaerobic, taurine-degrading bacterium *Bilophila wadsworthia* RZATAU to apparent homogeneity. The enzyme is involved in energy conservation by reducing sulfite, which is formed during the degradation of taurine as an electron acceptor, to sulfide. According to its UV-visible absorption spectrum with maxima at 392, 410, 583, and 630 nm, the enzyme belongs to the desulfoviridin type of DSRs. The sulfite reductase was isolated as an $\alpha_2\beta_2\gamma_n$ ($n \geq 2$) multimer with a native size of 285 kDa as determined by gel filtration. We have sequenced the genes encoding the α and β subunits (*dsrA* and *dsrB*, respectively), which probably constitute one operon. *dsrA* and *dsrB* encode polypeptides of 49 (α) and 54 kDa (β) which show significant similarities to the homologous subunits of other DSRs. The *dsrB* gene product of *B. wadsworthia* is apparently a fusion protein of *dsrB* and *dsrD*. This indicates a possible functional role of DsrD in DSR function because of its presence as a fusion protein as an integral part of the DSR holoenzyme in *B. wadsworthia*. A phylogenetic analysis using the available Dsr sequences revealed that *B. wadsworthia* grouped with its closest 16S rDNA relative *Desulfovibrio desulfuricans* Essex 6.

Bilophila wadsworthia is a strictly anaerobic, gram-negative bacterium (2) which belongs to the family *Desulfovibrionaceae* in the delta subdivision of the *Proteobacteria*, but does not reduce sulfate (2, 25). *B. wadsworthia* has been found quite frequently in patients with appendicitis and its complications and is the third most common anaerobic isolate in such infections (11), but can also be isolated from a wide variety of other infections, e.g., biliary tract infection (41), liver abscess (41), and ear infections (39). *B. wadsworthia* has also been found in the normal fecal flora (2). The organism lacks classical virulence factors like capsules, fimbriae, and extracellular enzymes (2). However, preliminary studies have indicated that *B. wadsworthia* exerts cytotoxic effects on two cell lines, and endotoxic activity of *B. wadsworthia* has been described (2, 34).

We recently isolated from a communal sewage plant a strain of *B. wadsworthia* which utilizes organic sulfonates (e.g., taurine [2-aminoethanesulfonate]) as a carbon source and electron sink. *B. wadsworthia* respires taurine anaerobically with electrons derived mainly from formate oxidation and oxidation of the taurine carbon (25). Taurine is transaminated to sulfoacetaldehyde (22), which is cleaved to sulfite and an unidentified organic product (K. Denger and A. M. Cook, unpublished). Finally, sulfite is reduced to sulfide by a dissimilatory sulfite reductase (DSR) (6, 25). In addition, sulfite or thiosulfate serves as an electron acceptor for anaerobic respiration with formate as the electron donor in *B. wadsworthia* (25).

DSR, a key enzyme in dissimilatory sulfate reduction, occurs in all organisms capable of reducing sulfite during anaerobic respiration investigated so far (9, 33). Otherwise, DSRs are rare. An apparently dissimilatory type of sulfite reductase inducible in the presence of sulfite under anoxic conditions has been found in *Salmonella enterica* serovar Typhimurium, but the function of dissimilatory sulfite reduction by this organism is not clear (16). The sulfite reductase characterized in *Clostridium pasteurianum* was also proposed to be of the dissimilatory type but differs in its properties from DSRs of sulfate-reducing organisms (12). In contrast to DSRs, assimilatory sulfite reductases are involved in assimilation of sulfate in many organisms.

DSRs are multisubunit enzymes (167 to 225 kDa) that catalyze the six-electron reduction of sulfite to sulfide. They all contain siroheme and [4Fe-4S] prosthetic centers and are classified according to their spectroscopic properties in four major groups (47). Sulfite reductases of the desulfoviridin type are found in *Desulfovibrio* species (27). Their subunit structure was initially described as $\alpha_2\beta_2$, with a molecular mass of 50 kDa for the α and 40 kDa for the β subunits (28), but a third subunit, γ (11 kDa), was discovered, and an $\alpha_2\beta_2\gamma_2$ structure was proposed for *Desulfovibrio vulgaris* (Hildenborough), *D. vulgaris oxamicus* (Monticello), *D. gigas*, and *D. desulfuricans* ATCC 27774 (36).

The *dsrA* (α subunit) and *dsrB* (β subunit) genes of DSR have been sequenced completely in six organisms: the sulfate-reducing bacterium *D. vulgaris* (19), the sulfate-reducing archaea *Archaeoglobus fulgidus* (9) and *Archaeoglobus profundus* (21), the thermophilic, gram-positive bacterium *Desulfotomaculum thermocisternum* (21), the sulfur-reducing archaeon *Pyrobaculum islandicum* (33), and the "reverse sulfite reductase" of the phototrophic *Allochromatium vinosum* (14). The γ subunit (*dsrC*) is apparently encoded in a separate locus (18). In

all cases except for *A. vinosum* (14), a third gene, *dsrD*, encoding a protein of unknown function, was found downstream of *dsrB*. Recently, Wagner et al. developed a PCR assay for the specific amplification of large parts of the *dsrA* and *dsrB* genes, which allows the detection of many organisms capable of dissimilatory sulfate reduction (45).

We report here on purification and properties of the DSR that is involved in energy conservation from taurine metabolism in the anaerobic respiration of *B. wadsworthia* RZATAU as well as the relationship of the DsrA and DsrB sequences of *B. wadsworthia* to those of its sulfate-reducing relatives.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. wadsworthia* RZATAU (DSM 11045) was routinely grown in batch culture (0.1 or 10 liters) in an anoxic freshwater mineral salts medium containing 12 mM taurine and 80 mM formate (25). Alternatively, 12 mM isethionate (2-hydroxyethanesulfonate) or 12 mM cysteate (2-amino-3-sulfopropionate) plus formate, 12 mM taurine plus 25 mM pyruvate, or 12 mM thiosulfate plus 20 mM DL-lactate was used. *B. wadsworthia*[T] was grown in taurine-plus-formate medium. *D. vulgaris* was grown in the same salts medium in the presence of 20 mM sulfate and 20 mM DL-lactate. *Desulfovibrio* sp. strain RZACYSA was grown in salts medium containing 10 mM cysteate and 20 mM DL-lactate (25). The sources of the chemicals and gases (N_2 and CO_2) used are given elsewhere (25).

Preparation of cell extracts and enzyme purification. Cells for the purification of DSR were harvested and stored as described elsewhere (23). Preparation of crude extracts by disruption of suspended cells in a French pressure cell, removal of membrane particles by ultracentrifugation, and precipitation of DNA with streptomycin sulfate are detailed elsewhere (17, 23).

DSR from *B. wadsworthia* RZATAU was purified from the cytosolic fraction of the crude extract in a two-step protocol involving anion-exchange and gel filtration chromatography. The 2.5-fold-diluted crude extract was applied to a Mono Q column (HR 10/10; Pharmacia) equilibrated with 20 mM MOPS (morpholinepropanesulfonic acid, pH 6.5) at a flow rate of 2 ml min^{-1}. Proteins were eluted with an increasing linear gradient to 1 M Na_2SO_4 and collected in 5-ml fractions. DSR was identified by its green color, and the identity was confirmed spectroscopically and by red fluorescence under alkaline conditions (37). Enrichment of the protein was monitored by the A_{630}/A_{280} ratio (purity index derived from reference 9; defined as $A_{630}/A_{280} \times 10^{-3}$). DSR eluted at about 130 mM Na_2SO_4 in one fraction and had a purity index of 174. Concentrated protein was loaded onto a Superose 12 column (HR 10/30; Pharmacia) equilibrated at a flow rate of 0.4 ml min^{-1} with 50 mM MOPS (pH 6.5) containing 150 mM Na_2SO_4, and 0.5-ml fractions were collected. The molecular masses of the proteins used to calibrate the column are described elsewhere (23). The purity index of the purified DSR was estimated to be 249.

The presence of DSR in crude extract of *B. wadsworthia* RZATAU grown with different substrates was detected qualitatively by its red fluorescence (37).

Sulfite reductase activity. Formation of sulfide from taurine was investigated in cell extracts from *B. wadsworthia* RZATAU grown with taurine plus formate. The assay was performed in 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM taurine, 5 mM pyruvate, 0.5 mM NAD$^+$, 0.1 mM pyridoxal-5′-phosphate, 0.1 mM thiamine pyrophosphate, and 20 mM formate under anoxic conditions in 16-ml serum bottles closed with butyl rubber septa. The center of the bottle contained a tube with a filter soaked with cadmium(II) acetate (10%, wt/wt) and NaOH (10%, wt/wt). The reaction was started by addition of 0.5 mg of protein, and the bottles were incubated on a shaker at 30°C. Sulfide was determined in aliquots of the reaction mixture or in the filter by the formation of methylene blue (5).

Gel electrophoresis and N-terminal sequence analysis. Proteins were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) according to the method of Laemmli (20) or of Schägger and Jagow (38) and subsequently stained with Coomassie brilliant blue G-250 (35). Blotting and N-terminal sequencing were done as described elsewhere (23).

Analytical methods. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard. UV-visible (UV/VIS) spectra were recorded by an Uvikon 922 spectrophotometer (Kontron). Taurine and alanine were quantified by high-pressure liquid chromatography after derivatization with 2,4-dinitrofluorobenzene (10, 26). The G+C content of *B. wadsworthia*[T] was determined by the German Culture Collection (Braunschweig, Germany) in a sample of 0.8 g (wet weight) of cells.

Isolation of nucleic acids and DNA amplification procedures. Total DNA was prepared from stationary-phase cultures of *B. wadsworthia* RZATAU (0.5 liter), *D. desulfuricans* (0.5 liter), or *Desulfovibrio* sp. strain RZACYSA (0.2 liter) by the cetyltrimethylammonium bromide precipitation method (1). The primer pair DSR1F and DSR4R was used to amplify by PCR a 1.9-kb DNA fragment encoding most of the α and β subunits of DSR (45). PCR was performed as described previously (45) except for the buffer [2.25 mM MgCl$_2$, 50 mM Tris-HCl (pH 9.2), 14 mM (NH$_4$)$_2$SO$_4$, 10% dimethyl sulfoxide] and for the *Taq* polymerase (MBI) in a Master Cycler gradient thermocycler (Eppendorf). Washed and concentrated cells of *B. wadsworthia* RZATAU were also added directly to the PCR mixtures instead of purified DNA as the template.

PCR amplification of the adenosine phosphosulfate (APS) reductase genes with degenerate primer sets (wh53, wh54, and wh62) involved the thermal profile described by Hipp et al. (14). The reaction buffer described above for the DSR primer set was used. DNAs from *D. desulfuricans* and from *Desulfovibrio* sp. strain RZACYSA were used as positive controls.

DNA sequencing and analysis. The nucleotide sequence of the 1.9-kb PCR product from the *dsr* region was determined by cycle sequencing and primer walking using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 DNA sequencer (GATC GmbH). We then used adaptor-ligated PCR to obtain the complete sequences of the genes encoding the α and β subunits of DSR. Genomic DNA was digested with different restriction enzymes and ligated to an adaptor of known sequence (Universal Genome Walker kit; Clontech). Nested PCR was done with the Advantage *Tth* polymerase mix (Clontech) and primer deduced from specific *dsrA* or *dsrB* gene sequences in combination with the adaptor primer. The *dsr* primer sequences for amplification of the upstream region were CAT GCA CGG TTC CAC CGG CGA CAT CGT GCT (primary PCR) and GGA GCC ACG GAG TTC CCA AAT GTC GCA CAG (nested PCR). To amplify the 3′ end of *dsrB*, two adaptor-ligated PCRs were done successively using primers GCG CCG TGC ACT GCT CCG ACA TCG GTA TCG (primary PCR) and GGT ATC CAC CGC AAG CCT CCG ATG ATC GAC (nested PCR) for the first and AGG ACT TCC TTG AAC TCT TCC CCA CCA AGG (primary PCR) and GTC CGT GCT CGT GTC CGA AGA GTC TCT GGA (nested PCR) for the second PCR. Upstream of the 1.9-kb sequence, a 1.8-kb amplification product was obtained, while downstream a 0.9-kb DNA fragment was amplified and sequenced. Sequence alignments were done using ClustalX (44).

Phylogeny of DsrA and DsrB. The phylogenetic analysis (i.e., sequence alignments and treeing) was performed by using the ARB software package (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München, Munich, Germany [http://www.biol.chemie.tu-muenchen.de/pub/ARB/]). Deduced DSR amino acid sequences were fitted manually into an alignment of DSR sequences retrieved from public databases (3) using the Genetic Data Environment (version 2.2) as implemented in the ARB software package. Prior to treeing analysis, amino acid frequency filters (20 to 100% sequence similarity) were generated for a concatenated data set comprising the amino acid sequences of the α (364 positions) and β (238 positions) subunit data sets. Treeing was performed on the concatenated α and β subunit data sets using distance matrix analysis [FITCH (PHYLIP version 3.5) and neighbor-joining (ARB)], parsimony [PROTPARS (PHYLIP version 3.5)], and maximum likelihood [PROTML (PHYLIP version 3.5)] as outlined previously (45). Bootstrap analysis (100 resamplings) was performed using parsimony analysis as implemented in the PHYLIP package.

Nucleotide sequence accession numbers. The sequences encoding the α and β subunits of DSR from *B. wadsworthia* RZATAU (accession no. AF269147) and *D. desulfuricans* (accession no. AF273034) have been deposited in GenBank.

RESULTS

Activity and purification of DSR from *B. wadsworthia* RZATAU. In cell extracts of *B. wadsworthia* RZATAU, 1.6 mM alanine and 80 μM sulfide were formed from taurine (5 mM) and formate (20 mM). However, sulfide was only detectable when trapped from the gas phase with cadmium acetate. In the absence of taurine, negligible amounts of sulfide were formed. We thus presume that there is DSR activity in the extract but that sulfide reacts with other components in the aqueous mixture (13) and that formation of sulfide is underestimated. The enzyme rapidly lost activity, so its purification depended on assaying its physical properties. DSR was detected by fluorescence in crude extracts of cells grown with taurine plus formate
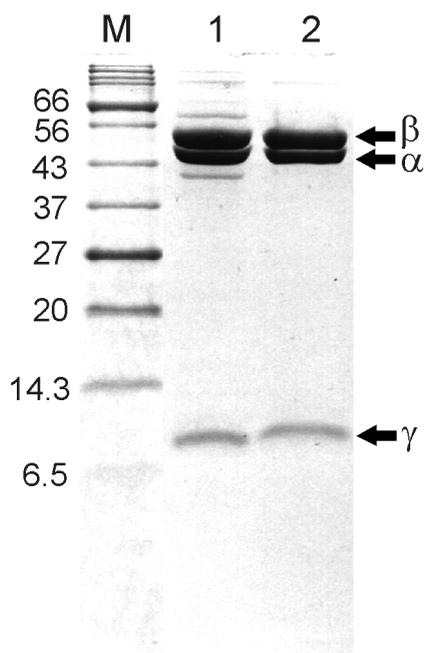
FIG. 1. Purification of DSR from *B. wadsworthia* RZATAU monitored by SDS-PAGE. Protein was separated on an SDS–12% PAGE gel and subsequently stained with Coomassie brilliant blue. Lanes: M, molecular mass standards (in kilodaltons); 1, fraction from Mono Q chromatography (10 μg); 2, fraction from gel filtration on Superose 12 (10 μg).

or pyruvate, cysteate or isethionate plus formate, thiosulfate plus lactate, or pyruvate as the sole carbon and energy source. We purified DSR from a soluble extract of *B. wadsworthia* RZATAU grown with taurine and formate. The protein from anion-exchange chromatography was about 90% pure, and apparent homogeneity was obtained by gel filtration chromatography (not shown). Based on the purity index (A_{630}/A_{280}), it can be calculated that DSR was purified 11-fold and represents about 9% of the soluble protein of *B. wadsworthia*.

Molecular properties. SDS-PAGE of the protein showed three bands with apparent molecular masses of 53, 49, and 11 kDa (Fig. 1), so DSR seems to be composed of three different subunits. The protein eluted from gel filtration under nondenaturing conditions with an apparent molecular mass of 285 kDa.

DSR had a UV/VIS spectrum with absorption maxima at 392, 410, 583, and 630 nm (not shown), characteristic of de-

sulfoviridin (27). The molar extinction coefficients of *B. wadsworthia* DSR at 392, 410, 583, and 630 nm were estimated to be 5.5×10^5, 5.8×10^5, 1.1×10^5, and 2.0×10^5 M^{-1} cm^{-1}, respectively. Under alkaline conditions, the enzyme showed red fluorescence, also characteristic of desulfoviridin (37).

The N-terminal sequences of the three subunits were determined by Edman degradation (Table 1). A comparison was done with the amino acid sequence of the subunits of the DSRs from *D. vulgaris* and *D. desulfuricans* (Table 1). The N-terminal sequences of the α, β, and γ subunits of the three organisms were each highly conserved. The data indicated, however, that the largest subunit (53 kDa) from *B. wadsworthia* exhibited similarities to the β subunit from *D. vulgaris* (40 kDa) and *D. desulfuricans* (45 kDa), whereas the 49-kDa subunit of the *B. wadsworthia* enzyme was similar to the α subunits (50 kDa) from the same organisms.

Nucleotide sequence analysis. A 1.9-kb DNA region encoding most of the α and β subunits of DSR was amplified by PCR from *B. wadsworthia*. The sequence showed high similarities to those determined for *D. vulgaris* and other organisms. This was in contrast to the different sizes of the β subunits (53 kDa rather than 40 to 45 kDa; Table 1), so we amplified and sequenced the complete *dsrAB* region. A total of 4.3 kb of double-stranded sequence was examined. It comprised three open reading frames, ORF1, *dsrA*, and *dsrB*. The gene *dsrA* (1,317 bp) was identified because the deduced amino acid sequence included the N-terminal sequence that we observed in the α subunit. Similarly, *dsrB* (1,452 bp) was identified because the N-terminal amino acid sequence of the β subunit corresponded to the deduced sequence.

In contrast to the published value for the G+C content of *B. wadsworthia*^{T} (39 to 40 mol% [2]), we now report the G+C content to be 59.2%, so the G+C content of *dsrA* and *dsrB* (59.4 and 60.2%, respectively) corresponds to the overall G+C content of the organism. A putative promoter sequence was located 123 nucleotides upstream of the translational start of the *dsrA* gene, and a putative transcription terminator was found downstream of the stop codon of *dsrB*. The start codons of *dsrA* and *dsrB* are both preceded by a putative ribosome-binding site (not shown). The intergenic distance between the 3′ end of the *dsrA* and the 5′ end of the *dsrB* gene comprised 18 nucleotides, analogous to the *dsr* operons of *D. vulgaris* and *A. fulgidus* (19).

The amino acid sequences deduced from *dsrA* and *dsrB* consisted of 438 and 483 residues, respectively, but the initiat-

TABLE 1. Alignment of the N-terminal amino acid sequences of DSR subunits (α, β, and γ) determined by Edman degradation from *D. vulgaris* Hildenborough, *D. desulfuricans* Essex 6, and *B. wadsworthia* RZATAU[a]

Subunit	Size (kDa)	Organism	Sequence	Reference
α	50	*D. vulgaris*	A—K H A T P K L D Q L E S G P W X S F V v d k k	36
	50	*D. desulfuricans*	A—K H A T P L L D Q L E S G P W P S F V	40
	49	*B. wadsworthia*	A I K H P T P L L D Q L E T G P W P X F V S	This study
β	40	*D. vulgaris*	A F I S S G Y N P E K p m a n	36
	45	*D. desulfuricans*	A F I P T G Y N P X K P M	40
	53	*B. wadsworthia*	A F V S S G Y N P E K P M E G R I S D I	This study
γ	11	*D. vulgaris*	A E V T Y K G K S F E V D E D G F L L R F D D W	18
	11	*D. desulfuricans*	A E I T Y K G K	40
	11	*B. wadsworthia*	A E V T Y K G K T F E V D E D G F L L K F D d	This study

[a] Identifications in lowercase letters are uncertain. X, residue not identified; —, alignment gap.

TABLE 2. Sequence similarities of DSR α and β subunit gene fragments (*dsrA* and *dsrB*) and their deduced amino acid sequences (DsrA and DsrB)[a]

Strain	Sequence similarity (%)						
	B. wadsworthia	*D. desulfuricans*	*D. vulgaris*	*Desulfovibrio* sp. strain PT-2	*D. multivorans*	*D. sapovorans*	*D. latus*
B. wadsworthia	100	82.4/81.4	83.6/82.3	80.1/81.1	74.7/72.2	72.8/70.1	71.4/69.2
D. desulfuricans	88.3/86.0	100	82.9/81.1	82.1/80.7	70.6/71.4	71.0/68.1	69.2/67.8
D. vulgaris	86.4/83.9	84.1/83.3	100	89.9/86.7	75.2/73.8	76.1/70.4	73.3/70.7
Desulfovibrio sp. strain PT-2	80.9/82.0	81.6/83.7	87.2/87.3	100	71.8/73.1	73.4/71.7	71.3/71.4
D. multivorans	74.4/73.3	75.1/73.9	77.1/75.3	76.7/75.3	100	85.8/77.8	81.2/73.8
D. sapovorans	73.3/71.3	72.5/72.7	76.6/74.1	73.5/72.9	76.3/71.2	100	83.0/76.5
D. latus	73.3/70.4	70.0/70.2	74.4/70.9	72.4/70.4	78.0/73.5	76.5/73.3	100

[a] Alignment positions of unambiguously determined nucleotides represented in all sequences were used for pairwise comparisons of *dsrA* (968 positions), *dsrB* (668 positions), DsrA (322 positions), and DsrB (224 positions). Values for DsrA/*dsrA* are below the diagonal, and those for DsrB/*dsrB* are above the diagonal.

ing methionine residue was obviously removed from each protein after synthesis. The derived sizes of the α (49.0 kDa) and β (53.6 kDa) subunits are in good agreement with the data determined for the purified sulfite reductase (49 and 53 kDa, respectively). The isoelectric points calculated for DsrA and DsrB were 5.3 and 6.5, respectively.

The third open reading frame, ORF1 (711 bp), was located at the 5′ end of the 4.3-kb DNA fragment, 618 bp upstream of the translational start codon of *dsrA*. Parts of the deduced amino acid sequence exhibited similarities to the rare lipoprotein A (*rlpA*) from *Escherichia coli* (39% identity) (42). The region between ORF1 and *dsrA* contained no open reading frames of more than 130 bp in length.

Sequence similarities. The deduced amino acid sequences of *dsrA* and *dsrB* were highly similar to the α and β subunits of DSRs from other sulfate-reducing microorganisms (Table 2). Analysis of partial DSR sequence data (available via the ~1.9-kb DSR PCR fragment) revealed that DsrA of *B. wadsworthia* RZATAU was most similar to that of *D. desulfuricans* Essex 6 on both the nucleotide and deduced amino acid levels (88.3 and 86.0%, respectively; Table 2), whereas DsrB was slightly more similar to that of *D. vulgaris* (83.6 and 82.3%, respectively; Table 2).

Full amino acid sequences (all positions) of DsrA were 84% identical to DsrA from *D. vulgaris* (19) and 57% identical to DsrA from *A. fulgidus* (9). Amino acids 1 to 381 of DsrB exhibited 83% identity to DsrB from *D. vulgaris* (19) and 56% identity to DsrB from *A. fulgidus* (9), while the C-terminal amino acids exhibited no similarity to other DsrBs. However, the C-terminal amino acids 405 to 483 of DsrB were 59% identical to DsrD from *D. vulgaris* (19), 42% identical to DsrD from *Desulfotomaculum thermocisternum* (21), and 46% identical to DsrD from *A. fulgidus* (9) (Fig. 2). Amino acids 382 to 404 of the *B. wadsworthia* DsrB showed similarities neither to other DsrBs nor to other DsrDs. Between the 3′ end of *dsrB* and the 5′ end of *dsrD* in *D. vulgaris* and *A. fulgidus* are 58 and 41 bp of intergenic sequence, respectively.

Phylogeny of DsrA and DsrB. Phylogenetic trees for the DSR α and β subunits (not shown) and for a region representing α and β subunits (Fig. 3) were estimated from deduced amino acid data sets of the ~1.9-kb PCR product by distance matrix, parsimony, and maximum-likelihood methods. Consistently, *B. wadsworthia* RZATAU grouped with its closest 16S rDNA relative *D. desulfuricans* Essex 6 (91.5% 16S rDNA sequence similarity). This tree topology was supported by all

treeing algorithms utilized, by high bootstrap scores, and by individual analyses of both subunits as well as analysis of the concatenated α and β subunit data sets.

Investigation of presence of genes encoding APS reductase. PCR conditions and primer sets (14) to amplify regions of the *apr* genes (encoding APS reductase) were applied to investigate the presence of these genes in *B. wadsworthia*. However, we did not obtain specific PCR fragments of the *apr* gene in *B. wadsworthia*, though in the *Desulfovibrio* control strains the corresponding 1.6- or 2.2-kb products were detected. The presumed absence of the *apr* genes is also reflected by the fact that *B. wadsworthia* is not able to reduce sulfate (6).

DISCUSSION

DSR was purified from *B. wadsworthia* as an inactive protein which we initially identified by its spectral and fluorescence properties and confirmed by sequence homologies. No other green protein was detected in separated extracts (H. Laue, unpublished results). We detected three subunits, α (49 kDa), β (53 kDa), and γ (11 kDa) (Fig. 2), and we interpret the native structure to be $\alpha_2\beta_2\gamma_n$ ($n \geq 2$), as suggested for *D. desulfuricans* Essex 6 (40). The function of the 11-kDa γ subunit that was copurified in most sulfite reductases (36) except that from *A. fulgidus* (9) is not clear at present. Whereas Pierik et al. reported that the γ subunit was tightly associated in the DSR of *D. vulgaris* (36), it was shown that in *D. desulfuricans* Essex 6, this subunit can be separated during gel filtration, which indicated a less tight association of DsrC with the α and β subunits in that organism (40). The presence and position of the putative promoter 123 nucleotides upstream of the translational start of the *dsrA* gene and putative termination sequences downstream of the stop codon of *dsrB* indicate that *dsrA* and *dsrB* constitute a single transcription unit and that the genes are coordinately expressed.

The order and sequences of the *dsrAB* genes of DSR from bacteria and archaea are highly conserved. Whereas in all organisms sequenced so far except *A. vinosum*, the operon consists of *dsrA, dsrB,* and *dsrD*, in *B. wadsworthia* there are only two genes, *dsrA* and *dsrB*. Apparently, the *dsrB* gene product of *B. wadsworthia* is a fusion of *dsrB* and *dsrD*. A function for DsrD has not been detected in earlier biochemical work (19); however, the presence of DsrD as an integral part of the DSR holoenzyme in *B. wadsworthia* as a DsrB-DsrD fusion suggests a possible involvement in DSR function. This hypothesis is

A

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MAFVSSGYNPEKPMEGRISDIGPRKYDSFFPEVIKKNFGKWLYHEILEPGVLVHVAESGD 60
MAFISSGYNPEKPMANRITDIGPRKFDEFFPPVIAKNFGSWLYHEILEPGVLMHVAESGD 60
***:********** **:******:* *** ** **** ************:*******

KVYTVRCGGTRTMSVTNIREICEIADKYCDGHVRWTTRNNIEFMVTDEATLKALKEDLAG 120
KVYTVRVGAARLMSITHIREMCDIADKYCGGHLRFTTRNNVEFMVADEASLKALKEDLAS 120
****** * :* **:*:***:*:****** **:*:***** :****:***:********

RKFAAGSYKFPIGGTGAGVSNIVHTQGWVHCHTPATDASGPVKAVMDTMFEEFKNMRLPA 180
RKFDGGSLKFPIGGTGAGVSNIVHTQGWVHCHTPATDASGPVKAIMDEVFEDFQSMRLPA 180
*** ** ***************************************:** :**:*: *****

PVRISLACINMCGAVHCSDIGIVGIHRKPPMIDDQWVDQLCEIPLAVAACPTAAVRPVK 240
PVRISLACINMCGAVHCSDIGVVGIHRKPPMIDHEWTDQLCEIPLAVASCPTAAVRPTK 240
********************:*********** :* **********:.******* *

SEHDGKKVNSVAIKQDRCMYCGNCYTMCPALPISDGEGDGIALMVGGKVSNRISMPKFSK 300
LEIGDKKVNTIAIKNERCMYCGNCYTMCPALPISDGEGDGVVIMVGGKVSNRISMPKFSK 300
 * ****:.***:.:**************************: .***************

VVVAYIPNEPPRWNTLTSTIKHIVEVYSENANKYERLGDWAERIGWESFFELTGLEFTHH 360
VVVAYIPNEPPRWPSLTKTIKHIIEVYSANAYKYERLGEWAERIGWERFFSLTGLEFSHH 360
************* :** *****:**** ** ******:********* ** ******:**

LIDDFRDPAYYTWRQSTQFKFSELALAAHGGEAHEAASAAEVTAEDKEIVVNFLKDKMSR 420
LIDDFRDPAYYTWRQSTQFKF--------------------------------------- 381
********************

PGAKTKYYFKDFLELFPTKGTRDVKNVLSVLVSEESLEYWSSGSTTMYGLKGAGKQASSE 480
------------------------------------------------------------
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GEN 483

B

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Bw   SELALAAHGGEAHEAASAAEVTAEDKEIVVNFLKDKMSRPGAKTKYYFKDFL----ELFP 437
Dv   --------------------MEEAKQKVVDFLN---SKSGSKSKFYFNDFT----DLFP 32
Dt   -MKTPAQVTSYIYELERVGLSVEEIKKAIIEFAS-----NSKKTRFYFKDMEKAVQEKIP 54
Af   ---------------MADYT--EEDKQKVLAHVS--------KKTWKIPELA-----KIL 30
Ap   --------------MAEEINKEELKQKVIEYLQ--------KKPWQVRDLA-----KIL 32
                              * *: ::              *    ::        :

Bw   TKGTRDVKNVLSVLVSEES-LEYWSSGSTTMYGLKGAGKQASSEGEN- 483
Dv   DMKQREVKKILTALVNDEV-LEYWSSGSTTMYGLKGAGKQAAAEHED- 78
Dt   TAKAREIKKAASELVNEGT-LIYFSTGSTTMYGLK--ERCASDEPQQ- 98
Af   KMDKKVVKKIVQDLINEGV-AGYWSSGSTTYVATKEYIEELEKKRAEG 77
Ap   RVKKKELDKIVQELINEGK-AAYWSSGSTTYITTPEKLKEMEEKRSGI 79
        : : :     *: :     *:*:****          :
```

FIG. 2. Sequence comparisons with DSR from *B. wadsworthia* RZATAU. (A) Amino acid sequences derived from *dsrB* (upper line) aligned with those of DsrB from *D. vulgaris* (lower line). The amino acid sequence positions are indicated in the margins. Highly conserved cysteine residues proposed to coordinate siroheme-[4Fe-4S] binding (9) are indicated by boxes. Amino acids which do not show any homologies are in boldface. (B) Alignment of amino acids 405 to 483 of DsrB from *B. wadsworthia* DSR (Bw), with DsrD from *D. vulgaris* (Dv), *Desulfotomaculum thermocisternum* (Dt), *A. fulgidus* (Af), and *A. profundus* (Ap). Conserved lysine and arginine residues are indicated by boxes; a conserved stretch of eight amino acids is shown in boldface. ∗, positions with a conserved residue; :, positions with a conservative replacement.

further corroborated by the high degree of conservation of DsrD sequences (Fig. 2) among the microorganisms sequenced so far, which suggests an essential role of this protein in dissimilatory sulfite reduction in general. Because of its high content and significant conservation of Lys residues among DsrDs, a function as a sulfite-binding protein was proposed (19), but

spectroscopic analysis of DsrD indicated that it bound neither sulfite nor sulfide (15). Alignment of DsrD from five different organisms shows in addition to the conserved Lys residues, a short, remarkably conserved stretch of eight amino acids (Y W/F S S/T G S T T) (Fig. 2) (21). Recently, DsrD from *D. vulgaris* Hildenborough has been crystallized, and preliminary
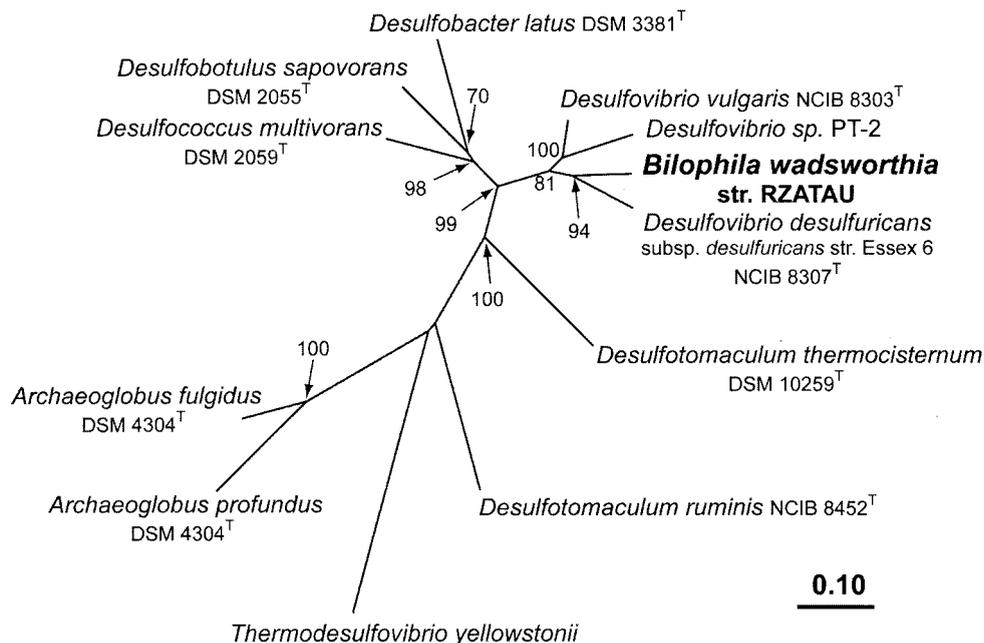
FIG. 3. Phylogenetic tree reflecting the relationship of the *B. wadsworthia* RZATAU DSR to the DSRs from *Desulfovibrio* sp. and other sulfate-reducing microorganisms (21, 45). Tree topology was estimated by using FITCH distance matrix analysis of the concatenated α and β subunit amino acid data sets (546 positions, including *D. vulgaris* positions 73 to 421 [DsrA] and 18 to 257 [DsrB]). Parsimony analysis was used to determine bootstrap values with an identical data set. Bootstrap values are only indicated for branches, which were recovered in the majority of bootstrap replicates (>50%). The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length. GenBank accession numbers used: U58114, U58115, U58118 to U58127, M95624, AF071499, AF074396, U16723, AF273034, and AF269147.

results concerning the crystal structure have been described (32). The forthcoming high-resolution three-dimensional structure may provide a clue to the function of DsrD.

The stretch of 23 residues between the sequences homologous to *dsrB* and *dsrD* in the DSR of *B. wadsworthia* revealed no homologies to these genes. The presence of several small residues like alanine (nine residues) and glycine (two residues) may indicate a function as a linker between the DsrB and DsrD domains, possibly necessary to allow correct assembly.

DSRs usually contain two sirohemes and additional iron-sulfur clusters, possibly four of the [4Fe-4S] type (9). By sequence alignment of different siroheme [4Fe-4S]-binding proteins, Dahl et al. identified highly conserved cysteine-containing clusters $(C-X_5-C)-X_n-(C-X_3-C)$ proposed to coordinate siroheme-[4Fe-4S] binding (9). This arrangement was also present in the predicted DsrA and DsrB amino acid sequence from *B. wadsworthia*, which indicates the same content of siroheme. Analogous to DsrB from *A. fulgidus* (9) and *D. vulgaris* (19), the first Cys residue of this motif in DsrB is replaced by a Thr residue (Fig. 2), so that the α subunit is most likely to bind siroheme, with no binding of the β subunit. Crane et al. showed that based on the crystallographic structure of *E. coli* sulfite reductase hemoprotein (8), key residues important for stability and function of siroheme-containing sulfite and nitrite reductases are clustered in five homology regions, H1 to H5 (7). As has been shown for the α and β subunits of DSR from *D. vulgaris* (7), the corresponding subunits of the *B. wadsworthia* enzyme also contained conserved sequences belonging to the homology regions.

The codon usage of the *dsr* genes of *B. wadsworthia* is very similar to the codon usage of the *dsr* genes of *D. vulgaris*, e.g.,

the arginine codons in *B. wadsworthia* are, as in *D. vulgaris* (19), almost exclusively CGT or CGC (41 of 43 codons). This is also reflected by the nearly identical G+C content of these genes (60.4% for *D. vulgaris* [19] and 59.8% for *B. wadsworthia*). *B. wadsworthia* differs in the prevalence of GAA for glutamate (65 of 70 codons) from *D. vulgaris*, which utilizes the codons GAA and GAG (36 and 24 of 60 codons, respectively).

The similar codon usage is perhaps not surprising, because *B. wadsworthia* is phylogenetically a member of the *Desulfovibrionaceae*, and its nearest defined neighbor is *D. desulfuricans*[T] (25). The phylogeny is based on the similarity of the 16S rDNA sequences, which is not reflected in the published G+C contents of these bacteria, 59% for *D. desulfuricans*[T] (46) and 40% for *B. wadsworthia*[T] (2). The latter value was obtained when cells had to be harvested from plates and worked up for melting points (2), whereas the recent discovery of ready growth in liquid culture facilitates high growth yields of metabolically active cells (22, 23), from which DNA was separated and hydrolyzed, and the monomers were subjected to chromatographic separation and determination. The newly obtained value (59.2%) for the G+C content corresponds to the G+C content of the *B. wadsworthia* genes sequenced in this work and elsewhere (22, 23) and is consistent with the values for the family *Desulfovibrionaceae*, e.g., 59% for *D. desulfuricans* and 66% for *D. vulgaris* (46).

One might speculate that *B. wadsworthia*, which does not reduce sulfate, was once a sulfate reducer and that the capacity for sulfate reduction was lost. The coupling of putative sulfite generation from organosulfonates with energy conservation involving DSR is well known in *Desulfovibrio* spp. (24, 29, 30) as it is in *B. wadsworthia* (25). What probably distinguishes

most *Desulfovibrio* spp. from *B. wadsworthia* is clinical importance (2, 11, 39, 41), though recent papers suggest pathogenic roles for some *Desulfovibrio* spp. (31, 43).

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