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Characterization of the desulforubidin operons from *Desulfobacter vibrioformis* and *Desulfobulbus rhabdoformis*

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Abstract

The genes encoding the desulforubidin type of dissimilatory sulfite reductase (Dsr) from the sulfate-reducing bacteria *Desulfobacter vibrioformis* and *Desulfobulbus rhabdoformis* were cloned and sequenced. Similar to the genes for dissimilatory sulfite reductase from the genera *Archaeoglobus*, *Desulfovibrio* and *Desulfotomaculum* the *dsr* genes were found to form an operon, *dsrABD*, where *dsrA* and *dsrB* encode the structural subunits, α and β, of Dsr, respectively. *dsrD* encodes a conserved unknown protein apparently restricted to sulfate-reducing species. In *Desulfobacter vibrioformis* a fourth gene, designated *dsrN*, was found downstream of *dsrD* forming a contiguous operon, *dsrABDN*. DsrN showed significant sequence homology to cobyrinic a,c-diamide synthase, which is involved in the biosynthesis of vitamin B12. A function for DsrN in amidation of siroheme is likely. Analysis of the *dsrAB*-encoded proteins confirmed that the high conservation observed for other types of dissimilatory sulfite reductase is also found in desulforubidin. The use of Dsr sequences in unravelling the phylogeny of sulfate-reducing bacteria is discussed.

Keywords: *Desulfobacter vibrioformis*; *Desulfobulbus rhabdoformis*; Dissimilatory sulfite reductase; Cobyrinic acid a,c-diamide synthase; Siroamide; Dsr

1. Introduction

Dissimilatory sulfite reductase (Dsr) is a key enzyme in sulfate-reducing prokaryotes catalyzing the reduction of sulfite to sulfide, an important step in the anaerobic sulfur cycle. Together with assimilatory sulfite reductase and nitrite reductase it forms a distinct family of proteins containing siroheme and iron–sulfur clusters as prosthetic groups [1]. The recently characterized sulfite reductases from *Allochromatium vinosum*, a phototrophic sulfur bacterium, and *Pyrobaculum islandicum*, a hyperthermophilic thiosulfate-reducing archaeon, also belong to this protein family although they are clearly separated phylogenetically from the Dsr sequences of sulfate reducers [2,3].

Despite biochemical differences, sequencing of *dsrAB* has revealed that Dsr is a highly conserved enzyme, with very high sequence identities between distant genera [4–6]. Dsr sequences thus have a potential as molecular markers for detection of sulfate reducers in environmental samples. Partial sequence information from a growing number of sulfate reducers is now becoming available using conserved primers for amplification of parts of *dsrA* and *dsrB* by PCR [5]. Recent characterization of Dsr sequences amplified directly from environmental samples has clearly demonstrated the usefulness of the conserved *dsr* genes for analysis of natural communities of sulfate reducers [7,8]. The entire *dsr* operon, however, has so far only been completely sequenced for four sulfate-reducing species, *Archaeoglobus fulgidus* [9], *Archaeoglobus profundus* [6], *Desulfovibrio* (*Dvi.*) *vulgaris* [4], and *Desulfotomaculum* (*Dtm.*) *thermocisternum* [6]. To further characterize Dsr operons and proteins we have cloned and sequenced the genes encoding the desulforubidin type of Dsr from *Desulfobacter* (*Dba.*) *vibrioformis* and *Desulfobulbus* (*Dbu.*) *rhabdoformis*, thereby providing the first complete sequences of desulforubidin. A phylogenetic analysis of Dsr, including partially sequenced PCR fragments, has been performed.

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2. Materials and methods

2.1. Bacterial strains and plasmids, media and growth conditions

Dbu. rhabdoformis M16 (DSM 8777^T) was grown as previously described [10]. *Escherichia coli* XL1-Blue MRF′ (Stratagene) was used as host for λZAP Express (Stratagene) and pGEM-T (Promega) derivatives. *E. coli* XLOLR (Stratagene) was the host for pBK-CMV phagemid derivatives and *E. coli* TOP10 (Invitrogen) was used as host for pCR®4-TOPO (Invitrogen) derivative. All *E. coli* strains were grown as recommended by the suppliers. A previously constructed λZAP Express library [11] was used for cloning of *dsrAB* from *Dba. vibrioformis* B54 (DSM8776^T).

2.2. Cloning and sequencing strategy

The presence of a single copy of *dsr* in *Dba. vibrioformis* and *Dbu. rhabdoformis* was confirmed by Southern analysis using a PCR fragment of *dsr* from *Dvi. vulgaris* as a probe (results not shown). Chromosomal DNA from *Dbu. rhabdoformis* was isolated as previously described [12]. The λZAP Express libraries were screened for recombinant phages as previously described [6]. The 5′-ends of the *dsr* operon could, however, not be isolated from these libraries. In order to obtain the 5′-end of the *dsr* operon from *Dba. vibrioformis*, genomic DNA was digested with various restriction endonucleases. Then a probe, BR10, was derived from the λZAP fragment and used in Southern analysis. It was found that the *dsr* operon was located on a 5-kb *Pst*I fragment (not shown). This fragment was extracted from an agarose gel using the QIAEX II DNA extraction kit (Qiagen) and ligated into pUC19 and sequenced. The 5′-end of the *dsr* operon from *Dbu. rhabdoformis* was obtained through a combination of PCR using conserved primers against *dsr* [5] and Southern analysis using a λZAP-derived probe specific for *Dbu. rhabdoformis*. A 3.5-kb *Pst*I fragment harboring *dsr* was isolated as above and self-ligated using T4 DNA ligase (Promega). This circular DNA fragment was then used as template in inverse PCR. The amplified PCR product was purified using the Stratagene PCR purification kit, cloned into pCR®4-TOPO (Invitrogen) and sequenced. DNA sequencing was carried out using the BigDye Terminator Cycle Sequencing Ready Reaction Kit with an ABI Prism 377 DNA Sequencer (PE Applied Biosystems) and analyzed using the Wisconsin program package v. 8.1 (Genetics Computer Group (GCG), Madison, WI, USA).

The nucleotide sequences have been deposited in the EMBL nucleotide sequence database with the accession numbers AJ250472 and AJ250473.

2.3. Phylogenetic analysis

Phylogenetic analyses were performed on partial sequences. A total of 187 and 179 unambiguously aligned amino acid residues were used for constructing phylogenetic trees for DsrA and DsrB, respectively. Parsimony analysis was conducted using the PROTPARS program included in the PHYLIP package [13]. Protein distances were calculated using the ClustalX program [14]. Protein maximum-likelihood trees were estimated with the PUZZLE 4.0.2 program with the JTT protein replacement model and a mixed eight-category discrete gamma-plus-invariant-site heterogeneity rate model [15]. Bootstrap analyses were performed with 1000 resamplings.

3. Results and discussion

3.1. Molecular characterization of the dsr operons

A total of 6189 bp were sequenced from *Dba. vibrioformis* (Fig. 1). BLASTX [16] searches identified three open reading frames (ORFs) with high sequence identity to *dsrA*, *B* and *D* from other sulfate reducers and to *dsrA* and *B* from *A. vinosum* and *P. islandicum* (Table 1). Immediately downstream of *dsrD* a fourth gene, *dsrN*, was identified. DsrN showed significant homology to cobyrinic acid a,c-diamide synthase, with a *P*-value of 8×10^{-58} to the enzyme from *Pseudomonas denitrificans*. Only 18 bp separate *dsrD* and *dsrN*, indicating that they are transcriptionally coupled. Two putative σ^{70} promoter sequences, TTGACAN$_{17}$TATATC and TTGAAAN$_{16}$TATTGA,

Table 1
Percent sequence similarities of complete DsrA and DsrB subunits (α subunit/β subunit)

	Dvivul	Dbavib	Dburha	Dtmthe	Aglful	Aglpro	Achvin
Dbavib	82/86						
Dburha	78/74	77/74					
Dtmthe	74/76	72/76	72/77				
Aglful	66/68	66/65	67/67	63/67			
Aglpro	63/66	62/63	63/65	61/67	81/87		
Achvin	56/56	56/55	59/55	53/56	60/59	57/58	
Pyrisl	41/46	42/44	43/47	40/49	47/48	45/48	41/44

Dvivul, *Desulfovibrio vulgaris*; Dbavib, *Desulfobacter vibrioformis*; Dburha, *Desulfobulbus rhabdoformis*; Dtmthe, *Desulfotomaculum thermocisternum*; Aglful, *Archaeoglobus fulgidus*; Aglpro, *Archaeoglobus profundus*; Achvin, *Allochromatium vinosum*; Pyrisl, *Pyrobaculum islandicum*.

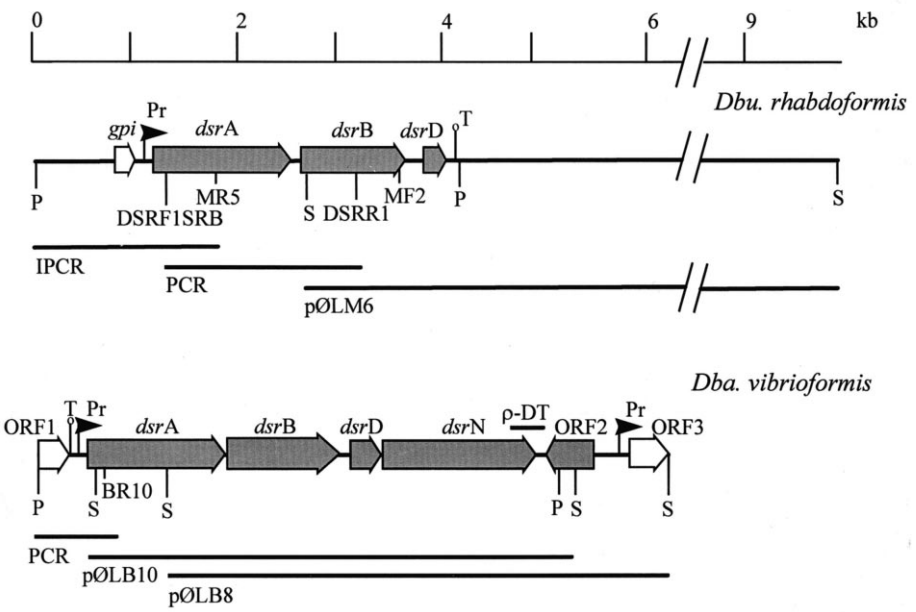

Fig. 1. Schematic representation of the *dsr* locus from *Dbu. rhabdoformis* and *Dba. vibrioformis*. *Pst*I (P) and *Sau*3AI (S) restriction sites used in construction of the λZAP and plasmid clones are indicated. Putative promoters (Pr), ρ-independent terminators (T) and a ρ-dependent terminator (ρ-DT) are indicated. DNA fragments obtained by PCR, inverse PCR (IPCR), or cloning into λZAP (pØLM6, pØLB8 and pØLB10) are indicated by bars. Primers (DSRF1SRB+DSRR1 and MF2+MR5) used in amplification of *dsr* fragments and probes (MR5 and BR10) used in Southern analyses are also indicated.

could be recognized at 150 and 60 nt upstream of *dsrA*, respectively. No ρ-independent terminator could be identified downstream of *dsrN*, but a CG pattern typical of ρ-dependent terminators [17] was found. Three ORFs flanking the *dsrABDN* operon were identified (Fig. 1). ORF1 shared the highest similarity with an unknown ORF from *Dtm. thermocisternum*, which is also closely associated with *dsr* [6]. This ORF shares weak homology with *O*-linked GlcNAc transferases. For ORF2 and ORF3 no significant matches were found in the databases.

A total of 3265 bp were sequenced from *Dbu. rhabdoformis* (Fig. 1). A putative σ^{70} promoter sequence, TTGACAN$_{15}$TTTATT, was found 126 nt upstream of *dsrA* and a typical ρ-independent termination sequence (GCAGAGGTTCCTGAAAGGGCCTCTGCTTTTTTATTTT) was found 27 nt downstream of *dsrD*. No additional ORFs were identified downstream of *dsrD*. Upstream of *dsrA* part of an ORF sharing homology with glucose isomerase (*gpi*) was identified.

3.2. Molecular characterization of the dsr gene products

The *dsrA* genes encode 48–50-kDa polypeptides while the *dsrB* genes encode 42–43-kDa polypeptides, which is in good agreement with the molecular masses of the desulforubidin α and β subunits from *Desulfosarcina* (*Dsa.*) *variabilis*, which have been estimated to be 50 and 42.5 kDa, respectively [18]. Furthermore, the homology regions, H1–H5, found in all siroheme-containing enzymes [19] and the ferredoxin motif, Fe, found in all Dsr [9] is also present in desulforubidins. We thus conclude that the genes reported here are the structural genes for desulforubidin.

The *dsrD* genes encode 9.8- and 8.7-kDa polypeptides, and show significant homology to the *dsrD* gene products from other sulfate reducers (Table 2). In *A. vinosum* and *P. islandicum* additional genes were found to be associated with *dsrAB*, but the *dsrD* gene was not present in the *dsr* operon [3,20]. A function in sulfite binding has been proposed [4], but this remains to be experimentally verified.

The *dsrN* gene encodes a 53.2-kDa polypeptide, and shows significant homology to cobyrinic a,c-diamide synthase, CbiA, from various microorganisms. CbiA is part of the vitamin B12 biosynthetic pathway where it catalyzes the amidation of cobyrinic acid to cobyrinic acid a,c-diamide [21]. Sequence comparison revealed a similar organization of DsrN and CbiA with respect to ATP and glutamine binding motifs (Fig. 2). Thus, DsrN might be a cobyrinic a,c-diamide synthase. However, a coupling of a biosynthetic gene to a catabolic operon seems unlikely. An amidated siroheme, siroamide, has been detected in *Desulfovibrio* species [22] and, thus, DsrN might be the protein

Table 2
Percent sequence similarities of DsrD

	Dvivul	Dbavib	Dburha	Dtmthe	Aglful
Dbavib	70				
Dburha	46	52			
Dtmthe	48	45	52		
Aglful	45	42	31	31	
Aglpro	45	45	44	38	75

Abbreviations as in Table 1.

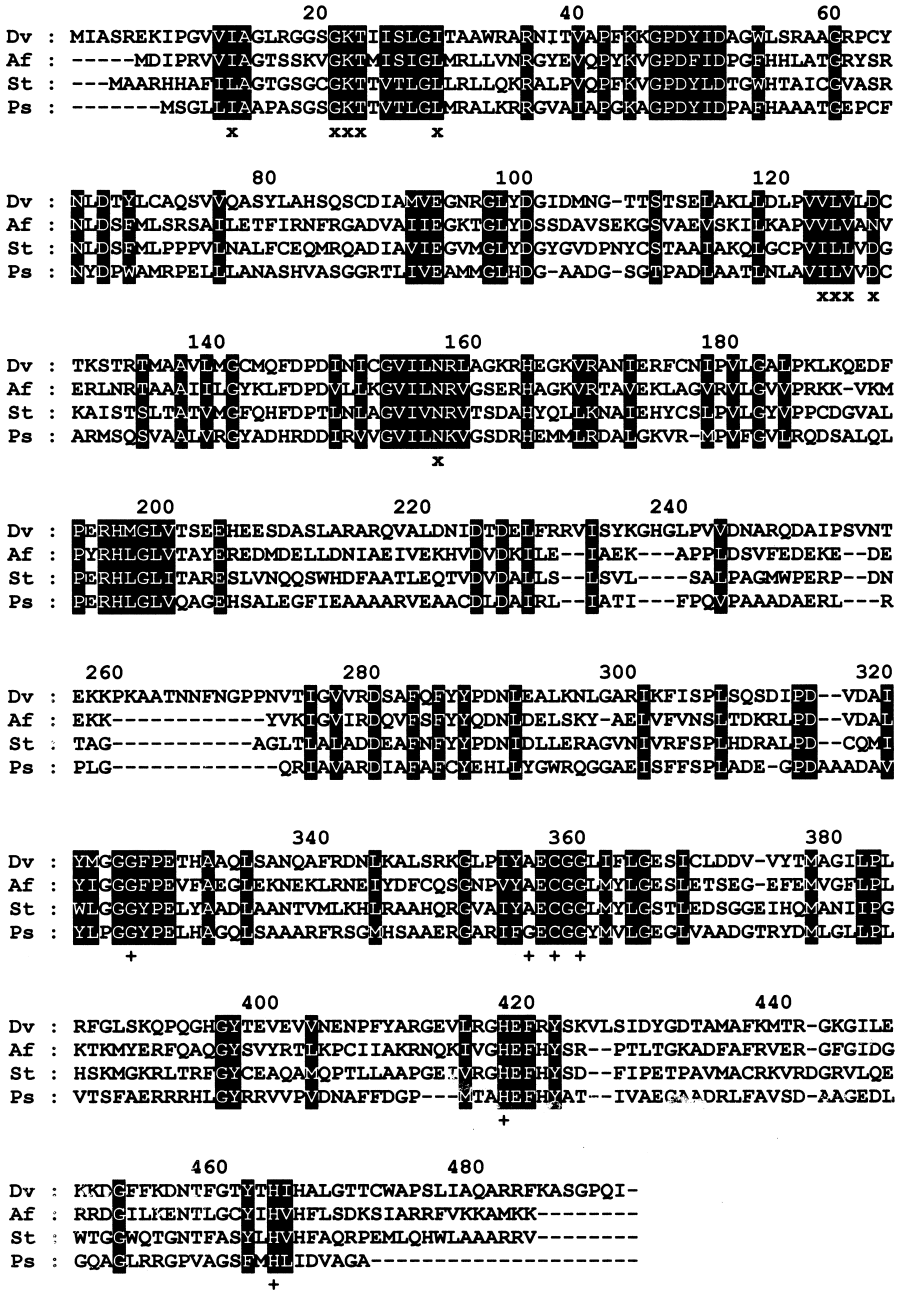

Fig. 2. Alignment of DsrN from *Dba. vibrioformis* (Dv) to CbiA from *Agl. fulgidus* (Af, accession number AAB89025) and *Salmonella typhimurium* (St, accession number AAA27252) and to CobB from *Paracoccus denitrificans* (Pd, accession number AAA25774). Conserved residues are shaded. Amino acid residues similar to the ATPase and glutamine transferase domains are marked by 'x' and '+', respectively.

responsible for this amidation. A coupling between *dsrD* and *dsrN* has also been detected in *Dba. postgatei* and *Dba. hydrogenophilus* (not shown). Whether DsrN can function both as a cobyrinic a,c-diamide synthase and as a siroheme amide synthase remains to determined.

3.3. Phylogenetic analyses

A phylogenetic analysis using partial DsrAB sequences has been done by Wagner et al. [5] concluding that Dsr can be useful in inferring phylogenetic relationships between sulfate-reducing bacteria. We have extended this analysis by inclusion of the *Dba. vibrioformis* and *Dbu. rhabdoformis* sequences. Distance, maximum-likelihood and parsimony analysis all firmly grouped *Dba. vibrioformis* next to *Dba. latus* forming a sub-cluster with other species belonging to the Desulfobacteriaceae family (Fig. 3). *Dbu. rhabdoformis*, however, forms a separate cluster and shows a weak affiliation to *Dtm. thermocisternum*. A recent 16S rRNA phylogeny analysis has shown that *Desulfobulbus* spp. might form a separate family [23], and the Dsr phylogeny might thus confirm this.

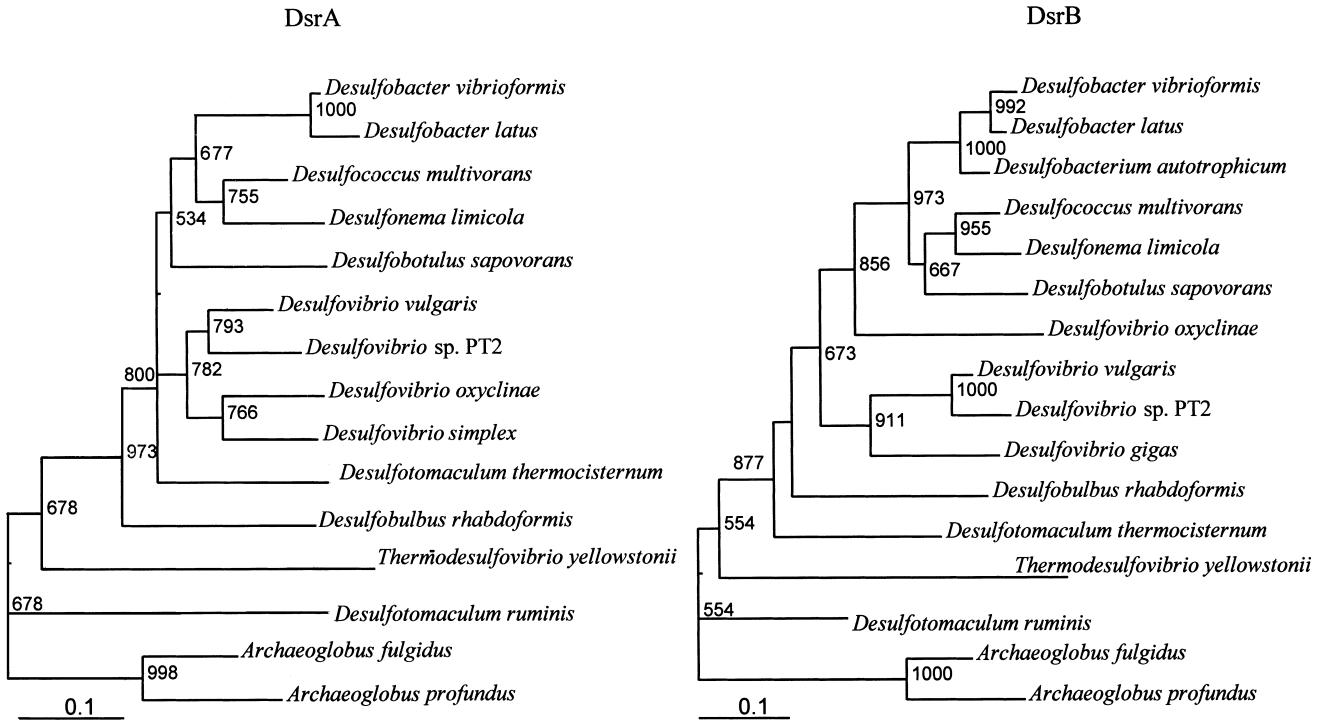

Fig. 3. An unrooted neighbor-joining tree of DsrA and DsrB. The numbers at the branches represent bootstrap values calculated with the ClustalX program. Dsr sequences used in constructing the trees were retrieved from the NCBI database (url: http://www.ncbi.nlm.nih.gov/). The scale bars indicate amino acid substitutions per residue per unit of branch length.

The assumption that Dsr phylogeny resembles the 16S rRNA phylogeny [5] does not apply to the genus *Desulfotomaculum* (Fig. 3). *Dtm. thermocisternum* Dsr seems to cluster with Gram-negative mesophilic sulfate-reducing bacteria whilst *Dtm. ruminis* branches off deeply in the Dsr phylogenetic tree and represents the deepest bacterial branch. This might indicate a gene exchange in the genus *Desulfotomaculum*. We are currently sequencing *dsr* genes from additional *Desulfotomaculum* species and thermophilic Gram-negative sulfate-reducing bacteria to clarify this.

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