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Lateral Gene Transfer of Dissimilatory (Bi)Sulfite Reductase Revisited

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In contrast to previous findings, we demonstrate that the dissimilatory (bi)sulfite reductase genes (*dsrAB*) of *Desulfobacula toluolica* were vertically inherited. Furthermore, *Desulfobacterium anilini* and strain mXyS1 were identified, by *dsrAB* sequencing of 17 reference strains, as members of the donor lineage for those gram-positive *Desulfotomaculum* species which laterally acquired *dsrAB*.

Dissimilatory (bi)sulfite reductase catalyzes the energy-generating step during the anaerobic respiration of sulfite or sulfate and thus represents a key enzyme of all sulfite- and sulfate-reducing prokaryotes (11, 22, 34). Recently, the genes encoding the alpha- and beta-subunits of this enzyme (*dsrAB*) have been used to infer the evolutionary history of dissimilatory (bi)sulfite reductases. For this purpose, a *dsrAB* database containing 75 entries for described sulfate-reducing prokaryotes (SRPs) (representing all known major evolutionary lineages of this guild) and four for sulfite-reducing microorganisms has been established (9, 12, 14–17, 23, 24, 28, 32, 33). Comparison of 16S rRNA- and DsrAB-based phylogenetic trees revealed congruent topologies for many SRP lineages, suggesting an ancient origin of dissimilatory (bi)sulfite reductase (33). This finding is consistent with isotopic evidence for biological sulfate reduction at 3.47 Gyr ago (31). However, we now recognize that the distribution of *dsrAB* among sulfate-reducing species reflects a combination of divergence through speciation (vertical descent) and acquisition via lateral gene transfer from distantly related prokaryotes (15). The archaeal SRPs of the genus *Archaeoglobus*, the deep-branching thermophilic SRPs of the genus *Thermodesulfobacterium*, as well as a large number of thermophilic gram-positive *Desulfotomaculum* species, possess laterally-acquired (bi)sulfite reductases. In addition, the deltaproteobacterial SRP *Desulfobacula toluolica* was postulated to have laterally acquired its (bi)sulfite reductase relatively recently, since its *dsrAB* genes differed significantly from those of its close relatives, including *Desulfobacter latus*, which have vertically transmitted (bi)sulfite reductase genes. In the DsrAB tree, the putative DsrAB sequence of *D. toluolica* formed a well-supported monophyletic cluster with the laterally acquired DsrAB sequences of *Desulfotomaculum* species. Therefore, it was speculated that *D. toluolica* and the *Desulfotomaculum* species received their *dsrAB* from a common but so far unidentified deltaproteobacterial donor lineage (15). However, no information on transfer mechanism or donor lineages is available for any of the recognized *dsrAB* lateral gene transfer events.

In an attempt to determine which additional genes might have been cotransferred with the *dsrAB* genes of *D. toluolica* and to reveal genetic traces indicative of the responsible transfer mechanism, the *dsr* operon (and flanking regions) of this SRP was sequenced. In a first step, a digoxigenin (DIG)-labeled 152-bp polynucleotide probe targeting *dsrA* was generated from *D. toluolica* DNA by using the primers DsrA415F (5′-TATCARGATGAGCTKCATCGYCC-3′) and DsrA542R (5′-ACYGCDTCCTGATCAATVCGGATAT-3′) and the PCR DIG probe synthesis kit (Roche, Mannheim, Germany). Using this probe, it was demonstrated by Southern hybridization of DNA restriction fragments from *D. toluolica* at low stringency that this organism contains a single *dsrA* in its genome (data not shown). After cloning of genomic DNA of *D. toluolica* into a lambda vector, phages containing the *dsrA* gene were identified by plaque hybridization with the polynucleotide probe (29). An 8.9-kb insert of *D. toluolica* DNA in a phage clone which hybridized with the *dsrA* polynucleotide probe was sequenced by primer walking (GenBank accession no. AJ457136). Gene sequence comparison revealed that this fragment contained the *dsr* operon consisting of the genes *dsrA*, *dsrB*, *dsrD*, and *dsrN* (Fig. 1A). The *dsrABDN* operon structure of *D. toluolica* has previously been detected in other deltaproteobacterial SRPs (Fig. 1B). Surprisingly, comparative sequence analysis of *dsrA* and *dsrB* of *D. toluolica* revealed that these genes were clearly different (less than 66% nucleic acid similarity) from the *dsrAB* gene fragments of this organism which were previously published (15). Sequence analysis of the target sites of the PCR primers used for *D. toluolica dsrAB* gene fragment amplification by Klein et al. (15) revealed that *dsrA* has three mismatches with primer DSR1F and *dsrB* has one mismatch with primer DSR4R. Therefore, the *dsrAB* gene fragment of *D. toluolica* could not be amplified with these primers (data not shown). Thus, the *dsrAB* sequence which was previously reported (15) most likely originated from a laboratory contamination and the *dsr* operon sequence reported in the present paper is the actual *dsr* sequence of *D. toluolica*. The

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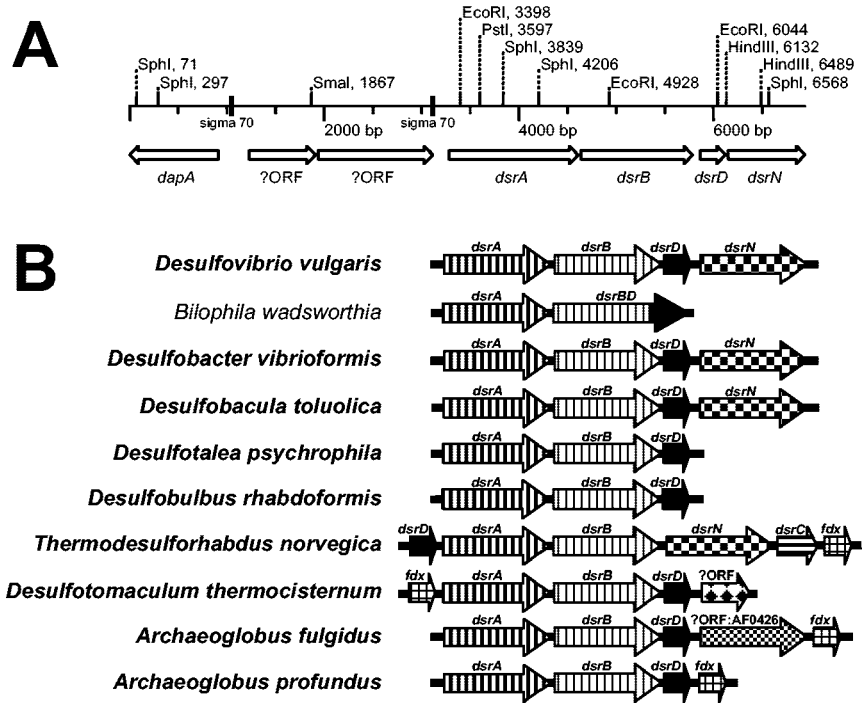
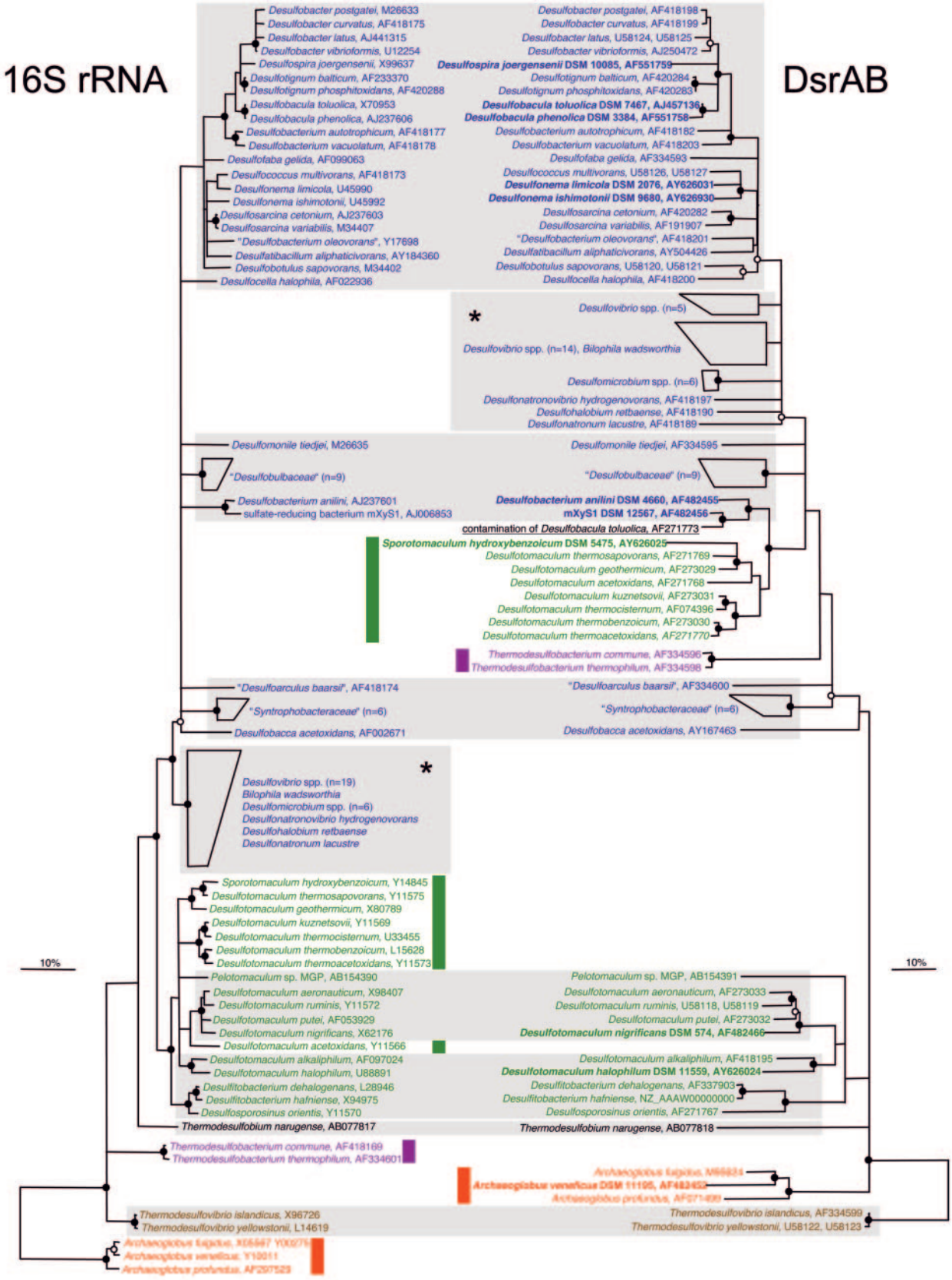


FIG. 1. (A) Schematic map showing the genetic organization of a *dsr* operon-containing genomic fragment of *D. toluolica*. Restriction sites of common endonucleases and sequence motifs similar to *Escherichia coli* sigma 70 promoters are shown. The following abbreviations represent fully sequenced open reading frames: *dsrA* and *dsrB*, alpha and beta subunits, respectively, of the dissimilatory (bi)sulfite reductase; *dsrD*, dissimilatory (bi)sulfite reductase D; which has a possible role in the regulation of *dsr* gene transcription (21); *dsrN*, putative siroheme amidase; *dapA*, dihydrodipicolinate synthase; ?ORF, unidentified open reading frame. (B) Genetic organization of all known *dsr* operons from SRPs and *Bilophila wadsworthia* (18). Prokaryotes which are able to use sulfate as an electron acceptor for anaerobic respiration are indicated in boldface type. Open reading frames: *dsrC*, gamma subunit of the dissimilatory (bi)sulfite reductase; *fdx*, ferredoxin. Accession numbers: *Desulfovibrio vulgaris*, AE017285; *B. wadsworthia*, AF269147; *Desulfobacter vibrioformis*, AJ250472; *D. toluolica*, AJ457136; *D. psychrophila*, NC_006138; *Desulfoblulbus rhabdoformis*, AJ250473; *Thermodesulforhabdus norvegica*, AJ277293; *Desulfotomaculum thermocisternum*, AF074396; *A. fulgidus*, NC_000917; and *Archaeoglobus profundus*, AF071499.

newly determined *dsrAB* sequence of *D. toluolica* phylogenetically clusters together with *dsrAB* sequences of the genus *Desulfobacter* (Fig. 2) independently from the treeing method applied (15, 19). Since this affiliation is consistent with the respective 16S rRNA gene tree topology, *D. toluolica* contains a vertically transmitted *dsr* operon. Two additional experiments were undertaken to further support this finding. First, the sequence of a 1.9-kb *dsrAB* PCR fragment of *Desulfobacula phenolica*, the closest known relative of *D. toluolica*, was determined and found to be almost identical (97.5% and 99.5% *dsrA* and *dsrB* nucleic acid similarity, respectively) to the respective gene sequences of *D. toluolica* (Fig. 2). Second, the DsrA and DsrB enzyme subunits were purified from cell extracts (7) of *D. toluolica* (Fig. 3) and N-terminal sequencing (6) of the DsrA subunit (N-terminal sequencing of DsrB failed) revealed 100% accordance with the respective amino acid stretch (AKHETPFL) predicted from the *dsrA* sequence. All predicted N-terminal amino acid sequences of DsrA from other SRPs differ in at least one amino acid from this sequence.

FIG. 2. Comparison of 16S rRNA- and DsrAB-based phylogenetic consensus trees. Sequences determined in this study are in boldface. 16S rRNA phylogenetic analyses were performed on alignment positions conserved in at least 50% of all bacteria. Alignment regions of insertions and deletions were omitted in DsrAB amino acid sequence analyses. Polytomic nodes connect branches for which a relative order could not be determined unambiguously by using distance matrix, maximum-parsimony, and maximum-likelihood methods. Filled circles indicate branch points highly supported by maximum-parsimony bootstrap analysis (>90% in 1,000 resamplings). Open circles at nodes indicate 75 to 90%, while nodes without circles showed <75% bootstrap support. The bars represent 10% sequence divergence as estimated from maximum-likelihood and distance matrix analysis for the 16S rRNA and DsrAB trees, respectively. "*Deltaproteobacteria*," low G+C gram-positive bacteria (*Firmicutes*), *Thermodesulfobacterium* species, *Thermodesulfovibrio* species, *Thermodesulfobium narugense*, and *Archaeoglobus* species are depicted in blue, green, violet, brown, black, and red, respectively. The wrong *dsrAB* sequence of *D. toluolica* published by Klein et al. (15) is underlined. Colored bars indicate species which harbor laterally acquired *dsrAB* genes. Consistent groups between both trees are shaded gray. Note that the apparently inconsistent positions of SRP groups that are labeled by an asterisk are not well resolved in the respective trees and thus cannot be interpreted as indicators of lateral gene transfer events. The strain *A. veneficus* SNP6 (DSM 11195) (containing plasmid XY), had been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) by K. O. Stetter, Lehrstuhl für Mikrobiologie, Universität Regensburg, Regensburg, Germany. An ungrouped version of this figure (supplementary web Fig. 1) can be downloaded from our web site (http://www.microbial-ecology.net/supplements.asp) together with the respective ARB *dsrAB* database.


16S rRNA
DsrAB
Desulfobacter postgatei, M26633
Desulfobacter curvatus, AF418175
Desulfobacter latus, AJ441315
Desulfobacter vibrioformis, U12254
Desulfospira joergensenii, X99637
Desulfotignum balticum, AF233370
Desulfotignum phosphitoxidans, AF420288
Desulfobacula toluolica, X70953
Desulfobacula phenolica, AJ237606
Desulfobacterium autotrophicum, AF418177
Desulfobacterium vacuolatum, AF418178
Desulfofaba gelida, AF099063
Desulfococcus multivorans, AF418173
Desulfonema limicola, U45990
Desulfonema ishimotonii, U45992
Desulfosarcina cetonium, AJ237603
Desulfosarcina variabilis, M34407
"Desulfobacterium oleovorans", Y17698
Desulfatibacillum aliphaticivorans, AY184360
Desulfobotulus sapovorans, M34402
Desulfocella halophila, AF022936
Desulfobacter postgatei, AF418198
Desulfobacter curvatus, AF418199
Desulfobacter latus, U58124, U58125
Desulfobacter vibrioformis, AJ250472
Desulfospira joergensenii DSM 10085, AF551759
Desulfotignum balticum, AF420284
Desulfotignum phosphitoxidans, AF420283
Desulfobacula toluolica DSM 7467, AJ457136
Desulfobacula phenolica DSM 3384, AF551758
Desulfobacterium autotrophicum, AF418182
Desulfobacterium vacuolatum, AF418203
Desulfofaba gelida, AF334593
Desulfococcus multivorans, U58126, U58127
Desulfonema limicola DSM 2076, AY626031
Desulfonema ishimotonii DSM 9680, AY626930
Desulfosarcina cetonium, AF420282
Desulfosarcina variabilis, AF191907
"Desulfobacterium oleovorans", AF418201
Desulfatibacillum aliphaticivorans, AY504426
Desulfobotulus sapovorans, U58120, U58121
Desulfocella halophila, AF418200
*
Desulfovibrio spp. (n=5)
Desulfovibrio spp. (n=14), Bilophila wadsworthia
Desulfomicrobium spp. (n=6)
Desulfonatronovibrio hydrogenovorans, AF418197
Desulfohalobium retbaense, AF418190
Desulfonatronum lacustre, AF418189
Desulfomonile tiedjei, M26635
"Desulfobulbaceae" (n=9)
Desulfobacterium anilini, AJ237601
sulfate-reducing bacterium mXyS1, AJ006853
Desulfomonile tiedjei, AF334595
"Desulfobulbaceae" (n=9)
Desulfobacterium anilini DSM 4660, AF482455
mXyS1 DSM 12567, AF482456
contamination of Desulfobacula toluolica, AF271773
Sporotomaculum hydroxybenzoicum DSM 5475, AY626025
Desulfotomaculum thermosapovorans, AF271769
Desulfotomaculum geothermicum, AF273029
Desulfotomaculum acetoxidans, AF271768
Desulfotomaculum kuznetsovii, AF273031
Desulfotomaculum thermocisternum, AF074396
Desulfotomaculum thermobenzoicum, AF273030
Desulfotomaculum thermoacetoxidans, AF271770
Thermodesulfobacterium commune, AF334596
Thermodesulfobacterium thermophilum, AF334598
"Desulfoarculus baarsii", AF418174
"Syntrophobacteraceae" (n=6)
Desulfobacca acetoxidans, AF002671
"Desulfoarculus baarsii", AF334600
"Syntrophobacteraceae" (n=6)
Desulfobacca acetoxidans, AY167463
*
Desulfovibrio spp. (n=19)
Bilophila wadsworthia
Desulfomicrobium spp. (n=6)
Desulfonatronovibrio hydrogenovorans
Desulfohalobium retbaense
Desulfonatronum lacustre
Sporotomaculum hydroxybenzoicum, Y14845
Desulfotomaculum thermosapovorans, Y11575
Desulfotomaculum geothermicum, X80789
Desulfotomaculum kuznetsovii, Y11569
Desulfotomaculum thermocisternum, U33455
Desulfotomaculum thermobenzoicum, L15628
Desulfotomaculum thermoacetoxidans, Y11573
10%
Pelotomaculum sp. MGP, AB154390
Desulfotomaculum aeronauticum, X98407
Desulfotomaculum ruminis, Y11572
Desulfotomaculum putei, AF053929
Desulfotomaculum nigrificans, X62176
Desulfotomaculum acetoxidans, Y11566
Desulfotomaculum alkaliphilum, AF097024
Desulfotomaculum halophilum, U88891
Desulfitobacterium dehalogenans, L28946
Desulfitobacterium hafniense, X94975
Desulfosporosinus orientis, Y11570
Thermodesulfobium narugense, AB077817
Thermodesulfobacterium commune, AF418169
Thermodesulfobacterium thermophilum, AF334601
Thermodesulfovibrio islandicus, X96726
Thermodesulfovibrio yellowstonii, L14619
Archaeoglobus fulgidus, X05567 Y00275
Archaeoglobus veneficus, Y12011
Archaeoglobus profundus, AF297523
Pelotomaculum sp. MGP, AB154391
Desulfotomaculum aeronauticum, AF273033
Desulfotomaculum ruminis, U58118, U58119
Desulfotomaculum putei, AF273032
Desulfotomaculum nigrificans DSM 574, AF482466
Desulfotomaculum alkaliphilum, AF418195
Desulfotomaculum halophilum DSM 11559, AY626024
Desulfitobacterium dehalogenans, AF337903
Desulfitobacterium hafniense, NZ_AAAW00000000
Desulfosporosinus orientis, AF271767
Thermodesulfobium narugense, AB077818
Archaeoglobus fulgidus, M59934
Archaeoglobus veneficus DSM 11195, AF482452
Archaeoglobus profundus, AF271400
Thermodesulfovibrio islandicus, AF334599
Thermodesulfovibrio yellowstonii, U58122, U58123
10%

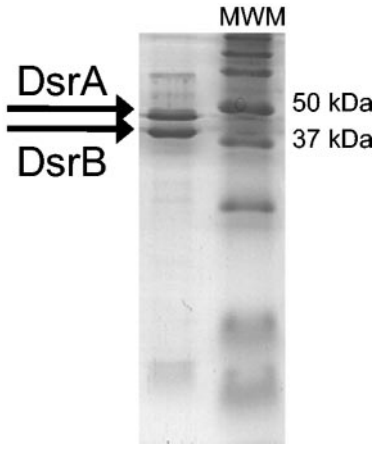


FIG. 3. Denaturing polyacrylamide gel electrophoresis analysis of the dissimilatory (bi)sulfite reductase purified from *D. toluolica*. The predicted sizes of DsrA and DsrB according to the determined gene sequences are 49.9 and 42.5 kDa, respectively. MWM, molecular weight marker.

The sequence of *D. toluolica* extends the number of available complete *dsrAB* sequences from reference cultures to 11. These sequences can be used to validate the suitability of the commonly used PCR primers for *dsrAB* amplification from SRP pure cultures and from environmental or clinical samples (Table 1). With the exception of the *dsrAB* of *D. toluolica* and *Desulfotalea psychrophila* (28), the primers DSR1F and DSR4R (33) and their recently published variants (19) perfectly match the available complete *dsrAB* genes. For improved coverage of SRPs in future experiments, it is recommended that primer pairs DSR1Fc-DSR4Rd and DSR1Fd-DSR4Re, targeting *dsrAB* of *D. toluolica* and *D. psychrophila*, respectively, are added to the primer variant mixture (Table 1). Furthermore, PCR annealing stringency should be kept low in environmental *dsrAB* diversity surveys (e.g., references 1–5, 8, 13, 20, 25, and 26) because it is likely that additional sequence variants in the *dsrAB* PCR primer binding sites exist.

In the DsrAB tree, none of the SRPs which received the genes for this enzyme by lateral transfer group with species possessing vertically transmitted enzyme genes. This suggests that the *dsrAB* donor lineages have yet to be described or, alternatively, are no longer extant. In order to more fully describe the evolutionary history of the dissimilatory (bi)sulfite reductase, PCR-amplified *dsrAB* gene fragments (19, 33) of 15 SRPs and the syntrophic gram-positive bacterium *Sporotomaculum hydroxybenzoicum* were cloned, sequenced, and phylogenetically analyzed (Fig. 2). The identity of the analyzed reference cultures was confirmed by comparative 16S rRNA gene sequence analysis (27).

Interestingly, the DsrAB sequences of the deltaproteobacterial SRPs *Desulfobacterium anilini* (30) and strain mXyS1 (10) formed a well-supported monophyletic branch with the laterally acquired sulfite reductases of the gram-positive *Desulfotomaculum* species (Fig. 2). This affiliation, which is consistently inferred by different treeing methods, suggests that *D. anilini* and strain mXyS1 either acquired their (bi)sulfite reductase genes from the same unknown donor lineage as the gram-positive SRPs or that these two organisms are members

TABLE 1. DSR1F and DSR4R primer binding sites as recognized from completely sequenced *dsrAB* genes of SRPs and *B. wadsworthia*

dsrAB-containing prokaryotes	*dsr4*-targeted forward primer binding site (5′→3′)[a]	Perfectly matching forward primer (5′→3′)	Forward primer reference	*dsrB*-targeted reverse primer binding site (5′→3′)[a]	Perfectly matching reverse primer (5′→3′)	Reverse primer reference
D. vulgaris AE017285	ACCC**ACTGGAAGCACG**	DSR1F: ACSCACTGGAAGCACG	33	**TGCGGTAACTGCTACAC**	DSR4R: GTGT AGCAGTTACCGCA	33
D. desulfuricans AJ249777	ACCC**ATTGGAAACACG**	DSR1Fa: ACCCAYTGGAAACACG	19	**TGCGGAAACTGCTACAC**	DSR4Rc: GTGTAGCAGTTK CCGCA	19
B. wadsworthia AF269147	ACG**CACTGGAAGCACG**	DSR1F: ACSCACTGGAAGCACG	33	**TGCGGTAACTGCTACAC**	DSR4R: GTGTAGCAGTTACCGCA	33
D. vibrioformis AJ250472	ACCC**ACTGGAAACACG**	DSR1Fa: ACCCAYTGGAAACACG	19	**TGCGGTAACTGTTACAC**	DSR4Rb: GTGTAACAGTTAC CGCA	19
D. toluolica AJ457136	ACCC**ATTGGAAACATG**	DSR1Fc: ACCCATTGGAAACATG	This study	**TGTGGTAACTGCTACAC**	DSR4Rd: GTGTAGCAG TTACCACA	This study
D. psychrophila NC_006138	ACT**CACTGGAAGCACG**	DSR1Fd: ACTCACTGGAAGCACG	This study	**TGTGGTAACTGTTACAC**	DSR4Re:GTGTA ACAGTTACCACA	This study
D. rhabdoformis AJ250473	ACCC**ATTGGAAACACG**	DSR1Fa: ACCCAYTGGAAACACG	19	**TGCGGTAACTGCTACAC**	DSR4R: GTGTAGCAGTTACCG CA	33
T. norvegica AJ277293	GGC**CACTGGAAGCACG**	DSR1Fb: GGCCACTGGAAGCACG	19	**TGCGGAAACTGCTACAC**	DSR4Rc: GTGTAGCAGTTKCCGCA	19
D. thermocisternum AF074396	ACCC**ACTGGAAACACG**	DSR1Fa: ACCCAYTGGAAACACG	19	**TGCGGCAACTGCTACAC**	DSR4Rc: GTGTAGCAGTT KCCGCA	19
A. fulgidus NC_000917	ACG**CACTGGAAGCACG**	DSR1F: ACSCACTGGAAGCACG	33	**TGCGGTAACTGCTACAC**	DSR4R: GTGTAGCAGTTACCGCA	33
A. profundus AF071499	ACG**CACTGGAAGCACG**	DSR1F: ACSCACTGGAAGCACG	33	**TGTGGAAACTGTTACAC**	DSR4Ra: GTGTAACAGTTTCCACA	19

[a] Highly conserved nucleic acid positions are in boldface.

of the *dsrAB* lineage, which served as donor for the gram-positive SRPs. Since *D. anilini* and strain mXyS1, which are marine mesophilic bacteria, form an independent lineage within the "*Deltaproteobacteria*" in the 16S rRNA and DsrAB trees (with the exception of the gram-positive bacteria with the laterally acquired *dsrAB*) (Fig. 2), this lineage is the most parsimonious *dsrAB* donor candidate for the gram-positive SRPs.

As *S. hydroxybenzoicum* forms a monophyletic branch in the 16S rRNA tree together with *Desulfotomaculum* species known to have received deltaproteobacterial *dsrAB*, it was not unexpected that *S. hydroxybenzoicum* also contains laterally acquired *dsrAB*. Similarly, *Archaeoglobus veneficus* possesses, like the other two species of this genus, laterally transferred *dsrAB*. The phylogenetic affiliations of the 12 remaining novel *dsrAB* sequences were found to be largely congruent with the respective 16S rRNA phylogeny of the organisms (Fig. 2). This observation further supports our current perception that the dissimilatory (bi)sulfite reductase is an ancient enzyme whose evolutionary history is largely consistent with vertical transmission but has also been influenced by periodic lateral gene transfer events. To avoid publication of incorrectly assigned *dsrAB* sequences in the future, we recommend careful checking of the purity of reference cultures. Furthermore, PCR-independent control experiments (e.g., Southern hybridization with genomic DNA using a *dsrAB*-targeted highly specific oligonucleotide probe) should be implemented for those reference cultures whose DsrAB sequences do not phylogenetically cluster with DsrAB sequences of recognized close relatives (as inferred from phylogenetic 16S rRNA trees).

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