Transposon Mutagenesis Identified Chromosomal and Plasmid Genes Essential for Adaptation of the Marine Bacterium *Dinoroseobacter shibae* to Anaerobic Conditions

Matthias Ebert, a Sebastian Laaß, b Melanie Burghartz, a Jörrn Petersen, b Sebastian Koßmehl, c Lars Wöhlbrand, c Ralf Rabus, c Christoph Wittmann, d Petra Tielen, e Dieter Jahn a

Institute for Microbiology, Technische Universität Braunschweig, Braunschweig, Germany a; Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany b; Institut für Chemie und Biologie des Meeres, Carl von Ossietzky Universität Oldenburg, Oldenburg, Germany c; Institute of Biochemical Engineering, Technische Universität Braunschweig, Braunschweig, Germany d

Anaerobic growth and survival are integral parts of the life cycle of many marine bacteria. To identify genes essential for the anaerobic life of *Dinoroseobacter shibae*, a transposon library was screened for strains impaired in anaerobic denitrifying growth. Transposon insertions in 35 chromosomal and 18 plasmid genes were detected. The essential contribution of plasmid genes to anaerobic growth was confirmed with plasmid-cured *D. shibae* strains. A combined transcriptome and proteome approach identified oxygen tension-regulated genes. Transposon insertion sites of a total of 1,527 mutants without an anaerobic growth phenotype were determined to identify anaerobically induced but not essential genes. A surprisingly small overlap of only three genes (*napA, phaA*, and the Na+/P, antiporter gene *Dshi_0543*) between anaerobically essential and induced genes was found. Interestingly, transposon mutations in genes involved in dissimilatory and assimilatory nitrate reduction (*nap, nasA*) and corresponding cofactor biosynthesis (genomic *moaB, moeB*, and *dsbC* and plasmid-carried *dsbD* and *ccmH*) were found to cause anaerobic growth defects. In contrast, mutation of anaerobically induced genes encoding proteins required for the later denitrification steps (*nirS, nirJ, nosD*), dimethyl sulfoxide reduction (*dmsA1*), and fermentation (*pdhB1, arcA, aceE, pta, acs*) did not result in decreased anaerobic growth under the conditions tested. Additional essential components (ferredoxin, *ccA*) of the anaerobic electron transfer chain and central metabolism (*pdhB*) were identified. Another surprise was the importance of sodium gradient-dependent membrane processes and genomic rearrangements via viruses, transposons, and insertion sequence elements for anaerobic growth. These processes and the observed contributions of cell envelope restructuring (*lysM, mipA, fadK*), C4-dicarboxylate transport (*dctM1, dctM3*), and protease functions to anaerobic growth require further investigation to unravel the novel underlying adaptation strategies.

The Roseobacter clade is one of the most abundant groups of bacteria in oceans. The ecological success of the *Roseobacter* clade can be attributed to its broad metabolic capabilities (1, 2). One of the model organisms of the *Roseobacter* clade is *Dinoroseobacter shibae*. It is a mixotrophic bacterium that can utilize various organic carbon sources, including several carboxylic acids, glucose, glycerol, and succinate (1–3). Fluxome analyses showed that *D. shibae* lacks phosphofructokinase activity during growth on glucose and preferentially uses the Entner-Doudoroff pathway (4). Moreover, *D. shibae* can gain additional energy by aerobic anoxygenic photosynthesis but is unable to grow phototrophically. Annotation of the 4.4-Mb genome of *D. shibae* DFL12T discovered genes that indicated the use of alternative electron acceptors such as nitrate and dimethyl sulfoxide in the absence of molecular oxygen (5). In agreement, anaerobic growth by denitrification was reported recently (6). The bacterium possesses *nap, nir, nor*, and *nos* operons encoding the nitrate reductase NapAB, the nitrite reductase NirS, the nitric oxide reductase NorCB, and the nitrous oxide reductase NosZ (7). Notably, *D. shibae* possesses the genes encoding the periplasmic nitrate reductase NapAB instead of the genes for the membrane-localized nitrate reductase NarGHI (5, 7). Additionally, genes for high-affinity cbb3-type cytochrome *c* oxidases and various alternative NADH dehydrogenase systems were identified. These might also be involved in energy conversation under low-oxygen conditions (5). Various electron-donating primary dehydrogenase genes were annotated (*gcd* for glucose, *gld* for gluconate, *ldl* and *dld* for lactate, *glp* for glycerol-3-phosphate, and *fda* for formate). Moreover, the capacity for substrate level phosphorylation processes, including the arginine deiminase pathway and a mixed-acid-type fermentation, can be deduced from the *D. shibae* genome (5).

However, the members of the anaerobic modulon remain to be experimentally defined for this important class of marine bacteria. The contribution of the five plasmids of *D. shibae* to these processes is completely unknown.

Here we present the identification of genes involved in the process of *D. shibae* adaptation to anaerobic conditions via transposon mutagenesis and combined transcriptome and proteome analyses. Chromosomal and plasmid genes were found to be essential. Only a small overlap between the genes found necessary
for anaerobic growth and those induced under these conditions was detected. A novel type of anaerobic adaptation strategy was deduced.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and plasmid transfer.** The type strain _D. shibae_ DFL12² (5) was cultured aerobically in Marine Bouillon (MB; Roth, Karlsruhe, Germany) at 30°C in bottle flasks shaking at 200 rpm in the dark. The mariner transposon located on plasmid pBT20 (8) (see Fig. S1 in the supplemental material) was used for transposon mutagenesis of _D. shibae_ DFL12². For selection of _D. shibae_ mutants, 80 µg/ml gentamicin was added after conjugation to half-concentrated MB (hMB) (6). Escherichia coli ST18, a ΔhemA mutant of _E. coli_ S17, served as the donor strain for the conjugal transfer of plasmid DNA (9). Luria-Bertani (LB) medium (Roth, Karlsruhe, Germany) supplemented with 50 µg/ml aminolevulinic acid and adjusted to pH 7 was used for its cultivation at 37°C and 200 rpm. For solid medium, agar was added to a final concentration of 1.5% (wt/vol).

Conjugative plasmid transfer into _D. shibae_ DFL12² was performed as described previously (6), with modifications (see the supplemental material). For selection of _D. shibae_ transposon mutants with an anaerobic growth deficiency, all clones were cultivated aerobically and anaerobically at 30°C in 96-well plates with hMB supplemented with 80 µg/ml gentamicin, respectively. For anaerobic cultivation, 25 mM nitrate was added. Growth was monitored by measurement of optical density at 595 nm (OD₅₉₅) in a microtitr plate reader (model 680; Bio-Rad, Munich, Germany). Strains showing growth deficiencies under anaerobic conditions were isolated for further study. The growth behavior of the selected _D. shibae_ DFL12² transposon mutants was analyzed aerobically and anaerobi-

**Identification of transposon integration site.** First an arbitrary PCR protocol was established as described by O’Toole and coworkers (11). For this purpose, two different PCR analyses were performed. The first PCR included the genomic DNA from a grown transposon mutant colony and primer 1 (5′-TCT ACG TGC AAG CAG ATT ACG GTG AC-3′), which hybridized to the transposon DNA. Random primer 2 (5′-GGC CAC GCG TCG ACT AGT CAT CGN NNN NNN GAT AT-3′) and primer 3 (5′-GGC CAC GCG TCG ACT AGT CAN NNN NNN GAT CC-3′) were added. The initial incubation at 95°C (5 min) was followed by six cycles of DNA denaturation at 94°C (30 s), annealing at 30°C (30 s), and elongation at 70°C (1 min). In the second part of the PCR, the annealing temperature was increased to 45°C for a further 30 cycles, followed by a final elongation phase at 72°C (5 min). The second PCR involved primer 4 (5′-GGC ATC GAC CCA AGT ACC GCC ACC TA-3′) and primer 5 (5′-GGC CAC GCG TCG ACT AGT AC-3′). The conditions used were chosen according to the first PCR protocol. PCR products were subjected to DNA sequence determination. The resulting FASTA sequences were aligned with the genome sequence of _D. shibae_ DFL12² (GenBank accession numbers NC_009952 and NC_009955-59).

**Cultivation of _D. shibae_ in a chemostat.** Continuous cultivation of _D. shibae_ DFL12² for transcriptome and proteome analyses was performed with SWM (10) and an Infors HT Multifor 2 bioreactor (Infors, Bottmingen, Switzerland) at 30°C, pH 8.0, with aeration at 0.7 liter of air/min and a stirring speed of 150 rpm. The bioreactor had a working volume of 1 liter. The pH was adjusted automatically with 500 mM H₂PO₄ and 500 mM NaOH. At steady state, the oxygen saturation of the culture in the bioreactor was stabilized at approximately 85%. To avoid aerobic anoxenic photosynthesis of _D. shibae_ during the experiment, the chemostat was protected from light by covering with aluminum foil. The bioreactor was inoculated to a starting OD₅₇₈ of 0.02 with an appropriate preculture. Feeding with fresh medium was started after the culture reached an OD₅₇₈ of 0.5. The dilution rate was 0.1 h⁻¹, establishing a half-maximum growth rate of _D. shibae_ in the exponential phase. The anaerobic shift was initialized after 20 h of continuous cultivation by stopping the aeration. The oxygen concentration in the reactor was determined with an InPro 6820 oxygen electrode (Mettler Toledo, Gießen, Germany), as well as with a sensor spot O₂ (PreSence, Regensburg, Germany). Anaerobic conditions were reached after approximately 20 min.

**DNA microarray experiments and data analysis.** A customized whole-genome DNA microarray (8,000-by-15,000 format; Agilent, Santa Clara, CA) containing three different 60-nucleotide oligonucleotides covering 96% of the genes of _D. shibae_ DFL12² was designed with the eArray platform from Agilent and used as described before (10). The investigated time points were 0 and 30 min after the oxygen supply had been switched off. Two micrograms of isolated total cellular RNA was labeled with either Cy3 or Cy5 with the ULS fluorescent labeling kit for Agilent arrays (Kreatch, Amsterdam, the Netherlands) according to the manufacturer's manual. Subsequently, 300 ng of each labeled RNA was pooled, fragmented, and hybridized according to the "two-color microarray" protocol from Agilent. The DNA microarrays were scanned with an Agilent C scanner with the Agilent scan control 8.4.1 software and the feature extraction 10.7.3.1 software. Data processing was performed in the R environment (http://www.cran.r-project.org/) with the limma package, the BioBASE package, and the gplots package of Bioconductor project q (http://www.bioconductor.org/) (12, 13). Three biological and three technical replicates were performed. Only genes with a logarithmic change of >0.8 in their expression between aerobic (0 min) and anaerobic (30 min) conditions and a P value of <0.05 were considered in subsequent analyses.

**Shotgun proteome analysis by nanoliquid chromatography (nanoLC)-electrospray ionization (ESI) tandem mass spectrometry (MS).** Cell pellets of approximately 50 mg (wet weight) from bioreactor growth were resuspended in 200 µl lysis buffer, and cells were disrupted with the PlusOne grinding kit (GE Healthcare, Munich, Germany) as described before (14). Protein concentrations were determined as described before (15). Following the reduction and alkylation of 50 µg total cellular protein, proteolytic digestion was performed overnight with 0.5 µg trypsin GOLD (Promega, Mannheim, Germany). Finally, 1 µg of digested protein was separated with an UltiMate 3000 nanoLC system (Thermo Scientific, Bremen, Germany) by applying a linear gradient of increasing acetonitrile concentrations over 215 min coupled online to an ESI ion trap mass spectrometer (amaZon ETD; Bruker Daltonik GmbH, Bremen, Germany) as described before (14). Three biological replicates were analyzed. Protein identification was performed with ProteinScape (version 3.0; Bruker Daltonik GmbH) on a Mascot server (version 2.3; Matrix Science Ltd., London, United Kingdom) by searching against a genomic database of _D. shibae_ DFL12² translated into amino acid sequences by using a target-decoy strategy. Searching was restricted to doubly and triply charged peptides. A false-discovery rate of <1.0% was set. Only peptides with a mascot score of ≥25 were considered for protein identification.

**Analysis of the membrane protein-enriched fraction by nanoLC-ESI MS.** Preparation and SDS-PAGE separation of the membrane protein-enriched fraction were performed as described recently (14). For each sample, one gel lane was cut into 11 slices that were further cut into smaller pieces for washing, reduction, alkylation, and tryptic digestion as described before (14). Separation of the peptides generated was performed with UltiMate 3000 nanoLC (Thermo Scientific, Bremen, Germany) with a 95-min linear gradient of increasing acetonitrile concentrations (14). Mass spectrometric analysis of the LC eluent was performed with an online-coupled ion trap mass spectrometer (amazon ETD; Bruker Daltonik GmbH) as described before (14). Protein identification was performed as outlined above.
Plasmid curing of *D. shibae*. *D. shibae* DSM 16493<sup>T</sup> was cured of 191-kb plasmid pDSH101 (NC_009955.1) as recently described (16). The RepABC-9-type 4,500-bp replication module, which encodes the replicase gene, the origin of replication (*oriV*), the parAB partitioning operon, and the putative cis-acting palindromic anchor sequence 5′-AAATCTCA ATCTTGAGCCGGCTTCAAGTTGAGTTT-3′ (17), was amplified with primers P046 (5′-GACGGGGCGTGTCCTACCTCAC-3′) and P047 (5′-TCACAAAAACCGAGGAGACACT-3′). The PCR product was cloned into the Smal site of a pBluescript SK<sup>+</sup> vector containing an additional gentamicin resistance cassette (18). Complete DNA sequencing of the 4.5-kb insert revealed the integrity of the replication module. The preparation of electrocompetent *D. shibae* cells and transformation of the plasmid containing the RepABC-9-type replication module construct were conducted as described before (19). The transformants were plated on marine broth medium with 40 μg/ml gentamicin and streaked to obtain a single colony. The successful elimination of the original 191-kb plasmid was verified via PCR with purified plasmid DNA (NucleoSpin Plasmid DNA kit; Macherey-Nagel) and the following primer combinations for all five extrachromosomal elements of *D. shibae*: pDSH01 (191 kb), P430 (5′-TCTGCTGGTGTTGCGTTT-3′) and P431 (5′-TGCGCTATAGTGCTCGGCTGCGTGGTGGCTTTC-3′); pDSH03 (126 kb), P432 (5′-GGCACCATCGTCCGGAACCAT-3′) and P433 (5′-TGATGATGCCGACCTGCTGTTCTGTC-3′); pDSH04 (103 kb), P422 (5′-TATAGAATTTCGCGGATAGAAGGCTGTTTT-3′) and P421 (5′-ATCCTGACACCAAGGCTCTCTCATTT-3′); pDSH01 and pDSH04 and tested for aerobic versus anaerobic growth (Table 1). Clearly, complementation experiments are required to ultimately confirm that the observed loci of transposon integration are responsible for the observed phenotype. For details, see the supplement in the material.

For transcriptome and proteome analyses, chemoautotrophic cultivation with a standardized protocol for the shift from aerobic to anaerobic conditions was developed. The analysis revealed 474 genes differentially expressed during the shift from aerobic to anaerobic conditions, with 207 showing an increase in expression and 267 showing a decrease. The proteome analyses detected 878 different proteins by the whole-cell protein shotgun approach and 1,215 different proteins in the membrane fraction covering approximately 25% of the predicted *D. shibae* proteins. The results of the various experimental approaches were interpreted and are discussed in the light of their functional consequences below.

Plasmids are essential for anaerobic growth of *D. shibae*. Besides the chromosome, *D. shibae* DFL12<sup>T</sup> contains five plasmids (5). The results of plasmid transmutation revealed an unexpected impact of these plasmids on the anaerobic growth of *D. shibae*. Both sister plasmids pDSH101 and pDSH103 and plasmid pDSH102 seemed to be essential for anaerobic growth (Table 1). No plasmid mutation affecting anaerobic growth was found in plasmid pDSH105.

In order to unambiguously demonstrate the contribution of the plasmid genes to aerobic growth, plasmid-deficient *D. shibae* strains were generated. The strains were cured of plasmids pDSH101 and pDSH104 and tested for aerobic versus anaerobic growth. Both plasmid-cured *D. shibae* strains had lost the ability to grow anaerobically. These observations clearly demonstrate the requirement of plasmid-provided genetic information for anaerobic growth.

Denitrification is induced, but only nitrate reduction is essential for anaerobic growth. Under anaerobic conditions, *D. shibae* is able to grow via denitrification with nitrate, nitrite, NO, and N<sub>2</sub>O as terminal electron acceptors (6). The first step of denitrification is the reduction of nitrate to nitrite (7). Accordingly, napA (*Dshi_3163*), which encodes the catalytic subunit of the periplasmic dissimilatory nitrate reductase NapAB, was identified by transposon mutagenesis as one of the essential genes under anaerobic denitrifying conditions (Table 1). Nap is encoded by the napFDAGHBC operon (*Dshi_3161* to *Dshi_3167*). The expression of the operon was found to be slightly induced upon oxygen depletion in the transcriptome analysis. The NapA protein was also detected under aerobic, as well as under anaerobic, conditions in the proteome analyses (see Table S2 in the supplemental material). *D. shibae* possesses only periplasmic NapAB and not the membrane-spanning NarGHI nitrate reductase (5). Obviously, in addition to further analyses. For details, see the supplemental material.

A total of 4,500 *D. shibae* transposon mutants were isolated and further screened for growth defects under anaerobic denitrifying conditions. Random integration of the transposon was observed (Fig. 1). Of the 1,580 transposon mutants sequenced, 1,134 showed different loci of integration (see Table S1 in the supplemental material). Taking approximately 12% of the essential genes into account, the saturation of mutagenesis reached 82% of the genome. Fifty-three mutants, 35 with transposon integration into chromosomal genes and 18 with transposon integration into plasmid genes, showed significantly decrease or even loss of anaerobic growth (Table 1). Consequently, complementation experiments are required to ultimately confirm that the observed loci of transposon integration are responsible for the observed phenotype. For details, see the supplement in the material.
the two napA mutants obtained, energy conservation via nitrate respiration became limiting. The later steps of denitrification cannot substitute for the process because nitrite, NO, and N₂O production is missing from this mutant. Consequently, mutations in this gene led to a lethal phenotype under anaerobic conditions (Table 1). Similar observations of an essential role for the NapAB nitrate reductase for denitrification and anaerobic growth were recently made for the Magnetospirillum gryphiswaldense (21).

Several transposon insertions in other genes encoding enzymes of the denitrification pathway were found to have no effect on anaerobic growth (see Table S2). For example, mutations in the nitrite reductase-encoding gene nirS (Dshi_3180) and the nitrous oxide reductase maturation protein-encoding gene nosD (Dshi_3195) failed to cause a lethal phenotype under anaerobic denitrifying conditions. Similarly, the defect in the nitrous oxide regulator-encoding gene nosR2 (Dshi_3181) did not lead to any growth defect. However, all of these genes (napH, napF, nirS, and nosD) were found to be induced under denitrifying conditions. The corresponding proteins were also found to be abundant under anaerobic conditions in the proteomic investigation (see Table S2).

Surprisingly, mutation of the assimilatory NADH-dependent nitrate reductase-encoding gene nasA (Dshi_1669) also led to a significant decrease in anaerobic growth (Table 1). The nasA gene is localized upstream of the nasDE genes, which encode the assimilatory nitrite reductase. During nitrogen assimilation, ammonium is generated through the reduction of nitrate via nitrite in the cytoplasm (22). However, the growth medium was supplemented with sufficient ammonium during the selection process, excluding a general defect in nitrogen metabolism. In agreement, no aerobic phenotype was observed. Furthermore, mutation of the nasD gene (Dshi_1667), which encode one of the assimilatory nitrite reductase subunits, did not result in an anaerobic growth phenotype under denitrifying conditions (see Table S2). Nevertheless, the amount of transcript of the whole nas operon did not change between aerobic and anaerobic conditions. In conclusion, the observed anaerobic growth phenotype of the nasA mutant in the presence of ammonium underscores the importance of this enzyme for dissimilatory denitrifying growth. Alternatively, ni-
**TABLE 1** *D. shibae* DFL127 transposon mutants showing decreased anaerobic growth under denitrifying conditions

<table>
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<tr>
<th>Locus tag</th>
<th>Function of gene product</th>
<th>Gene name</th>
<th>Position in ORF</th>
<th>Integration position(s)</th>
<th>TD</th>
<th>Growth&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Anaerobic</th>
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<td><strong>Nitrate reductases and electron transfer</strong></td>
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<td>Putative glutathione S-transferase</td>
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<td>(511) 516</td>
<td>2444388, chromosome F</td>
<td>F</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dshi_3356</td>
<td>Transposase</td>
<td></td>
<td>(424) 267</td>
<td>3529671, chromosome F</td>
<td>F</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3556</td>
<td>Transposase</td>
<td></td>
<td>(870) 267</td>
<td>3529225, chromosome F</td>
<td>F</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dshi_3655Dshi_3988</td>
<td>Type VI secretion system protein, TraG/TraD family protein</td>
<td><em>virD1</em></td>
<td>(911) 2004 (912) 2001</td>
<td>56453, pDSHI01; 49682, pDSHI03</td>
<td>R</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3758Dshi_4034</td>
<td>Hypothetical protein</td>
<td></td>
<td>(80) 390</td>
<td>162102, pDSHI01; 98681, pDSHI03</td>
<td>F</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3679Dshi_3875Dshi_4082</td>
<td>Integrase catalytic region (transposase)</td>
<td></td>
<td>(1411) 1494</td>
<td>81922, pDSHI01 95640, pDSHI02 14388, pDSHI04</td>
<td>F, R</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

(Continued on following page)
trite may have a novel, as-yet-unknown, function under anaerobic growth conditions.

Molybdopterin cofactor biosynthesis for nitrate reductase formation is essential under anaerobic growth conditions. The nitrate reductase NapAB contains a molybdopterin cofactor (Moco), iron-sulfur clusters, and a cytochrome c subunit. The nitrate reductase NapAB and the nitrite reductase NirS both require cytochrome c as a cofactor and electron transfer molecule. The nitrate reductase NapAB was shown to be essential for aerobic growth without MoeB and MoaB.

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Function of gene product</th>
<th>Gene name</th>
<th>Position in ORF</th>
<th>Integration position(s)</th>
<th>TD</th>
<th>Growth *</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dshi_3678</td>
<td>ATP-binding protein, putative transposase</td>
<td>repA</td>
<td>(782)825</td>
<td>81061, pDShi101; 94779, pDShi102; 19250, pDShi104</td>
<td>F, R, R</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3678</td>
<td>ATP-binding protein, putative transposase</td>
<td>repA</td>
<td>(223)825</td>
<td>81620, pDShi101; 95338, pDShi102; 18691, pDShi104</td>
<td>F, R, R</td>
<td>0–1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_4023</td>
<td>Plasmid partitioning protein</td>
<td>repA</td>
<td>(17) 1188</td>
<td>91461, pDShi103</td>
<td>R</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

- **Cell envelope**
  - Dshi_0027
  - Dshi_0808
  - Dshi_1766
  - Dshi_2238
  - Dshi_3403
  - Dshi_3576
  - Dshi_3628

- **Transport**
  - Dshi_3624/Dshi_3962
  - Dshi_3626/Dshi_3964
  - Dshi_3796

- **Other**
  - Dshi_0750
  - Dshi_1277
  - Dshi_2726
  - Dshi_3364
  - Dshi_3673

*ORF, open reading frame.

*TD, transposon direction; F, forward; R, reverse.

*The number 2 stands for normal growth, 1 stands for decreased growth, and 0 stands for no growth.

Mutations affecting cytochrome c and disulfide bond formation. The nitrate reductase NapAB and the nitrite reductase NirS both require cytochrome c as a cofactor and electron transfer molecule. The nitrate reductase NapAB was shown to be essential for anaerobic growth (see above). Mutations in Dshi_1072 (dbsC), plasmid-carried Dshi_3606/3944 (potential dbsD), and Dshi_3777/4053 (cmnH) had defects in genes involved in disulfide bond formation and cytochrome c formation. The disulfide bond formation machinery is part of cytochrome c formation. Mutations in these genes produced a loss of anaerobic growth, two of them with no influence on aerobic growth (dbsC, cmnH). One
Plasmid-encoded cytochrome c is essential for anaerobic growth in *D. shibae*. One transposon was found integrated in Dshi_3887 localized on plasmid pDSHI02, which contains the class I cytochrome-encoding gene *ccA*. *ccA* gene expression was found to be slightly enhanced under anaerobic growth conditions, indicating a role under denitrifying conditions. Class I cytochrome c molecules are small soluble cytochromes that are needed for electron transfer reactions during denitrification in other bacteria (7). In *Neisseria gonorrhoeae*, the *ccA* gene product cytochrome *c* is essential for the shuttling of electrons toward the denitrification machinery (29). Several other unclassified class I cytochromes of *D. shibae* (Dshi_0508, Dshi_2868) did not influence anaerobic growth (see Table S1). The expression of these genes was found to be downregulated or unaffected (Table S2). Obviously, the transposon has identified an essential cytochrome involved in the initial steps of denitrification.

**One of three pyruvate dehydrogenases is essential for anaerobic growth.** A transposon mutation in gene *pdh2* (Dshi_2159), which encodes the E1 component of one of the three pyruvate dehydrogenase complexes, resulted in decreased growth under anaerobic conditions.

### Table S1 Comparison of growth phenotypes, fold changes in gene expression after 30 min of oxygen depletion, and presence of cytoplasmic and membrane proteins under anaerobic denitrifying conditions

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene name</th>
<th>Function of gene product</th>
<th>Transposon insertion</th>
<th>Growth phenotype</th>
<th>Fold change in transcription under anaerobic conditions</th>
<th>Presence of protein under anaerobic conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dshi_3180</td>
<td><em>nirS</em></td>
<td>Nitrate reductase precursor</td>
<td>+</td>
<td>2</td>
<td>3.16</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_3192</td>
<td>Hypothetical protein</td>
<td>+</td>
<td>2</td>
<td>14.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_3195</td>
<td><em>nosD</em></td>
<td>Nitrous oxide maturation protein</td>
<td>+</td>
<td>2</td>
<td>12.8</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_2278</td>
<td><em>dnxA</em></td>
<td>Dimethyl sulfoxide reductase precursor</td>
<td>+</td>
<td>2</td>
<td>11.7</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_0542</td>
<td>Phosphate transporter</td>
<td>+</td>
<td>2</td>
<td>8.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_3173</td>
<td><em>nirF</em></td>
<td>Putative nitrite reductase heme biosynthesis</td>
<td>+</td>
<td>2</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_2304</td>
<td>Putative regulator of cell morphogenesis and NO signaling</td>
<td>+</td>
<td>ND</td>
<td>6.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_0664</td>
<td><em>fixP</em></td>
<td>Cytochrome c oxidase, <em>cbb</em>, type, subunit III</td>
<td>+</td>
<td>2</td>
<td>5.7</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_3165</td>
<td><em>napA</em></td>
<td>Nitrate reductase catalytic subunit</td>
<td>+</td>
<td>ND</td>
<td>2.0</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_0543</td>
<td>Na⁺/Pi cotransporter</td>
<td>+</td>
<td>ND</td>
<td>3.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_3152</td>
<td>Protein DUF1445 of unknown function</td>
<td>+</td>
<td>1</td>
<td>3.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_1449</td>
<td>TonB-dependent receptor</td>
<td>+</td>
<td>ND</td>
<td>3.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3163</td>
<td><em>napH</em></td>
<td>Ferredoxin-type protein NapH</td>
<td>+</td>
<td>2</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_2233</td>
<td><em>phbC</em></td>
<td>Poly-beta-hydroxybutyrate polymerase</td>
<td>+</td>
<td>2</td>
<td>3.1</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_3558</td>
<td>Hypothetical protein</td>
<td>+</td>
<td>2</td>
<td>3.1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_1968</td>
<td><em>aceE</em></td>
<td>Pyruvate dehydrogenase subunit E1</td>
<td>+</td>
<td>2</td>
<td>3.0</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_3066</td>
<td><em>atoB</em></td>
<td>Acetyl-CoA acetyltransferase</td>
<td>+</td>
<td>2</td>
<td>2.9</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_2363</td>
<td><em>ureE</em></td>
<td>UreE urease accessory domain-containing protein</td>
<td>+</td>
<td>2</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_0432</td>
<td><em>arcA</em></td>
<td>Arginine deiminase</td>
<td>+</td>
<td>2</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_3590</td>
<td>NADH dehydrogenase (ubiquinone)</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_0563</td>
<td><em>irpA</em></td>
<td>Iron-regulated protein</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_2052</td>
<td>Hypothetical protein</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_2966</td>
<td><em>panB</em></td>
<td>3-Methyl-2-oxobutanolate hydroxymethyltransferase</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_0426</td>
<td>Hypothetical protein</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dshi_3805</td>
<td><em>phaA</em></td>
<td>NADH dehydrogenase</td>
<td>+</td>
<td>ND</td>
<td>2.0</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_3249</td>
<td><em>fliE</em></td>
<td>Flagellar hook-basal body protein FliE</td>
<td>+</td>
<td>2</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>Dshi_2965</td>
<td>Hypothetical protein</td>
<td>+</td>
<td>2</td>
<td>1.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_3553</td>
<td><em>acs</em></td>
<td>Acetate-CoA ligase</td>
<td>+</td>
<td>2</td>
<td>1.8</td>
<td>+</td>
</tr>
<tr>
<td>Dshi_0540</td>
<td>NnrU family protein</td>
<td>+</td>
<td>2</td>
<td>1.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Dshi_1399</td>
<td><em>acsA</em></td>
<td>Acetate-CoA ligase</td>
<td>+</td>
<td>2</td>
<td>1.8</td>
<td>+</td>
</tr>
</tbody>
</table>

---

a The number 2 stands for normal growth, 1 stands for decreased growth, a minus sign stands for no growth, a 0 stands for not detected, and a plus sign stands for detected. In bold are the induced genes that produced an anaerobic growth phenotype upon transposon insertion.

b ND, not done.
aerobic conditions and no growth under anaerobic conditions (Table 1). In general, pyruvate dehydrogenase is converted into acetyl coenzyme A (acyt-l-CoA). D. shibae possesses three loci for pyruvate dehydrogenase, namely, pdhA2B1C2 (Dshi_0534-Dshi_0536), accEFlpADA (Dshi_1968-1970), and pdhA1B2C1 (Dshi_2158-2160). In contrast to pdhB2, inactivation of pdhB1 (Dshi_0535) did not influence anaerobic growth. However, both the pdhA1B2C1 and pdhA2B1C2 operons were not found to be differentially expressed. Consequently, the functional basis of the observed phenotype remains to be determined.

Sodium-dependent transport processes are essential for anaerobic growth. The identification of the Na\(^+\)-dependent NADH dehydrogenase PhaA and Na\(^+\)-dependent C4-dicarboxylate TRAP transporters (DctM1 and DctM3) as essential for the anaerobic growth of D. shibae pointed toward an important role for the Na\(^+\) gradient (Table 2; for a detailed description and discussion, see the supplemental material). Furthermore, Dshi_0543 encodes a type II Na\(^+\)/P\(^-\) cotransporter similar to transporters found in Methylobacterium, Campylobacter, and Helicobacter species and Pseudomonas stutzeri (30, 31). Finally, the mechanosensitive ion channel encoded by Dshi_2998 showed 33% amino acid sequence identity to E. coli YbiO (32). YbiO of E. coli revealed NaCl-induced channel activity (33). Finally, Dshi_3675 encodes a Na\(^+\)-H\(^+\) exchange protein. Overall, Na\(^+\) gradient-dependent membrane-associated processes are essential for the anaerobic growth of D. shibae. This might reflect an adaptation of D. shibae to its marine habitat.

Potential genome rearrangement as part of the anaerobic adaptation process. Unexpectedly, several genes that encode phase-related proteins and transposons were found to be essential for the anaerobic growth of D. shibae. The gene Dshi_2174 encodes a phase capsid protein and is part of a large operon (Dshi_2176 to Dshi_2161) that encodes a complete HK97-type (pro)phage (34). These phages were reported to carry so-called morons, DNA elements that increase host fitness (35). A corresponding lambda prophage of E. coli increased mammalian host cell binding and resistance to killing (36, 37). Salmonella phages Fels-2 and GIPSY-2 carried morons encoding superoxide dismutase, which sustained bacterial fitness during host infection (38). Many morons provide resistance to phase superinfection (39–41). Inspection of the genes downstream of Dshi_2174 identified genes of unknown function between the classical phase genes, however, without providing an explanation for the observed anaerobic growth phenotype.

Another surprising observation was that the transposon mutations found in Dshi_3356, which encodes IS1 insertion element protein A3 (42), and in Dshi_3655, which encodes a type IV secretion TRAG-type family protein involved in DNA transfer, were located in the vicinity of numerous genes whose products are predicted to be involved in DNA transport function. Similarly, Dshi_3628 and Dshi_3678 are part of inserted transposons. Furthermore, the anaerobically essential gene Dshi_3758 encodes a transposase of an IS\(^{4}\) element. The gene Dshi_2313 encodes an HsdR family type I DNAse as part of a restriction-modification system (43). In Mycoplasma, the HsdSMR enzyme system has been shown to be activated by high-frequency gene rearrangements (44) and the whole system is controlled by proteolysis (45). Overall, genetic mobility and rearrangement, most likely involving the highly conserved areas of the plasmids are an integral part of the strategy of D. shibae adaptation to anaerobic conditions.

Anaerobic growth requires proteases, peptide transport, restructuring of the cell envelope, cation efflux proteins, and FliK. The rest of the transposon mutants found are described and discussed in the supplemental material.

Strategy of D. shibae adaptation to anaerobic growth conditions. Obviously, solely nitrate reductases and the corresponding cofactor formation (Moco, cytochrome c) are the crucial parts of energy conversation under anaerobic growth conditions. The residual denitrification machinery, which is significantly induced under anaerobic conditions, further sustains anaerobic growth without being essential. Some of the anaerobically essential genes are plasmid encoded. Clear evidence of the importance of a Na\(^+\) gradient for anaerobic growth of D. shibae was found. Another surprise was the essential role of genome-restructuring genes localized on phages, transposons, and insertion sequence elements. The cell envelope has to be restructured, and because a set of proteases appears to be required for anaerobic growth, this might suggest that they are linked to cell wall restructuring. Overall, new surprising insights into the adaptation of the marine model bacterium D. shibae to oxygen-limiting conditions were obtained.

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