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ORIGINAL ARTICLE

The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea

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***Dinoroseobacter shibae* DFL12^T, a member of the globally important marine *Roseobacter* clade, comprises symbionts of cosmopolitan marine microalgae, including toxic dinoflagellates. Its annotated 4 417 868 bp genome sequence revealed a possible advantage of this symbiosis for the algal host. *D. shibae* DFL12^T is able to synthesize the vitamins B_1 and B_{12} for which its host is auxotrophic. Two pathways for the *de novo* synthesis of vitamin B_{12} are present, one requiring oxygen and the other an oxygen-independent pathway. The *de novo* synthesis of vitamin B_{12} was confirmed to be functional, and *D. shibae* DFL12^T was shown to provide the growth-limiting vitamins B_1 and B_{12} to its dinoflagellate host. The *Roseobacter* clade has been considered to comprise obligate aerobic bacteria. However, *D. shibae* DFL12^T is able to grow anaerobically using the alternative electron acceptors nitrate and dimethylsulfoxide; it has the arginine deiminase survival fermentation pathway and a complex oxygen-dependent Fnr (fumarate and nitrate reduction) regulon. Many of these traits are shared with other members of the *Roseobacter* clade. *D. shibae* DFL12^T has five plasmids, showing examples for vertical recruitment of chromosomal genes (*thiC*) and horizontal gene transfer (*cox* genes, gene cluster of 47 kb) possibly by conjugation (*vir* gene cluster). The long-range (80%) synteny between two sister plasmids provides insights into the emergence of novel plasmids. *D. shibae* DFL12^T shows the most complex viral defense system of all *Rhodobacterales* sequenced to date.**

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Introduction

One of the most abundant and metabolically versatile groups of bacteria in the world's oceans is the *Roseobacter* clade. Members of this alphaproteobacterial lineage are prominently involved in the global marine carbon and sulfur cycles (Moran *et al.*, 2004; Selje *et al.*, 2004; Buchan *et al.*, 2005; Wagner-Dobler and Biebl, 2006; Howard *et al.*, 2006; Moran and Miller, 2007; Brinkhoff *et al.*, 2008). Many *Roseobacter* species live as epibionts on marine algae where they reach high abundance during phytoplankton blooms (reviewed by Buchan *et al.* (2005)). Some phytoplankton blooms, the so-called

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red tides, are caused by toxic dinoflagellates, and *Roseobacter* species were frequently isolated from their laboratory cultures (Lafay *et al.*, 1995; Prokic *et al.*, 1998; Miller and Belas, 2004), and also detected on dinoflagellates in nature (Hasegawa *et al.*, 2007). A direct interaction between these algae and their associated bacteria is suggested by the fact that *Roseobacter* cells have been found attached to the surface of toxic *Pfiesteria* species using fluorescent *in situ* hybridization (FISH) (Alavi *et al.*, 2001).

The *Roseobacter* isolates from dinoflagellate cultures must be able to grow on metabolites excreted by the algae because they are heterotrophs (Moran and Miller, 2007) and there are no carbon sources present in the mineral media used for cultivating phototrophic algae. Many *Roseobacter* bacteria can degrade dimethylsulfoniopropionate (Moran *et al.*, 2007), an osmoprotectant released in large quantities during algal blooms, and they show positive chemotaxis toward this compound (Yoch 2002; Miller *et al.*, 2004; Miller and Belas, 2006). A strain of the abundant RCA (*Roseobacter* clade-affiliated) cluster could be cultivated in coculture with an axenic dinoflagellate (Mayali *et al.*, 2008). These studies show that *Roseobacter* bacteria can thrive on algal metabolites. However, it is presently not known whether this relationship provides an advantage to the dinoflagellate host.

In this study, we report the complete genome sequence of *Dinoroseobacter shibae* DFL12^T, the type strain of a species from the *Roseobacter* clade. It was isolated from *Prorocentrum lima*, a benthic dinoflagellate, by picking a single dinoflagellate cell from a culture, washing it several times and placing it on an agar surface (Allgaier *et al.*, 2003; Biebl *et al.*, 2005). *P. lima* produces okadaic acid, which can cause diarrhetic shellfish poisoning during red tides (Pan *et al.*, 1999). *D. shibae* DFL12^T was chosen for sequencing because of its symbiosis with dinoflagellates, its ability to perform light-driven ATP synthesis using bacteriochlorophyll *a* in the presence of oxygen (Allgaier *et al.*, 2003; Biebl and Wagner-Dobler, 2006; Wagner-Dobler and Biebl, 2006), the large number of extrachromosomal replicons (Pradella *et al.*, 2004) and its novel acylated homoserine lactone (AHL) compounds (Wagner-Dobler *et al.*, 2005). The complete genome sequence of *D. shibae* DFL12^T revealed traits that presumably are highly adaptive in the habitat of bloom-forming algae and that may be characteristic for the whole *Roseobacter* clade. Moreover, we discovered an essential gift provided to the dinoflagellate by the bacteria.

Materials and methods

Sequencing, assembly and finishing

The genome of *D. shibae* DFL12^T was sequenced at the Joint Genome Institute (JGI) Production Genomics Facility using a combination of 3 and 8 kb (plasmid), and 40 kb (fosmid) DNA libraries. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. The Phred/Phrap/Consed software package (http://www.phrap.com) was used for sequence assembly and quality assessment (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). After the shotgun stage, reads were assembled with parallel phrap (High Performance Software, LLC). Mis-assemblies were corrected with Dupfinisher (Han and Chain, 2006) or transposon bombing of bridging clones (Epicentre Biotechnologies, Madison, WI, USA). Gaps between contigs were closed by editing in Consed, custom primer walk or PCR amplification. A total of 742 additional reactions were necessary to close the gaps and to raise the quality of the finished sequence. The completed genome sequences of *D. shibae* DFL12^T contain 67 596 reads, achieving an average of a 11-fold sequence coverage per base with an error rate less than 1 in 100 000. The sequences of *D. shibae* DFL12^T, comprising a chromosome and five plasmids, can be accessed using the GenBank accession numbers NC_009952, NC_009955, NC_009956, NC_009957, NC_009958 and NC_009959. Manual curation and reannotation of the genome was carried out using The Integrated Microbial Genomes Expert Review System (img/er http://imgweb.jgi-psf.org) (Markowitz *et al.*, 2008) and the Artemis software package (http://www.sanger.ac.uk/Software/Artemis/v9).

Phylogenetic analysis

Phylogenetic analyses of 16S rRNA gene sequences were performed with the ARB software package (http://www.arb-home.de) (Ludwig *et al.*, 2004). A phylogenetic tree was constructed using neighbor-joining and maximum-likelihood analyses. Only sequences with more than 1200 bp were considered in these calculations. Sequences <1200 bp were added afterward using parsimony.

Design of D. shibae*-specific oligonucleotides for PCR and FISH*

Specific primers and probes were developed using the ProbeDesign function of the ARB software package (http://www.arb-home.de). Sequences of specific primers are DSH176f (5′-CGTATGTGGCC TTCGGGC-3′) and DSH1007r (5′-GCTTCCGTCTCC GGAAGCC-3′). Specificity of primer sequences was checked with the NCBI and RDP databases (http://www.ncbi.nlm.nih.gov and http://rdp.cme.msu.edu/) and resulted in at least one mismatch to 16S rRNA gene sequences of non-target organisms. Probe DSH176-HRP (5′-GCCCGAAGGCCACATACG-3′) was used for FISH, in combination with helper probes DSH176-H1 (5′-GTATTACTCCCAGTTTCC CA-3′) and DSH176-H2 (5′-ATCCTTTGGCGATAAA TCTTTC-3′).

Sample preparation for CARD-FISH
In total, 5 ml of the *Prorocentrum* culture were fixed with 5 ml paraformaldehyde (4% w/v) for 1 h and filtered onto a 0.2-μm filter (Nucleopore Track Etch PC MB 25 mm, Whatman Nr. 1110656, Whatman plc, Maidstone, Kent, UK) or a 10-μm filter (Polycarbonate Membrane Filters, TCTP04700, Millipore Corporation, Billerica, MA, USA). Cells were washed eight times with distilled water. Reference strains *D. shibae* DFL12^T (0 mismatches to probe DSH176) and *Sulfitobacter guttiformis* DSM11458^T (two mismatches to probe DSH176) were filtered onto 0.2-μm polycarbonate filters and fixed with paraformaldehyde (2% w/v) for 1 h, rinsed with 1 ml 1 × phosphate buffer solution and subsequently with 1 ml distilled water. Filters were air dried and stored at −20 °C until further processing.

CARD-FISH
CARD-FISH (Catalyzed reporter deposition FISH) was performed according to Sekar *et al.* (2003). Hybridization conditions were as follows: 2 h of hybridization at 35 °C (60% formamide), 30 min washing at 37 °C (14 mM NaCl) and 30 min amplification at 37 °C. Per 400 μl hybridization buffer, 15 μl of the HRP (horseradish peroxidase) probe DSH176-HRP and 15 μl of each unlabeled helper oligonucleotide (DSH176-H1 and DSH176-H2) were used (probe solutions 50 ng μl^{-1}): Tyramine-HCl was labeled with fluorescein-5-isothiocyanate as described by Pernthaler *et al.* (2002). To avoid unspecific accumulation of dye in the cells, the last washing step in phosphate buffer solution (1 ×) amended with TritonX-100 (0.05%) was extended to 30 min. Counterstaining was performed with Vectashield-mounting medium with 4′,6-Diamidino-2-phenylindole (1.5 μg ml^{-1}; Vector Laboratories, Peterborough, England).

Comparative genomics
The Integrated Microbial Genomes Expert Review System (img/er http://imgweb.jgi-psf.org (Markowitz *et al.*, 2008) was used for comparative analysis on the basis of the nine presently available completely sequenced genomes of the *Rhodobacterales*: *Jannaschia sp.* CCS1 (Moran *et al.*, 2007), *Roseobacter denitrificans* (Swingley *et al.*, 2007), *Silicibacter pomeroyi* (Moran *et al.*, 2004), *Silicibacter sp.* TM1040 (Moran *et al.*, 2007), three strains of *Rh. sphaeroides* (Choudhary *et al.*, 2007) as well as *Paracoccus denitrificans* (JGI Institute, unpublished).

CRISPR
Homologs of *cas* genes were detected using tools of the BLAST package (Altschul *et al.*, 1990). CRISPR arrays were detected by the CRISPR recognition tool (Bland *et al.*, 2007). Comparative analysis of repeat sequences was done both manually and by CRISPRdb (Grissa *et al.*, 2007). The origin of spacer sequences was searched for in the CAMERA databases (Seshadri *et al.*, 2007) (http://camera.calit2.net).

Regarding the methods for dinoflagellate cultivation and sampling, isolation of genomic DNA, PCR, cloning and sequencing, determination of vitamin B_{12} and coculture experiments, see Supplementary material 10.

Results and discussion

Ecological niche and phylogeny
D. shibae DFL12^T was isolated from *P. lima* (Biebl *et al.*, 2005). This phototrophic dinoflagellate occurs in sand and sediment and to some extent also in the water column, but reaches its highest densities in biofilms on marine macroalgae, macrophytes, corals, mussels and oysters (Levasseur *et al.*, 2003; Vershinin *et al.*, 2005; Okolodkov *et al.*, 2007; Parsons and Preskitt, 2007).

Several closely related strains were isolated from the dinoflagellate *Alexandrium ostenfeldii* (Allgaier *et al.*, 2003). Using PCR with *D. shibae*-specific primers for the 16S rRNA gene and sequencing of the obtained bands for confirmation, *D. shibae* was also detected in cultures of *Protoceratium reticulatum,* a dinoflagellate isolated from the North Sea. All three host species are cosmopolitan, toxic algae causing diarrhetic shellfish poisoning (Aasen *et al.*, 2005). Finally, *D. shibae* was isolated from *Isochrysis galbana*, a member of the class *Haptophyceae* (Genbank accession EF512132). This nontoxic alga is cultivated as food for bivalves in aquaculture. Thus, *D. shibae* is not restricted to toxic dinoflagellates, but is associated with a variety of cosmopolitan marine microalgae. Figure 1 shows the phylogenetic position of all currently known *D. shibae* strains within the *Roseobacter* clade and indicates from which algae they were isolated. The similarity of these sequences to *D. shibae* DFL12^T is between 97.2% and 99.8%, indicating that they probably belong to the same species, but additional data are needed (Rossello-Mora and Amann, 2001; Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005).

A physical association between host algae and bacteria is suggested by the fact that *D. shibae* DFL12^T was isolated from single washed cells of the dinoflagellate (Biebl *et al.*, 2005). Moreover, it can be seen attached to *P. lima* in coculture (Figure 2). In this study, dinoflagellates were separated from suspended bacteria using 10-μm polycarbonate filters, washed eight times and stained using CARD-FISH with probes specific for *D. shibae* DFL12^T. Many bacteria can be seen adhering to the surface of the dinoflagellate, and some are freely suspended in the medium. It remains to be seen if a genetic program coordinates the switch between planktonic growth and growth as an epibiont in *D. shibae*.

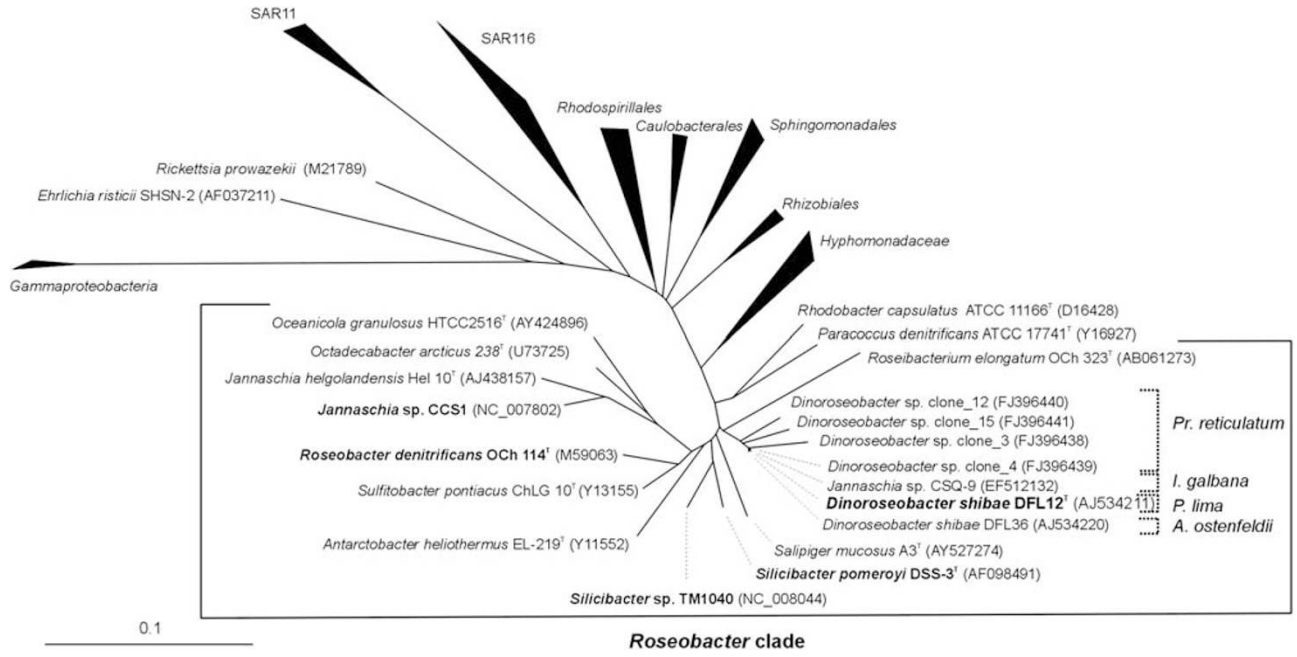


Figure 1 Phylogenetic position of *Dinoroseobacter shibae* DFL12^T and closely related strains and phylotypes within the *Rhodobacteraceae* (*Alphaproteobacteria*) based on 16S rRNA gene comparisons. All names written in boldface indicate organisms whose complete genome sequences have already been determined. The phylogenetic tree was generated using the maximum-likelihood method. Selected members of the *Gammaproteobacteria* were used as an outgroup (not shown). The eukaryotic host algae from which the strains were isolated are indicated next to the dotted brackets. *Pr., Protoceratium* (Dinophyceae); *I., Isochrysis* (Haptophyceae); *P., Prorocentrum* (Dinophyceae); *A., Alexandrium* (Dinophyceae). Bar = 0.1 substitutions per nucleotide position.

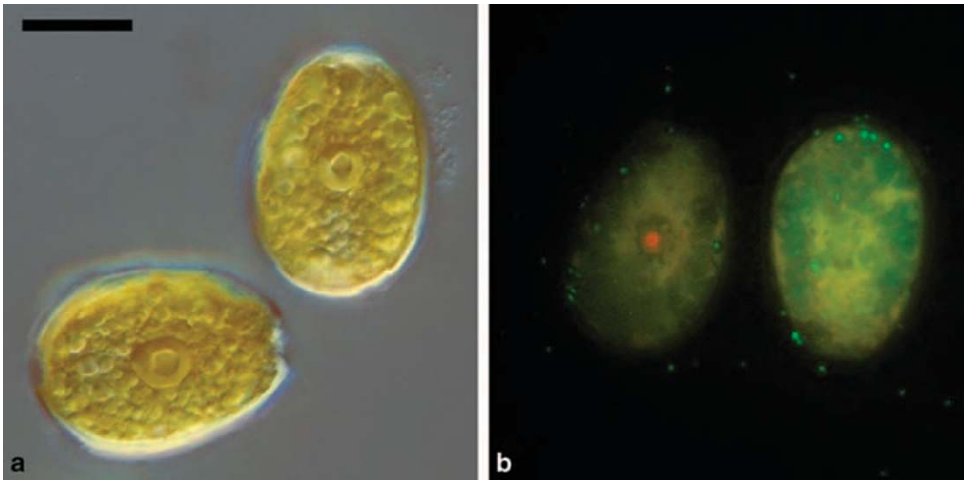


Figure 2 Specific attachment of *D. shibae* DFL12^T to its dinoflagellate host *Prorocentrum lima*. (**a**) Differential interference contrast (DIC) image of dinoflagellates from a non-axenic culture to which cells of strain DFL12^T had been added 2 months before; (**b**) Catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) with *Dinoroseobacter*-specific 16S rRNA probes showing most cells of *D. shibae* DFL12^T (green dots) closely attached to the dinoflagellate. Combined images generated by means of PICOLAY (http://www.picolay.de) from a series of six pictures taken at sequential focus levels. Bar = 20 μm. The central round structure in the cell center seen in (**a**) is the pyrenoid, a specialized area of the plastid that contains high levels of Rubisco. It is typically surrounded by a starch sheath that can also be seen. The crystal-like ultrastructure of the pyrenoid (Kowalik, 1969) may be the reason for its orange appearance in the left cell in (**b**).

General characteristics of the genome

The *D. shibae* type strain DFL12^T (= DSM 16493^T = NCIMB 14021^T) has a genome size of 4417 kbp, similar to the other fully sequenced *Roseobacter* genomes (Supplementary material S1). The genome contains 4198 protein-encoding genes. About 28% of the derived gene products have no predicted function. The finished genome sequence indicates a circular conformation of the chromosome and the five plasmids (Figure 3). Previously the presence of plasmids with linear conformation was hypothesized for two *D. shibae* strains and

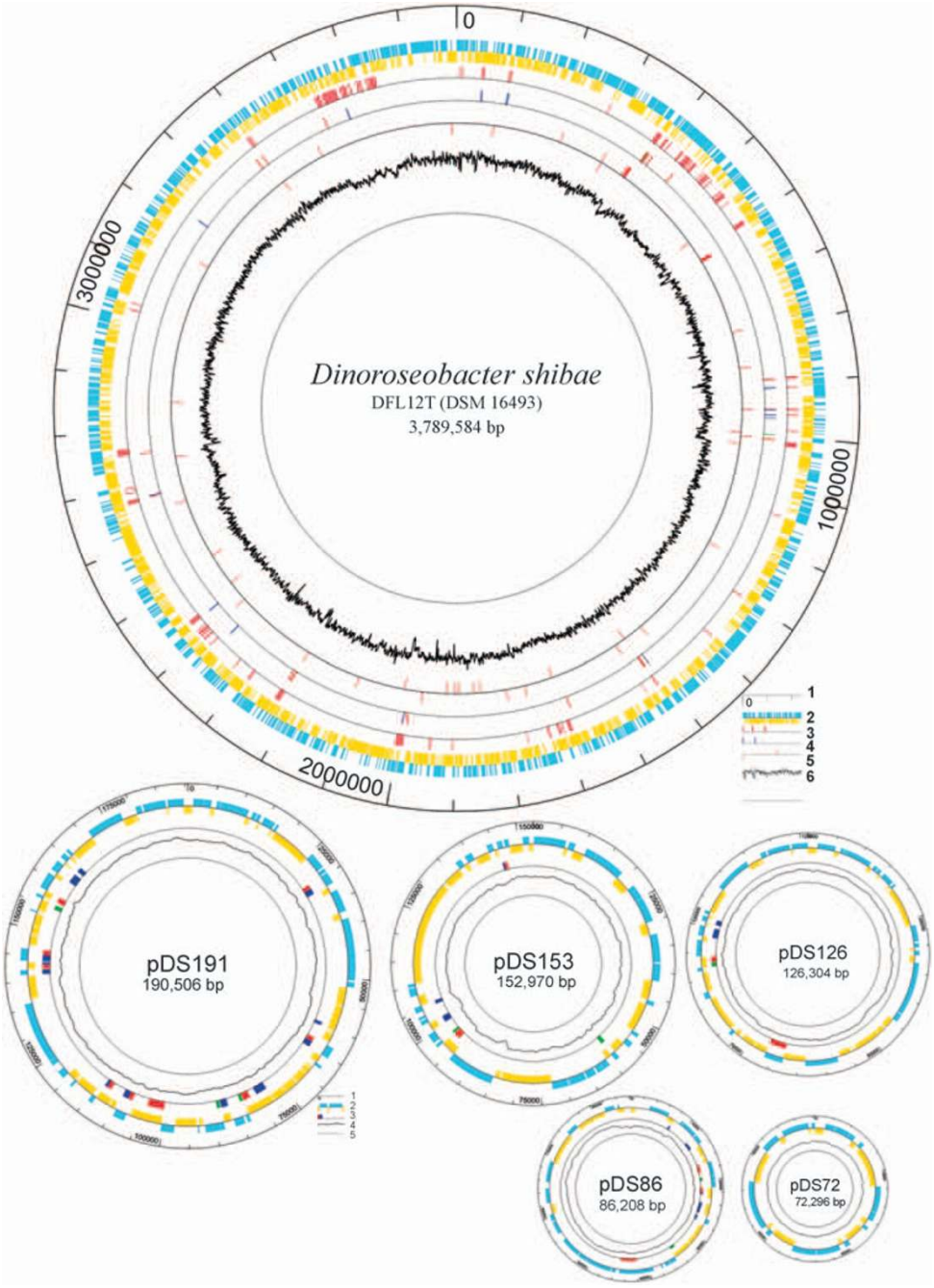


Figure 3 Circular representation of the chromosome and plasmids of *D. shibae* DFL12^T. The ring of the chromosome depicts (starting with the outer ring) (1) the scale in basepairs; (2) all protein-encoding genes, clockwise-transcribed genes in blue and counterclockwise-transcribed genes in yellow; (3) alien genes, that is, genes that are likely acquired by horizontal gene transfer; (4) genes related to DNA flexibility: transposases (blue), integrases (red) and recombinases (green); (5) rRNA and tRNA genes; (6) the GC content. The rings of the plasmids depict (1) the scale in base pairs; (2) all protein-encoding genes; (3) genes related to DNA flexibility; (4) rRNA and tRNA genes and (5) the GC content. Alien genes are not shown in the plasmids, because these contigs are too small for their calculation.

several related species (Pradella *et al*., 2004). We investigated the plasmid structure in *D. shibae* DFL12 by a restriction assay combined with length separation through pulsed-field gel electrophoresis and could confirm the circular conformation (Supplementary material S2). This is in agreement with the absence of genes typically associated with linear plasmids, for example, the telomere resolvase (Meinhardt *et al*., 1997; Kobryn and Chaconas, 2001).

Comparative genomics

D. shibae DFL12^T harbors all biogeochemically important traits reported previously from fully

sequenced *Roseobacter* isolates, namely anaerobic anoxygenic photosynthesis (*pufLM* genes), carbon monoxide oxidation (*cox* genes), aromatic compound degradation (*pcaGH* and *boxC* genes), sulfur oxidation (*soxB*), denitrification (*nirS/K*), nitrate assimilation (*nasA*), phosphonate use (*phn*), type IV secretion (*vir* genes) and dimethylsulfoniopropionate degradation (*dmdA*) (Supplementary material S3). Several families of transposases/integrases (61 and 43 copies, respectively) and site-specific recombinases/resolvases (11 copies) belong not only to the largest gene families, but also they are more frequent than in other members of the *Roseobacter* clade, indicating a large potential for DNA exchange in *D. shibae* DFL12^T. Proteins involved in information processing usually represent the role categories showing the least variation between closely related organisms. Their comparative analysis is described in Supplementary material S1.

The central energy metabolism and its regulation

Central carbon metabolism

The central carbon metabolism is reconstructed in Supplementary material S4. Glucose breakdown is potentially possible through glycolysis (Embden-Meyerhoff-Parnas pathway) and the Entner-Doudoroff pathway, the latter being closely connected to the oxidative pentose phosphate pathway. Interestingly, a fluxom analysis using ^{13}C labeling techniques showed that glucose breakdown is carried out exclusively using the Entner-Doudoroff pathway; thus, the alternative routes are used only for anabolic purposes (Fürch *et al.*, 2009). The Calvin cycle is lacking, indicated by the absence of the key enzyme Rubisco, as in all *Roseobacter* strains (Swingley *et al.*, 2007). Carbon dioxide might be fixed by anapleurotic enzymes either through (1) a two-step reaction involving pyruvate-orthophosphate dikinase and phosphoenolpyruvate carboxylase or through (2) pyruvate carboxylase. Genes for both carboxylases are present in all fully sequenced *Roseobacter* strains. Pyruvate carboxylase requires biotin as a cofactor, and hence the need to supply *D. shibae* DFL12^T with this vitamin when it is grown on minimal medium (Biebl *et al.*, 2005). A fluxom analysis revealed that, indeed, this enzyme displays the major CO_2-assimilating route in *D. shibae* (Fürch *et al.*, 2009). *D. shibae* DFL12^T is the only fully sequenced *Roseobacter* strain harboring the arginine deiminase pathway, which allows one to carry out a survival fermentation process known from the *Bacillus* and *Pseudomonas* species (Eschbach *et al.*, 2004; Williams *et al.*, 2007).

Electron transport chains

Complex electron transport systems were found (Supplementary material S5), including seven electron-donating primary dehydrogenases, such as for L- and D-lactate, glycerol-3-phosphate, NADH and succinate, and respiratory dehydrogenases for glucose and gluconate. The presence of the aerobically performed anoxygenic photosynthesis further complicates the electron transport machinery. Just as in the mitochondria, which are derived from *Alphaproteobacteria*, a cytochrome bc_1 complex is available. Electron flux can occur from ubiquinol, the reduced form of ubichinone, through the bc_1 complex and different cytochrome c molecules to two types of cytochrome c oxidases to reduce molecular oxygen to water. Consequently, a close coordination between light-dependent cyclic electron transport (photosynthesis) and respiration is required, the mechanism of which remains to be elucidated.

A complete denitrification pathway and a dimethylsulfoxide (DMSO) reductase are available for the reduction of the alternative electron acceptors DMSO and nitrate. Interestingly, the organism uses the Nap (periplasmic) type nitrate reductase, which is oriented toward the periplasmic space and is active both under aerobic and anaerobic conditions, instead of the Nar (respiratory) type facing the cytoplasm, and is induced only under anaerobic/microaerophilic conditions. Anaerobic growth using nitrate as an electron acceptor was confirmed experimentally in a defined mineral medium (data not shown).

Oxygen-dependent regulation of the central energy metabolism

We deduced the regulon for the global oxygen-dependent transcriptional regulator for fumarate and nitrate reduction, Fnr (Supplementary material S3). Six Fnr/Crp (cAMP receptor protein) type regulators are encoded by the genome of *D. shibae* DFL12^T. However, only one of the deduced proteins carries the cysteine residues required for iron sulfur cluster formation. Two others resemble Fnr-type regulators such as Dnr and DnrD (transcription factor for denitrification gene expression). Interestingly, Dnr is located directly upstream of the *nos* operon encoding a nitrous oxide reductase, the enzyme catalyzing the transformation of N_2O to N_2, the last step of denitrification. Such a gene arrangement has not been found before in denitrifying bacteria, for example, in *R. denitrificans.*

Using a position weight matrix approach (Munch *et al.*, 2005), the genome was searched for potential Fnr-binding sites. In agreement with findings for other Gram-negative bacteria, the promoter region of genes for high-affinity oxygen-dependent cytochrome c oxidases (cbb_3-type), alternative anaerobic systems including the NADH dehydrogenase, the quinone oxidoreductase and the corresponding cofactor biosynthesis genes (*hemN1*, *hemN2*, *hemA* and *moaC*) are all carrying Fnr-binding sites. No obvious Fnr boxes were found upstream of the various operons involved in denitrification and DMSO reduction. Interestingly, three universal

stress protein-like genes and eight potential regulator genes are also carrying Fnr boxes. Two of these regulators are suppressor proteins of the heat shock chaperone DnaK (DksA), and the function of the other six regulators is unknown. The Fnr box is also present in promoters of bacteriochlorophyll biosynthesis genes, but it is not possible to predict whether Fnr induces or represses the expression of the corresponding genes.

The Fnr regulator is not a unique feature of *D. shibae* DFL12^T. Searching the complete genome sequences of the *Roseobacter* clade with the *D. shibae* Fnr by reciprocal best hit BLAST showed that one copy is present in all of them. Thus, the ability to fine-tune their metabolism to anoxic conditions is a general trait of the *Roseobacter* clade.

Nutrients provided by the algal host and their uptake
The pathways for the known growth substrates of *D. shibae* DFL12^T (Biebl *et al.*, 2005) could be reconstructed from the genome sequence. Interestingly, most of them are Krebs cycle intermediates (fumarate, succinate, pyruvate and citrate). They may be released from the algal host after cell death or during photosynthesis. Photosynthate release from phytoplankton can be up to 40% of primary production (Wang and Douglas, 1997) and forms the basis of the microbial loop (Azam *et al.*, 1983). *D. shibae* DFL12^T also uses acetate and glucose, important components of the marine dissolved organic carbon (Aluwihare *et al.*, 2002; Biersmith and Benner, 1998). Growth also occurs on fructose and glyceraldehyde-3-phosphate, products of the photosynthetic Calvin–Benson cycle. The dimethylsulfoniopropionate breakdown product DMSO can be used as an alternative electron acceptor by *D. shibae* DFL12^T.

For the uptake of nutrients from the environment, tripartite ATP-independent periplasmic transporters (TRAP) seem to be preferentially used by *D. shibae* DFL12^T rather than ABC-type transporters, which are only about half as frequent as in other members of the *Roseobacter* clade. TRAP transporters are composed of a periplasmic solute receptor (DctP or TAXI) and a secondary transporter (DctM(Q)) (Forward *et al.*, 1997). Transport is driven by an electrochemical ion gradient rather than by ATP hydrolysis, and this may be the reason why TRAP systems have their highest prevalence among marine bacteria (Rabus *et al.*, 1999; Mulligan *et al.*, 2007). Their design allows the coupling of high-affinity solute reception (formerly attributed only to primary transporters) with energy-efficient ion gradient-driven permeases. The substrate range of TRAP systems also comprises, besides C4-dicarboxylates, pyruvate and two other oxoacids, glutamate, sialic acid, ectoine and 2,3-diketogulonate. With 27 complete TRAP systems encoded in its genome, *D. shibae* DFL12^T is second only to *S. pomeroyi* (28 complete systems). Efficient uptake of organic nutrients available only in the sub-micromolar concentration range could provide members of the *Roseobacter* clade with a competitive advantage.

Plasmid biology

Characteristic features of the plasmids
The most striking observation regarding the extrachromosomal elements is the long-range synteny between the 191 and the 126 kb plasmid (Figure 4). About 80% of the sequence of the smaller replicon, including a gene cluster of 47 kb, a *vir* operon and a short inverted region (shown in blue), are highly conserved. This pattern provides strong evidence for a common origin, thus justifying their designation as sister plasmids. The differences among the non-conserved regions result from genomic rearrangements likely assisted by transposition events, an explanation that is supported by the frequent occurrence of transposases and integrases (Supplementary material S6A). Moreover, comparative sequence analyses indicate that a common ancestor of the two sister plasmids may have been recruited through conjugational gene transfer. The *D. shibae* DFL12^T chromosome has a GC content of 66%, which is in the upper range of *Roseobacters*, and the respective values for the plasmids vary between 60% and 69% (Supplementary material S6A). The two sister plasmids show the lowest GC content and the analysis with the Artemis software revealed a poor so-called 'GC Frame Plot' (Supplementary material 6B–D). This finding probably reflects their relatively recent recruitment in an evolutionary time scale and an early stage of sequence adaptation within the new host bacterium. This scenario is supported by the observation that in *D. shibae*, DFL12^T *vir* operons are exclusively present on the sister plasmids (Figure 4). The *vir* gene cluster encoding the type IV secretion system is the structural prerequisite for the formation of sex pili required for conjugation. It is structurally highly conserved among *Roseobacter* strains and also present in *Silicibacter* sp. TM1040 (Moran *et al.*, 2007).

Plasmid replication and maintenance
We identified the core functions for plasmid replication initiation, partitioning and stability representing the 'functional heart' of a plasmid (Supplementary material 7A-C). They provide the structural basis for compatibility versus incompatibility (Petersen *et al.*, 2009). We found three different types for replication initiation including the *repABC* module that is characteristic for the sister plasmids (Figure 4). Our phylogenetic analyses document that their crucial replicases (*repC*) are only distantly related, indicating that at least one of them has been exchanged by a gene transfer event. The respective *repABC* modules hence belong to

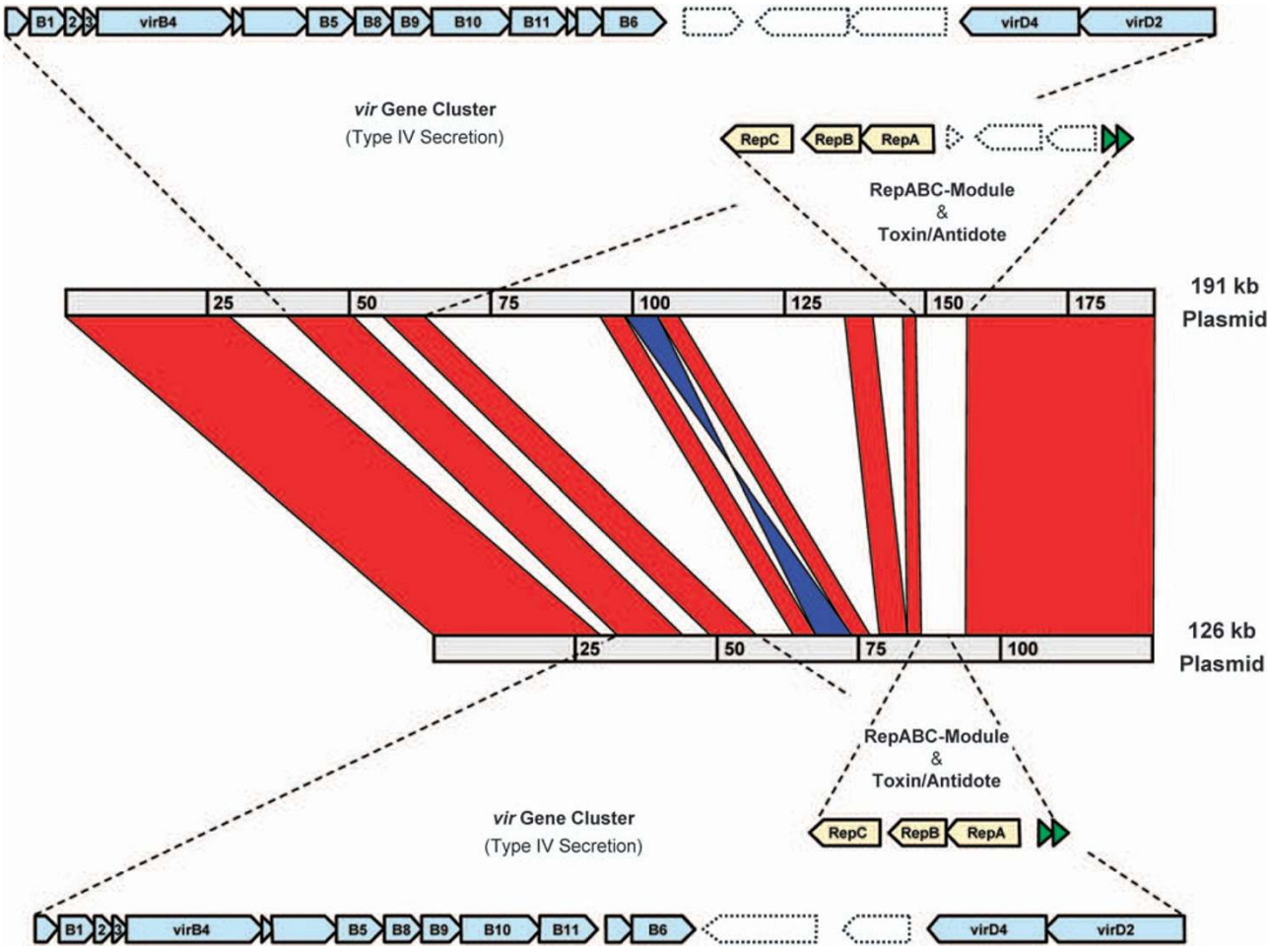


Figure 4 Synteny plot of the 191 and 126 kb plasmids of *D. shibae* DFL12^T. Long-range homologies are shown with red bars and an inverted region is printed in blue. The left and right borders of the plasmids are completely conserved. Homologous *vir* clusters and the distantly related *repABC*-type replicons as well as the adjacent toxin/antidote addiction systems for plasmid stability are shown in detail. Adjacent genes that are not involved in plasmid core functions are indicated with dotted lines. The analysis was calculated with the Artemis Comparison Tool (WebACT, http://www.webact.org/WebACT/generate).

different compatibility groups (Petersen *et al.*, 2009), the prerequisite for a stable coexistence of the syntenous plasmids. Finally, at least four of the five replicons contain putative addiction systems for plasmid maintenance (Zielenkiewicz and Ceglowski, 2001). They are represented by operons encoding a stable toxin as well as a less stable antidote (Figure 4). These selfish units indirectly ensure the maintenance of the low copy replicons in the offspring, as plasmid loss would be lethal for the host cell.

Metabolic capabilities and localization of genes
The extrachromosomal replicons of *D. shibae* DFL12^T can roughly be classified according to their genetic composition (Supplementary material S7D). The two sister plasmids are characterized by the *vir* gene cluster (see above), whereas the other plasmids harbor gene clusters for the degradation of aromatic compounds (153 kb), *cox* operons that presumably contribute to energy production (72 kb) and a wealth of genes for sugar metabolism (86 kb). The latter replicon represents an aged and possibly dying plasmid owing to its conspicuous amount of pseudogenes and transposases. Transposition may dominate intracellular genetic rearrangements as documented for *thiC*, an essential gene for thiamine (vitamin B_1) biosynthesis (Vander Horn *et al.*, 1993). In contrast to all other genes of this pathway that are localized on the *D. shibae* DFL12^T chromosome, altogether three copies of *thiC* are located on different plasmids (191 kb, 153 kb and 86 kb). The genomic neighborhood of an integrase, resolvase and transposase strongly supports an underlying dispersal mechanism based on DNA transposition.

Interaction with other organisms

Quorum sensing
D. shibae DFL12^T produces three different quorum-sensing signals from the group of AHLs, namely a

C_8-homoserine lactone (C8-HSL), and two AHLs with a side chain of 18 carbon atoms, C_{18}-en-HSL and C_{18}-dien-HSL (Wagner-Dobler *et al.*, 2005). This is the maximum side chain length for AHLs and it was, for the first time, detected in *Sinorhizobium meliloti,* a root nodule-forming symbiont (Marketon *et al.*, 2002), where this signal downregulates motility at high cell density (Hoang *et al.*, 2008). The unsaturations found in the C_{18} side chain of DFL12^T are novel and may be critical for signal specificity. The genome analysis predicted three autoinducer synthases (*luxI* type) and five *luxR* type genes for AHL-controlled transcriptional regulators (Supplementary material S3). $luxI_1$ and $luxI_2$ of *D. shibae* DFL12^T are located on the chromosome adjacent to a *luxR* gene with an interspacer region of appr. 100 kb, whereas $luxI_3$ is on the 86-kb plasmid without an adjacent *luxR* gene. *Roseobacter* strains usually have one or two autoinducer synthases, whereas up to four have been found in root nodule-forming *Rhizobia* (Case *et al.*, 2008). The orphan *luxR* genes may allow eavesdropping on competing bacterial species in the environment (Case *et al.*, 2008).

Production of secondary metabolites

Polyketide synthases (PKSs) catalyze the synthesis of polyketides, a large class of secondary metabolites, from acetyl-CoA precursors. *D. shibae* DFL12^T harbors a 12-gene cluster containing two adjacent modular type I PKS genes among hypothetical and putative proteins (Supplementary material S3). The first PKS gene is composed of only one module containing the domains essential for chain elongation (β-ketoacylsynthase domain, acyltransferase domain, and two ACP (acyl carrier protein) domains), a 2-nitropropane dioxygenase domain and a β-ketoacyl-ACP reductase domain. No reductive domain required for the modification of the keto-group could be identified. The second PKS gene contains two modules. In the N-terminal module, a β-ketoacyl-synthase domain and an acyltransferase domain were identified; the C-terminal module shows similarity to a phosphopantetheinyltransferase domain (PKS loading enzyme). The other genes in this cluster, such as the two putative short-chain dehydrogenases or the two putative NAD-dependent epimerases/dehydratases, may be involved in chain modification reactions to give the final active product.

Close homologs of these genes are not found in the *Roseobacter* clade, but a similar gene cluster is harbored by *Solibacter usitatus* (*Acidobacteria*) and *Gloeobacter violaceus* (*Cyanobacteria*), suggesting that these genes may have been acquired by horizontal gene transfer. Thus, *D. shibae* DFL12^T encodes a multiprotein complex that may catalyze the synthesis of a novel polyketide that could have a role in interactions with competing microbes. The dinoflagellate surface is colonized by a range of other bacteria, including *Vibrios* and *Flavobacteria*. Tropodithietic acid, an antibiotic produced by at least two *Roseobacter* isolates, is especially active against *Vibrios* (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005) and is encoded by a different pathway (Geng *et al.*, 2008).

Resistance against viral attack

Viruses are the most abundant biological entity on the planet (Williamson *et al.*, 2008). By lysing a significant fraction of bacterial communities, bacteriophages influence the cycling of organic matter in the sea (Fuhrman, 1999; Weinbauer, 2004; Brussaard *et al.*, 2008; Danovaro *et al.*, 2008) and the genetic diversity of their host populations (Muhling *et al.*, 2005). Continuous viral attack accompanies red tides (Tomaru and Nagasaki, 2004) and contributes to their sudden collapse (Nagasaki *et al.*, 2005; Rhodes *et al.*, 2008). Thus, resistance against phage attack must be highly adaptive in the habitat of *D. shibae* DFL12^T, which has evolved the most complex viral defense system of all *Roseobacter* strains sequenced to date.

CRISPRs (**c**lustered **r**egularly **i**nterspaced **s**mall **p**alindromic **r**epeats) are small, transcribed DNA spacers separated by short palindromic repeats that are located next to a cluster of *cas* (**C**RISPR **as**sociated) genes. Many spacer sequences have been shown to originate from bacteriophages, leading to the current hypothesis that CRISPRs are involved in an RNAi-like mechanism resulting in antiviral resistance (Haft *et al.*, 2005; Makarova *et al.*, 2006; Barrangou *et al.*, 2007). Two CRISPRs were detected in the genome of *D. shibae* DFL12^T, whereas no other completed genome from the *Rhodobacterales* contains more than one. Five organisms of this group revealed a CRISPR (Supplementary material S8). Remarkably, four of them lack the characteristical CRISPR marker gene *cas1*, which is reportedly present in all CRISPR-containing organisms, with *Pyrococcus abyssii*, as formerly, a single exception. Therefore, *cas1* is apparently no reliable marker for CRISPRs in the *Rhodobacterales*.

Figure 5 shows that the two CRISPR/*cas* gene clusters of *D. shibae* DFL12^T have totally different structures. Comparative sequence analyses indicated that they are complete and independent units derived from different microbial lineages. It is most likely that their acquisition occurred through horizontal gene transfer, as suggested earlier (Godde and Bickerton, 2006). To discover the origin of the CRISPR spacer sequences in *D. shibae* DFL12^T, these sequences were compared with the marine metagenomics database, CAMERA (Seshadri *et al.*, 2007), but no homologs were found, most likely because the habitats of *D. shibae* DFL12^T have not yet been searched for phages. Diversification of the CRISPR region is inferred to be a population-level response to the rapidly changing selective pressure of phage predation (Tyson and Banfield, 2008), and hence phage predation must represent a significant selective force in the habitat of *D. shibae* DFL12^T.

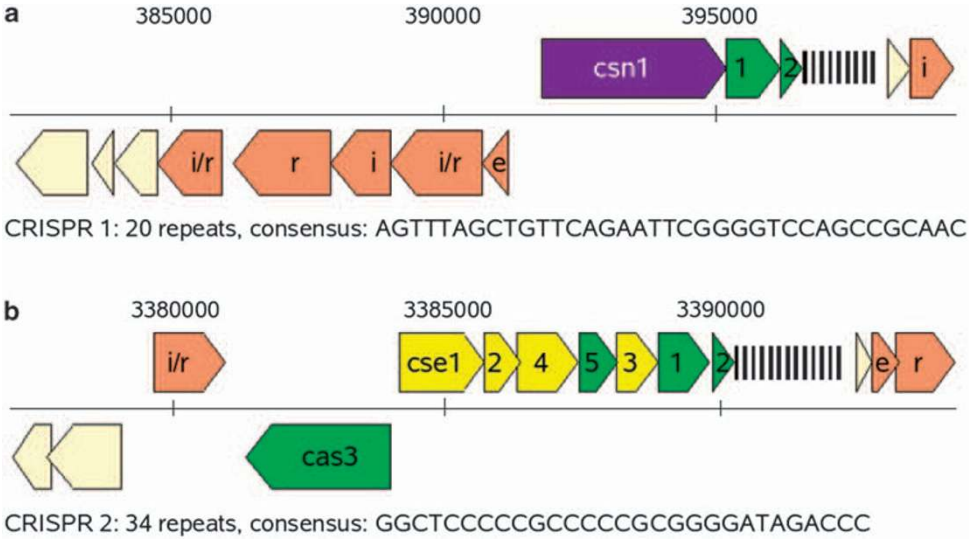


Figure 5 Genomic context of the two CRISPRs of *D. shibae* DFL12^T. The numbers indicate the base position in the genome. Barcode-like series of vertical black bars symbolize CRISPR arrays. The colored gene arrows show the orientation, length and assignment to functional gene groups: Green—*cas* core genes; 1 = *cas1*; 2 = *cas2*; 5 = *cas5*. Purple—*csn1* gene, specific for subtype *Neisseria meningitidis*. Yellow—*cas* genes specific for subtype *Escherichia coli*; 2 = *cse2*; 3 = *cse3*; 4 = *cse4*. Orange—genes for polynucleotide processing; i = integrase; r = recombinase; e = excisionase.

Synthesis and utilization of vitamin B_{12}

Vitamin B_{12}-dependent enzymatic reactions

Most Bacteria and Archaea including *D. shibae* DFL12^T have enzymes that require B_{12} as a cofactor. The genome revealed a gene encoding a B_{12}-dependent methionine synthase, catalyzing the final step in methionine biosynthesis. A gene encoding an enzyme that catalyzes the same reaction in a B_{12}-independent manner was also found. Genes encoding a B_{12}-dependent ribonucleotide reductase and a methylmalonyl-CoA mutase were also found, but genes for other B_{12}-dependent enzymes (glutamate mutase, ethanolamine ammonia lyase, diol dehydratase and glycerol dehydratase) were not present.

Cobalamin biosynthesis

Vitamin B_{12}, which is only synthesized *de novo* by some Bacteria and Archaea, is derived from uroporphyrinogen III, a precursor in the synthesis of heme, siroheme, cobamides, chlorophylls and the methanogenic F430 (Scott and Roessner, 2002; Warren *et al*., 2002; Rodionov *et al*., 2003). At least 25 enzymes are uniquely involved (Roth *et al*., 1996). The genome of *D. shibae* DFL12^T harbors all the genes required for *de novo* B_{12} synthesis (Supplementary material S9), similar to other fully sequenced members of the *Roseobacter* clade, for example, *R. denitrificans* and *S. pomeroyi*. However, it has an unusual route for vitamin B_{12} synthesis. Two different biosynthetic routes for cobalamin are known in Bacteria: (i) an oxygen-dependent (aerobic) pathway and (ii) an oxygen-independent (anaerobic) pathway (Martens *et al*., 2002; Scott and Roessner, 2002; Warren *et al*., 2002). They differ in the first part, the insertion of cobalt into the corrin ring, whereas the second part is common for both the routes. In the aerobic route, cobalt insertion is performed by an ATP-dependent cobalt chelatase, which is encoded in *P. denitrificans* by *cobN*, *cobS* and *cobT*. In the anaerobic pathway, single subunit ATP-independent cobalt chelatases such as CbiX of *B. megaterium* are performing the reaction. *D. shibae* DFL12^T contains putative genes for both types of chelatases and in this respect it is unique among the fully sequenced *Rhodobacterales*. Other pathway-specific genes were also present, but *cobG*, regarded as a signature gene for the aerobic route (Scott and Roessner, 2002), was absent. The gene for an alternative enzyme found in *Rhodobacter capsulatus*, CobZ, was also not present (McGoldrick *et al*., 2005). Thus, *D. shibae* DFL12^T might have the property to synthesize B_{12} by either the aerobic or the anaerobic pathway depending on the availability of oxygen. Alternatively, it might synthesize B_{12} by an unusual combination of both pathways.

We experimentally confirmed that the *de novo* pathway for vitamin B_{12} synthesis is functional in *D. shibae* DFL12^T. The bacteria can grow in a defined mineral medium, but to exclude that traces of vitamin B_{12} might be present even in such media, we compared the B_{12} content of cells grown in the presence and absence of methionine. *D. shibae* DFL12^T has two methionine synthases, one requiring B_{12} and another that is B_{12} independent. In the absence of methionine, the content of B_{12} in the cells should increase, as the B_{12}-dependent methionine synthase requires B_{12} as a cofactor. The vitamin B_{12} content of the cells was 340 ± 36 ng mg^{-1} protein in the presence of methionine and increased to 450 ± 26 ng mg^{-1} protein in the absence of methio-

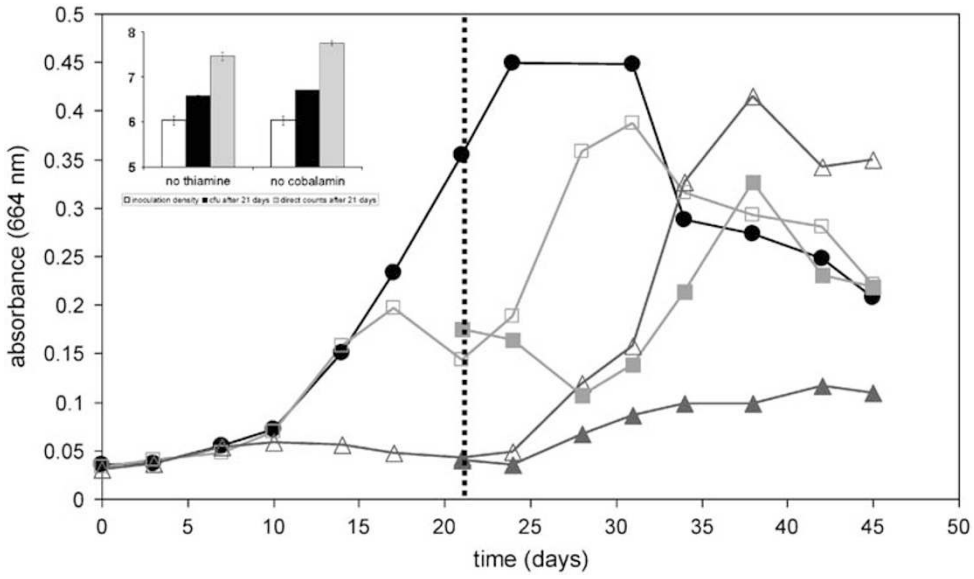


Figure 6 The bacterium *D. shibae* DFL12^T provides thiamine (B_1) and cobalamin (B_{12}) to the dinoflagellate *Prorocentrum minimum* in coculture. Growth of the algae is shown as the amount of chlorophyll *a* (absorbance at 664 nm). Symbols show cultures on L1 mineral salts medium Δ lacking thiamine; □ lacking cobalamin and ● control (complete L1 medium). On day 22 (dotted line), 1% of the cultures lacking thiamine or cobalamin were transferred to fresh medium of the same composition. One half of the culture was provided with the lacking vitamin (open symbols), and the other half was inoculated with *D. shibae* DFL12^T (filled symbols). The inset shows log cell densities of *D. shibae* DFL12^T at the time of inoculation and at the end of the experiment (cfu and direct microscopic counts). A full colour version of this figure is available at *The ISME Journal* online.

nine, showing that a complete *de novo* pathway for vitamin B_{12} synthesis is functional.

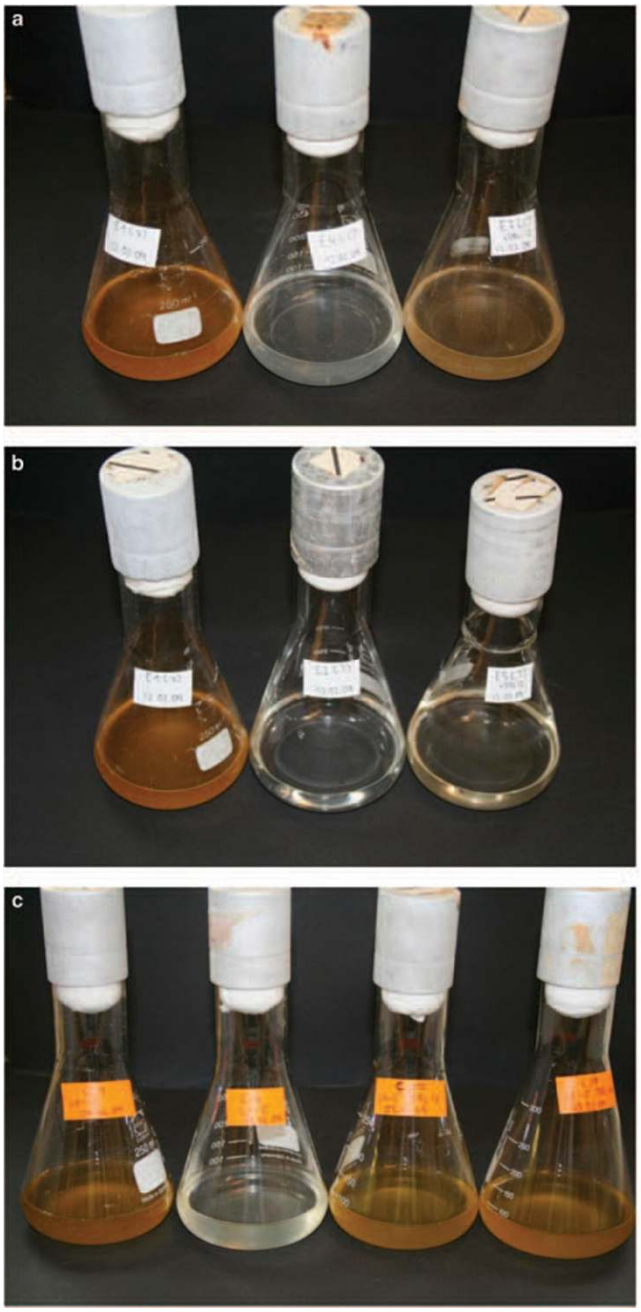
Symbiosis with dinoflagellates

Besides supplying its own B_{12}-dependent enzymes with the cofactor, the versatility in B_{12} production might also contribute to a successful symbiosis with the dinoflagellate. Axenic (bacteria-free) cultures of dinoflagellates can be obtained on defined mineral media, but supplementation with three vitamins is necessary, namely thiamine (B_1), cobalamin (B_{12}) and biotin (B_7 or vitamin H) (Guillard and Hargraves, 1993). *D. shibae* DFL12^T is able to synthesize two of these, B_{12} and B_1. It is auxotrophic for biotin, similar to many *Roseobacter* species (Shiba, 1991), and thus this vitamin must be provided by other sources in nature to both the dinoflagellate and the *Roseobacter* species.

To experimentally determine whether *D. shibae* DFL12^T can provide B_1 and B_{12} to its dinoflagellate host, we performed coculture experiments. Axenic cultures for *P. lima* are not available in culture collections, but as the symbiosis of *D. shibae* with algae is not strictly host specific, we reasoned that proof of principle might be obtained using the closely related dinoflagellate *P. minimum* CCMP1329.

First, auxotrophy of *P. minimum* for B_1 and B_{12} was investigated, as it had not been tested earlier (Croft *et al.*, 2005). Supplementary material S10 shows that *P. minimum* was not able to grow on a synthetic mineral medium from which either B_1 or B_{12} had been omitted. Next, either B_1 or B_{12} were replaced by 1.1×10^6 cells per ml of *D. shibae* DFL12^T. In the experiment shown in Figure 6, the dinoflagellate was first cultivated for 22 days without bacteria; there was no growth without thiamine, and cobalamin became growth limiting after about 17 days. On day 22, the cultures were divided. One half was provided with the lacking vitamin, and the other half was inoculated with *D. shibae* DFL12^T. Figure 6 shows that *D. shibae* DFL12^T stimulated the growth of the alga in the absence of B_1 or B_{12}, and that the alga allowed the bacterium to grow too (inlay in Figure 6). From an initial density of 1.1×10^6 cfu ml^{-1}, they increased to 5.1×10^6 cfu ml^{-1}. Bacterial densities determined by direct microscopic counts were even higher by more than one order of magnitude. The density of the dinoflagellate at the end of the experiment was appr. 2.5×10^6 cells ml^{-1}, as determined by microscopic counts. Supplementation with pure vitamins resulted in faster recovery of the dinoflagellate culture in the case of B_{12}, and in much stronger recovery in the case of B_1 compared with inoculation with *D. shibae* DFL12^T. The bacteria were entirely dependent on carbon sources excreted by the algae, and thus may have been initially starved in the non-growing algal culture. The concentration of thiamine present in the cultivation medium is 200-fold higher than that of B_{12}, and therefore the amount synthesized by the relatively low density of bacteria might not have been sufficient.

Experiments with the haptophyte *I. galbana* CCMP1323, from which *D. shibae* DFL12^T has also been isolated and which is known to be auxotrophic for B_{12} (Croft *et al.*, 2005), showed similar results for cobalamin, but this alga did not require thiamine for



***Prorocentrum minimum* CCMP1329.**
Media are (from left to right) L1 medium, L1 medium lacking cobalamin, and L1 medium lacking cobalamin inoculated with *D. shibae* DFL12^T.

***Prorocentrum minimum* CCMP1329.**
Media are (from left to right) L1 medium, L1 medium lacking thiamine, and L1 medium lacking thiamine inoculated with *D. shibae* DFL12^T.

***Isochrysis galbana* CCMP1323.**
Media are (from left to right) L1 medium, L1 medium lacking cobalamin, L1 medium lacking cobalamin inoculated with 10 µl *D. shibae* DFL12^T and L1 medium lacking cobalamin inoculated with 100 µl *D. shibae* DFL12^T

Figure 7 Cultures of the axenic dinoflagellate *Prorocentrum minimum* CCMP1329 (**a** and **b**) and the axenic haptophyte *Isochrysis galbana* CCMP1323 (**c**) inoculated with *D. shibae* DFL12^T in the absence of vitamin B_{12} and (**a** and **c**) or vitamin B_1 (**b**).

growth. Figure 7 shows the effects of cocultivation with *D. shibae* DFL12^T on cultures of *P. minimum* and *I. galbana* in media lacking cobalamin or thiamine.

Discussion

Adaptation to periodic anoxia

D. shibae DFL12^T is clearly optimized for respiratory modes of energy conservation. Its electron transport systems are fueled both by organic carbon compounds and light, with ubiquinone as a central intermediate pool. However, the arginine deiminase survival fermentation pathway and the ability to use alternative electron acceptors (nitrate, DSMO) allow *D. shibae* DFL12^T to sustain an active energy metabolism in the complete absence of oxygen. Several of the key genes involved are also found in other *Roseobacter* species. Therefore, the current paradigm that the *Roseobacter* clade comprises only obligate aerobes needs to be revised. Periodic anoxia is regularly encountered in photosynthetic biofilms at night as a result of intense respiration, as shown,

for example, for microbial mats (Steunou *et al.*, 2008). Moreover, steep gradients of oxygen and nitrate are present in marine sediments (Lorenzen *et al.*, 1998), and as microorganisms are easily transported by bioturbation to deeper layers (Pischedda *et al.*, 2008), the ability of *D. shibae* DFL12^T to switch between alternative electron acceptors is crucial.

D. shibae *DFL12^T is able to provide the dinoflagellate host with vitamins*

The genome data suggest that *D. shibae* DFL12^T is able to synthesize two nutrients that are essential and potentially growth limiting for their hosts, vitamin B_{12} (cobalamin) and vitamin B_1 (thiamine). The pathways for both of them have unique features: Altogether, three copies of the thiamine synthetase, *thiC*, are present on different plasmids (191 kb, 153 kb and 86 kb), whereas all the other thiamine synthesis genes are located on the chromosome. Synthesis of vitamin B_{12} should be possible by an oxygen-dependent as well as an oxygen-independent pathway in *D. shibae* DFL12^T, whereas organisms studied to date use only one of these pathways, pointing to both the importance of anaerobic conditions in the life of *D. shibae* DFL12^T and to the importance of B_{12}.

We experimentally confirmed that a pathway for *de novo* synthesis of B_{12} is functional in *D. shibae* DFL12^T and that the bacteria can provide both B_1 and B_{12} to the dinoflagellate *P. minimum* in a defined coculture. Thus, a symbiotic relationship between *D. shibae* DFL12^T and its dinoflagellate host may exist, which is based on an exchange of micronutrients synthesized at extremely low concentrations but high metabolic costs by the bacteria against photosynthate leaking from the algae during photosynthesis. As *D. shibae* is associated with several phylogenetically diverse species of algae, this symbiosis is not host specific. The dinoflagellate may replace *D. shibae* with other bacteria, provided they carry out the same essential functions, for example, synthesize B_{12}. Conversely, *D. shibae* may live with another algae if it excretes photosynthate, dimethylsulfoniopropionate and so on A variety of symbioses ranging from loose mutualistic interactions to obligate endosymbiotic relationships are increasingly discovered on the basis of genomic data and novel experimental methods (Moran, 2006). However, *D. shibae* DFL12^T could also be a scavenger of dead algae during the collapse of algal blooms. The metabolic flexibility as shown in the genome suggests that it might even switch from a symbiotic to a parasitic mode of life.

Growth limitation of phytoplankton by vitamins in the sea

Although 50% of all dinoflagellate species require B_{12}, they may not be dependent on a symbiosis with bacteria to obtain it (Croft *et al.*, 2005), as the requirements are so low that ambient concentrations in seawater may suffice (Droop, 2007). New methods have been used to determine the pmolar concentrations of B_1 and B_{12} *in situ* (Okbamichael and Sanudo-Wilhelmy, 2005). B_{12} concentrations correlate with bacterial densities at certain times and in some locations and decreased 90% during dinoflagellate blooms, relative to pre-bloom values (Gobler *et al.*, 2007). B_{12} colimitation with iron was, for the first time, shown in coastal waters of the Antarctic peninsula (Panzeca *et al.*, 2006) and later on in the Ross Sea, one of the most productive areas in the Southern Ocean (Bertrand *et al.*, 2007). In this study, the bacterial densities are low; cyanobacteria, which are able to synthesize B_{12}, are absent, and UV irradiance is high, all accounting for low ambient levels of B_{12}. These phytoplankton communities were iron limited, but adding B_{12} together with iron resulted in an additional increase in biomass. One of the dominant dinoflagellates present at the time was *P. minimum*, which was shown here to be auxotrophic for B_{12}, supporting the results of Bertrand *et al*. Thus, B_{12} can clearly be growth limiting in the sea under certain conditions. The composition of the bacterial flora may therefore have consequences for the succession of phytoplankton species (Panzeca *et al.*, 2008). Field investigations of bacterial communities associated with algae at such a level of detail, deciphering not only phylogenetic composition but also metabolic function, have not yet been carried out, but the breathtaking progress of sequencing whole microbial communities (Nealson and Venter, 2007) and, in the future, even single cells will eventually change this.

Horizontal and vertical gene transfer and the adaptive Roseobacter *gene pool*

The members of the *Roseobacter* clade are characterized by a significant fraction of extrachromosomal elements in their genomes (Pradella *et al.*, 2004), with *D. shibae* DFL12^T currently housing the largest number of plasmids among the fully sequenced strains. Conjugation obviously has a crucial role in the horizontal spread of these genetic elements, even if the experimental proof is still lacking. A conspicuous example is the presence of a 47-kb gene cluster on the two sister plasmids of *D. shibae* DFL12^T and on several other extrachromosomal elements of the *Roseobacter* clade, which also harbor the *vir* gene cluster required for conjugation. Genes that have been found on *Roseobacter* plasmids include not only typical plasmid-encoded traits such as degradation of aromatic compounds, but also key genes for biogeochemical cycles, for example, carbon monoxide oxidation in *D. shibae* DFL12^T and even the complete photosynthesis gene cluster in *R. litoralis* ((Pradella *et al.*, 2004) and integrated microbial genomes). The horizontal transfer of these traits within the *Roseobacter* clade is

consistent with their patchwork distribution in the phylogenetic tree (Buchan *et al*., 2005). Our data show that the boundary between the chromosome and the plasmid is dynamic, as outsourcing of chromosomal genes to plasmids can be observed and increases the shared adaptive gene pool in the lineage.

Besides plasmids, phages also may mediate horizontal gene transfer. In total, 5% of the genome of *S.* TM1040 consists of prophages (Chen *et al*., 2006; Moran *et al*., 2007). A phage-like structure, the so-called GTA (gene transfer agent), was discovered many years ago in *Rh. capsulatus.* GTA particles contain only a few kilobases of DNA, are not inducible by mitomycin and do not form plaques. They contain small random pieces of host DNA, rather than a phage genome, suggesting that their main activity is the lateral transfer of host DNA. A search for the 15 GTA genes from *Rh. capsulatus* in complete and draft bacterial genomes showed that they are exclusively present in *Alphaproteobacteria*, and that all but one of the genome sequences in the *Roseobacter* clade have a GTA-like gene cluster, including *D. shibae* DFL12^T (Biers *et al*., 2008). Thus, the genetic adaptability of the *Roseobacter* gene pool appears to be because of the richness of extrachromosomal elements, some of which are conjugative, the mobilization of genes from the chromosome to the plasmid, and a phage-like gene transfer mechanism unique in *Alphaproteobacteria*.

Outlook

Roseobacter bacteria are often associated with marine algae and reach high abundances during algal blooms. Our findings shed new light on this association, suggesting that it might be a symbiotic relationship in which a bacterium is required by the alga for the supply of essential nutrients, which can be growth limiting under certain conditions. Conversely, the bacteria may scavenge on dead algae and possibly switch between both types of interactions.

Studying the specificity and molecular mechanisms of this relationship in detail, both in the laboratory and in the sea, remains a fascinating field for future analyses.

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