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Fatal affairs – conjugational transfer of a dinoflagellate-killing plasmid between marine *Rhodobacterales*

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Abstract

The roseobacter group of marine bacteria is characterized by a mosaic distribution of ecologically important phenotypes. These are often encoded on mobile extrachromosomal replicons. So far, conjugation had only been experimentally proven between the two model organisms *Phaeobacter inhibens* and *Dinoroseobacter shibae*. Here, we show that two large natural RepABC-type plasmids from *D. shibae* can be transferred into representatives of all known major *Rhodobacterales* lineages. Complete genome sequencing of the newly established *Phaeobacter inhibens* transconjugants confirmed their genomic integrity. The conjugated plasmids were stably maintained as single copy number replicons in the genuine as well as the new host. Co-cultivation of *Phaeobacter inhibens* and the transconjugants with the dinoflagellate *Prorocentrum minimum* demonstrated that *Phaeobacter inhibens* is a probiotic strain that improves the yield and stability of the dinoflagellate culture. The transconjugant carrying the 191 kb plasmid, but not the 126 kb sister plasmid, killed the dinoflagellate in co-culture.

DATA SUMMARY

Raw sequence files have been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under accession number PRJEB46933. Strains are available from the authors upon request.

INTRODUCTION

Most bacterial species contain one large chromosome, but many strains carry additional smaller and autonomously replicating DNA molecules that are frequently exchanged between populations and even across species barriers by a process designated as conjugation. The effectiveness of these extrachromosomal replicons (ECRs) as vehicles for horizontal gene transfer is demonstrated by the rapid spread of antibiotic-resistance cassettes between pathogens and around the globe (e.g. [1–3]). ECRs can either be classified as chromids with a genetic imprint (G+C content, codon usage, tetranucleotide composition) similar to that of the chromosome or as genuine plasmids with a deviant genetic imprint [4, 5].

The roseobacters, a paraphyletic group within the *Rhodobacterales* that are adapted to the marine environment [6], represent an ideal system to study the ecological and evolutionary implications of conjugation in the ocean [7, 8]. Most roseobacters have compartmentalized genomes, harbouring one chromosome and up to 12 ECRs [5, 9, 10]. Many roseobacter plasmids harbour type IV secretion systems (T4SSs) that are required for conjugation [5, 11].

Comparative genome analyses have documented the natural transfer of plasmids in the ocean: homologues of the conjugative 126 kb plasmid of *Dinoroseobacter shibae* were discovered in two phylogenetically and geographically distant roseobacters [12].

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Abbreviations: ECR, extrachromosomal replicon; TDA, tropodithietic acid; T4SS, type IV secretion system.

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Impact Statement

Horizontal transfer of plasmids is a crucial mechanism for bacterial adaptation and evolution. Our findings suggest that conjugation can massively change the ecological niche of a species and is a potential driver of evolution in the roseobacter group. The library of transconjugant strains generated will allow further insights into the physiological consequences resulting from a differential content and interactions of extrachromosomal replicons.

Two roseobacter isolates from the phycosphere of the diatom *Skeletonema marinoi* carry virtually identical plasmids, indicating that conjugative transfer must have occurred recently [13]. We also provided experimental evidence for conjugation of plasmids from *D. shibae* DSM 16493^T into *Phaeobacter inhibens* DSM 17395 [14]. Donor and recipient are two well-studied roseobacter model organisms, both showing a 'Jekyll and Hyde'-behaviour in co-culture with microalgae, i.e. a switch from mutual symbiosis to pathogenicity [15, 16]. Furthermore, both species have been found to co-occur in the same algal bloom [17].

The donor *D. shibae* has been isolated from a culture of the dinoflagellate *Prorocentrum lima* [18]. Its 4.4 Mb genome consists of one chromosome, two chromids and three plasmids [19]. The two conjugative T4SS-carrying plasmids, 191 kb and 126 kb in size, share extensive syntenic regions and, therefore, were termed 'sister plasmids'. In co-culture with axenic *Prorocentrum minimum*, *D. shibae* first promotes growth of its host by providing the essential vitamins thiamine (B_1) and cobalamin (B_{12}); however, when the alga reaches stationary phase, it is killed by the bacterium [16, 19]. A *D. shibae* mutant that lacks the 191 kb plasmid, but still contains the 126 kb sister plasmid, has lost its ability to kill the dinoflagellate [20]. Therefore, the 191 kb plasmid was named 'killer plasmid'.

The recipient, *Phaeobacter inhibens*, was isolated from the Spanish Atlantic coast in Galicia and was originally named *Roseobacter gallaeciensis* [21, 22]. Its 4.2 Mb genome comprised one chromosome and three chromids, and the gene inventory suggests adaptation to life on surfaces [23]. The 65 kb chromid harbours genes for the metabolism of polysaccharides, and its curing results in a nearly complete loss of motility and surface attachment on the green alga *Ulva lactuca* [24]. The 262 kb chromid harbours the gene cluster for the antibiotic tropodithietic acid (TDA) [25], the synthesis of which is controlled by quorum sensing [26]. TDA acts as a redox shuttle and collapses the proton-motive force; therefore, the producing cell has to continuously counteract its own TDA activity [27]. Accordingly, maintenance of this chromid is extremely costly [28, 29].

Here, we show that the natural 191 kb killer plasmid of *D. shibae* and its 126 kb sister plasmid can be transferred into representative strains from the five major roseobacter clades and study the impact of xenologous plasmids on the co-cultivation of *Phaeobacter inhibens* with the dinoflagellate *Prorocentrum minimum*.

METHODS

Phylogenomic analysis

For multilocus sequence analyses (MLSA), the unique core genome of 42 *Rhodobacterales* genome comparisons was determined using the bidirectional BLAST+ approach, as previously described [30]. The final selection of 494 marker proteins with a combined length of 167 276 conserved amino acid residues was concatenated and clustered using the maximum-likelihood method implemented in RaxML [31], with the 'rapid bootstrap' method and 250 bootstrap iterations.

Strains and cultivation conditions

The bacterial strains used in this study, their source and genotype are listed in Table S1 (available with the online version of this article). Strains were routinely cultivated in 0.5× marine broth (MB) medium at 28 °C with shaking in the dark, except for co-cultivation experiments with *Prorocentrum minimum* described below.

Plasmid conjugation

The *D. shibae* DSM 16493^T mariner transposon mutant 17-B12 was used as a plasmid donor strain for all conjugation experiments [32]. The transposon with a gentamicin-resistance cassette is located in the middle of the highly syntenic 60 kb region of the 191 kb plasmid pDSHI01 or the 126 kb plasmid pDSHI03 {Dshi_3777/Dshi_4053 [integration position(s) 176 565/113 144]}, which should allow conjugation of both sister plasmids independently (see [14]). The underlying mechanism is the frequent recombination between identical plasmid sequences resulting – due to the intermediary formation of a fused composite plasmid – in a reversible localization of the mariner transposon in one of the two sister plasmids. Accordingly, the *D. shibae* plasmid donor strain that was used for our conjugation experiments already represented a heterogeneous population.

Six distantly related strains from the Roseobacter group, i.e. *Phaeobacter inhibens* DSM 17395 (clade 1), *Sulfitobacter dubius* DSM 16472^T (clade 2), *Sulfitobacter* sp. DFL-23 DSM 107132 (clade 2), *Marinovum algicola* DG898 DSM 27768 (clade 3), *Limimaricola*

cinnabarinus DSM 29954^T (clade 4) and *Aliiroseovarius crassostreae* DSM 16950^T (clade 7), were chosen as recipient strains for conjugation experiments. They were mutagenized with the EZ-Tn5 <R6Kγori/KAN-2> transposon kit (Epicentre) to tag them with a kanamycin-resistance cassette, which was the prerequisite for the selection of transconjugants. The insertion sites of at least ten transposon mutants of each strain were determined via arbitrary PCR and, if possible, mutants where the transposon was integrated in an intergenic region of the chromosome were used for conjugation.

Conjugation experiments with the *D. shibae* donor 17-B12 and the respective recipient strains were performed at 28 °C in MB medium with a modified protocol compared to that published by Patzelt *et al.* [14]. Precultures of *D. shibae* and the recipient strains were grown in 0.5× MB medium with gentamicin (80 µg ml^{-1}) and kanamycin (120 µg ml^{-1}), respectively. Mating of the two roseobacter strains (500 µl donor, 50 µl recipient) from the exponential growth phase was conducted on MB plates without antibiotics for 36 h, bacteria were resuspended with InoculatorZ cotton swabs (Biolog) in liquid MB medium and transconjugants were selected on 0.5× MB plates with gentamicin (80 µg ml^{-1}) and kanamycin (120 µg ml^{-1}).

The experiments for the quantification of the conjugation efficiency were conducted as follows. Comparability of different mating experiments was ensured by the usage of the same *D. shibae* donor preculture and a comparable amount of recipient cells, whose actual volume was calculated based on OD_{600} values of the different strains. The mating was performed on MB plates for 22 h and the cells were then resuspended in 500 µl MB medium. A total of 50 µl cells and 450 µl MB medium were used for a logarithmic dilution series, including a short vortexing after each dilution step. A 50 µl aliquot of cell suspension was plated in parallel on (i) 0.5× MB plates with gentamicin and kanamycin (experiment), as well as (ii) 0.5× MB plates with kanamycin (reference). The respective ratio of counted colonies reflects the conjugation rate.

Plasmid stability tests

The stable maintenance of the conjugated *D. shibae* plasmids in *Phaeobacter inhibens* was investigated according to the work of Bartling *et al.* [33] with the following modifications. Cultivation of the transformants in MB medium without antibiotics was extended from 14 h to 7 days. MB plates (0.5×) with gentamicin (40 µg ml^{-1}) and kanamycin (120 µg ml^{-1}) were used for the detection of spontaneous plasmid loss. The presence of the respective xenologous plasmid was investigated for 50 independent colonies of each transconjugant with three biological replicates.

Plasmid curing

The presence of the 65 kb biofilm chromid impairs the measurement of *Phaeobacter inhibens* DSM 17395 growth in microplates due to rosette formation and surface attachment [24]. Moreover, this replicon had spontaneously been lost in both transconjugants (Δ65kb+126kb, Δ65kb+191kb) [14]. Therefore, we cured the 65 kb replicon from all strains used for comparison. We chose the well-characterized Δ*tdaE* transposon mutant [transposon insertion in position 104 157 on the minus strand of the 262 kb chromid (NC_018291.1)] [29] and generated a Δ65kbΔ*tdaE* curing mutant, as previously described [24].

PCR-based differentiation of curing mutants and transconjugants

DNA of the donor strains, plasmid curing mutants and transconjugants was isolated with the NucleoSpin plasmid DNA kit (Macherey-Nagel) and 5 ng was used for PCR amplification with OneTaq DNA polymerase (New England Biolabs). The presence of the three *Phaeobacter inhibens* DSM 17395 chromids and the *D. shibae* 191 and 126 kb plasmids was monitored with primers specific for the respective replicon (see Table S2 for a list of all primers used). Transconjugants were validated via PCR amplification and sequencing of the 16S-rRNA gene, PCR amplification of the gentamicin-resistance cassette, and specific PCR for the 126 and 191 kb plasmids.

DNA isolation and genome re-sequencing

Genomic DNA from all *D. shibae* DSM 16493^T strains (wild-type, Δ191kb) and *Phaeobacter inhibens* DSM 17395 strains (Δ65kb, Δ65kb+126kb, Δ65kb+191kb, Δ65kbΔ262kb+126kb, Δ65kbΔ262kb+191kb) was isolated with the Qiagen genomic DNA kit. Illumina Nextera library preparation and sequencing were performed as recently described [29]. PacBio library preparation, sequencing, genome assembly and error correction with Illumina reads were conducted as previously described [33]. The preparation of PCR-free low throughput single-stranded TruSeq Illumina DNA libraries was conducted according to the recommendations of the manufacturer.

Detection of sequence variants and coverage estimation

DNA sequences and annotation files for *Phaeobacter inhibens* DSM 17395 and *D. shibae* DSM 16493^T were obtained from the National Center for Biotechnology Information. Genomes of the transconjugant strains were generated by combining the FASTA files of corresponding replicons. The accession numbers are as follows: *D. shibae* chromosome, NC_009952.1; 191 kb plasmid, NC_009955.1; 153 kb chromid, NC_009956.1; 126 kb plasmid, NC_009957.1; 86 kb plasmid, NC_009958.1; 72 kb chromid,

NC_009959.1; *Phaeobacter inhibens* chromosome, NC_018290.1; 262 kb chromid, NC_018291.1; 78 kb chromid, NC_018287.1; 65 kb chromid, NC_018288.1.

To estimate the coverage (copy number) of the replicons and reveal potential variants, Illumina reads were mapped on the respective reference genomes with BWA version 0.6.2 [34]. From the mpileup files generated via SAMtools (v0.1.19) [35], the coverage per position was extracted, and all variants with a frequency above 10% were extracted applying VarScan [36]. The coverages were plotted with the R package ggplot2 [37] and the copy number was calculated as the ratio of the median replicon coverage to the median chromosomal coverage.

Cell counting

The cell numbers of algal and bacterial pre-cultures, as well as main bacterial cultures, were determined using a BD FACS Canto flow cytometer (BD Biosciences). *Prorocentrum minimum* was identified according to its autofluorescence of chlorophyll. *D. shibae* and *Phaeobacter inhibens* were identified using staining with SYBR Green I (Molecular Probes). Both chlorophyll and SYBR Green I are excited with the 488 nm excitation laser, and emit at 695 nm (far red) and 519 nm (green), respectively. Depending on the cell size and fluorescence of these two organisms, typical settings of the flow cytometer for *Prorocentrum minimum* were as follows: forward scatter (FSC)=300, side scatter (SSC)=250, far red fluorescence (PerCP-Cy5.5)=300. For *D. shibae* stained with SYBR Green I, the typical settings were as follows: FSC=700, SSC=400, green fluorescence (FITC)=400.

For cell counting, 1 ml culture was fixed with 25% glutaraldehyde to a final concentration of 2% for about 15 min at room temperature. For determining the population density of *Prorocentrum minimum*, a sample of 500 µl was analysed, while for bacterial culture the sample was diluted to an appropriate density (<1000 events s^{-1}) with PBS (pH 7.0). Prior to analysis, the SYBR Green I was added at a final concentration of 10^{-4} of the stock reagent. Each sample was analysed for approximately 60 s at a flow rate of 1.2 µl s^{-1} determined according to work by Marie *et al.* [38]. After analysis, the acquisition time and the number of cells acquired were recorded to calculate the population density for each sample.

Co-cultivation of roseobacters with *Prorocentrum minimum*

The axenic culture of *Prorocentrum minimum* strain CCMP 1329 used in this work was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA). *Prorocentrum minimum* CCMP 1329 was cultivated in L1−Si medium (Table S3) in 100 ml batches in 300 ml Erlenmeyer flasks at 22 °C under a 12:12 h light–dark cycle with a light intensity of about 40 µmol photons m^{-2} s^{-1}. The algal culture was maintained in our lab by transferring 1% of the culture volume to fresh medium every fourth week, lack of contaminating bacteria was checked by streaking aliquots on LB and MB plates. *Phaeobacter inhibens* strains and *D. shibae* DSM 16493^T wild-type strain were grown at 30 °C and agitated using a shaker at 160 r.p.m. in the dark in defined artificial sea water medium (SWM) supplemented with 5 mM succinate (Table S3).

For co-cultivation experiments, the bacterial pre-culture was grown to late exponential phase, washed once by centrifugation at 5000 r.p.m. for 5 min, and re-suspended in L1−Si medium lacking vitamin B_{12} (L1−Si−B_{12}). The cell numbers of bacterial and algal pre-cultures were determined by flow cytometry. The co-culture was obtained by adding bacterial cells up to a final density of 10^7, 10^8 and 10^9 cells ml^{-1} to the culture of *Prorocentrum minimum* immediately after subculturing it in fresh L1−Si−B_{12} medium, with an initial density of approximately 2000 cells ml^{-1}. In addition, *Prorocentrum minimum* alone in L1−Si medium with and without B_{12} was used as positive and negative controls, respectively. The co-cultures and the control cultures were downscaled into 200 µl in a microplate and prepared in eight replicates, and incubated under the same conditions as the algal culture. The growth of *Prorocentrum minimum* was examined according to the autofluorescence of its chlorophyll, which was measured using a TECAN Infinite 200 microplate reader at λex=466 nm and λem=678 nm.

RESULTS

Conjugation of *D. shibae* plasmids into roseobacters

Roseobacter strains from five major clades (Figs 1 and S1) were tagged with a kanamycin-resistance marker and served as recipients for conjugation experiments. *D. shibae* (mutant 17-B12) served as donor and harboured the gentamicin-resistance gene in the middle of the syntenic 60 kb region of either of the two sister plasmids. Due to frequent recombination, both replicons could be alternatively conjugated into the new recipient. Furthermore, a simultaneous transfer of both plasmids in the same conjugation experiment [14] is best explained by the transient formation of a composite plasmid resulting from the recombinatory fusion of the sister plasmids. Two transconjugants of each conjugation experiment were analysed for the identity of the transferred plasmids by two PCRs specific for the non-syntenic region of each sister plasmid (Figs S2 and S3). The analysis of the formerly established *Phaeobacter inhibens* transconjugants confirmed the presence of the 191 and 126 kb plasmid in strain 17-B12_21-II (Δ65kb+191kb) and 17-B12_24-II (Δ65kb+126kb), respectively [14]. Both brownish strains lacked the 65 kb biofilm chromid, but still harboured the 78 and 262 kb chromids (Fig. S2).

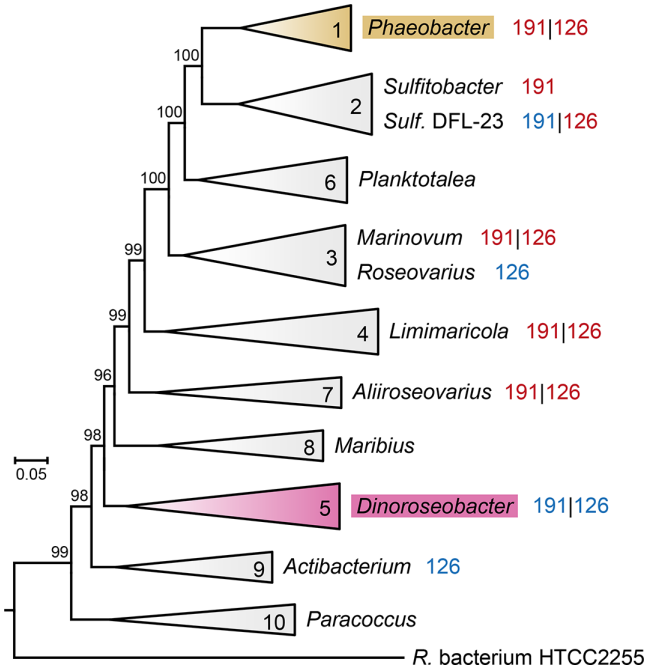

Fig. 1. Conjugational transfer of *D. shibae* 126 and 191 kb plasmids within the roseobacter group. Phylogenomic tree of *Rhodobacterales* clades 1 to 10 (as defined by Bartling *et al.* [30]); the branches for the clades hve been collapsed and the complete tree is shown in Fig. S1. Naturally occurring homologues of the 126 and 191 kb *D. shibae* plasmids are indicated in blue beside the respective leaf. Successful experimental conjugation of either plasmid is indicated in red. *Phaeobacter* and *Dinoroseobacter* are according to their natural pigmentation highlighted in brown and pink, respectively. Branch lengths show substitutions per site. Bootstrap values are shown near the branch nodes.

The two newly established *Sulfitobacter dubius* transconjugants harboured the 191 kb killer plasmid (Fig. S3). The 126 kb sister plasmid was conjugated into *Sulfitobacter* sp. DFL-23 twice. Interestingly, our PCR analysis of the DFL-23 wild-type and both transconjugants revealed an at first inexplicable band on the gel (Fig. S3), but genome analysis of this *Sulfitobacter* strain showed the presence of a natural 494 kb plasmid that shares several syntenic regions with the 191 kb killer plasmid (99.4% sequence identity over a total length of 36 kb). Both *D. shibae* plasmids were found in the transconjugants of *M. algicola* and *Aliiroseovarius crassostreae*. Finally, one *L. cinnabarinus* transconjugant had obtained the 126 kb plasmid, whereas the second transconjugant harboured a chimeric plasmid containing parts of the 191 and 126 kb plasmid (Fig. S3), which likely reflects illegitimate plasmid recombination before conjugation. Taken together, the current study provides experimental evidence for plasmid transfer via conjugation across phylogenetically distant *Rhodobacterales* (Fig. 1), i.e. from *D. shibae* (clade 5) to *Phaeobacter inhibens* (clade 1), *Sulfitobacter* sp. DFL-23 and *Sulfitobacter dubius* (clade 2), *M. algicola* (clade 3), *L. cinnabarinus* (clade 4) and *Aliiroseovarius crassostreae* (clade 7).

Efficiency of conjugation

In order to determine the efficiency of horizontal plasmid transfer, we performed conjugation experiments with one *D. shibae* preculture and four different recipient strains (Table S4). It should be mentioned that quantification of conjugation is probably hindered by the formation of a complex cellular matrix on surfaces that has previously been investigated for *Phaeobacter inhibens* DSM 17395 and *M. algicola* DSM 27768 [9, 24]. Moreover, aggregation and formation of the typical rosettes of *Phaeobacter*, which includes an active attachment of single cells at their cell poles [39], likely influences the dilution series that is required to obtain single colonies. However, our experimental set-up allowed us to roughly estimate the order of magnitude of conjugation efficiency. Colony counting showed a total number between 1.8 and 14.9 million transconjugants calculated for three of the recipient strains, while the experiment with *Sulfitobacter* sp. DFL23 resulted in only 200 transconjugants, which was below the detection level at the default dilution of 1×10^{-5} (Table S4). The transconjugant to recipient wild-type ratio of 1:1782, 1:1002 and 1:46 calculated for *Phaeobacter inhibens* DSM 17395, *Aliiroseovarius crassostreae* DSM 16950 and *M. algicola* DSM 27768, respectively, reflects the high efficiency of T4SS-mediated plasmid transfer.

Establishment of *Phaeobacter inhibens* reference strains by chromid curing

We next focused on transconjugants of *Phaeobacter inhibens* to understand the impact of introducing plasmids into non-native host strains. As a first step, we developed new reference strains varying in presence/absence of chromids. Both *Phaeobacter*

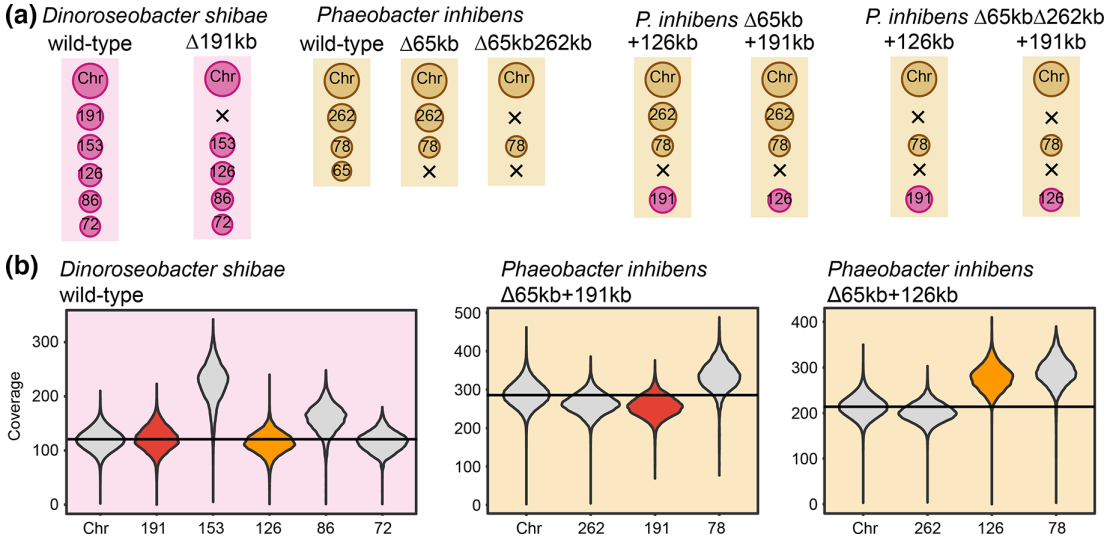

Fig. 2. Genome organization and ECR copy number of *D. shibae* and *Phaeobacter inhibens* strains. (a) Genome organization of *Phaeobacter inhibens* and *D. shibae* strains used for comparative analyses. Host cell and replicon affiliation is colour-coded in brown and pink for *Phaeobacter* and *Dinoroseobacter*, respectively. X indicates absence of the respective replicon. Chr, chromosome. ECRs are indicated by their size in kb. (b) The mean coverage was calculated via mapping of Illumina sequences on the different replicons. The coverage range of each replicon is illustrated in violin plots on the left-hand side and the chromosomal median indicated by a solid line. The 191 kb killer plasmid and the 126 kb sister plasmid are highlighted in red and orange, respectively.

inhibens transconjugants (Δ65kb+191kb; Δ65kb+126kb) had lost the 65 kb chromid during experimental conjugation, which probably results in major physiological changes in comparison to the wild-type [40]. Therefore, we decided to use two reference strains for comparative growth experiments that were also lacking this replicon (Figs 2a and S2). We generated a new Δ65kb reference strain with our established curing strategy that is based on plasmid incompatibility of low copy number replicons [5]. Co-cultivation experiments of *Phaeobacter inhibens* and the dinoflagellate *Prorocentrum minimum* should be conducted with strains that are lacking the 262 kb chromid; thus, excluding any influence of TDA on the eukaryotic host. This antibiotic has been shown to kill the coccolithophore (haptophyte) *Emiliania huxleyi* [15]. Accordingly, we cultivated the two *Phaeobacter* transconjugants in MB medium for 10 days, streaked them out on agar plates and identified white colonies that had spontaneously lost their 262 kb chromid (Figs 2a and S2). As a second reference strain for these experiments, we used a previously established double mutant that is lacking the 65 kb biofilm and the 262 kb TDA chromid (Δ65kbΔ262kb) [28].

Genome analysis of *D. shibae* and *Phaeobacter inhibens* strains

Complete genome (re-)sequencing of the wild-type genomes, curing mutants and transconjugants of *D. shibae* DSM 16493^T and *Phaeobacter inhibens* DSM 17395 documented the absence of large-scale inversions that might result in changes of the phenotype [41]. The plasmid-cured mutants and transconjugants accumulated several point mutations (Text S1, Table S5), but they were not of major relevance for the current study.

Copy numbers of extrachromosomal replicons

Recently, we determined the copy number of plasmids and chromids in different *Phaeobacter inhibens* strains based on Illumina genome sequencing with PCR-free libraries and subsequent mapping on the different replicons [42]. In the current study, we documented that the analysis of Illumina data from PCR-based libraries also allows a reliable determination of ECR copy number (Text S2). Mapping of Illumina reads from all *D. shibae* and *Phaeobacter inhibens* strains that were investigated in the current study showed that their ECRs are only represented by a single copy per cell; thus, indicating a very tight regulation of each replicon (Fig. 2b, Table S6). The 86 kb RepABC-1 plasmid of *D. shibae* exhibited in the wild-type and the Δ191kb mutant showed a ratio of 1.34 and 1.04, respectively, whereas the 78 kb RepB-I chromid of *Phaeobacter inhibens* occurred in ratios ranging between 0.75 and 1.36 in the five strains investigated in the current study. The observed deviations in ECR to chromosome ratios likely reflect slight cultivation-dependent differences during DNA sampling. The sole exception is the 153 kb RepA-I chromid of *D. shibae* with a calculated copy number of about two. This replicon seems to be indispensable for the cell as it encodes the genes for the biosynthesis of the compatible solute α-glucosylglycerol and has an essential role for the salt tolerance of *D. shibae* [43]. The 191 kb RepABC-9 killer plasmid showed a comparable ratio of 1.01 and 0.89 in *D. shibae* and *Phaeobacter inhibens*, respectively,

while the 126 kb RepABC-2 plasmid occurred with ratios of 0.95 and 1.29. The comparably low chromosome to plasmid ratio deviation of both sister plasmids is in the range of the natural fluctuation of ECRs in the same species [42].

Plasmid stability and genome integrity in *Phaeobacter* transconjugants

Stable inheritance of the xenologous plasmids in the new host is a crucial prerequisite for further experiments. After 7 days, representing approximately 14 generations, the 126 kb plasmid was present in all 150 tested *Phaeobacter* colonies and for the transconjugant harbouring the 191 kb plasmid only a single spontaneous loss was observed (Fig. S4), which is comparable with the stability of the recently described 57 kb RepC_soli plasmid from *Phaeobacter inhibens* P72 [42]. *Rhodobacterales*-specific RepABC modules that were cloned in a commercial vector showed a much higher frequency of loss. For example, about 5% of the cells lost their RepABC-type plasmid overnight during exponential growth under non-selective growth conditions [33]. Stable plasmid maintenance allowed us to conduct all further experiments without the supply of antibiotics in the main cultures.

Impact of ECR gain and loss on bacterial growth

Curing of the 191 kb killer plasmid in *D. shibae* and its conjugation into *Phaeobacter inhibens* provided the unique opportunity to compare the presence/absence of this replicon in the genuine and a new host. Carriage of plasmids usually constitutes a considerable metabolic burden to the cell [44]. However, loss of the 191 kb plasmid resulted in a prolonged lag phase of the respective *D. shibae* strain (Fig. S5). Loss of the 65 kb chromid resulted in a slightly prolonged lag phase and lower maximum cell density compared to the *Phaeobacter inhibens* wild-type. Loss of both the 65 and 262 kb chromid prolonged the lag phase, but did not affect the maximum cell density.

Interestingly, the influence of xenologous *D. shibae* plasmids on the growth of the new host *Phaeobacter inhibens* was strikingly different in the Δ65kb and Δ65kbΔ262kb background (Fig. S5). The transconjugants Δ65kb+126kb and Δ65kb+191kb grew comparably to *Phaeobacter inhibens* Δ65kb, but reached a higher maximum optical density. By contrast, transconjugant Δ65kbΔ262kb+191kb had a longer lag phase and slower growth than the parent strain, but finally reached the same maximum cell density. Transconjugant Δ65kbΔ262kb+191kb showed a strongly reduced growth rate and reached only half the optical density measured for the parent strain.

Co-cultivation of *Phaeobacter inhibens* strains with *Prorocentrum minimum*

We next sought to test whether plasmids of *D. shibae* conferred upon *Phaeobacter inhibens* similar capacity to affect the growth of *Prorocentrum minimum*. The experimental set-up for the co-cultivation between *Prorocentrum minimum* and roseobacters was described in detail previously [16, 20, 45]. Algal cultures from the exponential growth phase were inoculated with bacterial strains at densities of 10^7, 10^8 and 10^9 cells ml^{-1}, and algal growth was followed by measurement of chlorophyll *a* autofluorescence (Fig. S6). A representative experiment with *Phaeobacter inhibens* strains at initial densities of 10^8 bacteria ml^{-1} is shown in Fig. 3. The L1 medium with and without B_{12} served as positive and negative controls, respectively. Two independent co-cultivation experiments with different batches of algae and bacteria were performed.

Strains of *Phaeobacter inhibens* that did not carry the killer plasmid (Δ65kb, Δ65Δ262kb and Δ65kbΔ262kb+126kb) improved the growth of the dinoflagellate above that of the axenic control (L1+B_{12}). At inoculation densities of both 10^8 and 10^9 cells ml^{-1}, the final density of the dinoflagellate was about two to three times higher than in the absence of bacteria. At an inoculation density of 10^7 bacterial cells ml^{-1}, the increase in final algal abundance was slightly smaller. *Phaeobacter inhibens* also maintained stability of the algal culture for a longer time. The maximum cell density was already reached after 12 to 15 days in the axenic dinoflagellate culture (L1+B_{12}) and afterwards the fluorescence signal declined. When the alga was grown with *Phaeobacter inhibens* strains lacking the killer plasmid, the maximum chlorophyll fluorescence was obtained after 15 to 21 days and stayed stable afterwards. The virtually identical growth of *Prorocentrum minimum* co-cultivated with the *Phaeobacter inhibens* curing mutants Δ65kb and Δ65kbΔ262kb shows that the TDA biosynthesis pathway, which is encoded on the 262 kb chromid, had no adverse effect on the dinoflagellate.

By contrast, co-cultivation with the *Phaeobacter inhibens* transconjugant Δ65kbΔ262kb+191kb, carrying the killer plasmid had a strong negative impact on the growth of *Prorocentrum minimum* similar to co-cultivation with *D. shibae* (Figs 3, S6 and S7). The dinoflagellate never reached the chlorophyll fluorescence of the positive control. Its decline started after 6 days of co-cultivation and was below that of the negative control (L1–B_{12}). This is indicative of an active killing process occurring during exponential growth. Co-cultivation of *Prorocentrum minimum* with the transconjugant Δ65kbΔ262kb+126kb did not kill the dinoflagellate. Therefore, SNPs in the *Phaeobacter* host and the transconjugants are not associated with the killing effect. Rather, it must be encoded by one or several of the 82 genes that are specific for the 191 kb plasmid and not found on the 126 kb plasmid [45].

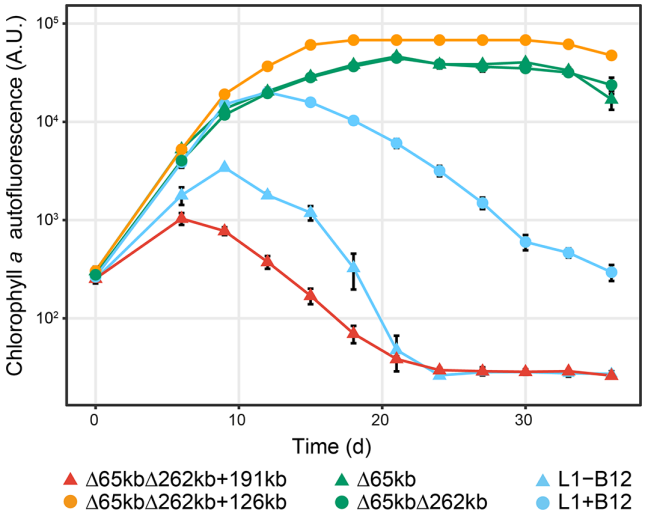

Fig. 3. Co-cultivation of *Prorocentrum minimum* and *Phaeobacter inhibens*. Axenic cultures of *Prorocentrum minimum* were inoculated with *Phaeobacter inhibens* control strains (*Phaeobacter inhibens* Δ65kb and *Phaeobacter inhibens* Δ65kbΔ262kb) or transconjugants carrying either the 191 kb killer plasmid of *D. shibae* (*Phaeobacter inhibens* Δ65kbΔ262kb+191kb) or the 126 kb sister plasmid of *D. shibae* (*Phaeobacter inhibens* Δ65kbΔ262kb+126kb). Cultures of *Prorocentrum minimum* in vitamin B_{12}-replete and -limited medium (L1+B_{12}, L1−B_{12}) served as positive and negative controls, respectively. Mean values and standard deviations of eight replicates are shown. Control experiments without bacteria are shown in blue, co-cultivation experiments with *Phaeobacter* reference strains and transconjugants are indicated in green and red/orange, respectively. d, Days. A.U.: arbitrary units

DISCUSSION

Experimental evidence for horizontal plasmid transfer across the phylogenetic diversity of marine *Rhodobacterales*

Here, we demonstrated plasmid transfer from *D. shibae* into five major lineages of the roseobacters. From the three routes of bacterial gene transfer, transformation, phage transduction and conjugation [46], the comparably frequent DNA uptake of large circular plasmids from the medium is extremely unlikely and their coincidental mis-packaging in large phage heads can be excluded. The genome of *D. shibae* is lacking any prophages beyond the well-characterized gene transfer agents [19, 47].

Thus, conjugation appears as a likely mechanism of horizontal gene transfer in this group, characterized by the patchy distribution of many adaptive traits together with widely present T4SSs [5, 7, 48]. The mode of rapid adaptation against florfenicol/chloramphenicol antibiotics, which are frequently used in marine aquaculture, was recently exemplified for different roseobacters by experimental conjugation of a natural resistance plasmid [42]. Conjugation is particularly important for complex genetic modules like the photosynthesis gene cluster (PGC), more than 40 kb in size, that comprises all structural components required for anoxygenic photosynthesis in the *Rhodobacterales*. The PGC is located on ECRs in several roseobacters [49–51] and has been horizontally transferred at least seven times [52]. Our data, thus, complement these phylogenomic investigations and point to the importance of conjugation as a mechanism of horizontal gene transfer in marine *Rhodobacterales*.

Furthermore, we also found evidence that recombination is the common mechanism for the emergence of mosaic plasmids in roseobacters. Transconjugants of *L. cinnabarinus* harboured either the 191 kb plasmid or a chimeric plasmid containing parts of the 191 and 126 kb plasmids. Taken together, the current study, which is the first quantitative assessment of conjugation between roseobacters, showed exceptional transfer rates for several recipient strains; thus, indicating frequent plasmid mobilization in natural biofilms. A high frequency of conjugational transfer in the ocean is also suggested by the discovery of identical replicons in different roseobacter genera [10]. These results support the idea that the marine pan-mobilome of roseobacters serves as a genetic backup for rapid adaptations to a changing environment.

The phycosphere as a hot spot for conjugation in the ocean

The microenvironment surrounding individual cells of the phytoplankton, known as the phycosphere, represents the aquatic analogue of the plant rhizosphere and is characterized by high bacterial densities, low convection and high nutrient concentrations [53]. It is one of the preferred habitats of roseobacters [7]. The newly discovered natural 494 kb plasmid in *Sulfitobacter* sp. DFL-23 is most likely derived from a recombination event after conjugation, as it shares an almost identical 36 kb fragment with the 191 kb plasmid of *D. shibae*. Both strains were isolated from the phycosphere of dinoflagellates, namely *Sulfitobacter* sp. DFL-23 from *Alexandrium lusitanicum* and *D. shibae* DFL12 from *Prorocentrum lima*. They may have co-occurred in the phycosphere in the

past, allowing for conjugation to occur. Similarly, in a culture of the diatom *Skeletonema marinoi*, two roseobacter strains were found carrying virtually identical plasmids [13]. Plasmid pSMR3-2 in *Roseovarius mucosus* strain SMR3 shared 100% sequence identity with interrupted regions of a *Loktanella* sp. plasmid found in the same culture [13]. Homologous RepC_soli-type plasmids have been found in several genera of roseobacters that have been isolated from the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Alexandrium minutum* in the East China Sea [42]. Taken together, these findings are strong indicators that the phycosphere of marine algae serves as a hot spot for conjugation.

Novel insights into the biology of RepABC-type plasmids from *Rhodobacterales*

The most prominent ECR-type of *Rhodobacterales* and rhizobia are RepABC-type plasmids, whose occurrence is restricted to *Alphaproteobacteria* [54]. Tumour-inducing (Ti) RepABC plasmids of *Agrobacterium* have been intensively studied since the 1970s, and their derivatives were later used as biotechnological workhorses for plant genetics in the pre-CRISPR-Cas9 era [55, 56]. However, the model system is still of scientific interest and mutations within the partitioning protein RepB have recently been correlated with the transition from a single copy to a higher copy number plasmid [57]. A tight copy control of RepABC replicons is of particular importance if the elements evolved from volatile plasmids to indispensable chromids [58], which are represented, for example, by the pSymB chromid of the model soil bacterium *Sinorhizobium meliloti* [59]. The current study demonstrated that both conjugative 126 kb RepABC-2 and 191 kb RepABC-9 replicons of *D. shibae* replicate as single copy number plasmids, regardless of their actual bacterial host. Our data, hence, suggest that the crucial factors for a tight regulation of the copy number of RepABC-type plasmids are intrinsically tied to the respective replicon and not determined by the host.

Future experiments are required to show whether the copy number of the sister plasmids is exclusively defined by the tripartite replication cassette and the palindromic sequences for plasmid partitioning [49, 60], or whether additional cis- and trans-acting factors are required to fully explain the functionality of *Rhodobacterales*-specific RepABC-type plasmids. The conspicuous observed stability of both conjugated plasmids in *Phaeobacter* correlates with the presence of characteristic toxin–antitoxin systems [19, 61]. The plasmid-cured *D. shibae* mutant, which is lacking the 191 kb killer plasmid, and the corresponding *Phaeobacter inhibens* transconjugant provide together with the respective wild-type strain a promising reference system to compare the functional role of this replicon in the genuine as well as the novel host. A first comparison showed clear differences of growth in minimal medium essentially reflected by individual lag phases. The observed holistic response suggests a significant role of conjugated plasmids in the regulatory network of the novel host, which should be further investigated in the future.

Phaeobacter inhibens as a probiotic for the dinoflagellate *Prorocentrum minimum*

Recently, it was shown that *Phaeobacter inhibens* kills calcifying cell types of the coccolithophore *Emiliania huxleyi* in co-culture [62] by inducing apoptosis-like programmed cell death [63]. In contrast, *Phaeobacter inhibens* stimulates the growth of the dinoflagellate *Prorocentrum minimum* in a cell-density-dependent fashion. Mutualistic symbiosis was most likely mediated by lack of vitamin B_{12} in the co-cultivation medium. Vitamin auxotrophy is widespread among eukaryotic algae. More than 50% of them require cobalamin (B_{12}), 22% require thiamine (B_1) and 5% depend on additional biotin (B_7) [64, 65], which explains why all three vitamins are provided in L1 medium.

However, there must be additional probiotic effects of *Phaeobacter inhibens* on the dinoflagellate that grew better in co-culture than in L1 medium. Genome analyses showed that *Phaeobacter inhibens* contains gene clusters for thiamine and biotin production (*thiCOSGEFD*, PGA1_c09110–09030; and *bioBFDAGC*, PGA1_c09120–09170; respectively), in addition to the cobalamin biosynthesis pathway [23]. *D. shibae* is also capable of cobalamin and thiamine biosynthesis, but it is auxotrophic for biotin [19]. The respective metabolites have been detected in the exometabolome of both species [66]. Mutualistic exchange of vitamins has also been observed between *D. shibae* and the abundant marine picoeukaryotic green alga *Ostreococcus* [67]. Thus, supply of biotin might contribute to the probiotic effect of *Phaeobacter inhibens* and could qualify the strain cured of the 262 kb chromid as a probiotic for marine algae. Positive effects of roseobacters beyond the supply of vitamins have been observed for the interaction between *Sulfitobacter* sp. with the diatom *Pseudonitzschia* sp. [68], the morphogenesis of the green alga *Ulva* [69] and growth of the cyanobacterium *Synechococcus* [70].

Horizontal transfer of pathogenicity towards eukaryotic algae by plasmid conjugation

The most dramatic observation of the current study was the horizontal transfer of the killer-phenotype across genus borders via plasmid conjugation. Active killing of the dinoflagellate *Prorocentrum minimum* was exclusively observed for the *Phaeobacter inhibens* transconjugant carrying the 191 kb plasmid. Our complementary experiments confirm the previous observation that the Δ191kb plasmid-cured mutant of *D. shibae* lacks the ability to kill the dinoflagellate *Prorocentrum minimum*. Thus, all genes required for pathogenicity are located on this plasmid. The lack of pathogenicity of the 126 kb plasmid shows that the killer genes must be located in regions specific for the 191 kb plasmid.

The 191 kb plasmid encodes 184 proteins, of which 43 are unique, i.e. not present on the 126 kb sister plasmid. A subset of these genes has been investigated for their influence on the growth of the dinoflagellate in co-culture [45]. The killing phenotype was

lost if genes of a putative biotin ABC transporter operon (*bioYMN*, Dshi_3685 to Dshi_3687) were inactivated. This operon is unique for the 191 kb plasmid. Biotin is an essential vitamin for dinoflagellates and it is one of the three vitamins provided in L1 medium. However, it is also essential for *D. shibae*. Thus, we hypothesize that bacteria and algae compete for biotin in co-culture, and that the BioYMN uptake system of the bacterium depletes biotin from the medium, resulting in auxotrophic death of the algae.

Horizontal transfer of natural plasmids across the phylogenetic depth of *Rhodobacterales* could considerably influence interactions between bacteria and algae in the ocean, and might even initiate bacterial speciation. Examples for such processes have been found in the rhizosphere and for certain pathogens. Conjugation of the Ti plasmid of *Agrobacterium tumefaciens* into distantly related rhizobia rendered them pathogenic for legumes [71]. The human pathogen *Shigella* evolved independently at least three times from an *Escherichia coli* ancestor, and its transition from a harmless gut microbe correlates with the acquisition of a virulence plasmid that is characteristic for all representatives of this pathovar [72]. Another example is the emergence of the Black Death. The pathogen *Yersinia pestis* evolved from the soil-dwelling bacterium *Yersinia pseudotuberculosis*, probably by the acquisition of two virulence plasmids [73]. The collection of well-characterized transconjugant strains created in the course of this study will allow further evaluation of the role of conjugational plasmid transfer on the interaction of roseobacters with other species in their natural habitat and their conquest of new ecological niches.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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