Title: Algal Exudates Promote Conjugation in Marine Roseobacters

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Abstract

Horizontal gene transfer (HGT) is a pivotal mechanism driving bacterial evolution, conferring adaptability within dynamic marine ecosystems. Among HGT mechanisms, conjugation mediated by Type IV secretion systems (T4SSs) plays a central role in the ecological success of marine bacteria. However, the triggers initiating conjugation events in the marine environment are not well understood. Roseobacters, abundant marine bacteria commonly associated with algae, possess a multitude of plasmids encoding T4SSs. Many Roseobacters are heterotrophic bacteria that rely on algal secreted compounds for supporting bacterial growth. Algal compounds therefore attract bacteria and promote colonization, including attachment to algal cells. Bacterial proximity, cell-to-cell contact, and attachment, can all foster HGT. Hence, we hypothesized that algal exudates, acting as chemoattractants for bacteria, may function as cues promoting bacterial HGT. Examination of various Roseobacters demonstrated that the genomic location of the T4SS impacts its functionality; a bacterial strain harboring a chromosomal-encoded T4SS does not perform conjugation, while a strain of the same species carrying a plasmid-encoded T4SS exhibits functional conjugation capability. Subsequently, we probed the influence of algal exudates on bacterial conjugation dynamics. Our findings revealed that algal exudates enhance plasmid transfer through conjugation but do not modulate the transcription of the conjugative machinery genes. These observations suggest that the bacterial responses to algal hosts evolved to correlate with an increased likelihood of encountering compatible partners for successful conjugation. Furthermore, since specific algae attract distinct bacterial populations, algae influence potential partners for genetic exchange and may shape the trajectory of bacterial evolution in the marine environment.

Introduction

Horizontal gene transfer (HGT), a key process in bacterial evolution, is the transfer of genetic material between a donor to an unrelated recipient. Bacteria, while maintaining small size genomes[1], display extraordinary genetic variations that enable them extreme flexibility and adaptability[1,2]. The ability of bacteria to obtain fragments of DNA in one single HGT event is a driving force enabling this flexibility[2]. HGT allows bacteria to adapt to environmental changes by acquiring new metabolic capabilities that facilitate various abilities such as symbiosis[3], antibiotic resistance[4], metabolic activity[5] and pathogenicity[6] thereby enabling bacteria to inhabit new and dynamic niches.

Horizontal transfer of genetic material is achieved by several main mechanisms, including 1) Transformation, which is the uptake of extracellular DNA[7], 2) Transduction, which is bacteriophage-mediated transfer[7], 3) Gene transfer agents (GTAs), that are bacteriophage-like particles that carry random pieces of the producing bacterial genome[8], and 4) Conjugation, which is the transfer of a plasmid between bacterial cells through direct cell-to-cell contact[7-9]. Conjugation typically occurs through the type IV secretion system (T4SS). T4SS is a versatile system found in both Gram-negative and Gram-positive bacteria. The T4SS facilitates cell-to-cell contact and enables the delivery of proteins and DNA between different bacterial strains, species, and even from bacteria to eukaryotic cells[10]. It is involved in a verity of processes such as toxin delivery, DNA release, DNA uptake, effector molecule translocation and conjugation[11]. The T4SS is mainly encoded by the *virB* operon while the *virD* operon carries several additional genes necessary for proper functioning of the T4SS[11,12].

Many bacteria carry the T4SS genes on a plasmid, but some harbor the T4SS genes on the chromosome[13-16]. Plasmid-encoded T4SSs can transfer plasmids in conjugation, however the role of the chromosome-encoded T4SS is less clear[13,16]. Chromosome-encoded T4SSs have rarely been shown to mobilize a plasmid to a recipient strain[17], and these systems are mainly involved in processes such as protein secretion, ssDNA secretion into the extracellular milieu and natural transformation[14,15,18-22]. Indeed, diverse configurations of the T4SS architecture exist. Interestingly, within the abundant and environmentally important Roseobacters group of marine bacteria[23], many species carry T4SS genes in different genomic locations[24]. Roseobacters harbor T4SS genes on plasmids, chromosomes, both plasmids and chromosomes, and even on multiple plasmids within a single species (Table S1). Only the transfer of plasmids by plasmid-encoded T4SSs has been demonstrated in Roseobacters[6,25,26], it therefore remains to be determined whether the genomic location impacts the function of the T4SS in these bacteria.

HGT, and specifically conjugation that was demonstrated between Roseobacters[6,25-27], has been suggested as a central reason for the ecological success of Roseobacters[26]. These bacteria exhibit diverse metabolic capabilities and a wide range of symbiotic lifestyles[28]. Many Roseobacters engage in various interactions with algal hosts[29-34]. Algae exude various metabolites that support bacterial growth in the nutrient-poor oligotrophic ocean[28,29,35]. Thus, planktonic bacteria that drift in the ocean can secure a nutrient flux through proximity to an algal host. Algal exudates were shown to influence bacterial physiology[28-30,35,36] and shape algal-bacterial interactions[29,37]. In the vicinity of algal hosts, bacteria experience spatial proximity to one another, with certain bacteria even attaching to the surface of the algal cells[29,36]. Previous studies have shown that bacterial proximity, cell-to-cell contact, and attachment promote HGT[27,38-40]. Algal exudates that attract bacteria may therefore serve as a signal that promotes bacterial HGT.

Here, we sought to explore Roseobacter conjugation in the ecological context of an algal host. Therefore, our study employed Roseobacter species that were identified in environmental samples of algae[29,41]. As a model algal host, we used the unicellular alga *Emiliania huxleyi* (also termed G*ephyrocapsa huxleyi*)[42], which is widespread in modern oceans and was shown to harbor a rich community of bacteria, including Roseobacters[29,41,43]. First, we tested the ability of various Roseobacters to successfully conduct conjugation, examining the influence of the T4SS genomic location. Next, we examined the impact of algal exudates on bacterial conjugation, specifically on the expression of T4SS-encoding genes and the resulting transfer of DNA.

Examination of two bacterial strains belonging to the same species revealed that a chromosome-encoded T4SS does not lead to successful conjugation, while a plasmid-encoded T4SS is functional. Interestingly, our investigation has demonstrated expression of the plasmid-encoded T4SS genes, which did not increase in response to algal exudates. However, we have observed enhancement in bacterial conjugation efficiency and the actual transfer of plasmids following exposure to algal exudates. These observations imply that the bacterial response to an algal host is interconnected with an increased probability of encountering a compatible partner for successful conjugation.

Results

The *P. inhibens* DSM 17395 model strain is unable to act as a donor strain for conjugation

The P. inhibens DSM 17395 model strain has been widely studied by us[29,44] and by others[45-47] as a representative Roseobacter strain that specializes in interactions with microalgae. To

112 investigate the algal impact on bacterial conjugation, we first attempted to establish a
113 conjugation assay using this model bacterial strain. The *P. inhibens* DSM 17395 strain harbors
114 three native plasmids of 65 kb, 78 kb, and 262 kb, and carries all the genes that encode a
115 complete T4SS on its chromosome (Fig. S1). In our study, a complete T4SS was defined as
116 consisting of all the *vir* genes that have been previously described in conjugative Roseobacter
117 T4SSs[25,26]. Interestingly, most reports on conjugation in bacteria involve plasmid-encoded
118 T4SSs[15,16,18,48], rather than chromosome-encoded T4SS. We examined and characterized the
119 ability of *P. inhibens* DSM 17395 to transfer a plasmid via conjugation. To evaluate
120 conjugation, a plasmid in the bacterial donor strain is marked with an antibiotic resistance
121 gene, while the recipient strain is marked with a second antibiotic resistance gene on its
122 chromosome. If the plasmid is transferred from donor to recipient, the resulting bacteria (called
123 transconjugants) will be resistant to both antibiotics following the mating and selection process
124 (Fig. 1A).
125
126 Since *P. inhibens* DSM 17395 harbors three plasmids, and the T4SS is located on the
127 chromosome, we conducted the conjugation assay with all three plasmids as possible donors.
128 Each of the three plasmids was marked with a kanamycin-resistance gene. As recipients, we
129 used representatives Roseobacter strains, including *D. shibae* and *M. algicola*, that were
130 marked with a chromosomal gentamicin-resistance gene. Our results show that under our
131 experimental conditions *P. inhibens* DSM 17395 is not capable of transferring any of its
132 plasmids to recipient bacteria (Fig. 1B). To further support this result, we conducted similar
133 conjugation assays on agar plates instead of liquid media (Fig. 1C). Previous studies have
134 shown that proximity, cell-to-cell contact, and attachment promote conjugation[27,38-40], thus a
135 solid surface like the agar plates should enhance the probability of plasmid transfer. Similar to
136 the results obtained in the liquid media, *P. inhibens* DSM 17395 was not capable of
137 transferring any of its plasmids to recipient bacteria during conjugation on plates (Fig. 1C). As
138 control, all experiments were accompanied by conjugation assays with a donor-recipient pair
139 that was previously shown to result in successful conjugation[26]. Our findings point to the
140 possibility that the chromosomally encoded T4SS in *P. inhibens* DSM 17395 is unable to
141 support conjugation.
142
143 **Conjugative transfer of *P. inhibens* P72 plasmid to various recipients**
144 Following the observation that the chromosome-encoded T4SS in *P. inhibens* DSM 17395
145 cannot execute conjugation, we investigated whether another strain of the same species, with
146 a plasmid-encoded T4SS, is able to perform conjugation and act as a donor strain. The *P.*
147 *inhibens* P72 strain carries complete T4SSs on both its 184 kb and 57 kb native plasmids,

148 with a similar genetic organization, namely the identity and order of genes, as the
149 chromosome-encoded T4SS in *P. inhibens* DSM 17395 (Fig. S1).
150
151 The 57 kb plasmid of *P. inhibens* P72 was marked with a kanamycin-resistance gene and was
152 used as a donor strain in a conjugation assay with three recipient Roseobacter strains, namely
153 *P. inhibens* DSM 17395, *D. shibae*, and *M. algicola* (Fig. 2A). These recipient strains were
154 marked with a gentamicin-resistance gene on their chromosome. Several of these donor-
155 recipient pairs were previously reported to execute successful conjugation under different
156 experimental conditions[26]. Following mating and selection, all assays resulted in colonies that
157 exhibit resistance towards both kanamycin and gentamicin. To confirm that the double-
158 resistance colonies were indeed transconjugants, several colonies were randomly chosen
159 from each donor-recipient pair and the presence of the acquired plasmid and recipient
160 chromosome was validated via PCR reactions. All tested transconjugants were confirmed to
161 be the recipient strains that acquired the 57 kb plasmid (Fig. 2B). These conjugation assay
162 demonstrated that under the same experimental conditions, *P. inhibens* P72 can serve as a
163 donor while *P. inhibens* DSM 17395 cannot. These observations further support a functional
164 difference between T4SSs that are encoded on a chromosome versus on a plasmid.
165
166 **Another Roseobacter species with plasmid-encoded T4SS can act as donor via**
167 **conjugation**
168 To investigate if another Roseobacter species that carry the T4SS on its plasmids can function
169 as plasmid donor in conjugation, we performed conjugation assays using *D. shibae* as the
170 plasmid donor. *D. shibae* was marked with a gentamicin-resistance gene on its 126 kb native
171 plasmid, harboring a complete T4SS (Fig. S1). For conjugation assays using *D. shibae* as the
172 plasmid donor, we selected two recipients: *P. inhibens* P72 and *P. inhibens* DSM 17395, both
173 tagged with a kanamycin-resistance gene on their chromosome (Fig. 3A). Following mating
174 and selection, all assays resulted in colonies that exhibit double resistance towards both
175 antibiotics. To confirm that the double-resistant colonies were indeed transconjugants, we
176 randomly selected several colonies from each pair and validated the presence of the acquired
177 plasmid and recipient chromosome using PCR reactions. All tested transconjugants were
178 confirmed to be the recipient strains that acquired a new plasmid (Fig. 3B). The conjugation
179 experiments demonstrated that *D. shibae* can act as a plasmid donor for both *P. inhibens* P72
180 and *P. inhibens* DSM 17395. These results are in line with a previous report that used *D.*
181 *shibae* and *P. inhibens* DSM 17395 under different experimental conditions and resulted in
182 successful conjugation[25]. Our findings indicate that plasmid-encoded T4SSs can facilitate the
183 transfer of plasmids among Roseobacters via conjugation.
184

185 **Expression of the conjugation machinery is evident in plasmid-encoded T4SSs and is**
186 **not influenced by algal exudates**
187 Now that we obtained various Roseobacters that successfully perform conjugation, we wished
188 to address our initial question and examine the algal impact on the bacterial conjugation
189 process. First, we assessed the expression of the bacterial T4SS, under the same
190 experimental conditions in which we observed a plasmid transfer in previous experiments (Fig.
191 2A, Fig. 3A). We analyzed expression levels of the T4SS genes in strains harboring plasmid-
192 encoded T4SS, namely *P. inhibens* P72 and *D. shibae*. Both strains have two complete T4SSs
193 on two different plasmids (57 kb and 184 kb plasmids for *P. inhibens* P72, and 126 kb and 191
194 kb for *D. shibae*). We assessed the expression of the T4SSs using qRT-PCR with primers that
195 target the T4SS central genes *virB4*, *virB8* and *virD4.* Each primer set was designed to assess
196 the expression of both T4SSs in each strain. In *P. inhibens* P72 bacteria, *virB4* and *virD4* gene
197 expression was assessed individually for each plasmid due to sequence differences (Fig.
198 S2A). As depicted in Fig. 4A, the expression levels of *virB4*, *virB8* and *virD4* were found to be
199 comparable to those of the housekeeping genes. This observation suggests that in the
200 experimental conditions that supports conjugation, the T4SS genes in both *D. shibae* and *P.*
201 *inhibens* P72 strains are actively expressed.
202
203 Next, we wished to evaluate the possible impact of algae on the expression of the T4SS. In
204 the nutrient-poor oligotrophic ocean, heterotrophic bacteria rely on micro-algae as an energy
205 source[28,35,49]. Algae exude nutrients and various algal metabolites that influence bacterial
206 physiology and play a role in algal-bacterial interactions[29,37]. Additionally, algae serve as an
207 attachment surface for bacteria[29,36]. Previous studies have shown that proximity, cell-to-cell
208 contact, and attachment promote HGT[27,38-40]. Therefore, algal exudates that are sensed by
209 bacteria may serve as a signal that promotes bacterial HGT. To explore this possibility, we
210 examined RNA expression levels of the bacterial T4SS machinery in bacterial cultures that
211 were supplemented with filtrates of algal cultures. These filtrates contain various algal
212 exudates that may be sensed by bacteria. All cultures, treated and control, were supplemented
213 with essential nutrients to eliminate the role of algal exudates in supporting growth. We
214 evaluated the expression of the T4SS in *P. inhibens* P72 and *D. shibae* bacteria. Both strains
215 were grown in pure cultures and qRT-PCR analyses compared expression levels of cultures
216 that were supplemented with algal filtrates versus non-treated cultures. Analyses were
217 performed using primers targeting the central T4SS genes, *virB4*, *virB8* and *virD4*. The results
218 demonstrated that gene expression levels are similar between untreated cultures and cultures
219 that were supplemented with algal filtrates, for both bacterial strains. Only expression of the
220 *virD4* gene in *P. inhibens* P72 treated with algal filtrates showed slightly higher expression
221 levels than untreated cultures (Fig. 4B, Fig. S2B). Collectively, these findings suggest that

genes of the T4SS are constantly transcribed, with similar expression patterns to housekeeping genes, and that algal secreted compounds do not influence the expression of the T4SS machinery.

Algal exudates enhance bacterial conjugation

Transcription of the T4SS machinery does not seem to be influenced by algal exudates (Fig. 4B, Fig. 4D). However, transcription is not the only factor affecting the actual transfer of plasmids via the T4SS between bacteria. Therefore, we investigated whether algal exudates influence plasmid transfer between Roseobacter bacteria. To achieve this, we performed conjugation assays in which bacteria were treated with algal filtrates and compared to untreated bacteria. For the conjugation assay *P. inhibens* P72 and *D. shibae* was chosen as plasmid donors. As recipients, we used *D. shibae, M. algicola*, and *P. inhibens* DSM 17395 (Fig. 5). Our results show that conjugation assays that were supplemented with algal filtrates resulted in increased conjugation efficiency (i.e., more transconjugants per donors) compared to untreated bacteria.

Notably, algal filtrates contain substrates that promote bacterial growth[28,35,49]. To determine whether the increased conjugation efficiency is due to enhanced bacterial growth, we quantified donors, recipients and transconjugants in the different conjugation assays (Fig. 5, Table S2). We calculated the ratio of colony-forming unit (CFU) between treated cells and untreated cells. A ratio larger than one indicates increased growth, while a ratio smaller than one indicates inhibition of growth due to algal filtrates. We then compared the ratio of transconjugants to the ratio of donors (Fig. S3A) and recipients (Fig. S3B). Our analysis revealed that transconjugants had a higher ratio compared to both donors and recipients, indicating that treatment with algal filtrates enhanced conjugation efficiency beyond any potential growth-promoting effect.

In summary, our data suggest that while algal exudates do not affect the transcription of the conjugation machinery (Fig. 4B, Fig. 4D), they do promote conjugation (Fig. 5). The enhanced conjugation is not the result of increased bacterial growth (Fig. S3), and the underlying mechanism remains to be explored.

Discussion

The current study reveals that algal exudates increase bacterial conjugation, and consequently HGT, in Roseobacters. High frequencies of HGT have been suggested to play important roles in the resilience of marine bacteria[50]. Since the marine environment is dynamic and rapidly changing[51], the acquisition of novel traits through HGT allows marine bacteria to adapt to this dynamic environment. Various mechanisms for HGT have been observed in

259 Roseobacters, including GTAs[50] and conjugation[27]. Moreover, Roseobacters have many
260 plasmids[23,52], up to a dozen plasmids in a single bacterium[52]. Many of these plasmids harbor
261 T4SSs[39] that may facilitate conjugative HGT, potentially supporting rapid adaptation. These
262 processes that allow Roseobacters to adapt to new niches are considered key in their
263 ecological abundance and success[23,52,53]. Indeed, 27% of sequenced Roseobacters genomes
264 harbor all genes needed for a functional conjugative T4SS (Fig. S4A). Conjugation initiation
265 and the successful transfer of DNA was shown to be impacted by factors such as signaling
266 molecules, temperature, cell physiology, population density, pH, and nutrients[39,54,55]. However,
267 in marine bacteria, little is known about the conditions required to induce HGT, particularly
268 conjugation. Here, we show that algal secreted metabolites promote HGT in Roseobacters.
269
270 In marine ecosystems, algae provide bacteria with nutrients[28,35,49] and an attachment
271 surface[29,36]. By attaching to an algal cell, bacteria can secure a steady flux of algal exudates
272 in the algal phycosphere, which is the immediate volume surrounding the algal cell. As multiple
273 bacteria can attach onto an algal host, they may engage in processes that require cell-to-cell
274 contact, such as conjugation. Therefore, proximity and attachment onto an algal host may
275 increase the chances for plasmid transfer events among bacteria in the algal phycosphere[27,38-
276 40]. Indeed, previous studies demonstrated increased conjugation following attachment to
277 abiotic surfaces[56]. Moreover, algal exudates support bacterial growth and metabolism[57],
278 further enhancing bacterial density, which in turn could augment the chances of bacterial HGT.
279 Interestingly, environmental experiments with phytoplankton-derived dissolved organic matter
280 demonstrated attraction of bacteria harboring genes for the T4SS, as shown by metagenomic
281 analysis[58]. Specifically, Roseobacter bacteria often co-occur with various algal hosts in the
282 environment and in laboratory cultures[29,43,59-61]. Our study demonstrates that algal filtrates
283 increase conjugation in Roseobacter bacteria (Fig. 5), even in the absence of an algal cell for
284 attachment. Whether a combination of algal exudates and an attachment surface further
285 enhance conjugation remains to be studied. The current study establishes the functional link
286 between algal exudates and bacterial HGT.
287
288 While conjugation was enhanced in response to algal exudates (Fig. 5), the expression of the
289 conjugative machinery was not affected by algal exudates (Fig. 4B), suggesting the
290 involvement of additional regulatory routes such as transcription of regulatory genes[62] and
291 post-transcriptional regulation. Reports on the importance of post-transcriptional regulation of
292 the conjugative process have been previously described[55] and were highlighted as a promising
293 regulatory mechanism of conjugation[63]. In line with our observations, previous work has
294 reported on substantial differences between levels of transcripts versus proteins of the T4SS,
295 with higher transcription levels in poor media compared to rich media. In contrast, western blot

296 of the corresponding proteins revealed elevated expression in rich media compared to the
297 poor media[64]. Taken together, algal exudates may impact post-transcriptional regulation of
298 genes involved in bacterial conjugation.
299
300 The functionality of the conjugative process appears to be affected by the genomic location of
301 the T4SS. Various bacteria carry the T4SS-encoding genes on plasmids, chromosomes, and
302 even on both[16,17]. While plasmid-encoded T4SSs are known to commonly mediate
303 conjugation[65], chromosomally encoded T4SSs rarely mobilize a plasmid to a recipient strain[17].
304 Chromosomally encoded T4SSs are mainly involved in processes such as protein secretion,
305 ssDNA secretion into the extracellular milieu, natural transformation, and symbiosis[13-16,18-22,66].
306 Of note, within sequenced Roseobacters genomes, 23% carry only plasmid-encoded T4SSs,
307 2% carry only chromosome-encoded T4SSs, and 1% carry T4SSs on both the chromosome
308 and a plasmid (Fig. S4A). Evaluating the genomic location of all T4SSs across Roseobacter
309 genomes reveals that 86% of T4SSs are found on plasmids, 8% are located on chromosomes,
310 and 6% cannot be conclusively assigned to a chromosome or plasmid (Fig. S4B). Therefore,
311 chromosome-encoded T4SSs appear to constitute a notable fraction of T4SSs in
312 Roseobacters, potentially hinting at other functions served by chromosomally encoded
313 secretion system[66]. Indeed, our data demonstrate that a strain carrying a complete
314 chromosomally encoded T4SS is unable to act as a plasmid donor in conjugation (Fig. 1B-C).
315 In contrast, a different strain from the same species, carrying a plasmid-encoded T4SS with a
316 similar genetic organization to the chromosome-encoded T4SS (Fig. S1), was able to serve
317 as a plasmid donor (Fig. 2).
318
319 The origin of transfer (oriT) is a key regulatory element needed for a successful conjugation
320 event. While a plasmid can be transferred *in-trans* by a T4SS apparatus, the oriT is required
321 for DNA mobilization initiation and functions *in-cis*[67]. Therefore, chromosomally encoded
322 T4SSs should be able to transfer plasmids that carry an oriT. Similarly, a chromosomally
323 encoded T4SS was demonstrated to transfer an adjacent DNA fragment harboring an oriT [15].
324 We demonstrate that under our experimental conditions the chromosomally encoded T4SS in
325 *P. inhibens* DSM 17395 cannot function as a plasmid donor (Fig. 1B-C). Therefore, the *P.*
326 *inhibens* DSM 17395 plasmids are not expected to have an oriT. However, identifying an oriT
327 is challenging. Although some oriTs have typical characteristics, most are unknown[67].
328 Moreover, the majority of oriTs have been identified in model bacteria[67-69], and knowledge on
329 environmental strains is therefore limited. Using a web-based tool designed for identifying
330 oriTs (oriTfinder)[70], we were unable to identify oriTs on the *P. inhibens* DSM 17395 plasmids.
331 Of note, when the same tool was applied to analyze plasmids that were successfully
332 transferred in our conjugation experiments, still an oriT was not identified. These results

333 underscore the limited molecular knowledge we currently have on key processes, such as
334 conjugation, in environmentally abundant bacteria.
335
336 The evolution of marine heterotrophic bacteria is linked to their micro-algal hosts, as
337 manifested by the many examples of algal-bacterial chemical exchanges that likely arose
338 during their co-evolution[29-31,44,45,71]. The current study reveals the impact that algal exudates
339 have on bacterial conjugation, which adds another layer to our understanding of bacterial
340 evolution in the ocean. Algal exudates represent a distinct chemical signature of a given algal
341 species[72], and consequently, attract phylogenetically and functionally discrete populations of
342 bacteria[57,58,73]. By attracting specific bacterial populations, algae have the potential to affect
343 the bacterial partners that perform genetic exchange, thus possibly shaping bacterial
344 evolution.
345
346 **Materials and Methods**
347 **Bacterial strains and growth conditions**
348 The bacterial strains *Phaeobacter inhibens* DSM 17395 and *Dinoroseobacter shibae* DFL-12
349 were purchased from the German Collection of Microorganism and Cell Cultures (DSMZ,
350 Braunschweig, Germany) (Table 1). *P. inhibens* P72 was kindly provided by Prof. Juan Barja
351 from University of Santiago de Compostela, Spain, and Dr. Jörn Petersen from the Leibniz
352 Institute, DSMZ, Germany (Table 1). The bacterial strains of *Phaeobacter inhibens* DSM
353 17395 with a mariner transposon (Tn)-tagged chromosome, *Marinovum algicola* DG898 DSM
354 27768 with a Tn-tagged chromosome, *Dinoroseobacter shibae* DFL-12 with a Tn-tagged
355 plasmid (pDSHI03, 126 kb), *Dinoroseobacter shibae* DFL-12 with a Tn-tagged chromosome
356 (Table 1), *P. inhibens* P72 with a Tn-tagged plasmid (pP72_e, 57 kbp), and *P. inhibens* P72
357 with a Tn-tagged chromosome were kindly provided by Dr. Jörn Petersen from the Leibniz
358 Institute, DSMZ, Germany.
359 *D. shibae* cultures were plated on ½ marine broth 2216 (MB) (Difco, USA) agar plates (MB,
360 18.7 g/L; agar, 16 g/L) with 30 µg/ml gentamicin antibiotic. *M. algicola* cultures were plated on
361 ½ YTSS agar plates (yeast extract, 2 g/L; trypton, 1.25 g/L; sea salts, 20 g/L; agar, 16 g/L, all
362 purchased from Sigma-Aldrich) with 30 µg/ml gentamicin antibiotic. *P. inhibens* DSM 17395
363 cultures were plated on ½ YTSS agar plates with 30 µg/ml gentamicin or 150 µg/ml kanamycin
364 antibiotic. *P. inhibens* P72 was plated on ½ YTSS agar plates with 150 µg/ml kanamycin
365 antibiotic. Transconjugants of *D. shibae* were plated on ½ MB agar plates with 30 µg/ml
366 gentamicin and 150 µg/ml kanamycin antibiotics. Transconjugants of *P. inhibens* DSM 17395,
367 *P. inhibens* P72 and *M. algicola* were plated on ½ YTSS agar plates with 30µg/ml gentamicin
368 and 150 µg/ml kanamycin antibiotics.

369 Cultures were grown in artificial seawater (ASW) prepared according to Goyet and Poisson[74].
370 ASW contained mineral salts (NaCl, 409.41 mM; Na_2SO_4, 28.22 mM; KCl, 9.08 mM; KBr,
371 0.82 mM; NaF, 0.07 mM; Na_2CO_3, 0.20 mM; $NaHCO_3$, 2 mM; $MgCl_2$, 50.66 mM; $SrCl_2$,
372 0.09 mM), L1 trace elements ($FeCl_3 \cdot 6H_2O$, 3.15 mg/L; $Na_2EDTA \cdot 2H_2O$, 4.36 mg/L;
373 $CuSO_4 \cdot 5H2O$, 9.8 µg/L; $Na_2MoO_4 \cdot 2H_2O$, 6.3 µg/L; $ZnSO_4 \cdot 7H_2O$, 22 µg/L; $CoCl_2 \cdot 6H_2O$,
374 10 µg/L; $MnCl_2 \cdot 4H_2O$, 180 µg/L), and L1 nutrients ($NaNO_3$, 882 µM; NaH_2PO_4, 36.22 µM). The
375 components were dissolved in Milli-Q water (IQ 7003; Merck, Darmstadt, Germany) and the
376 pH adjusted to 8 with HCl. Stock solutions of L1 trace elements and L1 nutrients were
377 purchased from the Bigelow Laboratory for Ocean Sciences (Boothbay, ME, USA).
378 Cultures of *P. inhibens* DSM 17395, *P. inhibens* P72, and *M. algicola* were grown in ASW
379 medium supplemented with additional carbon (glucose, 5.5 mM, Sigma Aldrich), nitrogen
380 (NH_4Cl, 5 mM, Sigma Aldrich), sulfur (Na_2SO_4, 33 mM, Merck), and f/2 vitamins (thiamine HCl,
381 100 µg/L; biotin, 0.5 µg/L; vitamin B_{12}, 0.5 µg/L, Bigelow Laboratory for Ocean Sciences).
382 Cultures of *D. shibae* were grown in ASW medium supplemented with additional carbon
383 (succinate, 5.5 mM, Sigma Aldrich), nitrogen (NH_4Cl, 5 mM), sulfur (Na_2SO_4, 33 mM), and
384 vitamins mix (adjusted from Gonzalez *et al.,*[75] Thiamine HCl, 0.148 µM/L; biotin, 0.082 µM/L;
385 Pyridoxine HCL, 0.486 µM/L; Nicotinic acid, 0.406 µM/L; Pantothenic acid, 0.228 µM/L;
386 Cyanocobalamin, 0.0006 µM/L; pABA, 0.364 µM/L). Bacteria were cultivated at 30 °C under
387 constant shaking at 130 rpm.
388
389 **Table 1:** List of bacterial strains and mutants.

Strain	Tagged locus	Insertion site	Reference	Lab collection identifier	Resistance	Comments
Phaeobacter inhibens DSM 17395			DSMZ, Germany			Wild type
Phaeobacter inhibens DSM 17395	65 kb plasmid	18,622	Current study	ES172	Kanamycin	Intergenic region mutant.
Phaeobacter inhibens DSM 17395	78 kb plasmid	26,249-30,284	Lab collection	ES171	Kanamycin	Knock-out mutant in both PGA1_RS19420 and PGA1_RS19415.
Phaeobacter inhibens DSM 17395	262 kb plasmid	138,002	Dr. Jörn Petersen from the Leibniz Institute, DSMZ, Germany.	ES170	Kanamycin	Insertion in PGA1_262p01260, generated with the <R6kyori/KAN-2> transposon-kit.
Dinoroseobacter shibae DFL-12	126 kb plasmid	27,457	Patzelt *et al.*[25]	ES94	Gentamicin	Insertion in Dshi_3964, generated using mariner transposon mutagenesis.
Phaeobacter inhibens P72	57 kb plasmid	38,628	Birmes *et al.*[26]	ES96	Kanamycin	Insertion in CP010740,

						generated with the <R6kyori/KAN-2> transposon-kit.
Dinoroseobacter shibae DFL-12	Chromosome	3,502,458	Birmes *et al.*[26]	ES95	Gentamicin	Intergenic region mutant, generated using mariner transposon mutagenesis.
Phaeobacter inhibens P72	Chromosome	1,678,662	Dr. Jörn Petersen from the Leibniz Institute, DSMZ, Germany and Prof. Juan Barja from University of Santiago de Compostela, Spain	ES168	Kanamycin	Insertion in PhaeoP72_RS07995, generated with the <R6kyori/KAN-2> transposon-kit.
Marinovum algicola DG898	Chromosome	3,256,562	Birmes *et al.*[26]	ES93	Gentamicin	Insertion in MALG_03188, generated using mariner transposon mutagenesis.
Phaeobacter inhibens DSM 17395	Chromosome	1,400,112	Lab collection	ES70	Gentamicin	Intergenic region mutant.
Phaeobacter inhibens DSM 17395	Chromosome	2,372,826	Dr. Jörn Petersen from the Leibniz Institute, DSMZ, Germany.	ES97	Kanamycin	Insertion in PGA1_RS11385, generated with the <R6kyori/KAN-2> transposon-kit.

Algal strain, growth conditions and monitoring

The axenic algal strain of *Emiliania huxleyi* CCMP3266 (also termed G*ephyrocapsa huxleyi*[42]) was purchased from the National Center for Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, Maine, USA). Algae were grown in ASW supplemented with f/2 vitamins, L1 nutrients and L1 trace elements. Algae were grown in standing cultures in a growth room at 18 °C under a light/dark cycle of 16/8 h. Illumination intensity during the light period was 150 mmoles/m^2/s. Absence of bacteria in axenic algal cultures was monitored periodically both by plating on ½ YTSS plates and under the microscope.

Cultures of *E. huxleyi* were grown as follows; algal cells concentrations from a late exponential phase culture were counted by a CellStream CS-100496 flow cytometer (Merck, Darmstadt, Germany) using 561 nm excitation and 702 nm emission. Algal cells were gated according to event size and fluorescence intensity. An inoculum of 10^5 algal cells was introduced into 100 ml of ASW and incubated as described above. Algal growth in cultures was monitored by a CellStream flow cytometer as described. For each biological replicate 50,000 events were recorded.

406

407 **Algal filtrates**

408 To obtain algal filtrates, algal cultures were grown as described above for 12 days. An initial
409 washing of the filters was conducted by passing 100 ml ASW medium through the filter
410 (Thermo scientific, 0.2 aPES, 250ml). Then, algal cultures were filtered, and filtrates pH was
411 adjusted to pH 8 (Thermo Scientific Eutech pH 700 Meter) with HCl, followed by a second
412 filtration. The ASW medium used as control was subjected to the same procedure.

413

414 **Monitoring bacterial growth**

415 Bacterial cultures were monitored daily by OD_{600} measurements using plastic cuvettes in a
416 spectrophotometer (Ultrospec 2100 pro, Biochrom).

417

418 **Conjugation assay**

419 *D. shibae*, *P. inhibens* P72*,* and *P. inhibens* DSM 17395 with plasmids carrying antibiotic
420 resistance genes were used as donor strains for conjugation. *P. inhibens* DSM 17395, *P.*
421 *inhibens* P72, *M. algicola*, and *D. shibae* with chromosomes carrying antibiotic resistance
422 genes were used as recipient strains for conjugation (Table 2).

423 **Pre-cultures:** Bacteria were plated on selective plates and incubated 2-4 days at 30 °C. Then,
424 bacteria were transferred from the plates to ASW medium and adjusted to an OD_{600} of 0.1.
425 Next, bacteria were diluted to an OD_{600} of 0.001 in the medium previously specified for each
426 strain. Pre-cultures were grown to an OD_{600} of 0.3, followed by centrifugation of 10 min at 4000
427 rpm. Bacteria were washed with ASW and centrifuged again for 10 min at 4000 rpm. Samples
428 were adjusted to an OD_{600} of 25 using ASW.

429 **Mating**: For each donor-recipient pair, four biological replicates were prepared for both ASW
430 control and algal filtrates treatment. The treatment consisted of 75% fresh algal filtrates diluted
431 in 25% ASW (v/v). Both control and treated cultures were supplemented with L1 trace
432 elements, L1 nutrients, vitamin mix, 5.5 mM Glucose, 33 mM Na_2SO_4, and 5 mM NH_4Cl. To
433 each breathing tube, 2.4 ml of the medium, 100 µl of the donor strain, and 20 µl of the recipient
434 strain were added. The breathing tubes were mixed and cultivated at 30 °C for 24 h without
435 shaking. For the mating on agar plates, 20 µl of donor cells and 4 µl of recipient cells were
436 mixed and plated on MB agar plates, followed by 24 h incubation at 30 °C.

437 **Selection and quantification**: Each breathing tube was plated in serial dilutions in two
438 technical replicates on three selective plates: one for the donor, one for the recipient and one
439 for the transconjugants. Plates were incubated for 48-96 h at 30 °C. Colonies were counted
440 and quantified. Importantly, transconjugants can grow on selective plates with a single
441 antibiotic that are used for quantifying the donors and recipients. However, our data indicated
442 2-5 orders of magnitude less transconjugants compared to donors and recipients. Therefore,

443 growth of transconjugants on single-antibiotic plates is negligible in comparison to cell
444 numbers of donors or recipients.
445 **Validation**: From each donor-recipient conjugation pair, 7 transconjugant colonies were re-
446 streaked on double-antibiotics plates. After three rounds of re-streaking, single transconjugant
447 colonies were subjected to DNA extraction using Wizard Genomic DNA Purification Kit
448 (Promega), and the DNA was used for PCR amplification using the PCRBIO HS Taq Mix Red
449 polymerase. For each transconjugant, three sets of primers were designed for validation: a
450 primer set specific for the donor, a primer set specific for the recipient and primer set specific
451 for the transferred plasmid (Table 3, Table 4).
452
453 **Table 2:** List of donor-recipient conjugation pairs. Donors carry an antibiotic resistance gene
454 on a plasmid (the plasmid is indicated by its size); recipients carry an antibiotic resistance
455 gene on the chromosome. IR- intergenic region. Tn- transposon insertion.

Donor	Recipient
P. inhibens DSM 17395, 262 kb	*D. shibae* DFL-12
P. inhibens DSM 17395, 78 kb	*D. shibae* DFL-12
P. inhibens DSM 17395, 65 kb	*D. shibae* DFL-12
P. inhibens DSM 17395, 262 kb	*M. algicola* DG898
P. inhibens DSM 17395, 78 kb	*M. algicola* DG898
P. inhibens DSM 17395, 65 kb	*M. algicola* DG898
P. inhibens P72, 57 kb	*D. shibae* DFL-12
P. inhibens P72, 57 kb	*M. algicola* DG898
P. inhibens P72, 57 kb	*P. inhibens* DSM 17395^{IR}
D. shibae DFL-12, 126 kb	*P. inhibens* DSM 17395^{Tn}
D. shibae DFL-12, 126 kb	*P. inhibens* P72

456
457 **Table 3:** Primers for transconjugant validation. Each transconjugant was validated using three
458 primer sets identifying the donor, the recipient, and the transferred plasmid. Primer sequences
459 are listed in table 4.

Strain	Target of the primer set	Primer name
D. shibae DFL-12	Donor	802+803
P. inhibens P72	Donor	794+777
P. inhibens DSM 17395^{Tn}	Recipient	126+92
D. shibae DFL-12	Recipient	802+803
M. algicola DG898	Recipient	804+805
P. inhibens DSM 17395^{IR}	Recipient	47+290
P. inhibens P72	Recipient	796+797
D. shibae DFL-12, 126 kb	plasmid	772+773
P. inhibens P72, 57 kb	plasmid	808+809

460
461

462 **Table 4:** Primer list for PCR reactions and construction of mutant strain. IR- intergenic region
463 mutant. Tn- transposon insertion mutant.

Primer name	Sequence	Strain	Comments	Origin
47	CAGGATGAGTTATTGCACCA	*P. inhibens* DSM 17395 ^{IR}	Validation of transconjugants	Current study
290	GAGCCTACATGTGCGAATGATGC	*P. inhibens* DSM 17395 ^{IR}	Validation of transconjugants	Current study
92	CCAGAGAACAATAGTAAGAGCG	*P. inhibens* DSM 17395 ^{Tn}	Validation of transconjugants	Current study
126	GCTTGATGTCGAGGTGCTGT	*P. inhibens* DSM 17395 ^{Tn}	Validation of transconjugants	Current study
772	GGCACCATCGTCGGAACCAAT	*D. shibae* DFL-12, 126 kb plasmid	Validation of transconjugants	Patzelt *et al.*[25]
773	TGGTATCAGGCATTCGCTTCA	*D. shibae* DFL-12, 126 kb plasmid	Validation of transconjugants	Patzelt *et al.*[25]
777	AGATCGCCAAATTGCAGCAG	*P. inhibens* P72	Validation of transconjugants	Current study
794	AGACGACCCCTTGAGGCAC	*P. inhibens* P72	Validation of transconjugants	Current study
802	ACGGCATCGACCACTTCTTC	*D. shibae* DFL-12	Validation of transconjugants	Birmes *et al.*[26]
803	ACGGAAGATCGGGTACATGG	*D. shibae* DFL-12	Validation of transconjugants	Birmes *et al.*[26]
804	CAATCCGATCGACAGCTACG	*M. algicola* DG898	Validation of transconjugants	Birmes *et al.*[26]
805	TGATCGGCATGTAGAGCAGC	*M. algicola* DG898	Validation of transconjugants	Birmes *et al.*[26]
808	GAACCAGGAATGCATCGGTC	*P. inhibens* P72, 57b kb plasmid	Validation of transconjugants	Birmes *et al.*[26]
809	CCTGATCCGGCTATGACCAT	*P. inhibens* P72, 57b kb plasmid	Validation of transconjugants	Birmes *et al.*[26]
1169	GTACAAAAAAGCAGGCTCCGAATTCGCCCTTAACCAGTCCTTCCGCAACAAGACGCGCAAGG	*P. inhibens* DSM 17395	Construction of the 65 kb plasmid mutant	Current study
1170	CAGTTTACGAACCGAACAGGCTTATGTCAAGCGAAATATCACTATCTCCACACTACCACC	*P. inhibens* DSM 17395	Construction of the 65 kb plasmid mutant	Current study
1171	CTTCTATCGCCTTCTTGACGAGTTCTTCTGAGAGGATTAATTTAAATCGCACTCTTATTCTC	*P. inhibens* DSM 17395	Construction of the 65 kb plasmid mutant	Current study
1172	CTTTGTACAAGAAAGCTGGGTCGAATTCGCCCTACCATTCATAAGCACGTGCTGTGGAAATGTC	*P. inhibens* DSM 17395	Construction of the 65 kb plasmid mutant	Current study

464
465
466 **Quantitative Real Time-PCR (qRT-PCR)**
467 Pre-cultures of *P. inhibens* P72 and *D. shibae* bacteria were grown as previously described.
468 At OD_{600} of 0.3, cells were harvested by centrifugation at 4,000 rpm for 10 min at room-
469 temperature and transferred into ASW (control) and algal filtrates (treatment). The treatment
470 consisted of 75% fresh algal filtrates diluted in 25% of ASW (v/v). Both control and treated
471 cultures were supplemented with L1 trace elements, L1 nutrients, vitamin mix, 33 mM Na_2SO_4,
472 and 5 mM NH_4Cl. *P. inhibens* P72 cultures were additionally supplemented with 5.5 mM
473 Glucose, and *D. shibae* cultures were supplemented with 5.5 mM succinate**.** After 30 min of
474 incubation at 30 °C with shaking at 130 rpm, the cells were centrifuged at 4,000 rpm for 10

475 min at 4 °C. Cells were transferred into 450 µL of RLT buffer containing 1% β-mercapto-
476 ethanol and kept at -80 °C until RNA extraction. RNA was extracted using the Isolate II RNA
477 mini kit (Meridian Bioscience, London, UK) according to the manufacturer instructions. Cells
478 were ruptured by adding low Binding Silica Grinding Beads (100 µm) (SPEX, Metuchen,
479 Netherland) to the thawed sample and were then beaten for 5 min at 30 mHz. For further
480 removal of genomic DNA, RNA samples were treated with 2 µL Turbo DNAse (ThermoFisher)
481 in a 60 µL reaction volume. RNA samples were cleaned and concentrated using RNA Clean
482 & Concentrator TM-5 kit (Zymo Research, Irvine, CA, USA) according to the manufacturer
483 instructions. Equal concentrations of RNA were utilized for cDNA synthesis using Superscript
484 IV (ThermoFisher), according to manufacturer instructions. qRT-PCR was conducted in 384-
485 well plates using the SensiFAST SYBR Lo-ROX Kit (Meridian Bioscience) in a QuantStudio 5
486 (384-well plate) qRT-PCR cycler (Applied Biosystems, Foster City, CA, USA). The qRT-PCR
487 program ran according to enzyme requirements for 40 cycles. Primers are listed in table 5.
488 Primer efficiencies were calculated through standard curves using the QuantStudio 5
489 software. Gene expression ratios were analyzed as previously shown[76], by geometric
490 averaging of the housekeeping genes *gyrA*, and *recA*. To validate the absence of genomic
491 DNA contamination, the same procedure and program were applied on RNA samples that
492 were not reverse transcribed.
493
494 **Table 5:** Primer list of bacterial genes for qRT-PCR.

Strain	Genetic locus	Gene name	Accession number	Forward Primer	Reverse Primer
D. shibae DFL-12	Chromosome	*recA*	DSHI_RS08365	CCGTAATCTCGAGAGCCTGC	GAAAGTTCCGGCAAGACCAC
	Chromosome	*gyrA*	DSHI_RS07515	GTGAAGCGGTAGAGCTGGTTc	CTCGACGAGATCCCCTACCA
	191 kb and 126 kb plasmids	*virB8*	DSHI_RS18440 and DSHI_RS20035	TTCCGCTATGTGACCGACC	CGATCCGGTTGATGCTGAG
	191 kb and 126 kb plasmids	*virB4*	DSHI_RS18425 and DSHI_RS20020	CGATCTGACCGGCATTCTC	GTCACCAGCCAGTTCGACAG
	191 kb and 126 kb plasmids	*virD4*	DSHI_RS18490 and DSHI_RS20080	GAGCATGATCATCACGGGc	CGGTGTCGGACTTTGATTTC
P. inhibens P72	Chromosome	*recA*	PhaeoP72_RS07860	GCTGACACCCAAGTCGGAG	AGCCGAACATAACGCCAATCT
	Chromosome	*gyrA*	PhaeoP72_RS07800	TCAGCTTATGTCGGGCTTCG	CGGTTCCTGACCTCCTTCC
	18 kb and 57 kb plasmids	*virB8*	PhaeoP72_RS19740 and PhaeoP72_RS20905	GGGTTCGTCCAGACCTCATC	CCGGCTCGATGTGGAAAT
	184 kb plasmid	*virB4*	PhaeoP72_RS19755	AAATCCGTAGGTGTTTGCCG	GCCGGATCGACTCAAGGA
	184 kb plasmid	*virD4*	PhaeoP72_03985	TTTTGCCACTCTCCTCCTGC	CGTTCAAACTGTTGTCGCC
	57 kb plasmid	*virB4*	PhaeoP72_RS20885	GAAGAACAGCTTTCCAGCATC	GCACCTGTTCCGCGTTC
	57 kb plasmid	*virD4*	PhaeoP72_RS20940	TTACGGTGAAGCAAGCCAGC	ACATCTGCATCGGTCACTTTC
	18 kb and 57 kb plasmids	*virB4*	PhaeoP72_RS19755 and PhaeoP72_RS20885	ACCGGCATTCTCGACAGC	GCGTATTGCGTCATCATCAC

	18 kb and 57 kb plasmids	*virD4*	PhaeoP72_03985 and PhaeoP72_RS20940	GCTTCGACCAATAACGACACG	GAAGAGGCGGATCAGCGG

495

496 **Genetic manipulation of Roseobacter bacteria**

497 To insert an antibiotic resistance gene into the 65 kb plasmid of *P. inhibens* DSM 17395, the
498 mutant strain ES172 (Table 1) was generated by inserting a kanamycin-resistant gene in an
499 intergenic region between gene PGA1_RS20320 and gene PGA1_RS19930. Regions of
500 approximately 1000 bp upstream and downstream of the intergenic region were PCR-
501 amplified using primers 1169, 1170, 1171 and 1172, respectively (Table 4). The amplified
502 fragments were assembled and cloned into the pYDR8 vector (Table 6) using restriction-free
503 cloning[78,79]. The resulting plasmid (pYDR9, Table 6) was introduced into competent *P.*
504 *inhibens* by electroporation. Preparations of competent cells was performed as previously
505 described[80], with slight modifications. Shortly, cells were grown to an OD_{600} of 0.7 in ½ YTS
506 with 40 g/L of sea salts (also termed full salts medium). Bacteria were washed 3 times using
507 10% (v/v) ice-cold glycerol, centrifuged each time for 5 min at 4 °C and 6000 rpm. The
508 competent cells were subsequently adjusted to an OD_{600} of 20 using 10% (v/v) ice-cold
509 glycerol, frozen in liquid nitrogen and stored at -80°C. Electroporation was conducted with 300
510 µl aliquot of electrocompetent cells, using 10 µg of DNA in a 2 mm cuvette at 2.5 V, followed
511 by 4 h recovery in ½ YTS with full salts. The transformed cells were then plated on ½ YTSS
512 plates containing 150 µg/ml kanamycin, and resistant colonies were validated by PCR and
513 DNA sequencing.

514

515 **Table 6:** Plasmids used in the study.

Plasmid name	Origin	Comments
pYDR8	Lab collection	Constructed using pCR8/GW/TOPO vector (Invitrogen), and used in the current study as a vector carrying the kanamycin resistant cassette
pYDR9	Current study	Constructed using pYDR8 vector for creating the 65 kb intergenic mutant of *P. inhibens* DSM 17395

516

517 **Generating the *Roseobacteraceae* T4SS dataset**

518 All *Roseobacteraceae* genomes (of any quality) available in the Integrated Microbial Genomes
519 & Microbiomes (IMG/M) database[81] were scanned for the presence of the *virB8* gene using
520 the IMG "Function search" tool and K03202 KEGG (Kyoto Encyclopedia of Genes and
521 Genomes[82]) Orthology (KO) designation for VirB8. The *virB8* genes (K03203) were found in
522 320 out of 898 *Roseobacteraceae* genomes. The respective scaffolds were scanned for the
523 presence of other *vir* genes (*virB1-B7, virB9-B11* and *virD4*) and were assessed as
524 representing either plasmids or chromosomes (Table S1). Within these 320 genomes a total
525 of 564 *virB8* genes were located on 553 scaffolds. Further assignment of the scaffolds to
526 plasmids or chromosomes was conducted using the presence or absence of plasmid

527 operational taxonomic unit (pOTU) designation that was assigned during genome annotation
528 in IMG and according to scaffold size. Scaffolds with no operational taxonomic unit designation
529 larger than 300 kb were marked as chromosomes. Scaffolds with no operational taxonomic
530 unit designation and smaller than 300 kb were marked as unknowns. Of note, the T4SS is an
531 extremely versatile apparatus. Conjugative T4SSs typically consist of the archetypal
532 VirB/VirD4 components (encoded by *virB1-11* together with *virD4*). Yet, the precise number of
533 needed genes and their essentiality for a functional T4SS remain poorly understood,
534 particularly in non-model bacteria[83-86]. Consequently, due to the difficulty in predicting which
535 T4SS is conjugative, we set stringent criteria and defined a potentially functional conjugative
536 T4SS (*i.e.,* complete T4SS) as one that includes all the *vir* genes that comprise the T4SSs
537 located on the conjugative plasmids of *D. shibae* and *P. inhibens* P72. Accordingly, T4SSs
538 homologous to Vir but possessing other KO assignments (e.g., Tra), that were not
539 experimentally shown to be conjugative in Roseobacters, were not included in this analysis.
540 In total, our analysis identified 387 out of the 553 scaffolds as carrying a complete set of T4SS
541 genes. However, it is possible that partially complete T4SSs in our analysis or homologous
542 T4SSs (e.g., Tra) may be functional as well[15].
543

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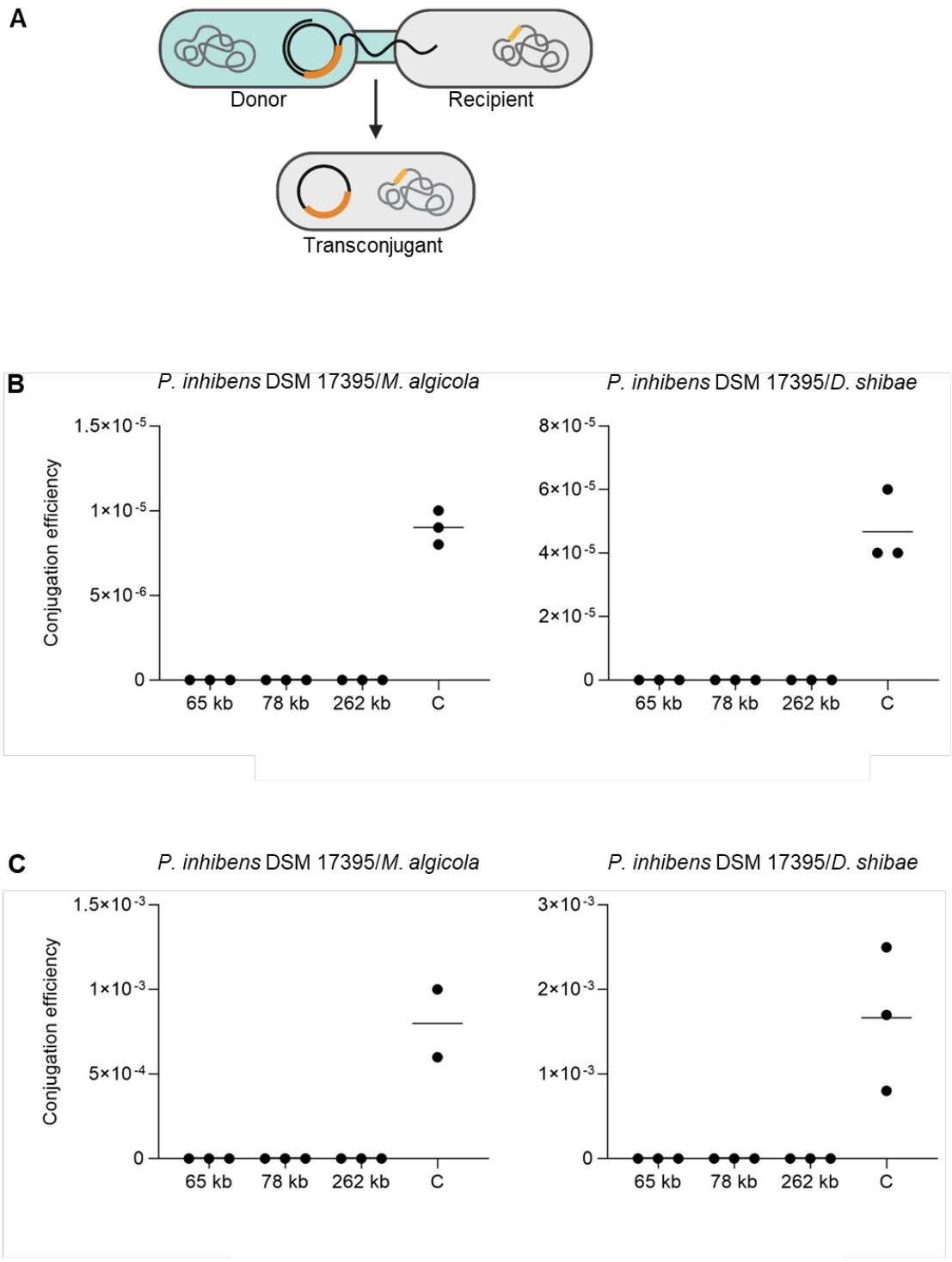

Fig. 1. The *P. inhibens* strain DSM 17395 is unable to serve as a plasmid donor. A) Schematic representation of the conjugation assay. The donor strain (light blue) carries an antibiotic resistance gene (orange) on a native plasmid, while the recipient strain (grey) harbors a chromosomal antibiotic resistance gene (yellow). Successful plasmid transfer from the donor to the recipient will result in transconjugants that are resistant to both antibiotics. Results of conjugations assays performed in **B)** liquid media and **C)** on plates. Conjugation

assays were carried out using *P. inhibens* DSM 17395 as the donor, with a kanamycin-resistance gene on one of its native 65 kb, 78 kb and 262 kb plasmids. Each donor strain was mated with recipient strains, *M. algicola* or *D. shibae*, marked with a gentamicin-resistance gene on their chromosome. As a control ("C" in the graphs), *P. inhibens* P72 carrying a kanamycin-resistance gene on its 57 kb native plasmid was used as a donor and mated with *M. algicola* or *D. shibae* recipients carrying a gentamicin-resistance gene on their chromosome. Dots represent individual biological replicate and lines indicate the mean value. Conjugation efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*.

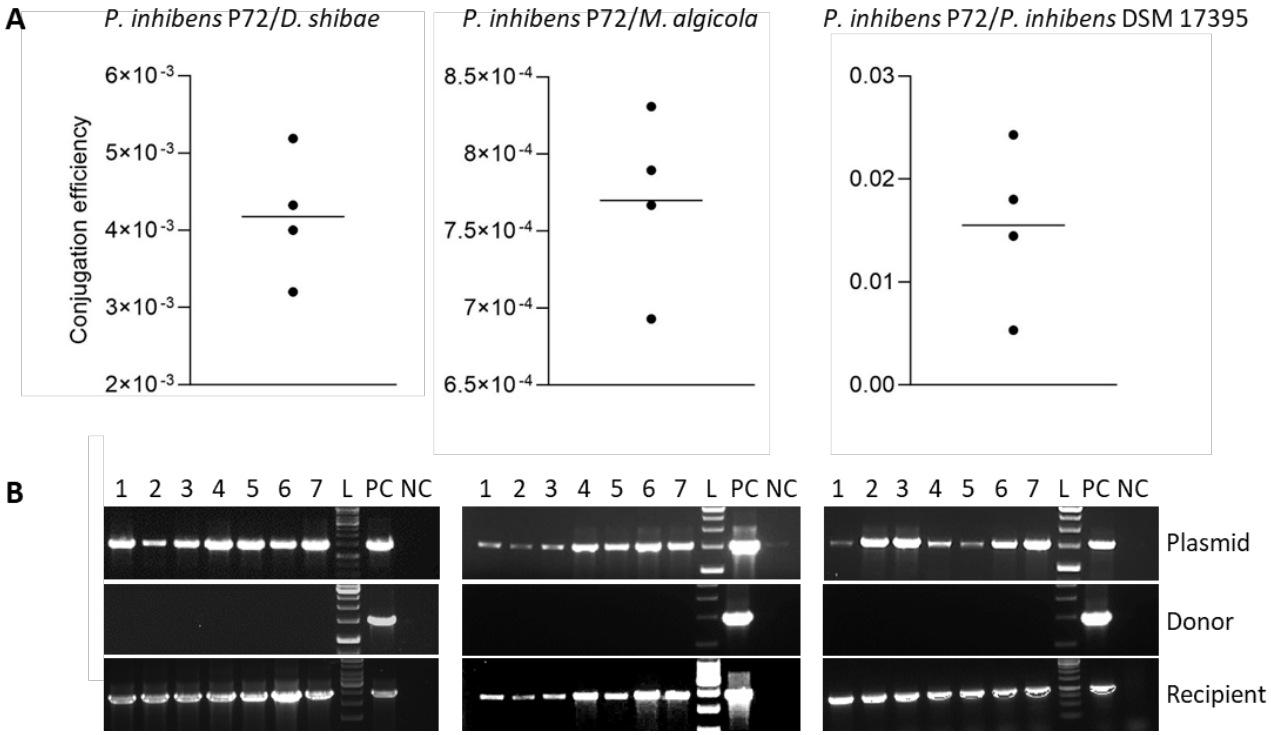

Fig. 2. The *P. inhibens* P72 strain serves as a plasmid donor to various recipients. A) Conjugation assays were performed in liquid media with *P. inhibens* P72 as a plasmid donor, carrying on its native 57 kb plasmid a kanamycin-resistance gene. The recipient strains *M. algicola, D. shibae* and *P. inhibens* DSM 17395, were marked with a gentamicin-resistance gene on their chromosome. Dots represent individual biological replicate and lines indicate the mean value. Conjugation efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*. **B)** Gel images of PCR products for validation of the transconjugants. From each donor-recipient pair, seven transconjugant colonies were randomly selected (marked 1-7). Each colony was streaked three times, followed by genomic extraction and PCR reactions. The transconjugants were tested using three sets of primers that identify the donor strain, recipient strain, and the transferred plasmid. Positive controls (PC) included primers and the corresponding strain DNA, and negative controls (NC) included the opposing strain DNA (i.e., primers targeting the donor strain with DNA of the recipient strain). Each gel image corresponds to the graph above it. L marks the 1 kb ladder incorporated in all gels.

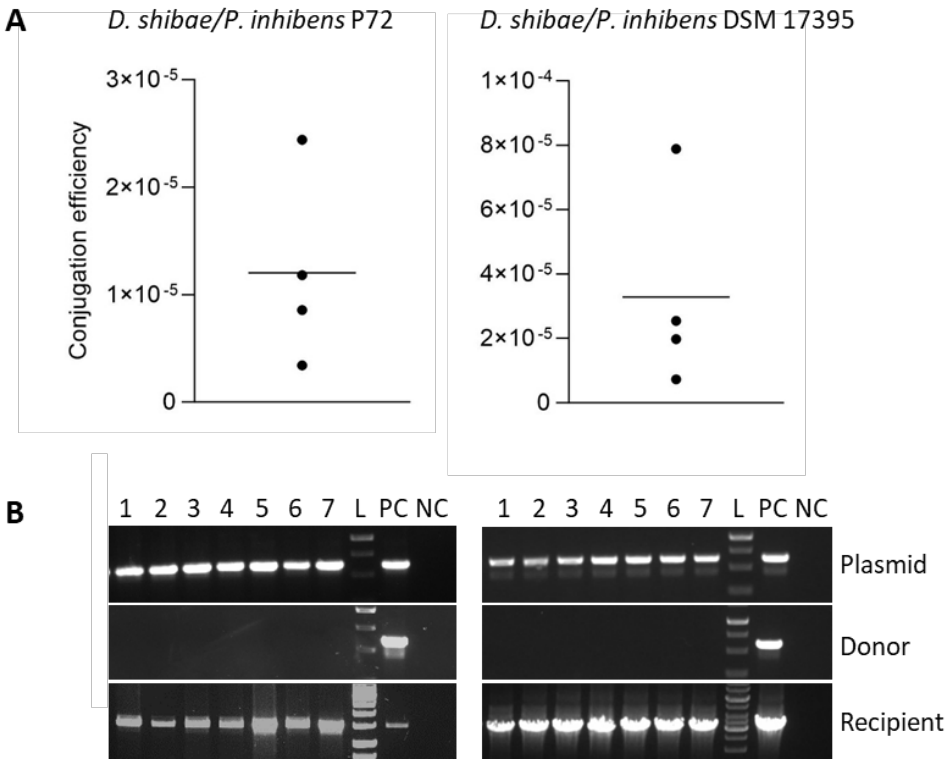

Fig. 3. Another Roseobacter strain that carries a plasmid-encoded T4SS can serve as a plasmid donor. A) Conjugation assays were performed with *D. shibae* as a donor strain, marked with a gentamicin-resistance gene on its 126 kb native plasmid. Recipient strains were *P. inhibens* DSM 17395 or *P. inhibens* P72, both marked with a kanamycin-resistance gene on their chromosome. Dots represent individual biological replicate and lines indicate the mean value. Conjugation efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*. **B)** Gel images of PCR products for validation of the transconjugants. From each donor-recipient pair, seven transconjugant colonies were randomly selected (marked 1-7). Each colony was streaked three times, followed by genomic extraction and PCR reactions. The transconjugants were tested using three sets of primers that identify the donor strain, recipient strain, and the transferred plasmid. Positive controls (PC) included primers and the corresponding strain DNA, and negative controls (NC) included the opposing strain DNA (i.e., primers targeting the donor strain with DNA of the recipient strain). Each gel image corresponds to the graph above it. L marks the 1 kb ladder incorporated in all gels.

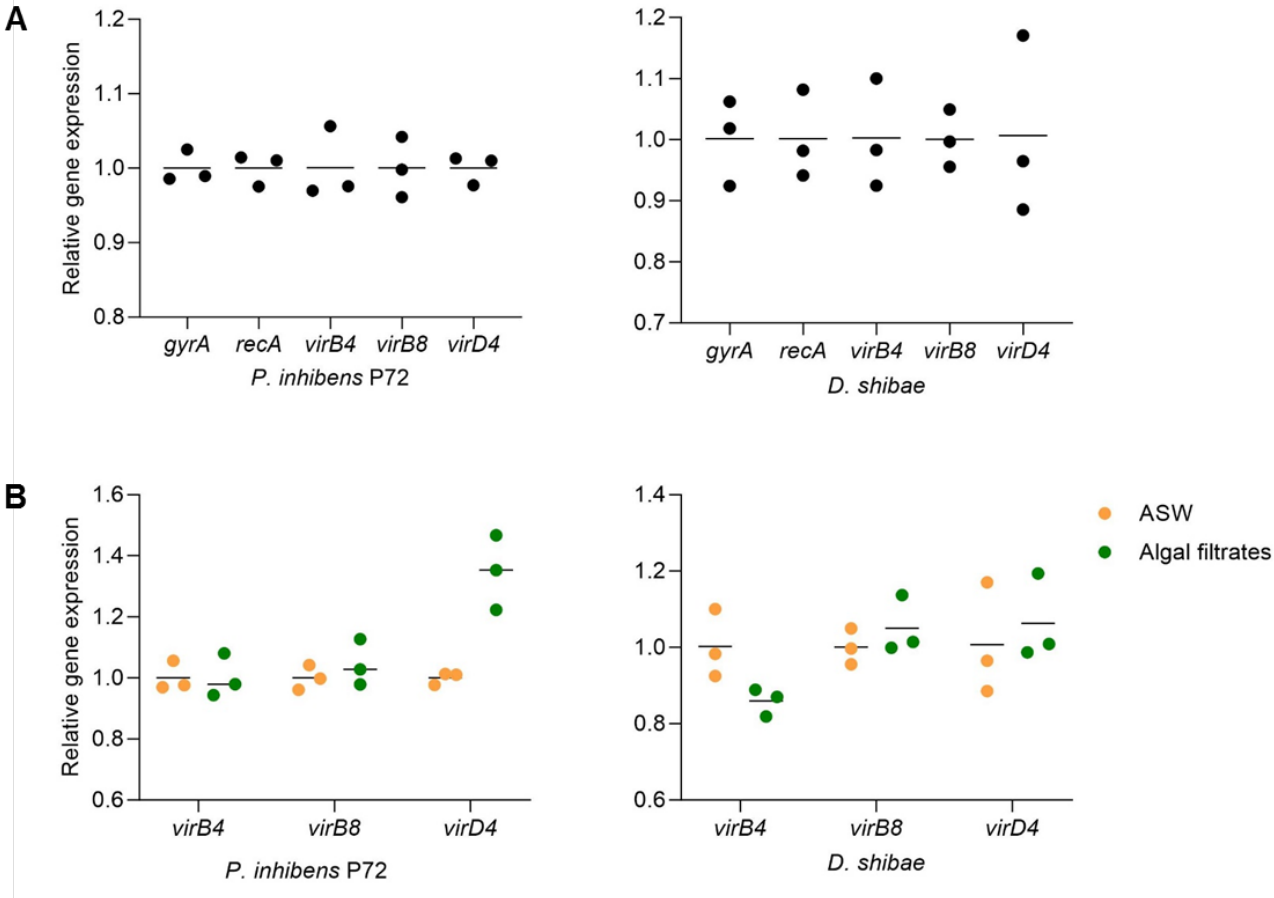

Fig. 4. Algal exudates do not influence the expression of genes encoding the T4SS. Relative gene expression of *virB4*, *virB8* and *virD4* genes was assessed using qRT-PCR. RNA was extracted from bacterial cultures of *D. shibae* and *P. inhibens* P72 strains under (**A**) control conditions and (**B**) following treatment either with artificial seawater as control (ASW, orange, data identical to panel A) or with algal exudates (Algal filtrates, green). Data points represent the mean of three technical replicates originating from one biological sample, lines represent replicates mean values.

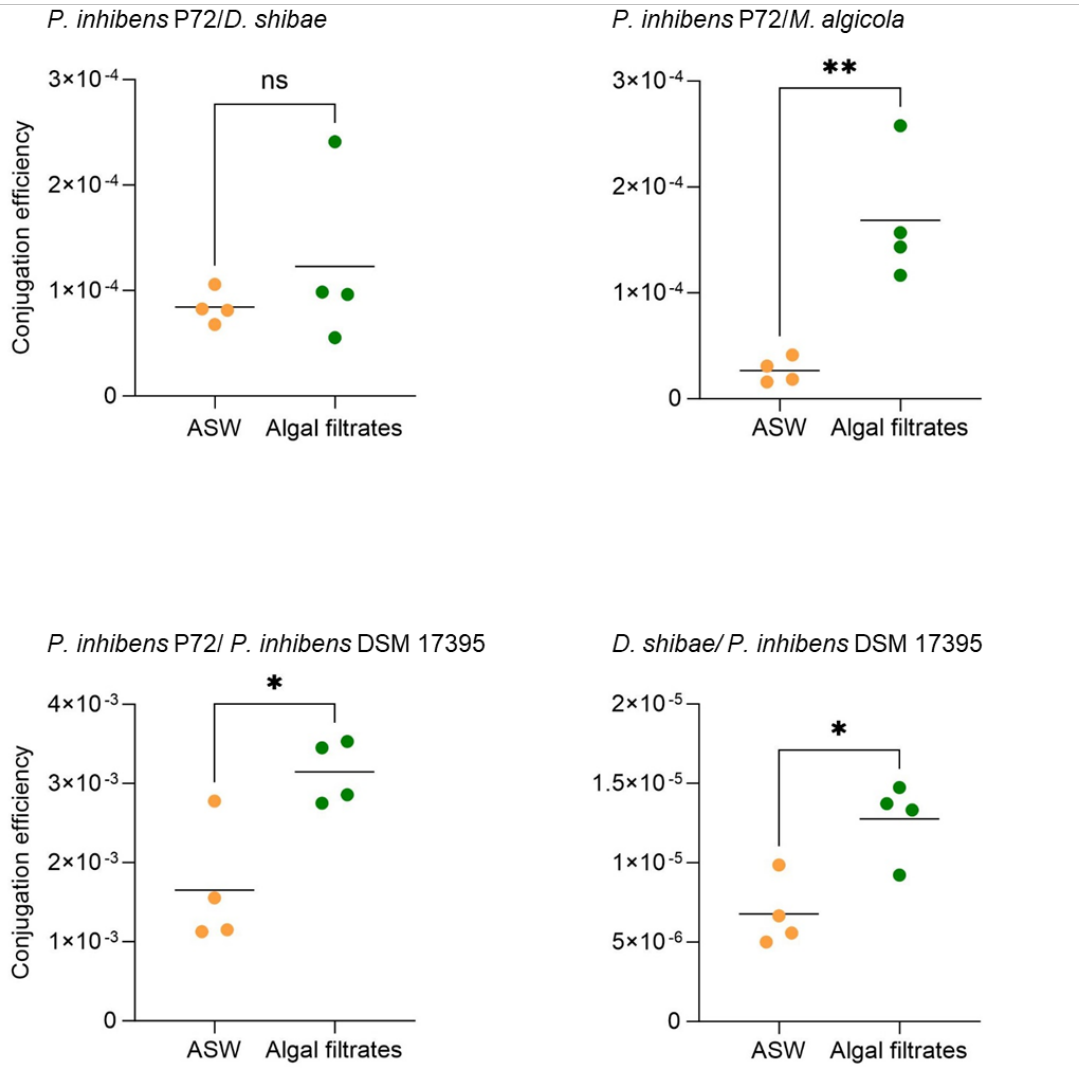

Fig. 5. Algal exudates increase conjugation efficiency. Conjugation assays were performed with either *P. inhibens* P72 as a donor, marked with a kanamycin-resistance gene on its native 57 kb plasmid, or *D. shibae* as a donor, marked with a gentamicin-resistance gene on its native 126 kb plasmid. For *P. inhibens* P72, three strains were tested as recipient: *M. algicola, D. shibae* and *P. inhibens* DSM 17395, and were marked with a gentamicin-resistance gene on their chromosome. For *D. shibae,* the recipient strain was *P. inhibens* DSM 17395, chromosomally marked with a kanamycin-resistance gene. Mating was performed with cultures treated with artificial seawater (ASW) as control (orange) or with algal exudates (algal filtrates, green). Conjugation efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*. Dots represents one biological sample and lines represent mean values. Statistical significance was calculated using a t-test, $p<0.05$ marked *, $p<0.01$ marked **, and not significant marked ns.