Title: Algal Exudates Promote Conjugation in Marine Roseobacters

Authors: Yemima Duchin Rapp, Valeria Lipsman, Lilach Yuda, Ilya V Kublanov, Dor Matsliyah and Einat Segev*

Affiliations:
Department of Plant and Environmental Sciences, Weizmann Institute of Science; Rehovot, 7610001, Israel.
*Corresponding author. Email: Einat.Segev@weizmann.ac.il

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 conditions promoting conjugation events in the marine environment are not well understood. Roseobacters, abundant marine bacteria commonly associated with algae, possess a multitude of T4SSs. Many Roseobacters are heterotrophic bacteria that rely on algal secreted compounds to support their growth. These compounds attract bacteria, facilitating colonization and attachment to algal cells. Algae and their metabolites bring bacteria into close proximity, potentially promoting bacterial HGT. Investigation across various Roseobacters revealed that algal exudates indeed enhance plasmid transfer through conjugation. While algal exudates do not influence the transcription of bacterial conjugative machinery genes, they promote bacterial attachment, potentially stabilizing proximity and facilitating HGT. Notably, under conditions where attachment is less advantageous, the impact of algal exudates on conjugation is reduced. These findings suggest that algae enhance bacterial conjugation primarily by fostering attachment and highlight the importance of studying bacterial HGT within the context of algal-bacterial interactions.

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 supports 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 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 also facilitates cell-to-cell contact and biofilm formation between bacteria. In turn, cell-to-cell contact and a sessile lifestyle in a biofilm can improve the efficiency of the conjugative transfer[12-20].

HGT, and specifically conjugation that was demonstrated between Roseobacters[6,21-23], has been suggested as a central reason for the ecological success of Roseobacters[22]. These bacteria exhibit diverse metabolic capabilities and a wide range of symbiotic lifestyles[24]. Many Roseobacters engage in various interactions with algal hosts[25-30]. Algae exude various metabolites that support bacterial growth in the nutrient-poor oligotrophic ocean[24,25,31]. Thus, marine bacteria can secure a nutrient flux through proximity to an algal host. Algal exudates were shown to influence bacterial physiology[24-26,31,32] and shape algal-bacterial interactions[25,33]. In the vicinity of algal hosts, bacteria experience spatial proximity to one another, with certain bacteria attaching to the surface of the algal cells[25,32]. Since bacterial proximity, cell-to-cell contact, and attachment can promote HGT[13,21,34,35], algal exudates may promote attachment and consequently 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[25,36]. As a model algal host, we used the unicellular alga *Emiliania huxleyi* (also termed G*ephyrocapsa huxleyi*)[37], which is widespread in modern oceans and was shown to harbor a rich community of bacteria, including Roseobacters[25,36,38]. First, we tested the ability of various Roseobacters to successfully conduct conjugation. Next, we examined the impact of algal exudates on bacterial conjugation. To understand the underlying mechanism, we monitored the expression of T4SS-encoding genes and bacterial attachment capabilities following exposure to algal exudates. Our findings demonstrate improved bacterial conjugation efficiency following exposure to algal exudates. While T4SS gene expression did

not increase in response to algal exudates, bacterial attachment capabilities did improve. Conducting conjugation assays under conditions in which attachment is not beneficial, resulted in a reduced impact of the algal exudates. Our data suggest that algal exudates enhance bacterial conjugation primarily by promoting bacterial attachment.

Materials & Methods

Bacterial strains and growth conditions

The bacterial strains *Phaeobacter inhibens* DSM 17395 and *Dinoroseobacter shibae* DFL-12 were purchased from the German Collection of Microorganism and Cell Cultures (DSMZ, Braunschweig, Germany) (Table S1). *P. inhibens* P72 was kindly provided by Prof. Juan Barja from University of Santiago de Compostela, Spain, and Dr. Jörn Petersen from the Leibniz Institute, DSMZ, Germany (Table S1). The bacterial strains of *Phaeobacter inhibens* DSM 17395 with a kanamycin-resistance cassette on its chromosome (*P. inhibens* DSM 17395$^{chr-kana}$), *Marinovum algicola* DG898 DSM 27768 with a gentamicin-resistance cassette on its chromosome (*M. algicola* DG898 $^{chr-gent}$), *Dinoroseobacter shibae* DFL-12 with a gentamicin-resistance cassette on its native 126 kb plasmid (*D. shibae* DFL-12 $^{p126-gent}$), *Dinoroseobacter shibae* DFL-12 with a gentamicin-resistance cassette on its chromosome (*D. shibae* DFL-12 $^{chr-gent}$), *P. inhibens* P72 with a kanamycin-resistance cassette on its native 57 kb plasmid (*P. inhibens* P72 $^{p57-kana}$), and *P. inhibens* P72 with a kanamycin-resistance cassette on its chromosome (*P. inhibens* P72 $^{chr-kana}$) were kindly provided by Dr. Jörn Petersen from the Leibniz Institute, DSMZ, Germany (Table. S1).

D. shibae cultures were plated on ½ marine broth 2216 (MB) (Difco, USA) agar plates (MB, 18.7 g/L; agar, 16 g/L) with 30 µg/ml gentamicin antibiotic. *M. algicola* cultures were plated on ½ YTSS agar plates (yeast extract, 2 g/L; trypton, 1.25 g/L; sea salts, 20 g/L; agar, 16 g/L, all purchased from Sigma-Aldrich) with 30 µg/ml gentamicin antibiotic. *P. inhibens* DSM 17395 cultures were plated on ½ YTSS agar plates with 30 µg/ml gentamicin or 150 µg/ml kanamycin antibiotic. *P. inhibens* P72 was plated on ½ YTSS agar plates with 150 µg/ml kanamycin antibiotic. Transconjugants of *D. shibae* were plated on ½ MB agar plates with 30 µg/ml gentamicin and 150 µg/ml kanamycin antibiotics. Transconjugants of *P. inhibens* DSM 17395, *P. inhibens* P72 and *M. algicola* were plated on ½ YTSS agar plates with 30µg/ml gentamicin and 150 µg/ml kanamycin antibiotics.

Cultures of algae and bacteria were grown in artificial seawater (ASW) prepared according to Goyet and Poisson[39]. ASW contained mineral salts (NaCl, 409.41 mM; Na_2SO_4, 28.22 mM; KCl, 9.08 mM; KBr, 0.82 mM; NaF, 0.07 mM; Na_2CO_3, 0.20 mM; $NaHCO_3$, 2 mM; $MgCl_2$, 50.66 mM; $SrCl_2$, 0.09 mM), L1 trace elements ($FeCl_3 \cdot 6H_2O$, 3.15 mg/L; $Na_2EDTA \cdot 2H_2O$, 4.36 mg/L; $CuSO_4 \cdot 5H2O$, 9.8 µg/L; $Na_2MoO_4 \cdot 2H_2O$, 6.3 µg/L; $ZnSO_4 \cdot 7H_2O$, 22 µg/L;

112 $CoCl_2 \cdot 6H_2O$, 10 µg/L; $MnCl_2 \cdot 4H_2O$, 180 µg/L) and L1 nutrients ($NaNO_3$, 882 µM; NaH_2PO_4,
113 36.22 µM). The components were dissolved in Milli-Q water (IQ 7003; Merck, Darmstadt,
114 Germany) and the pH adjusted to 8 with HCl. Stock solutions of L1 trace elements and L1
115 nutrients were purchased from the Bigelow Laboratory for Ocean Sciences (Boothbay, ME,
116 USA).
117 Cultures of *P. inhibens* DSM 17395, *P. inhibens* P72, and *M. algicola* were grown in ASW
118 medium supplemented with additional carbon (glucose, 5.5 mM, Sigma Aldrich), nitrogen
119 (NH_4Cl, 5 mM, Sigma Aldrich), sulfur (Na_2SO_4, 33 mM, Merck), and f/2 vitamins (thiamine HCl,
120 100 µg/L; biotin, 0.5 µg/L; vitamin B_{12}, 0.5 µg/L, Bigelow Laboratory for Ocean Sciences).
121 *D. shibae* cultures were cultivated with minor modifications including 5.5 mM succinate as a
122 carbon source (Sigma Aldrich) and different vitamins mix (adjusted from Gonzalez *et al.,*[40]
123 Thiamine HCl, 0.148 µM/L; biotin, 0.082 µM/L; Pyridoxine HCL, 0.486 µM/L; Nicotinic acid,
124 0.406 µM/L; Pantothenic acid, 0.228 µM/L; Cyanocobalamin, 0.0006 µM/L; pABA, 0.364
125 µM/L). This medium, ASW supplemented with vitamins and nutrients, is referred to as CNS.
126 Bacteria were cultivated at 30 °C under constant shaking at 130 rpm.
127
128 **Algal strain, growth conditions and monitoring**
129 The axenic algal strain of *Emiliania huxleyi* CCMP3266 (also termed G*ephyrocapsa huxleyi*[37])
130 was purchased from the National Center for Marine Algae and Microbiota (Bigelow Laboratory
131 for Ocean Sciences, Maine, USA). Algae were grown in ASW (as described above)
132 supplemented with f/2 vitamins. Algae were grown in standing cultures in a growth room at 18
133 °C under a light/dark cycle of 16/8 h. Illumination intensity during the light period was 150
134 mmoles/m^2/s. Absence of bacteria in axenic algal cultures was monitored periodically both by
135 plating on ½ YTSS plates and under the microscope.
136 Cultures of *E. huxleyi* were grown as follows; algal cells concentrations from a late exponential
137 phase culture were counted by a CellStream CS-100496 flow cytometer (Merck, Darmstadt,
138 Germany) using 561 nm excitation and 702 nm emission. Algal cells were gated according to
139 event size and fluorescence intensity. An inoculum of 10^5 algal cells was introduced into 100
140 ml of ASW and incubated as described above. Algal growth in cultures was monitored by a
141 CellStream flow cytometer as described. For each biological replicate 50,000 events were
142 recorded.
143
144 **Algal filtrates**
145 To obtain algal filtrates, algal cultures were grown as described above for 12 days (Fig. S6).
146 An initial washing of the filters was conducted by passing 100 ml ASW medium through the
147 filter (Thermo scientific, 0.22 µm PES, 250ml). Then, algal cultures were vacuum filtered, and
148 filtrates pH was adjusted to pH 8 (Thermo Scientific Eutech pH 700 Meter) with HCl, for

149 identical pH levels across both control and treatment. This step was followed by a second
150 filtration since the titration procedure was conducted in non-sterile conditions. The ASW
151 medium used as control was subjected to the same procedure. To validate that vacuum
152 filtration did not rupture the algal cells, the filters were suspended in ASW and the algal
153 population was assessed using flow cytometry.
154
155 **Monitoring bacterial growth**
156 Bacterial cultures were monitored daily by OD_{600} measurements using plastic cuvettes in a
157 spectrophotometer (Ultrospec 2100 pro, Biochrom).
158
159 **Conjugation assay**
160 *D. shibae* DFL-12 $^{p126\text{-}gent}$, *P. inhibens* P72 $^{p57\text{-}kana}$, *P. inhibens* DSM 17395 $^{p262\text{-}gent}$, *P. inhibens*
161 DSM 17395 $^{p78\text{-}gent}$ and *P. inhibens* DSM 17395 $^{p65\text{-}gent}$ with plasmids carrying antibiotic
162 resistance genes were used as donor strains for conjugation. *P. inhibens* DSM 17395$^{chr\text{-}gent}$,
163 *P. inhibens* DSM 17395$^{chr\text{-}kana}$, *P. inhibens* P72 $^{chr\text{-}kana}$, *M. algicola* DG898 $^{chr\text{-}gent}$, and *D. shibae*
164 DFL-12 $^{chr\text{-}gent}$ with chromosomes carrying antibiotic resistance genes were used as recipient
165 strains for conjugation (Table S2). The conjugation assay involved several stages: first,
166 preparing pre-cultures of pure donors and recipients; then, mating in liquid or agar plates; and
167 finally selection on selective agar plates.
168 **Pre-cultures:** Bacteria were inoculated from a frozen stock (-80 °C) onto selective agar plates
169 and incubated 2-4 days at 30 °C. Then, bacteria were transferred from the plates to ASW
170 medium and adjusted to an OD_{600} of 0.1. Next, bacteria were diluted to an OD_{600} of 0.001 in
171 the medium previously specified for each strain. Pre-cultures were grown to an OD_{600} of 0.3,
172 followed by centrifugation of 10 min at 3,200 g. Bacteria were washed with ASW and
173 centrifuged again for 10 min at 3,200 g. Samples were adjusted to an OD_{600} of 25 using ASW.
174 **Mating**: For conjugation assay in liquid media (Fig. 1, Fig. 2), the treatment consisted of 75%
175 fresh algal filtrates diluted in 25% ASW (v/v, ratio of 3:1). Both control (ASW) and treated
176 cultures were supplemented with L1 trace elements, L1 nutrients, 5.5 mM glucose, vitamin
177 mix, 33 mM Na_2SO_4, and 5 mM NH_4Cl. The final control and treatment medium are referred
178 to as CNS, with the treatment containing 75% volume of algal filtrates and 25% volume of
179 ASW (v/v), in contrast to the control which consists solely of ASW. To each 14 ml tube
180 (Corning Falcon Polypropylene round bottom tube), 2.4 ml of the medium, 100 µl of the donor
181 strain, and 20 µl of the recipient strain were added. The tubes were mixed and incubated at
182 30 °C for 24 h without shaking.
183 For mating on agar plates (Fig. 6), agar (16 g) was added to 250 ml of ASW medium (w/v) and
184 autoclaved. After cooling, 750 ml of algal filtrates or ASW were added as treatment or control
185 respectively (v/v). Both control and treated cultures were supplemented with L1 trace

186 elements, L1 nutrients, vitamin mix, Glucose, Na_2SO_4, and NH_4Cl (consisting of the CNS
187 medium). The mediums were mixed and poured onto the plates. Then, 20 µl of donor cells
188 and 4 µl of recipient cells were mixed and plated on the agar plates, followed by 24 h incubation
189 at 30 °C. For mating on MB agar plates (Fig. S3), 20 µl of donor cells and 4 µl of recipient cells
190 were mixed and plated on MB agar plates, followed by 24 h incubation at 30 °C.
191 **Selection and quantification**: for conjugation in liquid media, each tube was plated in serial
192 dilutions in two technical replicates on three selective plates: one for the donor, one for the
193 recipient and one for the transconjugants. For conjugation on agar plates, bacteria were
194 scraped from the plate, resuspended in 150 µl of ASW, and plated in serial dilutions as
195 described for the conjugation assay in liquid. Plates were incubated for 48-96 h at 30 °C.
196 Colonies were counted and quantified. Importantly, transconjugants can grow on selective
197 plates containing a single antibiotic, used for quantifying the donors and recipients, as they
198 possess resistance to both antibiotics. However, our data indicated that there were 2-5 orders
199 of magnitude fewer transconjugants compared to donors and recipients. Therefore, the growth
200 of transconjugants on single-antibiotic plates is negligible in comparison to cell numbers of
201 donors or recipients.
202 **Validation**: From each donor-recipient conjugation pair, seven transconjugant colonies were
203 re-streaked on double-antibiotics plates. After three rounds of re-streaking, single
204 transconjugant colonies were subjected to DNA extraction using Wizard Genomic DNA
205 Purification Kit (Promega), and the DNA was used for PCR amplification using the PCRBIO
206 HS Taq Mix Red polymerase. For each transconjugant, three sets of primers were designed
207 for validation: a primer set specific for the donor, a primer set specific for the recipient and
208 primer set specific for the transferred plasmid (Table S3, Table S4).
209
210 **Quantitative Real Time-PCR (qRT-PCR)**
211 Pre-cultures of *P. inhibens* P72 $^{p57\text{-}kana}$ and *D. shibae* DFL-12 $^{p126\text{-}gent}$ bacteria were grown as
212 previously described. At OD_{600} of 0.3, cells were harvested by centrifugation at 3,200 g for 10
213 min at room-temperature and transferred into ASW (control) and algal filtrates (treatment).
214 The treatment consisted of 75% fresh algal filtrates diluted in 25% of ASW (v/v). Both control
215 and treated cultures were supplemented with L1 trace elements, L1 nutrients, vitamin mix,
216 33 mM Na_2SO_4, and 5 mM NH_4Cl. The final control and treatment medium is referred to as
217 CNS, with the treatment containing 75% volume of algal filtrates and 25% volume of ASW
218 (v/v), in contrast to the control which consists solely of ASW. *P. inhibens* P72 $^{p57\text{-}kana}$ cultures
219 were additionally supplemented with 5.5 mM Glucose, and *D. shibae* DFL-12 $^{p126\text{-}gent}$ cultures
220 were supplemented with 5.5 mM succinate**.** After 30 min of incubation at 30 °C with shaking
221 at 130 rpm, the cells were centrifuged at 3,200 g for 10 min at 4 °C. Cells were transferred
222 into 450 µL of RLT buffer containing 1% β-mercapto-ethanol and kept at -80 °C until RNA

223 extraction. RNA was extracted using the Isolate II RNA mini kit (Meridian Bioscience, London,
224 UK) according to the manufacturer instructions. Cells were ruptured by adding low Binding
225 Silica Grinding Beads (100 µm) (SPEX, Metuchen, Netherland) to the thawed sample and
226 were then beaten for 5 min at 30 mHz. For further removal of genomic DNA, RNA samples
227 were treated with 2 µL Turbo DNAse (ThermoFisher) in a 60 µL reaction volume. RNA samples
228 were cleaned and concentrated using RNA Clean & Concentrator TM-5 kit (Zymo Research,
229 Irvine, CA, USA) according to the manufacturer instructions. Equal concentrations of RNA
230 were utilized for cDNA synthesis using Superscript IV (ThermoFisher), according to
231 manufacturer instructions. qRT-PCR was conducted in 384-well plates using the SensiFAST
232 SYBR Lo-ROX Kit (Meridian Bioscience) in a QuantStudio 5 (384-well plate) qRT-PCR cycler
233 (Applied Biosystems, Foster City, CA, USA). The qRT-PCR program ran according to enzyme
234 requirements for 40 cycles. Primers are listed in table S5. Primer efficiencies were calculated
235 through standard curves using the QuantStudio 5 software. Gene expression ratios were
236 analyzed as previously shown[41], by geometric averaging of the housekeeping genes *gyrA*,
237 and *recA*. To validate the absence of genomic DNA contamination, the same procedure and
238 program were applied on RNA samples that were not reverse transcribed.
239
240 **Reporter strains**
241 The T4SS reporter strains were constructed by cloning the *virB* promoter upstream of a gene
242 encoding the super-folded green fluorescent protein (sfGFP) in the pBBR1MCS-5 vector[42]
243 (kindly provided by Prof. Kenneth M. Peterson, Louisiana State University Medical Center,
244 USA) (Table 6).
245 **Construction of *P. inhibens* P72 reporter strains:** The *virB* promoter from the 184 kb
246 plasmid was amplified using primers 987 and 988 (Table S4). The *virB* promoter from the 57
247 kb plasmid was amplified using primers 989 and 990 (Table S4). The amplified fragments
248 were cloned separately into the pYDR1 vector (Table S6) upstream of a sfGFP encoding gene,
249 using restriction-free cloning[43,44]. The resulting plasmids, pYDR3 for the 184 kb plasmid and
250 pYDR10 for the 57 kb plasmid (Table S6) were introduced into competent *P. inhibens* P72
251 cells by electroporation. Preparations of competent cells was performed as previously
252 described[45], with slight modifications. Shortly, cells were grown to an OD_{600} of 0.7 in ½ YTS
253 with 40 g/L of sea salts (also termed full salts medium). Bacteria were washed 3 times using
254 10% (v/v) ice-cold glycerol, centrifuged each time for 5 min at 4 °C and 5,500 g. The competent
255 cells were subsequently adjusted to an OD_{600} of 20 using 10% (v/v) ice-cold glycerol, frozen
256 in liquid nitrogen and stored at -80°C. Electroporation was conducted with 300 µl aliquot of
257 electrocompetent cells, using 10 µg of DNA in a 2 mm cuvette at 2.5 V, followed by 4 h
258 recovery in ½ YTS with full salts. The transformed cells were then plated on ½ YTSS plates
259 containing 50 µg/ml gentamicin. Resistant colonies were re-streaked three times. Sequencing

260 and PCR validation of the reporter strains was conducted using primers 132 and 255 (Table
261 S5).
262 **Construction of *D. shibae* reporter strains:** The *virB* promoter from the 191 kb plasmid was
263 amplified using primers 974 and 975 (Table S4). The *virB* promoter from the 126 kb plasmid
264 was amplified using primers 972 and 973 (Table S4). The amplified fragments were separately
265 cloned into pYDR1 vector (Table S6) upstream of a gene encoding the sfGFP protein, using
266 restriction-free cloning[43,44]. The resulting plasmids, pYDR6 for the 191 kb plasmid and pYDR7
267 for the 126 kb plasmid (Table S6) were conjugated into *D. shiba*e as previously described[45],
268 with slight modifications. Shortly, the constructs were introduced into competent *E. coli* ST18
269 DSM 22074, carrying a *hemA* deletion that results in defective tetrapyrrole biosynthesis
270 (purchased from DSMZ). Hence, the mutant strain requires addition of 5-aminolevulinic acid
271 to grow. The donor *E. coli* ST18 containing the pYDR6 or pYDR7 plasmids was mated with
272 the recipient *D. shibae.* Cultures of *D. shibae* recipients were cultivated for 24 h at 30 °C to an
273 OD_{600} of 0.4 in MB medium. The donor *E. coli* ST18 was grown in LB medium supplemented
274 with 50 µg/ml 5-aminolevulinic acid (Sigma-Aldrich) at 37 °C to an OD_{600} of 0.3. Both cultures
275 were centrifuged, concentrated 25-fold, and mixed in a donor-to-recipient ratio of 10:1. Then,
276 50 µl drops of the mixed cultures were plated on ½ MB plates containing 50 µg/ml 5-
277 aminolevulinic acid, and incubated for 24 h at 30 °C. Cells were scraped from the plate,
278 resuspend in 100 µl of MB, and plated on ½ MB plates containing 80 µg/ml gentamicin.
279 Colonies of transconjugants were re-streaked three times. Sequencing and PCR validation of
280 the reporter strains was conducted using primers 132 and 255 (Table S5).
281 **Reporter strain assay:** Cultures of the reporter strains were adjusted to an OD_{600} of 0.001
282 and grown in ASW (control) and algal filtrates (treatment). The treatment consisted of 75%
283 fresh algal filtrates diluted in 25% of ASW (v/v). Both control and treatment cultures were
284 supplemented with L1 trace elements, L1 nutrients, vitamin mix, 33 mM Na_2SO_4, and 5 mM
285 NH_4Cl. *P. inhibens* P72 cultures were additionally supplemented with 5.5 mM Glucose, and
286 *D. shibae* cultures were supplemented with 5.5 mM succinate. The final control and treatment
287 medium is referred to as CNS, with the treatment containing 75% volume of algal filtrates and
288 25% volume of ASW (v/v), in contrast to the control which consists solely of ASW. Cultures
289 were grown at 30 °C with shaking at 130 rpm and harvested at an OD_{600} of 0.3 for GFP
290 visualization. Bacterial populations were monitored using a Merck CellStream flow cytometer,
291 excited at 561 nm, collected at 615–789 nm. GFP was evaluated by determining a GFP
292 fluorescence threshold (according to autofluorescence of control cultures) and calculating the
293 percentage of bacterial cells with fluorescence intensity higher than the threshold. For each
294 biological replicate 100,000 events were recorded. Results are presented as percentages of
295 the fluorescent bacteria ratio. Calculation involved dividing each control/treated sample by the
296 average value of the respective control/treated empty vector (EV). For example, the value for

297 each biological replicate of the 184 kb reporter strain control was divided by the average value
298 of biological samples from the EV control. Similarly, the value for each biological replicate of
299 the 184 kb reporter strain supplemented with algal filtrates was divided by the average value
300 of biological samples from the EV control supplemented with algal filtrates.
301
302 **Genetic manipulation of Roseobacter bacteria**
303 To insert an antibiotic resistance gene into the 65 kb plasmid of *P. inhibens* DSM 17395, the
304 mutant strain ES172 (Table S1) was generated by inserting a kanamycin-resistant gene in an
305 intergenic region between gene PGA1_RS20320 and gene PGA1_RS19930. Regions of
306 approximately 1000 bp upstream and downstream of the intergenic region were PCR-
307 amplified using primers 1169, 1170, 1171 and 1172, respectively (Table S4). The amplified
308 fragments were assembled and cloned into the pYDR8 vector (Table S6) using restriction-free
309 cloning[43,44]. The resulting plasmid (pYDR9, Table S6) was introduced into competent *P.*
310 *inhibens* by electroporation. Preparations of competent cells was performed as previously
311 described[45], with slight modifications. Shortly, cells were grown to an OD_{600} of 0.7 in ½ YTS
312 with 40 g/L of sea salts (also termed full salts medium). Bacteria were washed 3 times using
313 10% (v/v) ice-cold glycerol, centrifuged each time for 5 min at 4 °C and 5,500 g. The competent
314 cells were subsequently adjusted to an OD_{600} of 20 using 10% (v/v) ice-cold glycerol, frozen
315 in liquid nitrogen and stored at -80°C. Electroporation was conducted with 300 µl aliquot of
316 electrocompetent cells, using 10 µg of DNA in a 2 mm cuvette at 2.5 V, followed by 4 h
317 recovery in ½ YTS with full salts. The transformed cells were then plated on ½ YTSS plates
318 containing 150 µg/ml kanamycin, and resistant colonies were validated by PCR and DNA
319 sequencing.
320
321 **Attachment assay**
322 Bacterial cultures were diluted to an optical density of 0.01 at 600 nm (OD_{600}) using 150 µl of
323 75% fresh algal filtrates diluted in 25% of ASW (v/v) for treatment and ASW for control. Both
324 control and treated cultures were supplemented with L1 trace elements, L1 nutrients, vitamin
325 mix, 33 mM Na_2SO_4, and 5 mM NH_4Cl and 5.5 mM Glucose. The final control and treatment
326 medium is referred to as CNS, with the treatment containing 75% volume of algal filtrates and
327 25% volume of ASW (v/v), in contrast to the control which consists solely of ASW. The diluted
328 cultures were dispensed into 96-well flat-bottom plates to initiate the assay. To minimize
329 evaporation during incubation, the plates were securely covered with UV-treated Parafilm. The
330 bacterial cultures were incubated at 30 °C for 48 hours. The attachment assay was carried out
331 following established protocols[46,47]. After incubation, the plates were subjected to the following
332 steps: The culture medium was carefully aspirated, and the wells were rinsed twice with 1×
333 phosphate-buffered saline (PBS, obtained from Sartorius, Beit HaEmek, Israel). This process

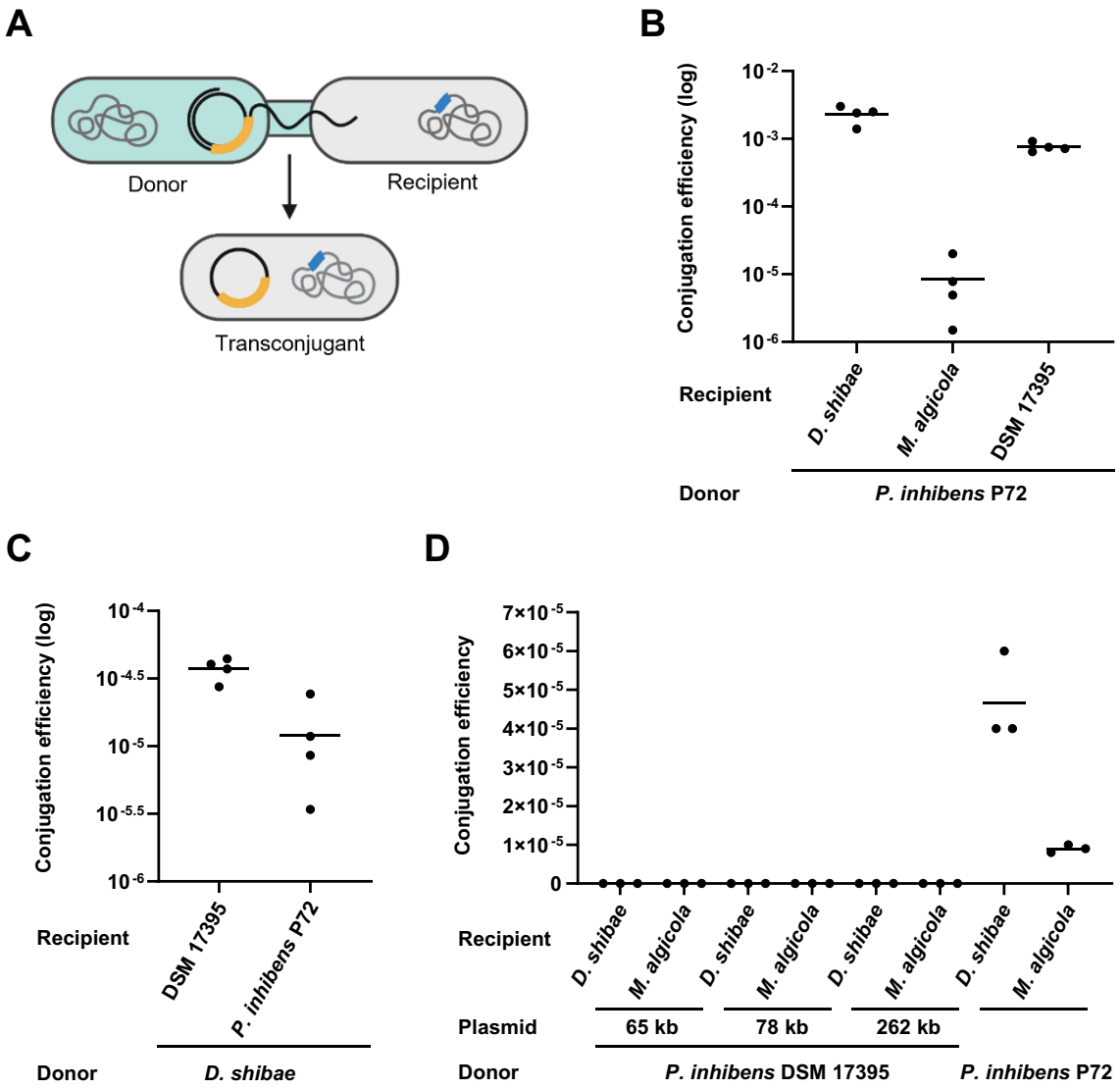
334 aimed to remove non-adherent bacterial cells. The plates were dried by exposing them to a
335 temperature of 55 °C for 20 minutes. The dried plates were submerged in a solution of 0.1%
336 crystal violet. The plates were then incubated at room temperature for 10 minutes to allow the
337 crystal violet to interact with the attached cells. The staining solution was discarded, and the
338 plates were washed twice with PBS to remove excess crystal violet. The plates were left to
339 dry overnight at room temperature to ensure complete fixation of the stained cells. To quantify
340 the attached cells, the remaining crystal violet was extracted by adding 200 µL of 33% acetic
341 acid to each well. The plates were incubated at room temperature for 15 minutes to facilitate
342 extraction. Absorbance measurements were conducted at a wavelength of 595 nm using the
343 TECAN microplate reader. The absorbance values were normalized to a blank consisting of
344 the same growth medium, without bacterial cells, that was subjected to the same protocol.
345

346 **Generating the *Roseobacteraceae* T4SS dataset**

347 All *Roseobacteraceae* genomes (of any quality) available in the Integrated Microbial Genomes
348 & Microbiomes (IMG/M) database[48] were scanned for the presence of the *virB8* gene using
349 the IMG "Function search" tool and K03202 KEGG (Kyoto Encyclopedia of Genes and
350 Genomes[49]) Orthology (KO) designation for VirB8. The *virB8* genes (K03203) were found in
351 320 out of 898 *Roseobacteraceae* genomes. The respective scaffolds were scanned for the
352 presence of other *vir* genes (*virB1-B7, virB9-B11* and *virD4*) and were assessed as
353 representing either plasmids or chromosomes (Table S7). Within these 320 genomes a total
354 of 564 *virB8* genes were located on 553 scaffolds. Further assignment of the scaffolds to
355 plasmids or chromosomes was conducted using the presence or absence of plasmid
356 operational taxonomic unit (pOTU) designation that was assigned during genome annotation
357 in IMG and according to scaffold size. Scaffolds with no operational taxonomic unit designation
358 larger than 300 kb were marked as chromosomes. Scaffolds with no operational taxonomic
359 unit designation and smaller than 300 kb were marked as unknowns. Of note, the T4SS is an
360 extremely versatile apparatus. Conjugative T4SSs typically consist of the archetypal
361 VirB/VirD4 components (encoded by *virB1-11* together with *virD4*). Yet, the precise number of
362 needed genes and their essentiality for a functional T4SS remain poorly understood,
363 particularly in non-model bacteria[50-53]. Consequently, due to the difficulty in predicting which
364 T4SS is conjugative, we set stringent criteria and defined a potentially functional conjugative
365 T4SS (*i.e.,* complete T4SS) as one that includes all the *vir* genes that comprise the T4SSs
366 located on the conjugative plasmids of *D. shibae* and *P. inhibens* P72. Accordingly, T4SSs
367 homologous to Vir but possessing other KO assignments (e.g., Tra), that were not
368 experimentally shown to be conjugative in Roseobacters, were not included in this analysis.
369 In total, our analysis identified 387 out of the 553 scaffolds as carrying a complete set of T4SS

370 genes. However, it is possible that partially complete T4SSs in our analysis or homologous
371 T4SSs (e.g., Tra) may be functional as well[54].
372
373 **Results**
374
375 **Establishing a conjugation assay using different Roseobacters**
376 To monitor conjugation among various Roseobacters, we examined the ability of four different
377 bacterial species - *Phaeobacter inhibens* P72, *Dinoroseobacter shibae, Marinovum algicola*,
378 and *Phaeobacter inhibens* DSM 17395 - to act as donors and recipients in conjugation assays.
379 These strains have been widely studied by us[25,55] and by others[22,56-61] as representative
380 Roseobacters that specializes in interactions with microalgae. To establish a conjugation
381 assay that can be followed in the lab, each strain was labeled with an antibiotic resistance
382 gene on either its native plasmid or chromosome, allowing us to track the different participants
383 using selection on antibiotics (Fig. 1A).
384 First, the *P. inhibens* P72 strain, carrying two complete T4SSs on both its 184 kb and 57 kb
385 native plasmids (Fig. S1), was assessed as a donor. For the conjugation assays, the 57 kb
386 plasmid was marked with a kanamycin-resistance gene and mated with three recipient strains:
387 *P. inhibens* DSM 17395, *D. shibae*, and *M. algicola*, which were marked with a gentamicin-
388 resistance gene on their chromosome (Fig. 1B). Second, the *D. shibae* strain, carrying two
389 complete T4SSs on both its 126 kb and 191 kb native plasmids (Fig. S1), was evaluated as a
390 donor. For the conjugation assays, the 126 kb plasmid was marked with a gentamicin-
391 resistance gene and mated with two recipient strains: *P. inhibens* P72 and *P. inhibens* DSM
392 17395, both marked with a kanamycin-resistance gene on their chromosome (Fig. 1C).
393 Following mating and selection, all assays resulted in colonies that exhibit resistance towards
394 both kanamycin and gentamicin. To confirm that the double-resistance colonies were indeed
395 transconjugants, several colonies were randomly chosen from each donor-recipient pair and
396 the presence of the acquired plasmid, and the recipient chromosome was validated via PCR
397 reactions. All tested transconjugants were confirmed to be the recipient strains that acquired
398 the 57 kb plasmid (Fig. S2). Of note, several of these donor-recipient pairs were previously
399 reported to execute successful conjugation under different experimental conditions[22,23].
400 Finally, we evaluated the ability of *P. inhibens* DSM 17395 to act as a donor. The *P. inhibens*
401 DSM 17395 strain harbors three native plasmids of 65 kb, 78 kb, and 262 kb, and carries a
402 complete T4SS on its chromosome (Fig. S1). We used three strains in which each of the native
403 plasmids is marked with a kanamycin-resistance gene. The three strains were used as donors
404 in conjugation assays with either *D. shibae* and *M. algicola* as recipients that were marked
405 with a chromosomal gentamicin-resistance gene (Fig. 1D). Our results show that under our
406 experimental conditions *P. inhibens* DSM 17395 is not capable of transferring any of its

407 plasmids to recipient bacteria. To further support this result, we repeated the conjugation
408 assays on agar plates instead of liquid media (Fig. S3), as previous studies demonstrated
409 enhanced conjugation efficiency on solid surfaces like agar plates[13,62]. However, *P. inhibens*
410 DSM 17395 was not capable of transferring any of its plasmids to recipient bacteria during
411 conjugation on agar plates. As control, all experiments were accompanied by conjugation
412 assays with a donor-recipient pair that was previously shown to result in successful
413 conjugation[22]. Our data thus demonstrated that the antibiotic resistance-marked plasmids of
414 *P. inhibens* P72 and *D. shibae* can be successfully transferred via conjugation to various
415 Roseobacter recipients.
416



417

418 **Fig. 1. Establishing a robust conjugation assay. A)** Schematic representation of the conjugation assay. The
419 donor strain (light blue) carries an antibiotic resistance gene (yellow) on a native plasmid, while the recipient strain
420 (grey) harbors a chromosomal antibiotic resistance gene (blue). Successful plasmid transfer from the donor to the
421 recipient will result in transconjugants that are resistant to both antibiotics. **B-D)** Results of conjugation assays in

422 liquid medium performed using different donor strains. **B)** *P. inhibens* P72 $^{p57\text{-}kana}$ served as a donor strain, carrying
423 a kanamycin-resistance gene on its native 57 kb plasmid. The recipient strains *M. algicola* $^{chr\text{-}gent}$, *D. shibae* $^{chr\text{-}gent}$
424 and *P. inhibens* DSM 17395$^{chr\text{-}gent}$, were marked with a gentamicin-resistance gene on their chromosome. **C)** *D.*
425 *shibae* $^{p126\text{-}gent}$ served as a donor strain, carrying a gentamicin-resistance gene on its native 126 kb plasmid.
426 Recipient strains were *P. inhibens* DSM 17395$^{chr\text{-}kana}$ and *P. inhibens* P72 $^{chr\text{-}kana}$, both marked with a kanamycin-
427 resistance gene on their chromosome. **D)** *P. inhibens* DSM 17395 served as a donor strain, carrying a kanamycin-
428 resistance gene on its native 65 kb, 78 kb or 262 kb plasmids (*P. inhibens* DSM 17395 $^{p65\text{-}gent}$, *P. inhibens* DSM
429 17395 $^{p78\text{-}gent}$ and *P. inhibens* DSM 17395 $^{p262\text{-}gent}$ respectively). Recipient strains were *M. algicola* $^{chr\text{-}gent}$ and *D.*
430 *shibae* $^{chr\text{-}gent}$, marked with a gentamicin-resistance gene on their chromosome. As a control, *P. inhibens* P72 $^{p57\text{-}}$
431 ^{kana} carrying a kanamycin-resistance gene on its native 57 kb plasmid was used as a donor and mated with the
432 same recipients. Dots represent individual biological replicates, and lines indicate the mean values. Conjugation
433 efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. DSM 17395 indicates
434 the *P. inhibens* DSM 17395 strain.
435

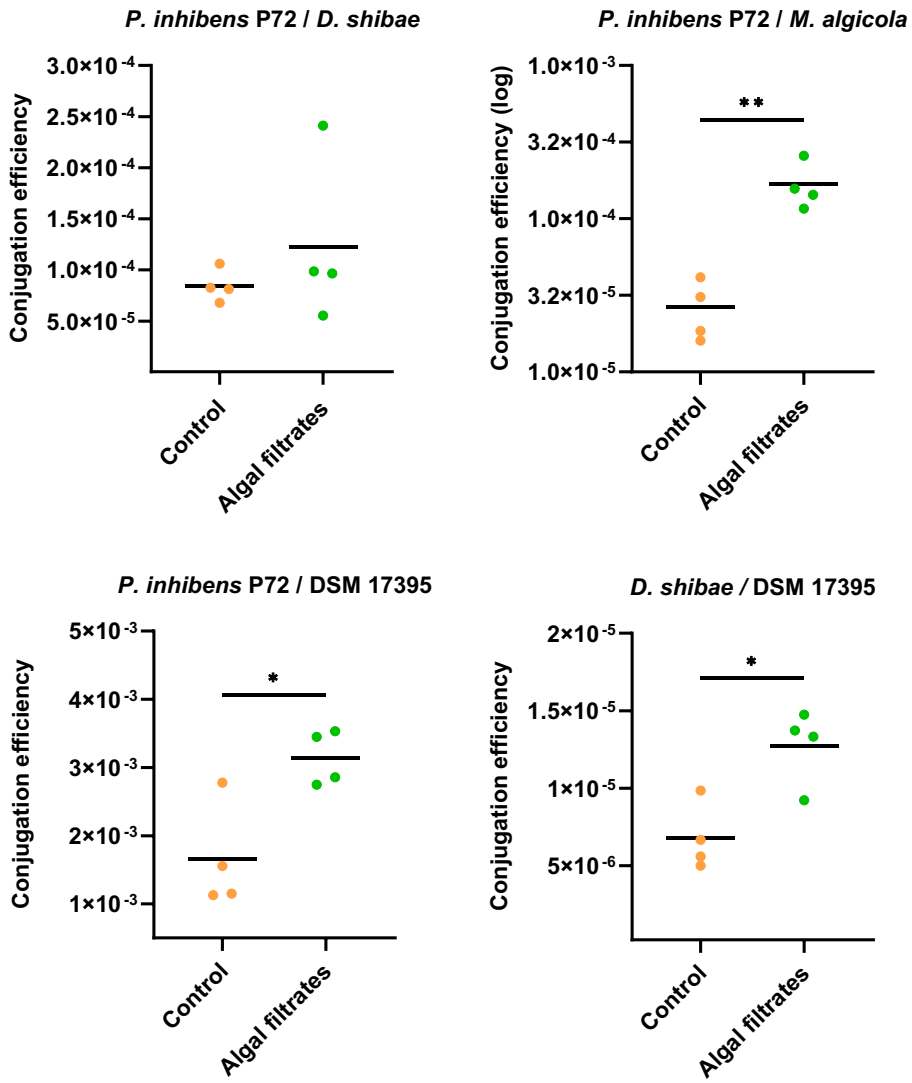
436 **Algal exudates enhance bacterial conjugation**

437 Next, we investigated whether bacterial conjugation is influenced by proximity to an algal cell.
438 To distinguish between proximity and the possible impact of algae as a surface for attachment,
439 we chose to expose bacteria to algal exudates and not to actual algae. This approach allowed
440 us to mimic the "chemical presence" of algae and probe its influence on conjugation between
441 Roseobacter bacteria. We performed conjugation assays in which bacteria were treated with
442 algal filtrates and compared them to control assays with untreated bacteria. Both treated and
443 control assays were supplemented with essential nutrients to support bacterial growth. The
444 conjugation assays were performed with *P. inhibens* P72 and *D. shibae* as plasmid donors to
445 the recipients *D. shibae, M. algicola*, and *P. inhibens* DSM 17395 (Fig. 2). Our results show
446 that conjugation assays that were supplemented with algal filtrates resulted in increased
447 conjugation efficiency compared to untreated bacteria.
448

449 **Algal exudates enhance conjugation efficiency beyond the bacterial growth-promoting**
450 **effect**

451 Algal exudates contain metabolites that are known to promote bacterial growth[24,31,63]. To
452 understand whether enhanced conjugation is merely the result of increased bacterial growth,
453 we monitored the growth of donor strains, recipient strains, and transconjugants in pure
454 cultures that were supplemented with algal filtrates, and compared them to untreated control
455 cultures (Fig. 3A). Both treated and control cultures were supplemented with essential
456 nutrients to support bacterial growth. Our data show that most bacteria displayed a shorter lag
457 phase upon treatment with algal filtrates, a phenomenon previously documented by our
458 laboratory[64]. Furthermore, although the assays were performed in nutrient replete conditions,
459 several strains exhibited higher yields upon treatment with algal exudates.

460 Now, we aimed to determine whether algal filtrates enhance conjugation efficiency beyond
461 their impact on bacterial growth dynamics. We therefore calculated the impact of algal filtrates
462 on the growth of donors and recipients and compared it to the impact on growth of the
463 transconjugants (Table 1). Our analysis demonstrate that algal exudates enhanced
464 transconjugants cell numbers beyond the exudate impact on both donors and recipients,
465 across all mated pairs. These findings suggest that algal filtrates enhance conjugative plasmid
466 transfer beyond the growth promoting effect.
467
468



469 **Fig. 2. Algal exudates increase conjugation efficiency.** Conjugation assays in liquid medium were performed
470 using either *P. inhibens* P72 $^{p57\text{-}kana}$ as a donor, marked with a kanamycin-resistance gene on its native 57 kb
471 plasmid, or *D. shibae* $^{p126\text{-}gent}$ as a donor, marked with a gentamicin-resistance gene on its native 126 kb plasmid.
472 For *P. inhibens* P72 $^{p57\text{-}kana}$, three strains were tested as recipients: *M. algicola* $^{chr\text{-}gent}$, *D. shibae* $^{chr\text{-}gent}$ and *P.*
473 *inhibens* DSM 17395$^{chr\text{-}gent}$, all marked with a gentamicin-resistance gene on their chromosome. For *D. shibae* $^{p126\text{-}gent}$, the recipient strain was *P. inhibens* DSM 17395$^{chr\text{-}kana}$, chromosomally marked with a kanamycin-resistance
475 gene. Mating was performed with cultures treated under control conditions in CNS medium (orange dots) or with

algal filtrates (green dots). Conjugation efficiency was calculated by normalizing transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*. Dots represents individual biological samples, and lines indicate the mean values. Statistical significance was calculated using a t-test, with $p<0.05$ marked as *, and $p<0.01$ marked as **. DSM 17395 indicates the *P. inhibens* DSM 17395 strain.

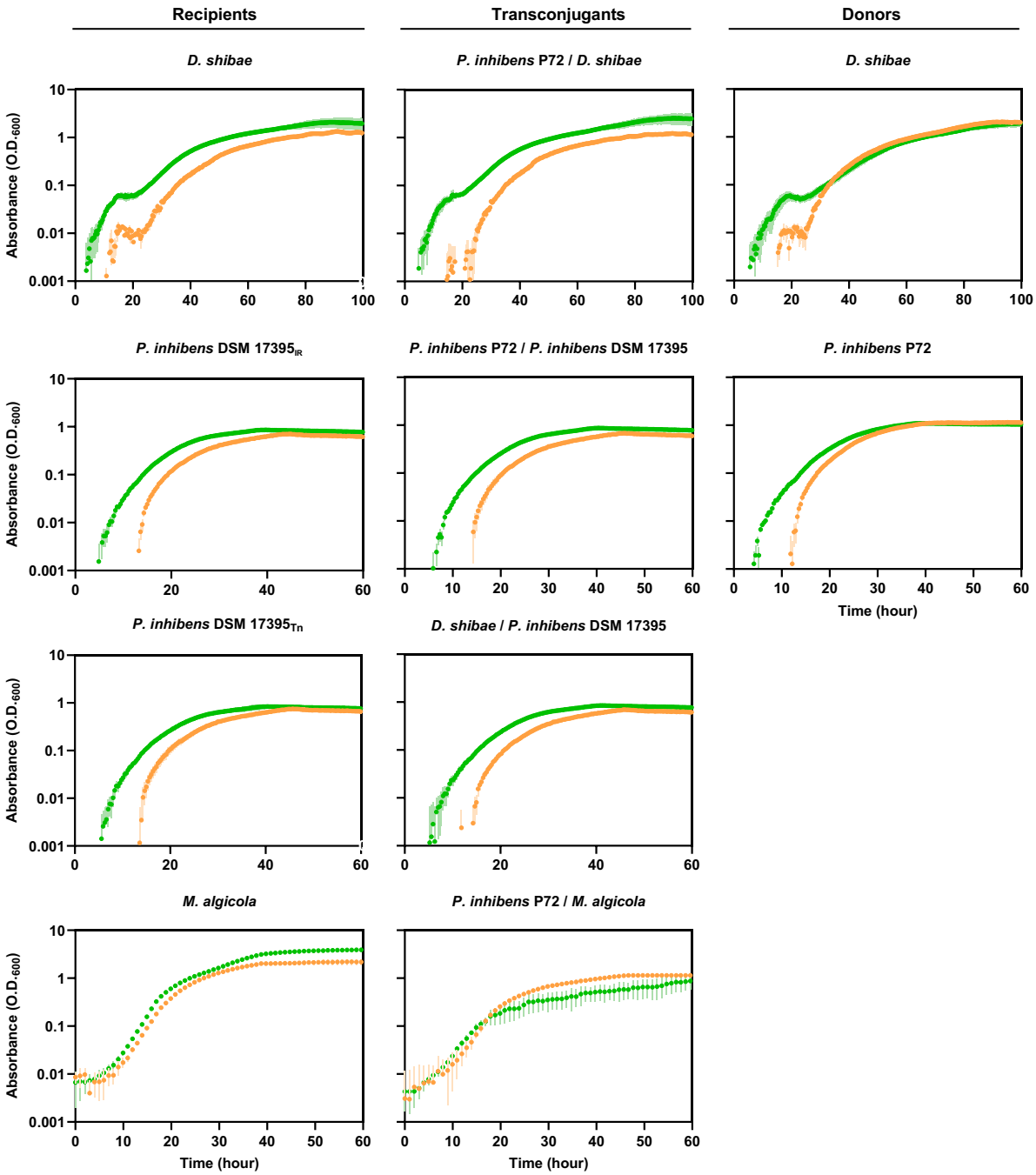


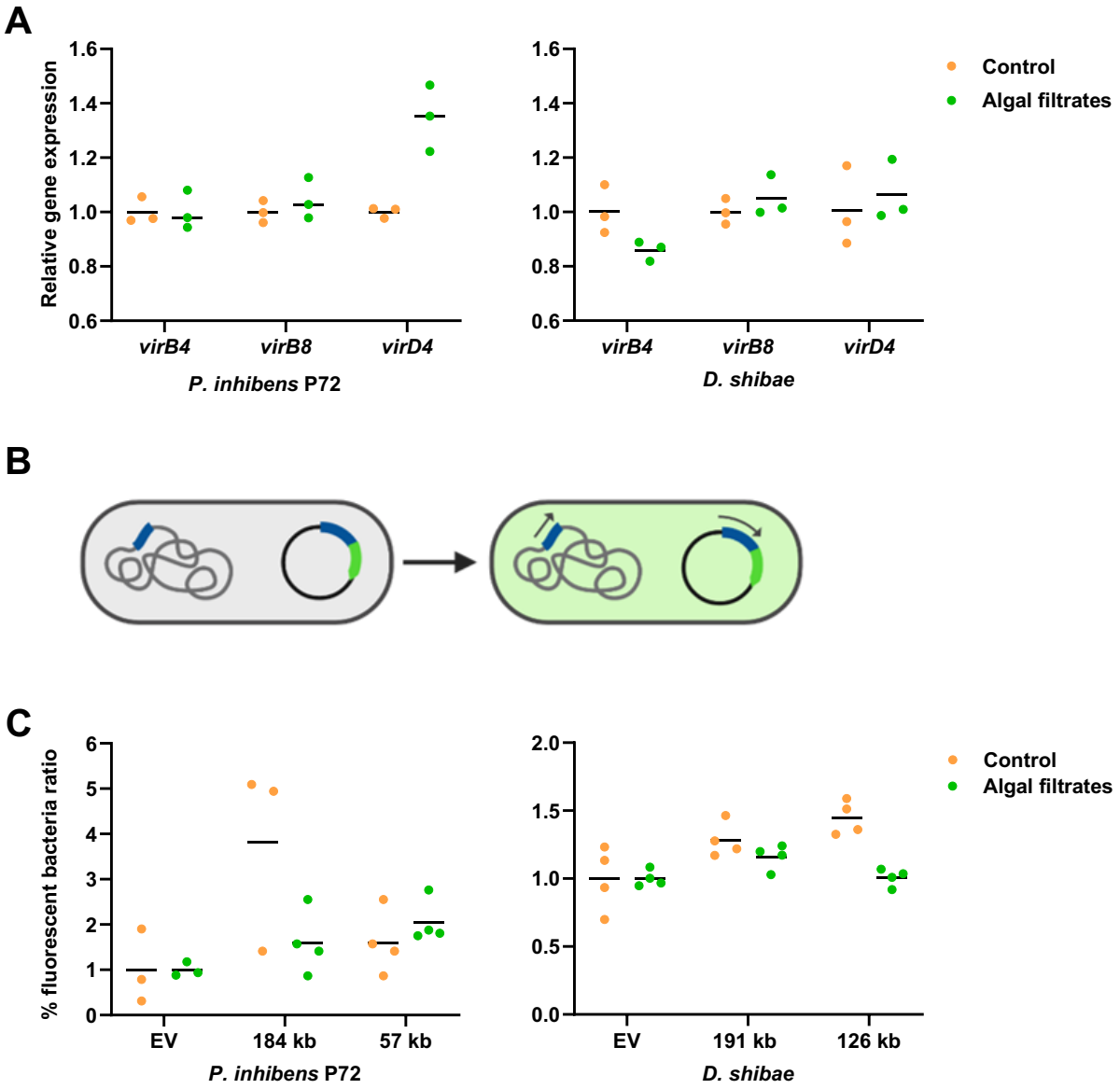
Fig. 3. Algal filtrates effect on bacterial growth. Bacterial cultures of recipients (left column), transconjugants (middle column) and donors (right column), were treated with either CNS medium as control conditions (orange dots) or with algal filtrates (green dots). Growth was evaluated by measuring the optical density using a wavelength

486 of 600 nm ($O.D._{600}$). Each dot represents the average of three biological replicates. The title above the column of
487 transconjugants denotes *Donor/Recipient.*
488
489 **Table 1. Algal filtrates enhance conjugation efficiency beyond bacterial growth promotion.** The effect of
490 algal filtrates on the growth of donors and recipients was calculated and compared to that of the transconjugants.
491 Percentages were calculated by averaging colony forming units (CFU) numbers of donors, recipients and
492 transconjugants separately (Table S8), for each conjugation pair (Fig. 2). Then, by dividing the averaged CFU
493 numbers under algal filtrate treatment to the control, we determined the increase induced by the treatment in each
494 group. For example, *P. inhibens* P72 $^{p57\text{-}kana}$ demonstrated 78% increase in CFU numbers due to the algal filtrates.
495 As can be seen, the increase in in transconjugants numbers exceeded the increase observed for both donors and
496 recipients across all conjugation pairs.

Donor	Recipient	Algal filtrate impact on bacterial cell numbers		
		Donors	Recipients	Transconjugants
D. shibae $^{p126\text{-}gent}$	*P. inhibens* DSM 17395$^{chr\text{-}kana}$	-14%	45%	59%
P. inhibens P72 $^{p57\text{-}kana}$	*D. shibae* $^{chr\text{-}gent}$	78%	79%	142%
P. inhibens P72 $^{p57\text{-}kana}$	*M. algicola* $^{chr\text{-}gent}$	15%	-26%	625%
P. inhibens P72 $^{p57\text{-}kana}$	*P. inhibens* DSM 17395$^{chr\text{-}gent}$	61%	36%	210%

497
498
499 **Expression of the conjugation machinery is not influenced by algal exudates**
500 To understand the mechanism underlying enhanced bacterial conjugation in response to algal
501 exudates, we monitored the expression of the bacterial T4SS genes. The T4SS is mainly
502 encoded by the *virB* operon while the *virD* operon carries several additional genes necessary
503 for proper functioning of the T4SS[11,65]. To evaluate the impact of algal exudates on the
504 expression of the T4SS, we analyzed expression levels in the donor strains *P. inhibens* P72
505 and *D. shibae* using qRT-PCR with primers that target the T4SS central genes *virB4*, *virB8*
506 and *virD4.* We compared expression levels of bacterial cultures that were supplemented with
507 algal filtrates versus non-treated cultures. Both treated and control cultures were
508 supplemented with essential nutrients to support bacterial growth.
509 The results demonstrated that gene expression levels are similar between untreated cultures
510 and cultures that were supplemented with algal filtrates, for both bacterial strains. Only
511 expression of the *virD4* gene in *P. inhibens* P72 treated with algal filtrates showed slightly
512 higher expression levels than untreated cultures (Fig. 4A). Additionally, the expression levels
513 of *virB4*, *virB8* and *virD4* were comparable to those of the housekeeping genes, suggesting
514 that under experimental conditions that support conjugation, the T4SS genes are expressed
515 in a basal level but are not upregulated (Fig. S4).

516 Due to the rarity of conjugation events[66,67], we questioned whether a qRT-PCR analysis, which
517 averages RNA from the entire population, would possess the necessary sensitivity to detect
518 such infrequent incidents. Instead, we aimed to observe the process at a single-cell resolution.
519 Therefore, we developed reporter strains that contain a *gfp* gene under the *virB* promoter (Fig.
520 4B). However, analysis of these reporter strains using flow cytometry, following exposure to
521 algal exudates, did not reveal increased expression (Fig. 4C). Taken together, these results
522 suggest that algal-secreted compounds do not affect the expression of the T4SS machinery.
523

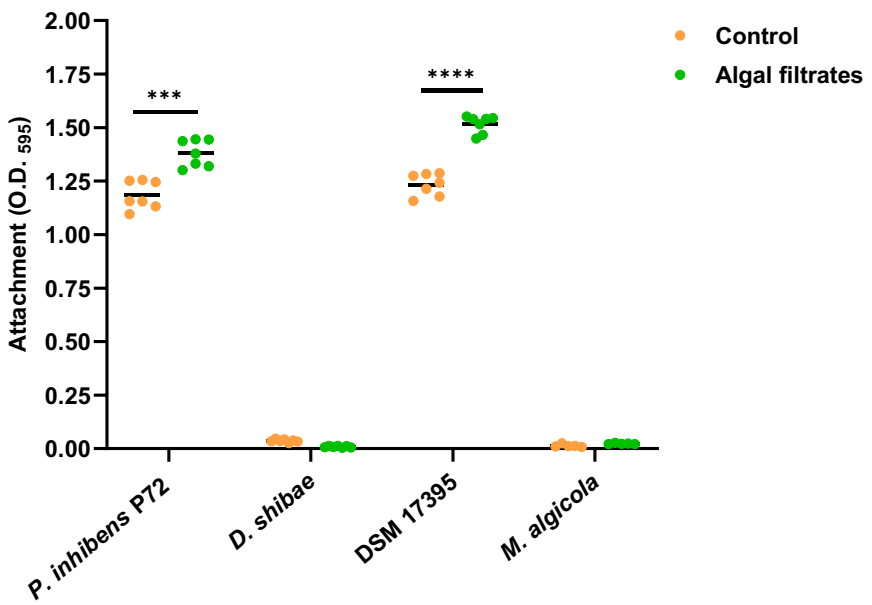


524
525 **Fig. 4. Algal exudates do not influence the expression of genes encoding the T4SS. A)** Relative gene
526 expression of *virB4*, *virB8* and *virD4* genes was assessed using qRT-PCR. RNA was extracted from bacterial
527 cultures of *D. shibae* $^{p126\text{-}gent}$ and *P. inhibens* P72 $^{p57\text{-}kana}$ donor strains following treatment with either CNS medium
528 as control conditions (orange dots) or with algal filtrates (green dots). RNA was extracted when bacterial cultures
529 reached OD_{600} of 0.3. Each data point represents a biological replicate, lines represent the mean values of

530 biological replicates. **B)** Schematic representation of the T4SS transcriptional reporter strains. The promoter of the
531 *virB* operon (blue) was fused to GFP (green), cloned into a replicative plasmid, and transformed into *D. shibae* and
532 *P. inhibens* P72. Activation of the native promoter is expected to lead to activation of the reporter construct, resulting
533 in the expression of GFP and emission of a fluorescent signal. **C)** Reporter strains were generated using the T4SS
534 promoters of the 57 kb and 184 kb native plasmids of *P. inhibens* P72, and the 126 kb and 191 kb native plasmids
535 of *D. shibae.* Bacteria carrying these constructs were supplemented either with CNS as a control (orange dots) or
536 algal exudates (green dots). Dots represent the percentage of fluorescent bacteria. For each biological sample, the
537 value was normalized to the average of its corresponding control (see Methods section). Each dot represents a
538 biological replicate, and lines represent the mean values.
539

540 **Algal exudates increase bacterial attachment capabilities**

541 In the absence of increased expression of T4SS genes upon exposure to algal exudates, the
542 mechanism behind the enhanced bacterial conjugation remains unclear. We hypothesized
543 that enhanced conjugation might be facilitated by increased bacterial adhesion. Previous
544 research in our laboratory has shown that algal exudates enhance the attachment of wild-type
545 bacteria from the *P. inhibens* DSM 17395 strain[46]. Now, we sought to investigate whether
546 similar effects are observed when the diverse Roseobacters in our current study are exposed
547 to exudates (Fig. 5). Using an attachment assay, we evaluated the adhesion capabilities of
548 the various donors and recipients, and tested whether algal exudates affect their adhesion.
549 Our results show that algal exudates significantly improve the attachment capabilities of *P.*
550 *inhibens* P72 and *P. inhibens* DSM 17395 strains. *D. shibae* and *M. algicola* in contrast, do
551 not appear to have attachment capabilities in our experimental conditions. Of note, in all our
552 conjugation assays, either the donor or recipient strain possessed attachment capabilities that
553 are enhanced following exposure to algal exudates. Therefore, increased attachment could
554 explain the enhanced conjugation efficiency.

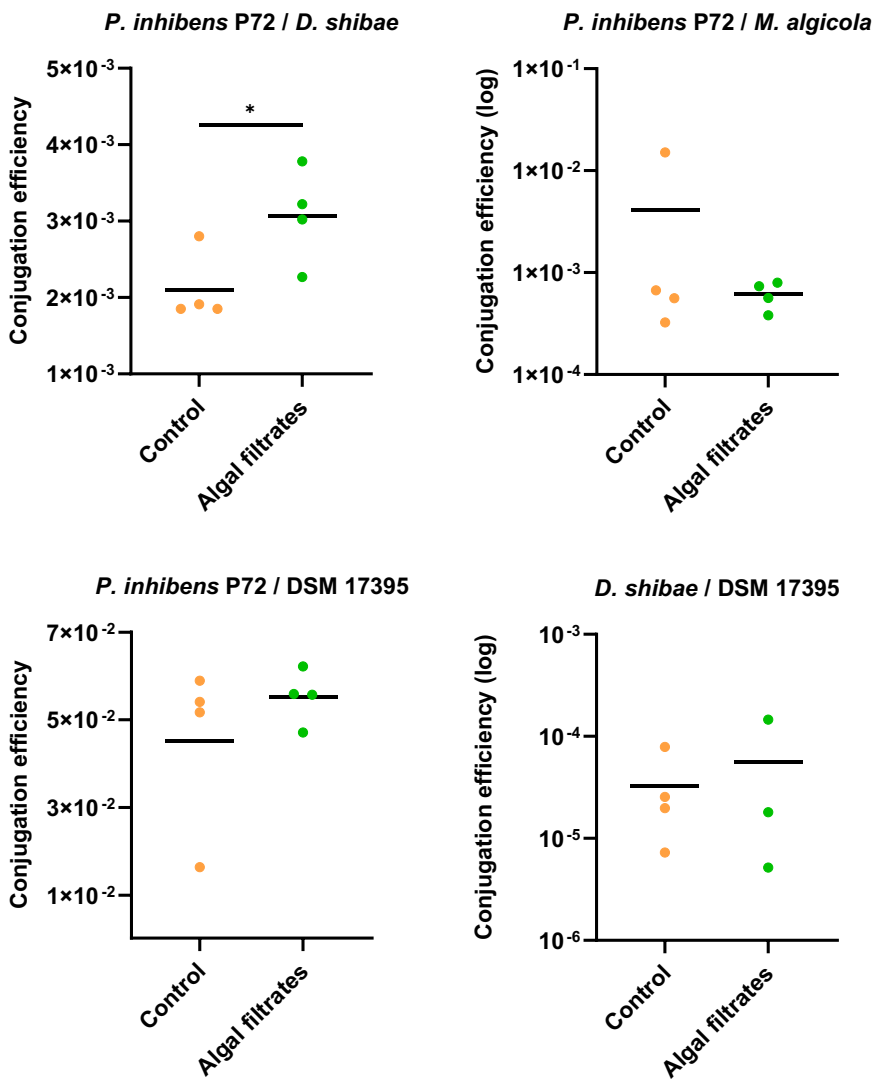


555

556 **Fig. 5. Algal exudates promote bacterial surface attachment.** Bacterial donor and recipient strains were treated
557 either with CNS as control (orange dots) or with algal filtrates (green dots). The bacterial donor strains were *P.*
558 *inhibens* P72 $^{p57\text{-}kana}$ marked with a kanamycin-resistance gene on its native 57 kb plasmid and *D. shibae* $^{p126\text{-}gent}$
559 marked with a gentamicin-resistance gene on its native 126 kb plasmid. The recipient strains were *P. inhibens*
560 DSM 17395$^{chr\text{-}gent}$ and *M. algicola* $^{chr\text{-}gent}$ marked with a gentamicin-resistance gene on the chromosome.
561 Attachment was assessed by measuring the absorbance at $O.D._{595}$ of the Crystal Violet dye from bacterial cells that
562 were strongly attached to 96-well plates following an attachment assay (see Methods section). Each sample was
563 normalized to Crystal Violet extracted from the same medium but without bacteria. Each strain and treatment
564 consisted of n=7 wells. Dots represent individual biological replicates, and lines indicate the mean values. Statistical
565 significance was calculated using a t-test, with $0.0001<p<0.001$ marked as ***, and $p<0.0001$ marked as ****.
566

567 **Conjugation enhancement by algal exudates is reduced when conducted on solid**
568 **surface**

569 We further investigated the correlation between enhanced conjugation and heightened
570 bacterial attachment triggered by algal exudates. To understand if the enhanced conjugation
571 efficiency resulted from increased attachment, we conducted the conjugation assay under
572 conditions where attachment no longer conferred an advantage. Thus, we performed
573 conjugation assays on agar plates using a dense, thoroughly mixed inoculum of both donors
574 and recipients. Unlike in liquid-based conjugation assays where bacteria swim and locate their
575 conjugation partners, assays on plates facilitate immediate contact between all bacteria,
576 thereby eliminating the advantage of attachment[13,62]. The conjugation assays on plates were
577 conducted using *P. inhibens* P72 and *D. shibae* as plasmid donors, while recipients included
578 *D. shibae, M. algicola*, and *P. inhibens* DSM 17395 (Fig. 6). Assays were treated with algal
579 filtrates and compared to untreated conditions, and all assays were supplemented with
580 essential nutrients to support bacterial growth. Our findings reveal that the majority of
581 conjugation assays supplemented with algal filtrates exhibited conjugation efficiencies
582 comparable to the control. Thus, when attachment no longer confers an advantage, the impact
583 of algal exudates on bacterial conjugation is reduced. These results support the role of algal
584 exudates in promoting bacterial attachment, thus facilitating enhanced conjugation.



585

586

587 **Fig. 6. Algal exudates exhibit a lower impact on conjugation when performed on agar plates.** Conjugation
588 assays on agar plates were performed using either *P. inhibens* P72 $^{p57\text{-}kana}$ as a donor, marked with a kanamycin-
589 resistance gene on its native 57 kb plasmid, or *D. shibae* $^{p126\text{-}gent}$ as a donor, marked with a gentamicin-resistance
590 gene on its native 126 kb plasmid. For *P. inhibens* P72 $^{p57\text{-}kana}$, three strains were tested as recipients: *M. algicola*
591 $^{chr\text{-}gent}$, *D. shibae* $^{chr\text{-}gent}$ and *P. inhibens* DSM 17395$^{chr\text{-}gent}$, all marked with a gentamicin-resistance gene on their
592 chromosome. For *D. shibae* $^{p126\text{-}gent}$, the recipient strain was *P. inhibens* DSM 17395$^{chr\text{-}kana}$, chromosomally marked
593 with a kanamycin-resistance gene. Mating was performed with cultures treated under control conditions with CNS
594 medium (orange dots) or with algal filtrates (green dots). Conjugation efficiency was calculated by normalizing
595 transconjugant cell numbers to donor cell numbers. The title above each graph denotes *Donor/Recipient*. Dots
596 represents individual biological samples, and lines indicate the mean values. Statistical significance was calculated
597 using a t-test, with $p<0.05$ marked as *. DSM 17395 indicates the *P. inhibens* DSM 17395 strain.

598

599

600 **Discussion**

601

602 *HGT and the ecological success of Roseobacters*

603 The current study reveals that algal exudates increase bacterial conjugation, and
604 consequently HGT, among Roseobacter bacteria. HGT has been suggested to play important
605 roles in the resilience of marine bacteria[68] and is considered key in the ecological abundance
606 and success of Roseobacters[69-71]. Various mechanisms for HGT have been observed in
607 Roseobacters, including GTAs[68] and conjugation[21]. Moreover, Roseobacters have many
608 native plasmids[70,71], up to a dozen plasmids in a single bacterium[70]. Many of these plasmids
609 harbor T4SSs[13] that may facilitate conjugative HGT, potentially supporting rapid adaptation.
610 Indeed, 27% of sequenced Roseobacters genomes harbor all genes needed for a functional
611 conjugative T4SS (Fig. S5). Conjugation initiation and the successful transfer of DNA was
612 shown to be impacted by factors such as signaling molecules, temperature, cell physiology,
613 population density, pH, and nutrients[13,66,72]. However, in marine bacteria, little is known about
614 the conditions required to induce HGT, particularly conjugation. Here, we show that algal
615 secreted metabolites promote HGT in Roseobacters.

616

617 *Bacterial HGT in the algal phycosphere*

618 In marine ecosystems, algae provide bacteria with nutrients[24,31,63] and an attachment
619 surface[25,32]. By attaching to an algal cell, bacteria can secure a steady flux of algal exudates
620 in the algal phycosphere, which is the immediate volume surrounding the algal cell. Indeed,
621 Roseobacter bacteria often co-occur with various algal hosts in the environment and in
622 laboratory cultures[25,38,73-75]. As multiple bacteria can attach onto an algal host, they may
623 engage in processes that require cell-to-cell contact, such as conjugation. Therefore, proximity
624 and attachment onto an algal host may increase the chances for plasmid transfer events
625 among bacteria in the algal phycosphere[13,21,34,35]. Interestingly, environmental experiments
626 with phytoplankton-derived dissolved organic matter demonstrated attraction of bacteria
627 harboring T4SS genes, as shown by metagenomic analysis[76]. Moreover, algal exudates
628 support bacterial growth and metabolism[77], further enhancing bacterial density, which in turn
629 could augment the chances of bacterial HGT.

630

631 *Algal exudates increase attachment and enhance HGT*

632 HGT mechanisms rely on proximity, with conjugation specifically requiring cell-to-cell
633 contact[78]. Therefore, conditions that stabilize and facilitate cell-to-cell contact will enhance the
634 opportunities for conjugation events[20]. Indeed, conjugation in biofilms was extensively
635 studied[13]. Conjugation is enhanced in biofilms, and further stimulates biofilm development[14-
636 19]. Specifically, it was shown that factors mediating formation and stabilization of cell-to-cell

637 contacts in biofilms promote T4SS mediated gene transfer[20]. Additionally, attachment to
638 abiotic surfaces was also demonstrated to increased conjugation[79]. Recent studies have
639 revealed that microalgae and Roseobacter bacteria create a shared algal-bacterial
640 extracellular matrix, fostering algal-bacterial aggregation[46]. Algal exudates stimulate bacterial
641 exopolysaccharide production, which contributes to the formation of a joint algal-bacterial
642 extracellular matrix. Our data illustrate that algal exudates primarily enhance Roseobacter
643 conjugation by promoting bacterial attachment. Consequently, it is plausible that algal-
644 bacterial aggregates may facilitate enhanced conjugation, although further investigation is
645 required to confirm this hypothesis.
646
647 *The T4SS genomic location*
648 Establishing a conjugation assay resulted in characterizing Roseobacter strains capable of
649 serving as donors in conjugation, as well as a strain with a chromosomally encoded T4SS
650 unable to donate a plasmid during conjugation (Fig. 1D). Various bacteria carry the T4SS-
651 encoding genes on plasmids, chromosomes, and even on both[80,81]. While plasmid-encoded
652 T4SSs are known to commonly mediate conjugation[82], chromosomally encoded T4SSs rarely
653 mobilize a plasmid to a recipient strain[80]. Chromosomally encoded T4SSs are mainly involved
654 in processes such as protein secretion, ssDNA secretion into the extracellular milieu, natural
655 transformation, and symbiosis[54,58,81,83-89]. Of note, within sequenced Roseobacters genomes,
656 23% carry only plasmid-encoded T4SSs, 2% carry only chromosome-encoded T4SSs, and
657 1% carry T4SSs on both the chromosome and a plasmid (Fig. S5). Given the notable fraction
658 of chromosome-encoded T4SSs in Roseobacters, the function of these secretion systems
659 merits further exploration[58].
660
661 *The algal impact on bacterial HGT and evolution*
662 The evolution of marine heterotrophic bacteria is linked to their micro-algal hosts, as
663 manifested by the many examples of algal-bacterial chemical exchanges that likely arose
664 during their co-evolution[25-27,55,57,90]. The current study reveals the impact that algal exudates
665 have on bacterial conjugation, potentially through bacterial attachment, which adds another
666 layer to our understanding of bacterial evolution in the ocean. Algal exudates represent a
667 distinct chemical signature of a given algal species[91], and consequently, attract
668 phylogenetically and functionally discrete populations of bacteria[76,77,92]. By attracting specific
669 bacterial populations, algae have the potential to affect the bacterial partners that perform
670 genetic exchange, thus possibly influencing bacterial evolution.
671
672

Acknowledgments

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Author contribution

YDR and ES designed the study. YDR, VL, LY, DM, and IVK performed and analyzed experiments. YDR and ES wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

Data availability

Strains and plasmids used in this study are listed in Tables S1 and S6, respectively. Engineered bacteria and plasmids are available from the corresponding author on reasonable request. Primers designed for bacterial identification and engineering and for qRT-PCR analysis, are available in Tables S4 and S5 respectively. Data used for supplementary Fig. S5 is presented in Table S7.

Competing interests

The authors declare no competing interest.

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