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Alterocin, an antibiofilm protein secreted by *Pseudoalteromonas* sp. 3J6

Jouault Albane ¹, Gobet Angelique ^{1,2,3}, Simon Marjolaine ¹, Portier Emilie ¹, Perennou Morgan ⁴,
 Corre Erwan ⁴, Gaillard Fanny ⁴, Vallenet David ⁵, Michel Gurvan ², Fleury Yannick ⁶, Bazire Alexis ¹,
 Dufour Alain ^{1,*}

¹ Université de Bretagne-Sud, EA 3884, LBCM, IUEM, F-56100 Lorient, France

² Sorbonne Université, CNRS, UMR8227, Integrative Biology of Marine Models (LBI2M), Station Biologique de Roscoff (SBR), F-29680 Roscoff, France

³ Univ Montpellier, CNRS, Ifremer, IRD, MARBEC, F-34203 Sète, France

⁴ Sorbonne Université, CNRS, FR2424, Station Biologique de Roscoff (SBR), F-29680 Roscoff, France

⁵ LABGeM, Génomique Métabolique, CEA, Genoscope, Institut François Jacob, CNRS, Université d'Evry, Université Paris-Saclay, F-91057 Evry, France

⁶ Université de Brest, EA 3884, LBCM, IUEM, IUT Quimper, F-29000 Quimper, France

* Corresponding author : Alain Dufour, email address : alain.dufour@univ-ubs.fr

Abstract :

The aim was to identify and study the antibiofilm protein secreted by the marine bacterium *Pseudoalteromonas* sp. 3J6. The latter is active against marine and terrestrial bacteria, including *Pseudomonas aeruginosa* clinical strains forming different biofilm types. Several amino acid sequences were obtained from the partially purified antibiofilm protein, named alterocin. The *Pseudoalteromonas* sp. 3J6 genome was sequenced and a candidate alt gene was identified by comparing the genome-encoded proteins to the sequences from purified alterocin. Expressing the alt gene in another non-active *Pseudoalteromonas* sp. strain, 3J3, demonstrated that it is responsible for the antibiofilm activity. Alterocin is a 139-residue protein including a predicted 20-residue signal sequence, which would be cleaved off upon export by the general secretion system. No sequence homology was found between alterocin and proteins of known functions. The alt gene is not part of an operon and adjacent genes do not seem related to alterocin production, immunity or regulation, suggesting that these functions are not fulfilled by devoted proteins. During liquid growth, the alt mRNA level peaked during the stationary phase. A single promoter was experimentally identified and several inverted repeats could be binding sites for regulators. alt genes were found in about 30% of the *Pseudoalteromonas* genomes, and in only few instances of other marine bacteria of the *Hahella* and *Paraglaciecola* genera. Comparative genomics yielded the hypotheses that alt gene losses occurred within the *Pseudoalteromonas* genus. Overall, alterocin is a novel kind of antibiofilm protein of ecological and biotechnological interest.

Importance

Biofilms are microbial communities that develop on solid surfaces or interfaces, and are detrimental in a number of fields including for example food industry, aquaculture, and medicine. In the latter, antibiotics are insufficient to clear biofilm infections, leading to chronic infections such as in the case of infection by *Pseudomonas aeruginosa* of the lungs of cystic fibrosis patients. Antibiofilm molecules are thus urgently

needed to be used in conjunction with conventional antibiotics, as well as in other fields of application, especially if they are environmentally friendly molecules. Here, we describe alterocin, a novel antibiofilm protein secreted by a marine bacterium belonging to the *Pseudoalteromonas* genus, and its gene. Alterocin homologs were found in about 30% of *Pseudoalteromonas* strains, indicating that this new family of antibiofilm proteins likely plays an important albeit non-essential function in the biology of these bacteria. This work opens up the possibility of a variety of applications.

Keywords : biofilm, antibiofilm protein, *Pseudoalteromonas*, *Pseudomonas aeruginosa*

58 INTRODUCTION

59 Biofilms are microbial communities of cells that are attached to a substratum, to an interface,
60 or to each other, and are embedded into a matrix of extracellular polymeric substances that
61 they have produced (1). The biofilm lifestyle is considered as predominant for many bacteria
62 in the environment and is adopted in the course of numerous host infections. Within biofilms,
63 bacteria are generally more resistant to antibiotics than their planktonic counterparts (2), and
64 antibiotics are therefore insufficient to clear biofilm infections, leading to chronic infections
65 (1, 3). The need for biofilm-specific therapies, in which antibiofilm compounds would be
66 used in conjunction with conventional antibiotics, leads to the search for antibiofilm
67 molecules active against pathogenic bacteria (3, 4). *Pseudomonas aeruginosa* is a target
68 organism of particular interest because it is not only one of the leading causes of nosocomial
69 infections (5), but also because the chronic infections it establishes in the lungs of cystic
70 fibrosis (CF) patients constitute their main cause of morbidity and mortality (6, 7). Biofilms
71 are not only detrimental in medicine, and environmentally friendly antibiofilm molecules
72 and/or their producer organisms are of great interest for a variety of other fields, including for
73 example food industry or aquaculture (8).

74 Antibiofilm agents include notably: quorum-sensing (QS) inhibitors of various nature
75 (e.g. inhibitors of the synthesis of signal molecules [autoinducers], autoinducer analogs,
76 enzymes degrading or modifying autoinducers); natural and synthetic antimicrobial peptides;
77 proteins such as matrix-degrading enzymes; surfactants; free fatty acids; and polysaccharides
78 (4, 9, 10). Marine bacteria constitute a non-negligible source of antibiofilm polysaccharides,
79 biosurfactants, and QS inhibitors (8, 11). Furthermore, the marine bacteria
80 *Pseudoalteromonas tunicata* D2 and *Marinomonas mediterranea* MMB-1 secrete the AlpP
81 and LodA (formerly named marinocine) enzymes, respectively, which are responsible for

82 both antimicrobial and antibiofilm activities due to the hydrogen peroxide they generate as a
83 byproduct of their amino acid oxidase activities (8, 12, 13). These enzymes led to the
84 discovery of a large family of proteins encoded by algae-associated marine bacteria and by
85 plant-associated terrestrial microorganisms (8, 14). More generally, marine bacteria from the
86 *Pseudoalteromonas* genus are often found in association with marine eukaryote organisms,
87 and their ability to produce compounds displaying a variety of biological activities has
88 attracted particular attention (15-18). This genus includes two phylogenetically distinct
89 groups: the pigmented group and the non-pigmented group, with the first group displaying a
90 stronger bioactive potential (16, 18).

91 The non-pigmented *Pseudoalteromonas* sp. 3J6 strain was isolated from the Morbihan
92 Gulf, Brittany, France (19), and we previously showed that its culture supernatant displayed
93 antibiofilm activities while being devoid of antimicrobial (biocidal or bacteriostatic) activity
94 against planktonic bacteria (20). Seventy-five percent of the tested marine strains were
95 sensitive to this antibiofilm activity (20-22). Sensitive strains belonged to various genera
96 (Gram negative: *Paracoccus*, *Vibrio*, *Colwellia*, *Algibacter*, *Alteromonas*,
97 *Pseudoalteromonas*, *Sulfitobacter*; Gram positive: *Bacillus*, *Micrococcus*), while most of the
98 non-sensitive Gram negative strains were other *Pseudoalteromonas* strains. The spectrum of
99 action also includes the three tested strains belonging to the human pathogen species *P.*
100 *aeruginosa*, *Salmonella enterica*, and *Escherichia coli* (20). The antibiofilm activity was lost
101 after proteinase K treatment, indicating that it is due to at least one proteinaceous molecule
102 (21). The first step of biofilm formation is the attachment of bacteria onto the substratum.
103 *Pseudoalteromonas* sp. 3J6 exoproducts did not impair the attachment of *Vibrio* sp. D66 and
104 strains belonging to the genera *Algibacter*, *Alteromonas*, *Colwellia*, *Micrococcus*,
105 *Sulfitobacter* and *Vibrio* (23), or they affected the attachment too mildly to explain the
106 subsequent defect in biofilm formation (*Paracoccus* sp. 4M6 and *Vibrio* sp. D01) (20). The

107 only exception was observed with the *Vibrio tapetis* CECT4600-GFP strain, the attachment of
108 which onto glass was reduced more than fivefold by the *Pseudoalteromonas* sp. 3J6 culture
109 supernatant (22). Except in the latter case, the *Pseudoalteromonas* sp. exoproducts are thus
110 thought to impair biofilm formation in our experimental conditions by adsorbing onto the
111 glass substrate and subsequently acting mainly at a post-attachment step (20). Two effects of
112 the *Pseudoalteromonas* sp. 3J6 culture supernatant were observed on sensitive strains: it
113 reduced the biomass of the resulting biofilms and/or led to biofilms in which cell viability was
114 decreased. These two phenomena could either occur together or independently from each
115 other (20). Its target in sensitive strains thus remains elusive, and we hoped that identifying
116 the protein/peptide responsible for this activity might provide some clues on its mode of
117 action. This antibiofilm protein/peptide was named alterocin (8, 22). Here, we show that the
118 *Pseudoalteromonas* sp. 3J6 exoproducts are active against clinical CF *P. aeruginosa* strains
119 and we report the sequencing of the genome of *Pseudoalteromonas* sp. 3J6, in which the *alt*
120 gene encoding alterocin was identified. Homologs of the *alt* gene were mostly found in
121 *Pseudoalteromonas* strains, and the evolutionary history of the *alt* gene in this genus was
122 examined by comparative genomics.

123

124 **RESULTS**

125 **Activity of *Pseudoalteromonas* sp. 3J6 exoproducts against clinical strains of**
126 ***Pseudomonas aeruginosa*.** The *Pseudoalteromonas* sp. 3J6 culture supernatant (SN_{3J6}) was
127 previously shown to be active against the laboratory *P. aeruginosa* PAO1 strain (20). Since
128 large variabilities were reported between biofilms of *P. aeruginosa* isolates (24, 25), SN_{3J6}
129 should be tested against other *P. aeruginosa* strains forming different types of biofilms.
130 Considering the critical *P. aeruginosa* role in lung infections of CF patients, we used

131 additional strains isolated from CF patients. For this purpose, a group of four such clinical
132 strains was constituted mainly on the basis of differences in the architecture of their biofilms,
133 including the distribution of matrix components (26), but also on the bases of their mucoid
134 phenotype (mucoid strains overproduce the alginate exopolysaccharide and are responsible
135 for chronic infections (6)), virulence level and antibiotic resistance (M. Simon, E. Pernet, A.
136 Jouault, E. Portier, A. M. Boukerb, S. Pineau, J. Vieillard, E. Bouffartigues, C. Poc-
137 Duclairoir, M. G. J. Feuilleley, O. Lesouhaitier, J. Caillon, S. Chevalier, A. Bazire, and A.
138 Dufour, submitted for publication). This panel is composed of the clinical strains MUC-N1,
139 MUC-P2, MUC-P4, and MUC-P5 (Table 1), the genomes of which were recently sequenced
140 (27). To test the effect of SN_{3J6} on these strains, the bacterial attachment step was performed
141 without or in the presence of SN_{3J6}, and biofilms were then grown under a flow of medium.
142 Without SN_{3J6}, the mucoid MUC-N1 strain formed biofilms with mushroom-like structures
143 containing alginate as the main matrix component (Fig. 1A, left). The MUC-N1 biofilms
144 obtained in the presence of SN_{3J6} were devoid of mushroom-like structures and their
145 biovolume was 8.6-fold lower (Fig. 1A). Biofilms of the non-mucoid MUC-N2 strain grown
146 without SN_{3J6} were devoid of mushroom-like structures, and the stained matrix components
147 were not abundant at the biofilm surface, as seen by the large predominance of the green
148 coloration (bacteria) over the blue (β -polysaccharides) and red (eDNA) colorations (Fig. 1B,
149 left). In the presence of SN_{3J6}, the MUC-N2 biofilm growth was prevented since only
150 dispersed cells and small cell clusters were visible, which led to a 27-fold lower biovolume
151 (Fig. 1B). When using the two other non-mucoid clinical strains of the panel, SN_{3J6} was active
152 against MUC-P4, but did not impair biofilm formation by MUC-P5, which is thus non-
153 sensitive to alterocin (Supplemental Fig. S1). Altogether, these data show that SN_{3J6} is not
154 only active against the *P. aeruginosa* laboratory strain PAO1, but also against three out of the
155 four tested CF clinical strains forming different biofilm types, including one mucoid and two

156 non-mucoid strains. Any of the sensitive strains would be suitable as indicators of antibiofilm
157 activity. In our subsequent experiments, the *P. aeruginosa* MUC-N1 strain was chosen
158 because it forms biofilms with higher biovolumes than the other strains in the absence of
159 SN_{3J6}, therefore yielding more consistent control biofilms.

160

161 **General characteristics of the *Pseudoalteromonas* sp. 3J6 genome.** We sequenced
162 the genome of *Pseudoalteromonas* sp. 3J6, which is about 4.1-Mpb long with an average GC
163 content of 39.93% and contains 3,789 coding DNA sequences (CDS) (Table 2). The sequence
164 seemed complete and not contaminated, as estimated using CheckM (28). To determine
165 whether the *Pseudoalteromonas* sp. 3J6 strain could belong to a novel species, we determined
166 its genomic relatedness with 58 other *Pseudoalteromonas* strains, the genome sequences of
167 which were available on the NCBI RefSeq complete genome section at the time of analysis
168 (February 2019), by estimating the average nucleotide identity (ANI) between genomes. The
169 ANI between *Pseudoalteromonas* sp. 3J6 genome and 57 out of the 58 other
170 *Pseudoalteromonas* genomes were lower than 96% (Supplemental Table S1), the typical
171 threshold to determine different bacterial species (29, 30). The only ANI above this threshold
172 was obtained when comparing the genomes of *Pseudoalteromonas* sp. 3J6 and
173 *Pseudoalteromonas undina* NCIMB 2128^T: the ANI of 96.7% (Supplemental Table S1)
174 indicates that *Pseudoalteromonas* is most closely related to a *P. undina* strain, but is not
175 sufficient to firmly conclude that strain 3J6 belongs to the *undina* species, and it could
176 alternatively belong to a new species. *P. undina* NCIMB 2128^T being part of the non-
177 pigmented *Pseudoalteromonas* group (31), it at least indicates that *Pseudoalteromonas* sp. 3J6
178 is a member of this group.

179

180 **The locus of the putative alterocin gene.** We observed that the *Pseudoalteromonas*
181 sp. 3J6 genome is devoid of *alpP*-like gene, the product of which could have been responsible
182 for antibiofilm activities (8). This is consistent with previous findings, since AlpP and related
183 proteins lead to both antibacterial and antibiofilm activities (8) whereas no antibacterial
184 activity was ascribed to *Pseudoalteromonas* sp. 3J6 (20). The antibiofilm activity of the latter
185 thus relies on another protein. To identify it, we concentrated the active molecules by loading
186 SN_{3J6} onto a Sep-Pak® Plus C18 cartridge and eluting the adsorbed molecules with stepwise
187 increased concentrations of acetonitrile. The fraction eluted with 50% acetonitrile was the
188 most active and was named the 3J6 extract (E_{3J6}). Upon SDS-PAGE analysis, a single 13-kDa
189 protein was observed in E_{3J6}. Peptide sequences were obtained from this protein as described
190 in the Materials and Methods section. None of these sequences matched a protein of known
191 function in Swissprot databases, but seven peptide sequences matched one protein encoded by
192 the *Pseudoalteromonas* sp. 3J6 genome (Fig. 2B) from a gene located on the second largest
193 contig (contig 11: 791,825 bp). This protein was considered as the putative alterocin and its
194 gene was named *alt*. The *alt* gene lies in the opposite orientation compared to the upstream
195 and downstream genes (Fig. 2A) and therefore cannot be part of an operon. The surrounding
196 genes P3J6_110063, P3J6_110065 and P3J6_110066 (Fig. 2A) are predicted to encode an
197 aspartate-semialdehyde dehydrogenase, which is involved in amino acids biosynthesis (32), a
198 protein of unknown function, and a TonB-dependent receptor/transporter (TBDR),
199 respectively (Supplemental Table S2). The latter belongs to a family of outer membrane
200 proteins responsible for the uptake of iron complexes and other substrates which are often rare
201 nutrients (33). There is thus no obvious relationship between the products of the *alt*-adjacent
202 genes and alterocin production. A larger view of the *alt* gene vicinity is provided in
203 Supplemental Fig. S2 and its accompanying text. In this genomic region, the only gene which
204 could be related to biofilm formation encodes a protein with a predicted diguanylate cyclase –

205 GGDEF domain (ORF P3J6_110075). This protein would contribute to the synthesis of cyclic
206 diguanylate (c-di-GMP), a near universal signaling molecule involved in the regulation of
207 bacterial biofilm formation (34, 35).

208

209 **The *alt* gene and its product.** The *alt* gene encodes a 139-residue protein predicted
210 by the SignalP 5.0 Server (36) to include a 20-residue N-terminal signal peptide (Fig. 2B)
211 with a likelihood of 0.9073. This signal peptide would be cleaved off by the signal peptidase I
212 in course of translocation across the plasma membrane by the general secretory pathway. The
213 resulting alterocin protein would be composed of 119 amino acids (calculated molecular
214 weight: 13,655.99 Da; pI: 4.82), including four cysteines in its C-terminal half (Fig. 2B),
215 which could lead to the formation of disulfide bridges. The DISULFIND tool (37) indeed
216 predicted two disulfide bridges, with the best ranking connectivity pattern linking residues 65-
217 81 and 109-188 (confidence of prediction: 6 for each bridge on a scale of 0=low to 9=high).
218 The alterocin sequence did not display significant similarities with proteins of known
219 functions using the protein Basic Local Alignment Search Tool (BLAST) program, and with
220 conserved domains from the Conserved Domain Database (CDD) (38). When searching for
221 alterocin homologs in the Prokaryotic Genome DataBase (PkgDB) from the MicroScope
222 platform (<http://mage.genoscope.cns.fr/microscope>), we identified 19 proteins (13 different
223 amino acid sequences since encoded by 18 *Pseudoalteromonas* strains (\geq 45% amino acid
224 sequence identities) and one *Hahella chejuensis* strain (44% aa sequence identity). The
225 sequence comparison of the *Pseudoalteromonas* sp. 3J6 alterocin and its 19 homologs showed
226 that the C-terminal two-third part of the protein (residues 38 to 119) is the most strongly
227 conserved with numerous residues found in 19 or all of the 20 proteins (Fig. 2B). The four
228 cysteine residues are conserved in all alterocin homologs, consistently with the hypothesis
229 that they could play an important structural role. When searching for alterocin homologs in

230 NCBI sequence databases using the protein BLAST program, 48 proteins displayed more than
231 40% identity with alterocin. These 48 protein sequences included those of all 19 alterocin
232 homologs found in the PkGDB from MicroScope (corresponding to 13 different sequences),
233 plus 35 additional sequences, in which the residues highlighted on Figure 2B are also highly
234 conserved. *Pseudoalteromonas* sp. strains encode the large majority of these proteins (44 out
235 of 48), and the four remaining strains are marine Gram-negative bacteria belonging to the
236 *Hahella* (two strains) and *Paraglaciecola* (two strains) genera (39, 40). Like the
237 *Pseudoalteromonas* genus, the *Paraglaciecola* genus belongs to the Alteromonadales order
238 within the *Gammaproteobacteria* class, whereas the *Hahella* genus is a member of the
239 Oceanospirillales order (*Gammaproteobacteria* class)
240 (<https://www.ncbi.nlm.nih.gov/Taxonomy>). By contrast, proteins similar to the product of the
241 P3J6_110065 gene located just downstream of the *alt* gene in the *Pseudoalteromonas* sp. 3J6
242 genome were only found in eight other *Pseudoalteromonas* sp. strains with the BLAST
243 program. Therefore, many *Pseudoalteromonas* genomes include an *alt*-like gene without any
244 P3J6_110065-like gene, indicating that the P3J6_110065 gene product is not necessary to the
245 production and/or activity of the alterocin protein. We therefore subsequently focused only on
246 the *alt* gene.

247

248 **Expression of the *alt* gene in the *Pseudoalteromonas* sp. 3J3 host.** To examine if the
249 *alt* gene indeed encodes an antibiofilm protein, we first tried to construct an *alt* deletion
250 mutant of *Pseudoalteromonas* sp. 3J6. Unfortunately, we were unable to obtain the expected
251 mutant (data not shown). We thus attempted to introduce the *alt* gene into another strain,
252 *Pseudoalteromonas* sp. 3J3 (Table 1), which was isolated from the same environment as the
253 3J6 strain (19). The 3J3 strain was chosen because: it belongs to the same genus as the 3J6

254 strain (19); we could not amplify by PCR an *alt* gene from 3J3 total DNA and, consistently,
255 we did not observe an antibiofilm activity in 3J3 culture supernatants; and the 3J3 strain is not
256 sensitive to SN_{3J6} (20, 21). *Pseudoalteromonas* sp. 3J3 was thus a good candidate strain to
257 express the *alt* gene and produce the encoded protein. The *alt* gene with its own promoter
258 region was cloned into the pOriT-4Em vector (designated thereafter as pOriT), yielding the
259 pOriTalt plasmid (Table 1). Both plasmids were introduced into *Pseudoalteromonas* sp. 3J3,
260 and we examined the effect of the culture supernatants of the two resulting strains, SN_{3J3(pOriT)}
261 and SN_{3J3(pOriTalt)}, on the *P. aeruginosa* MUC-N1 biofilm development. In the presence of
262 SN_{3J3(pOriTalt)}, the biofilm biovolume and average thickness were about 2 and 3-fold lower,
263 respectively, than in the presence of SN_{3J3(pOriT)} (Fig. 3). These data show that introducing the
264 *alt* gene from strain 3J6 into strain 3J3 provides an antibiofilm activity to the exoproducts of
265 the latter.

266

267 **Alterocin detection in *Pseudoalteromonas* sp. 3J3(pOriTalt) culture supernatants.**

268 SDS-PAGE analyses were performed to verify that *Pseudoalteromonas* sp. 3J3(pOriTalt)
269 secretes alterocin. Since no band was visible when loading the raw culture supernatants of
270 strains 3J3(pOriTalt) and 3J3(pOriT), we extracted and concentrated the antibiofilm molecule
271 from SN_{3J3(pOriTalt)} using a Sep-Pak Plus C18 cartridge and the fraction eluted with 50%
272 acetonitrile constituting the E_{3J3(pOriTalt)} extract was resuspended in a volume of water 1,000-
273 fold lower than the used SN volume. Similar extracts were made from SN_{3J3(pOriT)} and SN_{3J6}.
274 A single SDS-PAGE band at about 13 kDa was observed in E_{3J3(pOriTalt)} and E_{3J6}, whereas no
275 band was visible in E_{3J3(pOriT)} (Fig. 4A). To ascertain that this band was due to alterocin, we
276 performed Western blot experiments using rabbit antibodies produced upon inoculation of the
277 peptide P1 that had been chemically synthesized based on the alterocin sequence (Fig. 2B).

278 The antibodies detected the 13-kDa protein in E_{3J6} and E_{3J3(pOriTalt)}, whereas they did not detect
279 any protein in the negative control E_{3J3(pOriT)} (Fig. 4B). This confirms that *Pseudoalteromonas*
280 sp. 3J3(pOriTalt) is able to express the *alt* gene and to secrete alterocin, thereby
281 demonstrating that alterocin is responsible for the antibiofilm activity of the recombinant
282 strain against *P. aeruginosa* MUC-N1.

283

284 **The *alt* gene promoter and expression.** The bacterial σ^{70} promoter recognition
285 program BPROM (41) predicted a single promoter upstream of the *alt* gene (Fig. 5, P-35 and
286 P-10). We mapped the transcriptional start site of the *alt* gene site by 5'-RACE PCR
287 performed on RNAs extracted from *Pseudoalteromonas* sp. 3J6 transformed by pOriTalt in
288 order to increase the *alt* gene copy number and thus the *alt* mRNA level. A single PCR
289 product was obtained and its sequencing revealed three potential +1 bases: the GGC bases
290 located 54-56 bp upstream of the translation initiation codon (Fig. 5). This result did not
291 confirm that the BPROM-predicted promoter is functional since it is located too far away of
292 the mapped transcriptional start site (the putative -10 sequence is lying 50 bp upstream of the
293 first potential +1 base). Although the sequences TTAACC-17 bp-TACCAA were not
294 predicted to constitute a promoter by BPROM, they are at the appropriate positions relative to
295 the +1 bases (Fig. 5) and match loosely the consensus promoter sequences recognized by the
296 primary σ^{70} factor (TTGACA-16 to 19 bp-TATAAT). These sequences are thus proposed to
297 constitute a weak promoter where the transcription could be initiated by the RNA polymerase
298 containing σ^{70} . It should be noted that the *alt* promoter region contains several inverted
299 repeats (IR) located upstream and downstream of the two promoter-like sequences (Fig. 5)
300 and some of these IRs could be involved in transcription initiation regulation, by binding
301 repressor or activator proteins.

302 The above data suggesting that the *alt* gene expression is regulated at the
303 transcriptional level, we assayed the *alt* mRNA level by RT-qPCR at different time points
304 during the growth of *Pseudoalteromonas* sp. 3J6. The *alt* mRNA level was respectively 5, 11
305 and 2-fold higher at 5, 10 and 24h compared to 3h (Fig. 6). Under our culture conditions, the
306 *alt* mRNA level thus increased during the stationary phase (5 and 10h) before decreasing
307 almost down to the exponential phase level at 24h (Fig. 6). The transcription of the *alt* gene
308 seems therefore regulated to reach its peak during the first hours of the stationary phase.

309

310 **Evolutionary history of the *alt* gene in the *Pseudoalteromonas* genus.** To determine
311 the acquisition mode of the *alt* gene in the *Pseudoalteromonas* genus, we first searched for
312 alterocin homologs in the 4,418 bacterial genomes from the PkGDB on the MicroScope
313 platform. As mentioned above, *Hahella chejuensis* KCTC 2396 possesses a gene encoding an
314 alterocin homolog (44% amino acid sequence identity), as well as 18 out of 58 (31%)
315 *Pseudoalteromonas* genomes ($\geq 45\%$ aa sequence identity). Interestingly, the *P. undina*
316 NCIMB 2128^T strain, which was the most phylogenetically closely related to
317 *Pseudoalteromonas* sp. 3J6, is devoid of *alt* gene. Compared to *Pseudoalteromonas* sp. 3J6,
318 the synteny of the *alt* gene environment is not conserved in the *H. chejuensis* genome. Parts of
319 the synteny of the genes downstream of the *alt* gene (region P3J6_110066 to P3J6_110076
320 including genes for the TonB transporter complex and a TBDR) are conserved in all
321 *Pseudoalteromonas* genomes. This is the case of the genomes of the ten pigmented
322 *Pseudoalteromonas* strains belonging to the species *luteoviolacea* (nine strains) and *citrea*
323 (one strain) while the *alt* gene is located in another region of the genome. The three *P.*
324 *ruthenica* genomes and the six genomes of the non-pigmented *Pseudoalteromonas* strains
325 (including *Pseudoalteromonas* sp. 3J6) display seven versions of the *alt* gene environment

326 with different parts of the gene synteny conserved (Supplemental Fig. S2), which suggests a
327 differential loss of some genes in the region around the *alt* gene during the evolutionary
328 history of the *Pseudoalteromonas* genus.

329 The alterocin proteins from *H. chejuensis*, and the 19 *Pseudoalteromonas* strains
330 (including the 3J6 one) allowed computing a phylogenetic tree where *H. chejuensis* clustered
331 alone and the *Pseudoalteromonas* strains clustered together, forming four groups (Fig. 7).
332 Two groups are made only of pigmented *Pseudoalteromonas* strains: one group consists in *P.*
333 *citrea* DSM 8771 and the nine *P. luteoviolacea* strains, while the second one includes the
334 three *P. ruthenica* strains. The two other groups contain only non-pigmented
335 *Pseudoalteromonas* strains: one group with *P. haloplanktis* ATCC 14393 and *P. porphyrae*
336 UCD-SED14; and the *Pseudoalteromonas* sp. 3J6 group, which also includes two other
337 *Pseudoalteromonas* sp. strains, Bsi20495 and S2292, and *P. fuliginea* KMM216 (Fig. 7).

338 The evolutionary history of the *alt* gene in the *Pseudoalteromonas* genus was further
339 investigated using two phylogenetic approaches. The first was to construct a 16S rRNA gene
340 tree including the 58 strains with an available genome plus *Pseudoalteromonas* sp. 3J6, and
341 34 additional type strains to determine the position of the *alt*-containing *Pseudoalteromonas*
342 strains in the genus (Supplemental Fig. S3). The second was to compute a tree based on
343 pairwise genome distances calculated from Mash values between the 59 *Pseudoalteromonas*
344 genomes, including the 3J6 one (Supplemental Fig. S4). In both trees, the *P. luteoviolacea*
345 strains did not cluster with *P. citrea* whereas they belong to the same group in the alterocin
346 tree (Fig. 7). This suggests that they acquired the *alt* gene from two independent horizontal
347 gene transfer events but likely from the same organism. The other pigmented strains, i.e. the
348 three *P. ruthenica* strains, clustered together in both 16S rRNA gene and genome trees.
349 Whereas non-pigmented *Pseudoalteromonas* strains were distributed in two groups in the
350 alterocin tree (Fig. 7), the two corresponding groups were not observed in the 16S rRNA gene

351 tree (Fig. S3): *P. haloplanktis* ATCC14393 did not cluster with *P. porphyrae* UCD-SED14,
352 and *Pseudoalteromonas* sp. 3J6 did not cluster with the *Pseudoalteromonas* sp. strains
353 Bsi20495 and S2292 (Fig. S3). By contrast, the two corresponding clades were found in the
354 genome-based tree (Fig. S4). Therefore, the genome-based tree is more consistent with the
355 alterocin tree, suggesting that the genome-based tree may give a finer phylogenetic
356 description of the *Pseudoalteromonas* evolutionary history than a 16S rRNA-based tree, even
357 though less *Pseudoalteromonas* strains are considered in the genome-based tree. In the latter,
358 several *Pseudoalteromonas* genomes in the non-pigmented clade and the *P. citrea* clade do
359 not contain the *alt* gene. From the above observations and as the *alt* gene is located in a rather
360 conserved region of the genome, an ancestor common to the strains of the non-pigmented
361 group may have acquired the *alt* gene and non-pigmented strains lacking this gene may have
362 subsequently lost it.

363

364 **DISCUSSION**

365 We report here the genome sequence of the *Pseudoalteromonas* sp. 3J6 strain and the
366 identification of the alterocin gene, the product of which is secreted by this marine bacterium.
367 Alterocin, a predicted 119-residue protein after cleavage of a 20-residue signal peptide, is a
368 novel antibiofilm protein since it is devoid of sequence similarity with proteins of known
369 function and does not contain a known functional domain. Its mode of action thus cannot be
370 deciphered from protein sequence comparisons, and future works will be required to unravel
371 it, by combining in-depth analysis of biofilm formation in the presence of alterocin,
372 transcriptomic analyses of bacteria submitted to alterocin, the search for alterocin-insensitive
373 mutants, and their subsequent study.

374 In the case of ribosomally-synthesized peptides (known as RiPPs or RPNPs) secreted
375 by bacteria, the structural genes are generally clustered with other genes forming one or

376 several operons and encoding proteins specifically involved in the production of the mature
377 peptides (modification enzymes, proteases cleaving the leader peptide, dedicated ATP-
378 binding cassette [ABC] transporters); in the protection of the producer bacteria (immunity
379 systems in the case of antimicrobial peptides); and in dedicated systems regulating the
380 transcription of these genes (42, 43). By contrast, the *alt* gene is not part of an operon. The
381 P3J6_110065 gene lying immediately downstream of the *alt* gene in the *Pseudoalteromonas*
382 sp. 3J6 genome encodes a protein of unknown function, but most of the other
383 *Pseudoalteromonas* strains possessing an *alt*-like gene are devoid of P3J6_110065-like gene,
384 indicating that the P3J6_110065 gene product is unrelated, or at least non-essential, to
385 alterocin production and activity. The other genes of the *alt* locus encode proteins without
386 obvious relationship to production of an antibiofilm protein or transcriptional regulation.
387 Therefore, the genetic environment did not help to understand the alterocin production
388 pathway, suggesting that alterocin is not the object of maturation and export by dedicated
389 proteins. Consistently, the 20-residue signal peptide was strongly predicted to be cleaved off
390 by the signal peptidase I, meaning that alterocin is very likely translocated across the plasma
391 membrane by the general secretory pathway and not by a specific transporter. The secretion
392 pathway across the outer membrane remains to be identified. Furthermore, introducing only
393 the *alt* gene in the *Pseudoalteromonas* sp. 3J3 strain was sufficient to provide to the latter an
394 antibiofilm activity, which showed that no other gene found specifically in
395 *Pseudoalteromonas* sp. 3J6 (i.e. absent in the 3J3 strain) is required for alterocin production.

396 We observed that the *alt* gene expression is regulated, with the mRNA level increasing
397 during the 6 first hours of the stationary phase before decreasing. We obtained the
398 experimental evidence of a single transcription start site, which would result from a promoter
399 only weakly matching the consensus sequence of primary σ^{70} factor-dependent promoters (7
400 bases identical out of 12). This promoter could thus be a weak promoter recognized by the

401 primary σ^{70} factor. Alternatively, a secondary sigma factor could be responsible for the
402 transcription initiation at the mapped start site, as in the cases of genes for several pyocins and
403 tailocins, which are bacteriocins produced by *P. aeruginosa* strains (44). The
404 *Pseudoalteromonas* sp. 3J6 genome indeed encodes 12 putative secondary sigma factors
405 (Supplemental Table S3), each of them recognizing a specific unknown or hypothetical
406 promoter sequence. In both cases, regulations are expected to occur, either via the action of at
407 least an activator protein on a weak σ^{70} -dependent promoter, or via the regulation of a
408 secondary sigma factor activity. Several IRs in the *alt* promoter region could be binding sites
409 for regulatory proteins. Furthermore, a second promoter was predicted. Although its
410 functionality was not experimentally confirmed, it remains possible that it could be active in
411 other conditions than ours. The pattern of the *alt* mRNA level in the course of growth (higher
412 level during the first hours of the stationary phase) is consistent with a control by a QS system
413 in response to cell density. There are indeed examples of QS-regulated production of peptidic
414 antimicrobial molecules by Gram-negative bacteria (50, 51). Recent analyses of
415 *Pseudoalteromonas* genomes indicate that they encode homologs of proteins belonging to
416 three sets of cross-linked QS systems: the *Aliivibrio fisheri* and *P. aeruginosa* LuxI-LuxR
417 type systems producing and sensing autoinducers AI-1 (*N*-acyl homoserine lactones); the
418 *Vibrio* LuxPQ and CqsS sensing the autoinducers AI-2 (produced by LuxS) and CAI-1,
419 respectively, and both feeding the LuxU-LuxO pathway; and the *E. coli* QseC-QseB and
420 QseE-QseF two-component systems sensing AI-3 and/or the hormones Epinephrine and
421 norepinephrine (45, 46). Whereas several LuxI-LuxR type systems have been shown to be
422 functional in *Pseudoalteromonas* strains (46, 47), the two other types of systems are not
423 identical to those described in other genera since some proteins are missing in the studied
424 *Pseudoalteromonas* strains (45, 46). It can thus be speculated that a sophisticated QS network
425 integrating signals provided by several autoinducers is likely to take place in

426 *Pseudoalteromonas* strains, allowing the achievement of elaborate cell physiology (46). No
427 AI-1 and AI-2 autoinducers were detected in culture supernatants of the *Pseudoalteromonas*
428 sp. 3J6 strain (48, 49). Consistently, the *Pseudoalteromonas* sp. 3J6 genome is devoid of
429 genes encoding LuxI, LuxR, and LuxS homologs. However, it includes genes encoding
430 homologs of LuxU, LuxO, LuxQ, and of the two-component system QseC-QseB
431 (Supplemental Table S4). Future works will be required to define the QS functioning in
432 *Pseudoalteromonas* sp. 3J6 and to examine if it controls alterocin production.

433 The *alt* gene is not part of the core *Pseudoalteromonas* genome, but is nevertheless
434 present in a non-negligible proportion of strains (about 30%), indicating that it plays an
435 important, albeit non-essential, role in the physiology of bacteria belonging to this genus. The
436 *alt* gene is not restricted to one of the two *Pseudoalteromonas* phylogenetic groups:

437 *Pseudoalteromonas* sp. 3J6 is affiliated to non-pigmented strains, and the *alt* gene was also
438 found in other non-pigmented strains as well as in pigmented ones. We found *alt* genes in
439 only very few instances of non-*Pseudoalteromonas* bacterial strains. The latter are marine
440 bacteria belonging to the *Hahella* and *Paraglaciecola* genera. This is in sharp contrast for
441 example with the *alpP-lodA* family genes, which encode antibacterial/antibiofilm amino acid
442 oxidases (8, 12, 13) and were found in a large variety of marine bacteria and terrestrial
443 bacteria belonging to eight phyla and having in common the ability to associate with algae or
444 plants (14).

445 Consistently with the ecological roles proposed for *Pseudoalteromonas* such as
446 influencing the biofilm formation in different ecological niches or being defensive agents for
447 marine flora and fauna (16), alterocin could provide a competitive advantage to the producer
448 strains towards other bacteria to colonize living (algae) or inert surfaces and develop a
449 biofilm, and/or to invade a preformed biofilm. The *Pseudoalteromonas* sp. 3J6 strain tagged
450 with the green fluorescent protein (GFP)-producing plasmid pCJS10 has indeed been shown

451 to form biofilms and to exert its antibiofilm activity in the course of biofilm formation,
452 inhibiting the development of sensitive strains co-inoculated with *Pseudoalteromonas* sp.
453 3J6(pCJS10) in attempts to grow two-species biofilms (20). It is however puzzling that such
454 an advantage would be of benefit only for *Pseudoalteromonas* strains and a few other strains
455 of marine bacteria, and that the *alt* gene had not been more broadly disseminated. An
456 explanation for host limitation of the *alt* gene could come from the fact that no immunity
457 gene, which would protect the alterocin producer strain from its own antibiofilm protein, was
458 found next to the *alt* gene, and from our previous observation that most of the strains of
459 marine bacteria which were non-sensitive to alterocin belonged to the *Pseudoalteromonas*
460 genus (20, 21). This feature of at least some *Pseudoalteromonas* strains that are not producers
461 of alterocin could be due to the absence or modification(s) of the alterocin target. This would
462 allow the acquisition of an *alt* gene without detrimental effect on the ability of the strain to
463 form its own biofilms while providing a competitive advantage. Consistently, we were able to
464 introduce the *alt* gene into the *Pseudoalteromonas* sp. 3J3 strain, which was non-sensitive to
465 alterocin and became alterocin-producer. The resulting 3J3(pOriTalt) strain was still able to
466 form biofilms (data not shown). On the opposite, the gain of an *alt* gene by an alterocin-
467 sensitive strain would modify its lifestyle by preventing it to form biofilms, which would be
468 too detrimental, thereby limiting the *alt* gene host range. To test this hypothesis, discovering
469 the alterocin target and searching for its presence/variability in *Pseudoalteromonas* strains and
470 other bacteria will be required. *Pseudoalteromonas* sp. 3J6 itself is non-sensitive to alterocin
471 at concentrations found in its own culture supernatants (21), consistently with its ability to
472 form biofilms (20, 21). However, it would be interesting to examine if alterocin could
473 accumulate sufficiently within mature biofilms to affect its own producer cells. In this case,
474 alterocin might contribute to the life cycle of producer bacteria by favoring the dispersal of
475 cells from mature biofilm and the colonization of novel environments. Such an ecological role

476 has been documented for the AlpP and LodA enzymes in the marine bacteria *P. tunicata* and
477 *M. mediterranea*, respectively: the hydrogen peroxide synthesized by these proteins leads to
478 death of AlpP/LodA-producer cells inside biofilm microcolonies, which promotes the
479 dispersal of surviving cells (52, 53). Interestingly, the released bacteria displayed a high level
480 of phenotypic variation, which could be advantageous to colonize environments under
481 changing conditions.

482 Alterocin homologs produced by different strains are not identical to each other.
483 Amino acid sequence comparisons showed that the C-terminal part (residues 38 to 119) is
484 more strongly conserved than the N-terminal part (residues 1 to 37) (Fig. 2B). This can
485 provide the basis for future studies of structure-activity relationship: a mutagenesis work
486 could focus on the most conserved residues to examine which of them play key roles in the
487 antibiofilm activity. The presence of four cysteine residues suggest that two disulfide bridges
488 could be formed, which could be crucial for the tridimensional structuration and activity of
489 the protein. Consistently, these four residues are located within the C-terminal part and are all
490 highly conserved. They would thus be amongst the residues to mutagenize in priority. It
491 would also be interesting to examine if truncated alterocin versions lacking the whole N-
492 terminal part or portions of it could retain some antibiofilm activity.

493 In conclusion, we identified a novel antibiofilm protein. Our data raise numerous
494 questions, as discussed above, but they also open up the possibility of applications for
495 alterocin and/or its producer strains in antibiofilm strategies in various domains, including the
496 medical one since they are active against clinical strains of the human pathogen *P.*
497 *aeruginosa*.

498

499 **MATERIALS AND METHODS**

500 **Strains and culture conditions.** The strains used in this study are listed in Table 1.
501 *Pseudoalteromonas* sp. strains were grown in Vaatanen nine-salt solution (VNSS) medium at
502 20°C under agitation (54). The *P. aeruginosa* and *E. coli* strains were grown in LB medium at
503 37°C with shaking or on LB agar plates (1.5% agar). For conjugation, strains were grown in
504 modified LB medium (peptone, 10 g L⁻¹; yeast extract, 5 g L⁻¹; and sea salt 15 g L⁻¹) at 20°C.
505 Diaminopimelate (DAP) was added at a final concentration of 0.3 mM final to grow *E. coli*
506 GEB883 (55). When necessary, ampicillin was used at 100 µg mL⁻¹ for *E. coli* and
507 *Pseudoalteromonas* sp. strains.

508
509 **Biofilms growth and observation.** Biofilms were grown for 24h in flow cells under
510 dynamic conditions (2.5 mL.h⁻¹ of LB medium) at 37°C as previously described (56), bacteria
511 were stained with 5 µM SYTO 9 green (Molecular Probes, Life Technologies, Saint Aubin,
512 France) and observed by confocal laser scanning microscopy (CLSM) with a TCS-SP2
513 microscope (Leica Microsystems, Heidelberg, Germany) or a LSM 710 microscope (ZEISS,
514 Oberkochen, Germany) using a 63x or a 40x oil immersion objective, respectively. To
515 visualize matrix compounds, β1-3 and β1-4 polysaccharides (including alginate) were stained
516 with 100 µM CalcoFluor White (Sigma Aldrich) (57), Psl was labeled with 100 µM
517 Tetramethylrhodamine isothiocyanate (TRITC) conjugated Hippeastrum Hybrid Amaryllis
518 (HHA) lectin (Biovalley SA, Marne-la-Vallée, France) (58), and eDNA was detected by 1 µM
519 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) (Molecular Probes) (59).
520 Dyes in NaCl 0.9% were injected in flow cell chambers and incubated for 15 min. Biofilms
521 were then rinsed with a LB flow (2.5 mL.h⁻¹) for 10 to 15 min and observed immediately.
522 Excitation wavelengths for SYTO 9 green, CalcoFluor White, DDAO and TRITC conjugated

523 HHA lectin were 485, 400, 646 and 541 nm, respectively. Emissions wavelengths were 498,
524 410 to 480, 659 and 572 nm, respectively. Fluorescence signal of double- or triple-labelled
525 specimens were acquired simultaneously. DDAO and TRITC conjugated HHA lectin were
526 applied and imaged separately since they both emit red fluorescence. Images were taken every
527 micrometer throughout the whole biofilm depth. The Leica LCS or Zen 2011 software was
528 used to visualize and process three-dimensional (3D) image data (volume rendering with
529 shadow projection). Biovolumes and thicknesses were determined with the COMSTAT
530 software (<http://www.imageanalysis.dk/>) (60). Three image stacks from each of three
531 independent experiments were used for each analysis.

532

533 **DNA extraction, library preparation and genome sequencing of**

534 *Pseudoalteromonas* sp. **3J6**. After growth in 200 mL of VNSS medium until the stationary
535 phase, the bacteria were harvested by centrifugation (3,000 g for 15 min) and the genomic
536 DNA was extracted as in (61). The DNA was quantified on a Qubit ds DNA HS kit
537 (Invitrogen, Carlsbad, CA, USA). Libraries were prepared using the paired-end and the mate-
538 pair strategies. Paired-end libraries were done using the Nextera XT DNA sample preparation
539 kit (Illumina, San Diego, CA, USA) and mate-pair libraries were done using the Nextera Mate
540 Pair sample preparation kit (Illumina). Template size distribution was verified on a
541 Bioanalyzer 2100 (Agilent, Santa Clara, USA). Paired-end and mate-pair libraries were then
542 sequenced on a MiSeq sequencer using the kit v2 (300 cycles) of Illumina. The assembly
543 using the reads from the two libraries was done in SPAdes version 3.1.1 (62), resulting in a
544 genome of 4.1 Mb with 17 contigs (> 1 kb). Gene function was annotated as in (61). Briefly,
545 all the genes were subjected to the automatic annotation of the MicroScope platform (63).
546 Selected genes, including the alterocin gene as well as the surrounding genes, were annotated

547 manually by searching for homologs (at least 30% amino acid sequence identity and 80%
548 sequence coverage) with characterized proteins on the MicroScope platform.

549

550 **Preparation of *Pseudoalteromonas* culture supernatants and alterocin extract.**

551 Culture supernatants were recovered by centrifugation for 10 min at 10,000g and 18°C after
552 24h of growth of *Pseudoalteromonas* sp. 3J6, 3J3(pOriT) or 3J3(pOriTalt) strains in VNSS
553 medium without antibiotic at 20°C under agitation. For *Pseudoalteromonas* sp. 3J3 carrying
554 the pOriT or pOriTalt plasmid, ampicillin was added for the precultures, but was omitted in
555 the last culture. The stability of pOriT and pOriTalt in ampicillin-free cultures has been
556 verified as follows: after 24 h of liquid growth without ampicillin, the proportion of bacteria
557 with plasmid was determined by plating dilutions of the cultures and comparing the number
558 of colonies on LB plates with ampicillin and on ampicillin-free LB plates. The experiment
559 was carried out 3 times. Plasmids pOriT and pOriTalt were maintained in 83 and 66%,
560 respectively, of *Pseudoalteromonas* sp. 3J3 cells. The culture supernatants were filtered on
561 Steritop-GP 0.22 µm (Millipore, Molsheim, France) and were conserved either at 4°C for a
562 rapid use or at -80°C for a longer period. Culture supernatant of *Pseudoalteromonas* sp. 3J6
563 (SN_{3J6}) lacking antibiofilm activity was obtained by replacing the pancreatic digest of soy by
564 pancreatic digest of casein (Biokar Diagnostics, Groupe Solabia, Pantin, France) in the VNSS
565 medium.

566 The alterocin extracts E_{3J6} and E_{3J3(pOriTalt)} were obtained by eluting SN_{3J6} and
567 SN_{3J3(pOriTalt)}, respectively, from a Sep-Pak® Plus C18 cartridge with acetonitrile (10 or 20%,
568 and then 50%). The 50% acetonitrile fractions were evaporated using a Speed Vac apparatus
569 (Thermo Fisher Scientific, Bremen, Germany) and then resuspended in sterile ultrapure water.

570

571 **SDS-PAGE and Western blot analyses.** Culture supernatants or concentrated
572 extracts were suspended in one volume of sample buffer, boiled and electrophoresed on one-
573 dimensional 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The gels were
574 stained with Coomassie brilliant blue or used for protein blotting. In the latter case, the
575 proteins were electro-transferred from the polyacrylamide gel onto a nitrocellulose membrane.
576 The membrane was then incubated at room temperature for 30 min in blotting buffer (Tris
577 Buffer Saline [TBS] + 5% nonfat dry milk, w/v) and for 1h with a 1:100 dilution of anti-
578 alterocin rabbit antibodies in blocking buffer. After a brief washing with TBS, the membranes
579 were incubated with a 1:5000 dilution of alkaline phosphatase-conjugated antibodies against
580 rabbit immunoglobulin. The revelation was performed with Bromochloroindolyl phosphate
581 (BCIP) (Thermo Fisher Scientific, Bremen, Germany).

582 Anti-alterocin antibodies were produced by Eurogentec (Seraing, Belgium) using the
583 synthetic peptide P1 CEERGHNQEISGSTIT (Fig. 2B). This peptide was synthesized with
584 purity greater than 70%, conjugated to the carrier protein Keyhole Limpet Hemocyanin and
585 then injected into rabbits on days 0, 7, 10 and 18. Serum samples were collected on days 21
586 and 28. Antibody production was checked in the first serum sample with an ELISA test.
587 Antibody purification was performed on affinity column from the second serum sample. P1
588 was fixed on the AF-Amino matrix TOYO of the column. A volume of 5 ml of serum was
589 then loaded on the column and a washing with PBS was carried out. Antibodies attached to
590 the column were then eluted with 100 mM Glycine at pH 2.5. The antibodies were stored at -
591 20° C in a solution of 50% PBS buffer - 50% glycerol.

592

593 **Trypsin digestion and mass spectrometry sequencing of alterocin.** After SDS-
594 PAGE and Coomassie blue staining, the band of interest was cut out and the gel piece was

595 cut into smaller pieces, washed with distilled water and de-stained using acetonitrile. The
596 cysteine residues were reduced by 100 μ l of 10 mM DTT at 56°C and alkylated by 150 μ l of
597 55 mM iodoacetamide at room temperature. Iodoacetamide solution was replaced by 100 μ l
598 of 100 mM NH_4HCO_3 and gel dehydration was achieved with acetonitrile. After evaporation
599 in a Speed Vac apparatus (Thermo Fisher Scientific, Bremen, Germany), proteins were
600 digested overnight at 37°C by a solution containing 0.9 μ g of a modified porcine trypsin
601 (Promega, Madison, WI, USA) prepared in 25 mM NH_4HCO_3 . Finally, a double extraction
602 was performed, first with 5% (v/v) formic acid solution, and subsequently with 100% (v/v)
603 acetonitrile. The resulting peptide mixture was dried under vacuum and resuspended in 50 μ l
604 of 1% formic acid solution.

605 The peptides mixtures were analyzed by online nanoflow liquid chromatography
606 tandem mass spectrometry (LC-MS/MS) on an EASY-nLC IITM system (Proxeon, Odense,
607 Denmark) connected to the LTQ Orbitrap Discovery instrument (Thermo Fisher Scientific,
608 Bremen, Germany). 10 μ l of the peptide mixtures were concentrated onto the 2-cm pre-
609 analytical column (300- μ m inner diameter) packed with 5- μ m C_{18} beads (C18PepMap100,
610 Dionex). They were separated in a 15-cm analytical column (75- μ m inner diameter) packed
611 with 3- μ m C_{18} beads (AcclaimPepMap100, Dionex) with a 60-min gradient from 5% to 35%
612 acetonitrile in 0.1% formic acid. The effluent from the HPLC column was directly
613 electrosprayed into the mass spectrometer. The LTQ Orbitrap instrument was operated in
614 data-dependent mode to automatically switch between full scan MS and MS/MS acquisition.
615 Instrument control was through Tune 2.5.5 and Xcalibur 2.1. For the collision-induced
616 dissociation (CID)-MS/MS top5 method, full scan MS spectra (from m/z 400–2000) were
617 acquired in the Orbitrap analyzer after accumulation to a target value of 5e6 in the linear ion
618 trap with resolution $r = 30,000$. The 5 most intense peptide ions with charge states ≥ 2 were
619 sequentially isolated to a target value of 30,000 and fragmented in the linear ion trap by CID

620 with normalized collision energy of 35% and wideband-activation enabled. Ion selection
621 threshold was 500 counts for MS/MS, and the maximum allowed ion accumulation times
622 were 500 ms for full scans in the orbitrap and 200 ms for CID-MS/MS measurements in the
623 LTQ. An activation $q = 0.25$ and activation time of 30 ms were used. Standard mass
624 spectrometric conditions for all experiments were: spray voltage, 1.7 kV; no sheath and
625 auxiliary gas flow; heated capillary temperature, 200 °C; predictive automatic gain control
626 (AGC) enabled. Peptide sequences were deduced from the resulting fragment ion spectra
627 using the PEAKS software (Peaks Studio 5.3, Bioinformatics Solutions Inc.). The resulting
628 peptide sequences were submitted to Peaks Search option to perform protein identification
629 into Swissprot databases.

630

631 **DNA and protein sequence analyses.** The BPROM program
632 (<http://www.softberry.com/>) was used to predict σ^{70} -dependent promoters in the DNA
633 sequence lying upstream of the *alt* gene (41). The SignalP 5.0 server
634 (<http://www.cbs.dtu.dk/services/SignalP/>) and DISULFIND tool (<http://disulfind.dsi.unifi.it/>)
635 were used to predict a signal sequence and disulfide bridges, respectively, in the alterocin
636 protein (36, 37). The ProtParam tool on the ExPASy server
637 (<https://web.expasy.org/protparam/>) was used to analyze the alterocin protein sequence (64).
638 The protein Basic Local Alignment Search Tool (BLAST) program
639 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Conserved Domain Database (CDD)
640 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (38) were used to search for proteins
641 similar to alterocin and for functional domains within alterocin, respectively.

642 **Plasmid construction and introduction into *Pseudoalteromonas* sp. 3J3.** The *alt*
643 gene and its promoter region were PCR amplified as a DNA fragment extending from -129 to

644 +476 (relative to first possible mapped transcription initiation site [Fig. 5]) using
645 *Pseudoalteromonas* sp. 3J6 total DNA as template, the Q5[®] High-Fidelity DNA polymerase
646 (New England Biolabs, Ipswich, USA), and the primers Fpalt (5'-
647 TATgtcgacCTAAACTAGTGAATAAAGTCG-3') binding to bases -129 to -108 and Rpalt
648 (5'-TATgtcgacTTACTGACACTTTATAGTTGC-3') binding to bases +456 to +476,
649 respectively, the lower case letters corresponding to *SalI* restriction sites. The following step
650 were programmed: 94°C for 3 min, 35 cycles (94°C for 1 min, 51°C for 1 min, 72°C for 2
651 min), and 10 min at 72°C. The amplicon and the pOriT-4Em vector were both digested with
652 *SalI*, ligated and transformed into *E. coli* Top10 electrocompetent cells (Invitrogen, Carlsbad,
653 CA, USA). The resulting pOriTalt plasmid (Table 1) was verified by restriction analysis and
654 sequencing. *E. coli* GEB833 (55) was then transformed by pOriT-4Em and pOriTalt from
655 which these two plasmids were transferred into *Pseudoalteromonas* sp. 3J3 by conjugation, as
656 described by Wang *et al.* (65).

657

658 **Promoter mapping by 5' RACE-PCR.** Total RNAs were isolated from
659 *Pseudoalteromonas* sp. 3J6(pOriTalt) grown in VNSS medium by use of a MasterPure RNA
660 purification kit (Epicentre Biotechnologies, Madison, WI, USA). The 5' end of *alt* mRNA
661 was amplified using a 5'-RACE System for Rapid Amplification of cDNA Ends kit
662 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers
663 used for cDNA synthesis, the first PCR, and the second PCR were GSP1 *alt*
664 (5'-TTACTGACACTTTATAGTTGC-3'), GSP2 *alt*
665 (5'-CCATAATTGGTTATACCAATC-3'), and Nested GSP *alt*
666 (5'-CCCCACAAGTGATTGTACT-3'), respectively. The final PCR product of 5'-RACE
667 amplifications was sequenced (GATC Biotech, Cologne, Germany).

668

669 **mRNA assay by RT-qPCR.** RNA extraction, cDNA synthesis and quantitative PCR
670 were performed as previously described (66). The primers for the *alt* mRNA were 5'-
671 CTTTCAGCAAACACAATGGCA -3' and 5'- GCCTTGTCGTCCTCCACAT -3' and the
672 primers for the 16S rRNA used as endogenous control were 5'-
673 GACTGAGACACGGCCCAGAC -3' and 5'- GCAATATTCCCCACTGCTGC -3'. PCRs
674 were performed in triplicate, and the standard deviations were <0.15 threshold cycle (Ct).
675 Quantifications were obtained using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (67).

676

677 **Phylogenetic position of *Pseudoalteromonas* sp. 3J6 based on the 16S rRNA gene.**

678 We determined the phylogenetic position of *Pseudoalteromonas* sp. 3J6 among 92 other
679 *Pseudoalteromonas* strains (58 *Pseudoalteromonas* strains which have a genome available in
680 public databases and 34 taxonomically characterized *Pseudoalteromonas* type strains) and
681 one *Algicola bacteriolytica* strain as outgroup. The 16S rRNA sequences from the selected
682 species were aligned using the L-INS-i algorithm in MAFFT v.7 (68). The resulting multiple
683 sequence alignment was visualized using Bioedit v.7.0.5.3 (69) and non-aligned regions were
684 removed. A total of 1,341 nucleotide positions were used for the phylogeny, which was done
685 as in (61).

686

687 **ANI and genome phylogeny.** Pairwise genome distances were calculated under the
688 MicroScope platform using Mash, a method that estimates the mutation rate between two
689 sequences (70), after uploading onto the MicroScope platform the 58 *Pseudoalteromonas*
690 genome sequences which were available at the time of analysis (February 2019) on the NCBI
691 RefSeq complete genome section. The interest of using the Mash distance is that it strongly

692 correlates with the ANI, which allows to determine genomic relatedness between prokaryote
693 strains without having to perform the pairwise genome alignments or experimental DNA-
694 DNA hybridizations (71). The Mash distance and the ANI follow a relationship similar to the
695 equation: Mash distance $\approx 1 - \text{ANI}$. From the computed Mash distances, a tree was constructed
696 using a neighbor joining algorithm.

697

698 **Phylogeny of the alterocin gene and synteny comparison.** We searched for
699 homologs of the alterocin protein sequences of *Pseudoalteromonas* sp. 3J6 in PkGDB, the
700 database of the MicroScope platform, using the Blastp algorithm (similarity constrains: at
701 least 30% identity with a coverage of the query of at least 80%) and found 18
702 *Pseudoalteromonas* strains and the Oceanospirillales *Hahella chejuensis* KCTC 2396 strain
703 with an *alt* gene in their genome. A phylogenetic tree based on the 20 alterocin proteins
704 (including the 3J6 one) was then calculated with the *Hahella chejuensis* one as outgroup. The
705 alterocin protein sequences were aligned and non-aligned regions were trimmed as for the 16S
706 rRNA gene phylogeny. Evolutionary distances were computed using a Jones-Taylor-Thornton
707 evolutive model by assuming that a proportion of sites in the sequence are invariant while the
708 remaining sites are gamma-distributed (72). A total of 128 amino acid positions were used for
709 the phylogeny. In those 19 genomes, we searched for a set of homologous genes including the
710 *alt* gene with a same local organization (i.e. synteny conservation) as in the genome of
711 *Pseudoalteromonas* sp. 3J6 by using the MicroScope platform (63), with correspondence
712 relationships between genomes calculated using protein sequence similarity (parameters:
713 BlastP Bidirectional Best Hit or at least 30% identity on 80% of the shortest sequence).

714

715 **Statistical analyses.** *P*-values were determined using the Student's t-test when
716 comparing two groups or ANOVA when comparing more than two groups. The GraphPad
717 Prism and Origin softwares were used.

718

719 **Data availability.** The annotated part of *Pseudoalteromonas* sp. 3J6 genome including
720 the *alt* gene was deposited in the European Nucleotide Archive (ENA;
721 <https://www.ebi.ac.uk/ena>) under the accession number PRJEB37533.

722

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849 *Paraglaciecola* gen. nov. as *Paraglaciecola mesophila* comb. nov., *P. agarilytica* comb.
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958 **Figure legends**

959 **FIG 1** Antibiofilm activity of *Pseudoalteromonas* sp. 3J6 exoproducts (SN_{3J6}) on *P.*
960 *aeruginosa* clinical strains. *P. aeruginosa* MUC-N1 (A) and MUC-N2 (B) biofilms obtained
961 without SN_{3J6} (Control or C) or in the presence of SN_{3J6} (+ SN_{3J6}) during the attachment step.
962 Biofilms were then grown for 24h under a flow of LB medium and bacteria were stained with
963 SYTO 9 green, β -polysaccharides (including alginate) were visualized with Calcofluor white
964 (blue), and eDNA was stained with DDAO (red). Top views of the biofilms are displayed.
965 The white bars represent 50 and 30 μ m on the left and middle panels, respectively. The
966 biovolumes were determined by analyzing at least three image stacks from each of three
967 independent biofilms (at least nine stacks in total) using the COMSTAT software.
968 ***P<0.001 using Student's t-test.

969 **FIG 2** The *alt* gene locus and the alterocin protein. A. The *alt* gene encoding alterocin and the
970 surrounding genes are represented in red and grey, respectively, with the arrow head
971 indicating the transcription orientation. The gene lengths include the stop codons. B.
972 Sequence of the alterocin protein. The vertical arrow indicates a predicted cleavage site which
973 would allow the release of the protein (numbered +1 to +119) from its signal peptide (-20 to -
974 1). The four cysteine residues are underlined. Residues on a colored background are highly
975 conserved: they are found in all 19 alterocin homologs from the Prokaryotic Genome
976 DataBase (grey) or in all but one of these 19 homologs (yellow). The sequences underlined in
977 blue correspond to the seven amino acid sequences which were determined from the partially
978 purified alterocin extracted from an SDS-PAGE gel. The sequence underlined in red
979 corresponds to the peptide P1 which was synthesized to generate anti-alterocin antibodies.

980 **FIG 3** Antibiofilm activity of *Pseudoalteromonas* sp. 3J3(pOriTalt) exoproducts compared to
981 *Pseudoalteromonas* sp. 3J3(pOriT) exoproducts. After an attachment step in the presence of

982 culture supernatant of *Pseudoalteromonas* sp. 3J3(pOriT) (– *alt*) or of *Pseudoalteromonas* sp.
983 3J3(pOriTalt) (+ *alt*), *P. aeruginosa* MUC-N1 biofilms were grown for 24 h, stained with
984 SYTO 9 green, and observed by confocal laser scanning microscopy. A. 3D views of the
985 biofilms. The white bars represent 10 μ m. B. Biofilm biovolumes and average thicknesses
986 determined by analyzing at least three image stacks from each of three independent biofilms
987 (at least nine stacks in total) using the COMSTAT software. **P<0.01; ***P<0.001 using
988 Student's t-test.

989 **FIG 4** Alterocin detection in culture supernatants of *Pseudoalteromonas* sp. 3J3 carrying the
990 *alt* gene and *Pseudoalteromonas* sp. 3J6. A. Alterocin was extracted from culture supernatants
991 of *Pseudoalteromonas* sp. 3J3(pOriT) (strain without *alt* gene, lane noted “– *alt*”),
992 *Pseudoalteromonas* sp. 3J3(pOriTalt) (strain carrying the *alt* gene, lane noted “+ *alt*”), and the
993 wild type alterocin producer *Pseudoalteromonas* sp. 3J6, and the extracts were analyzed by
994 SDS-PAGE. M: molecular weight markers. B. Western blot analyses of the same extracts as
995 in A, using anti-alterocin antibodies.

996 **FIG 5** Mapping of the *alt* promoter. The transcriptional start site (+1) was identified by 5'-
997 RACE PCR, as shown by the relevant part of the chromatogram displaying the PCR product
998 sequence. There are three potential +1 bases (underlined GGC bases with vertical lines
999 reaching an arrow indicating the transcription direction) and the sequence is numbered +1
1000 from the first of the two Gs. The sequences noted -35 and -10 are proposed to constitute the
1001 σ^{70} -dependent promoter yielding the mRNA starting at the +1 bases. The sequences noted P-
1002 35 and P-10 were predicted by BPROM to constitute a promoter. The horizontal arrows
1003 indicate inverted repeat sequences. RBS is the ribosome binding site, and the first codon of
1004 the *alt* gene is translated (M).

1005 **FIG 6** Expression of the *alt* gene in the course of *Pseudoalteromonas* sp. 3J6 growth. A.
1006 Growth curve of *Pseudoalteromonas* sp. 3J6 representative of four different experiments. B.
1007 Relative *alt* mRNA levels quantified by RT-qPCR from total ARN extracted at 5, 10 and 24h
1008 of growth, compared to the level at 3h. * P<0.05 using the ANOVA test on four different
1009 experiments.

1010 **FIG 7** Phylogenetic tree of the *Gammaproteobacteria* based on the alterocin protein
1011 sequences. Only bootstrap values above 50% are shown. The gene label from the MicroScope
1012 annotation corresponding to each putative alterocin is indicated in parentheses. The eight
1013 *Pseudoalteromonas* strains that show a similar gene synteny around the *alt* gene in their
1014 genome to that of *Pseudoalteromonas* sp. 3J6 are in bold (Fig. S2). Non-pigmented
1015 *Pseudoalteromonas* strains (16, 18) are in orange boxes. Bar, 0.2 substitution per amino acid
1016 position.

1017

1018 **TABLE 1** Strains and plasmids used in this study.

Strain or plasmid	Characteristics ^a	Source or reference
<i>Pseudoalteromonas</i> sp.		
3J6	Wild type alterocin producer strain	19
3J3	Wild type strain devoid of antibiofilm activity and not sensitive to alterocin, Amp ^S	19
3J3(pOriT)	3J3 strain containing the pOriT-4EM vector, Amp ^R	This work
3J3(pOriTalt)	3J3 strain containing the pOriTalt plasmid, Amp ^R	This work
3J6(pOriTalt)	3J6 strain containing the pOriTalt plasmid, Amp ^R	This work
<i>P. aeruginosa</i>		
MUC-N1	Mucoid clinical strain, biofilms with mushroom-like structures, alginate predominant in matrix	27, Simon et al. ^b
MUC-N2	Nonmucoid clinical strain, flat biofilms with Psl and eDNA as main matrix components	27, Simon et al. ^b
MUC-P4	Nonmucoid clinical strain, biofilms with a few 3D structures, eDNA predominant in matrix	27, Simon et al. ^b
MUC-P5	Nonmucoid clinical strain, thin biofilms with filamentous cells	27, Simon et al. ^b
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> λ-	Invitrogen
GEB883	<i>E. coli</i> K12 Δ <i>dapA::erm pir RP4-2 ΔrecA gyrA462, zei298::Tn10</i> . DAP ⁻ strain used for cloning and as a donor strain for conjugation	55
Plasmids		

pOriT-4Em	<i>E. coli-Pseudoalteromonas</i> shuttle vector, Amp ^R Em ^R	73
pOriTalt	pOriT-4Em carrying a 605-pb DNA fragment including the <i>alt</i> gene and its promoter region, Amp ^R Em ^R	This work

1019

1020 ^a Amp, ampicillin; Em, erythromycin; DAP, diaminopimelate.

1021 ^b M. Simon, E. Pernet, A. Jouault, E. Portier, A. M. Boukerb, S. Pineau, J. Vieillard, E.

1022 Bouffartigues, C. Poc-Duclairoir, M. G. J. Feuilloley, O. Lesouhaitier, J. Caillon, S.

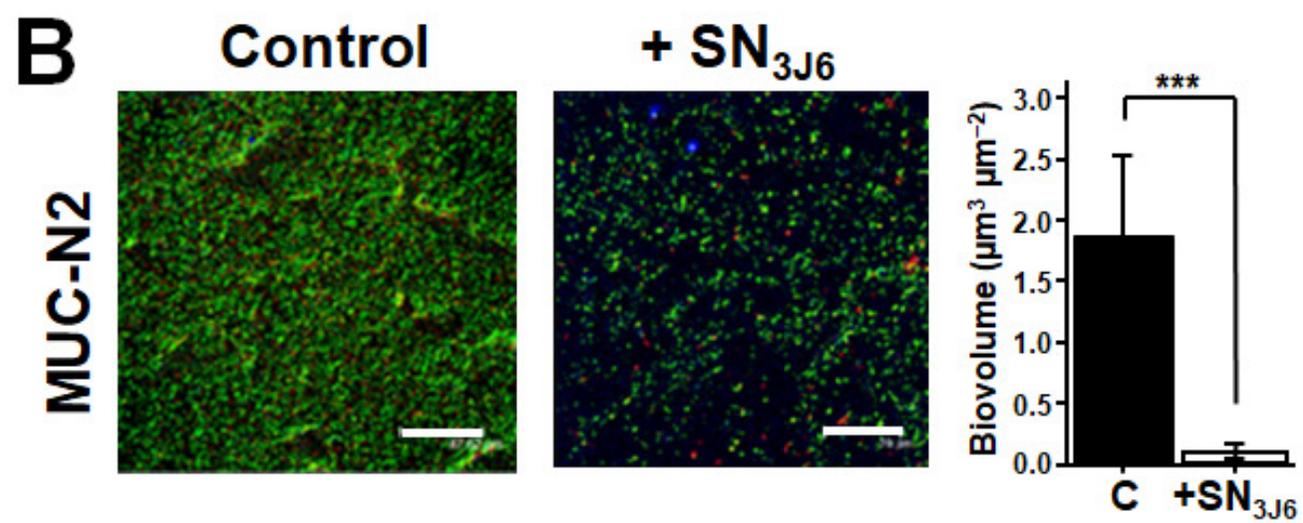
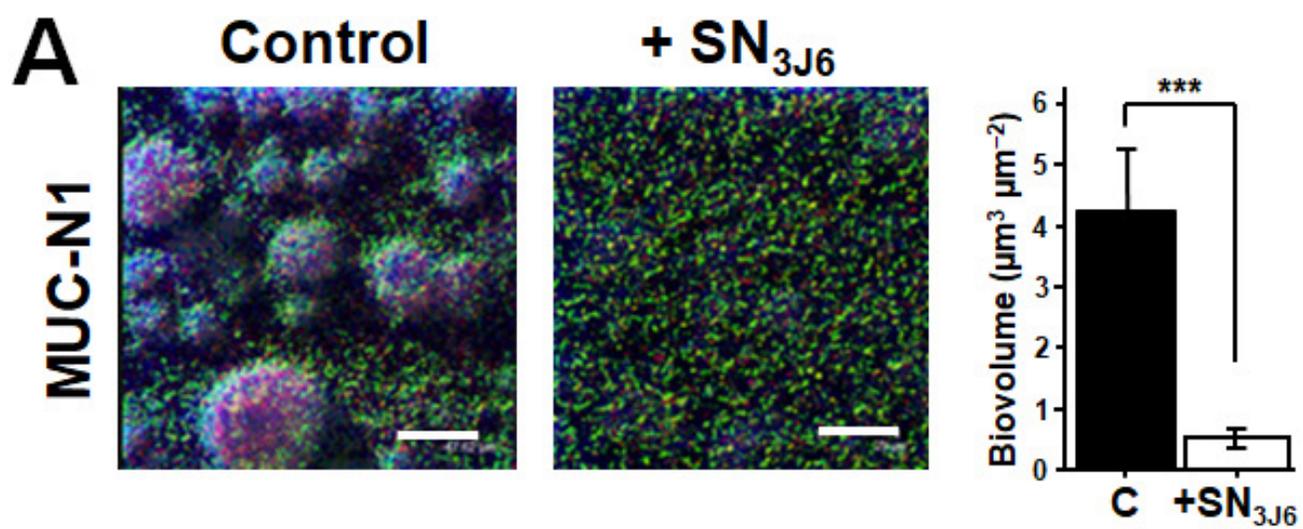
1023 Chevalier, A. Bazire, and A. Dufour, submitted for publication

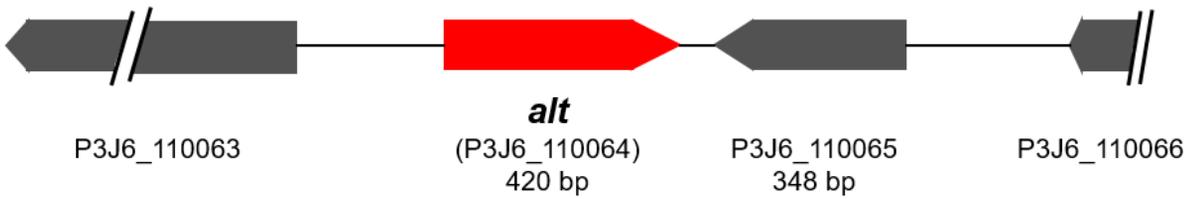
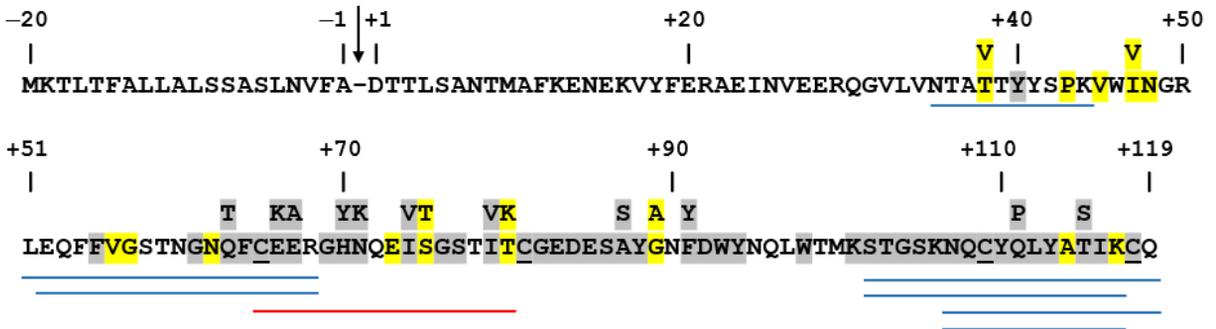
1024

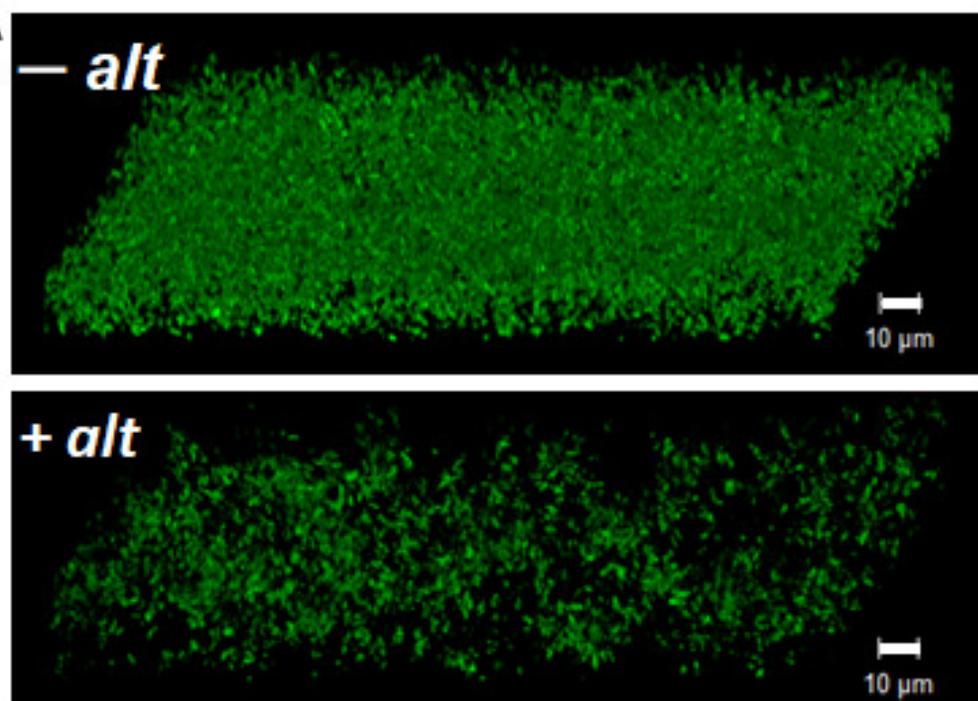
1025 **TABLE 2** General characteristics of the *Pseudoalteromonas* sp. 3J6 genome.

1026

Characteristic	Value
Size	4,099,791 bp
GC content	39.93%
Number of contigs	12
Total number of CDSs	3,789
Average CDS length	962.9 bp
Protein coding sequences	88.55%
Number of rRNA operon	1
Number of tRNAs	79



A**B**

A**B**