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1 **Antimicrobial properties of *Pseudomonas* strains producing the antibiotic mupirocin**

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20

21 **Abstract**

22 Mupirocin is a polyketide antibiotic with broad antibacterial activity. It was isolated
23 and characterized about 40 years ago from *Pseudomonas fluorescens* NCIMB 10586. To
24 study the phylogenetic distribution of mupirocin producing strains in the genus *Pseudomonas*
25 a large collection of *Pseudomonas* strains of worldwide origin, consisting of 117
26 *Pseudomonas* type strains and 461 strains isolated from different biological origins, was
27 screened by PCR for the *mmpD* gene of the mupirocin gene cluster. Five *mmpD*⁺ strains from
28 different geographic and biological origin were identified. They all produced mupirocin and
29 were strongly antagonistic against *Staphylococcus aureus*. Phylogenetic analysis showed that
30 mupirocin production is limited to a single species.

31 Inactivation of mupirocin production leads to complete loss of *in vitro* antagonism
32 against *S. aureus*, except on certain iron-reduced media where the siderophore pyoverdine is
33 responsible for the *in vitro* antagonism of a mupirocin-negative mutant. In addition to
34 mupirocin some of the strains produced lipopeptides of the massetolide group. These
35 lipopeptides do not play a role in the observed *in vitro* antagonism of the mupirocin producing
36 strains against *S. aureus*.

37
38 **Key words:** mupirocin; massetolide; pyoverdine, *Pseudomonas fluorescens* NCIMB 10586;
39 *Pseudomonas* sp. W2Aug9

40

41 1. Introduction

42 Mupirocin is a polyketide antibiotic which has been isolated and characterized from
43 the soil bacterium *P. fluorescens* NCIMB 10586 [1]. In fact mupirocin is a mixture of four
44 pseudomonic acids (A-D). The basic structure of mupirocin comprises a monic acid (a
45 heptaketide) containing a pyran ring, attached to 9-hydroxynonanoic acid via an ester linkage
46 [2]. Mupirocin has a broad spectrum activity against both Gram-positive and Gram-negative
47 bacteria, although most Gram-negative bacteria tested are less susceptible than Gram-positive
48 bacteria [3]. The antibiotic acts through the inhibition of bacterial isoleucyl-tRNA synthetase
49 [4]. Mupirocin is currently used topically for the treatment of skin infections, impetigo and for
50 the decolonization of patients with nasal carriage of *Staphylococcus* [5].

51 Although the biosynthetic pathway and the antimicrobial activity of mupirocin have
52 been extensively studied [6], the strain producing this antibiotic is not well characterized. The
53 taxonomic position of strain *P. fluorescens* NCIMB 10586 is not known since its 16S rRNA
54 gene nor any other housekeeping gene have been sequenced yet. It is also not known whether
55 the ability to produce mupirocin is widespread throughout the genus *Pseudomonas* or limited
56 to only a few species. Besides *P. fluorescens* NCIMB 10586, another two mupirocin
57 producing strains, strain D7 and G11 isolated from groundwater sediment samples, have been
58 reported [7]. Both strains were allocated to the *P. fluorescens* group [7] based on their 16S
59 rRNA sequence.

60 The aim of this study was dual; first to assess the distribution of mupirocin producing
61 *Pseudomonas* spp. throughout the genus *Pseudomonas* and to subsequently study their
62 diversity. Therefore a specific mupirocin primer set was developed based on the *mmpD* gene.
63 This multifunctional gene is involved in the synthesis of the backbone of monic acid. A large
64 collection of *Pseudomonas* type strains and partially identified environmental strains
65 representing a high phylogenetic diversity was screened using the mupirocin primer set.

66 Mupirocin production was confirmed through isolation and identification of the antibiotic and
67 *in vitro* antagonism tests against *Staphylococcus aureus*. The second part of the work focused
68 on the mupirocin producing strains themselves whereby it was investigated whether the
69 strains produce additional well-known antimicrobial metabolites and extracellular enzymes to
70 gain insight in their antimicrobial potential. The respective role of the antimicrobial
71 compounds in the *in vitro* antagonism against *S. aureus* was further investigated via
72 transposon mutagenesis.

73

74 **2. Material and Methods**

75 *2.1. Bacterial strains*

76 A total of 578 strains and isolates from different biological origins were studied. These
77 included 117 type strains of the genus *Pseudomonas* (listed in Supplementary Table S1) and a
78 collection of 198 *Pseudomonas* isolates from the River Woluwe, which were isolated from the
79 source (site W2) and the mouth of the river (site W15) (Supplementary Table S2 in [8]). In
80 addition, a collection of 48 *Pseudomonas* non type strains obtained from different culture
81 collections (Supplementary Table S2) were included together with 215 strains and isolates
82 received from different laboratories (Supplementary Table S3).

83

84 *2.2. Growth conditions*

85 The *Pseudomonas* strains (Table 1) were routinely grown at 28°C on an in house
86 medium, medium 853, composed of 10 g l⁻¹ bacto tryptone, 5 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl, 1
87 g l⁻¹ glucose, 0.7 g l⁻¹ K₂HPO₄ and 0.3 g l⁻¹ KH₂PO₄. *Escherichia coli* SM10 (λpir) was grown
88 in 853 at 37°C. Gentamicin (Gm) was used at a concentration of 50 µg ml⁻¹ (*P. fluorescens*
89 NCIMB 10586) or 200 µg ml⁻¹ (*Pseudomonas* sp. W2Aug9), chloramphenicol (Cm) was used
90 at a concentration of 25 µg ml⁻¹. Antagonism tests were performed on 4 different media;

91 medium 853, blood agar (BBL™ Blood agar Base [Becton, Dickinson and company] with 5
92 % of defibrinated sheep blood), Mueller-Hinton agar (MH) (Difco Laboratories) and GCA
93 medium [9]. When required GCA was supplemented with FeCl₃ to a final concentration of
94 100 µM (GCA+Fe medium). Pyoverdine detection and purification was done on iron-poor
95 casamino acids (CAA) medium [10].

96

97 2.3. Screening for mupirocin producing strains by PCR

98 During a PCR screening of 195 *Pseudomonas* strains for the antibiotic pyoluteorin
99 with the primer set PltBf and PltBr [11] two false positives were picked up of which the
100 nucleotide sequence aligned with part of the *mmpD* gene sequence (position 21309-22090) of
101 the mupirocin gene cluster of *P. fluorescens* NCIMB 10586 (accession number AF318063).
102 Comparing the sequence of the PltBf-r primer set with the complementary sequence of the
103 *mmpD* gene of *P. fluorescens* NCIMB 10586 showed that there was only 1 mismatch with the
104 PltBf primer and several for the PltBr primer (Supplementary Fig. S1). The combination of
105 one almost perfect primer with the relatively low annealing temperature during the
106 amplification can explain why *mmpD* fragments were obtained during the screening with the
107 pyoluteorin primer set. A specific mupirocin primer set (MUP-F1: 5'-
108 CGGATCATGGACCCCCAGC-3' and MUP-R1: 5'-CAGGCCTTGGATCTCGATAG-3')
109 was developed on the basis of the PltBf primer and the *mmpD* sequences of *P. fluorescens*
110 NCIMB 10586 and the 2 candidates obtained through the screening with the pyoluteorin
111 primer set. They amplify a fragment of 717 bp. All 578 strains and isolates were screened
112 with MUP-F1 and MUP-R1. The PCR was carried out in a final volume of 25 µl containing
113 PCR buffer (Qiagen) with 0.625 U *Taq* DNA polymerase (Qiagen), 5 µl Q-solution (Qiagen),
114 the deoxynucleotide mixture at 100 µM (Fermentas), each of the primers at 0.5 µM (Sigma)
115 and 1-2 µl template DNA. Template DNA for PCR reaction was prepared by inoculating a

116 fresh colony in 25 μ l water and heating it for 5 min at 95°C. The PCR program used was an
117 initial denaturation of 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s,
118 annealing at 50°C for 30 s and extension at 72°C for 50 s, followed by an incubation for 10
119 min at 72°C. *P. fluorescens* NCIMB 10586 was used as positive control. The obtained PCR
120 fragments were sequenced directly using the amplification primers at Beckman Coulter
121 Genomics (UK). The accession numbers of the *mmpD* fragment of *P. fluorescens* LMG
122 14677, *Pseudomonas* sp. W2Aug9, W2Jun17, W15Feb34 and B329 are KJ528554,
123 KJ528555, KJ528556, KJ528557 and KJ528558, respectively.

124

125 2.4. Mupirocin production

126 Mupirocin was purified from the culture supernatant as described in [12]. The
127 presence of the antibiotic was determined by HPLC with a reverse-phased C18 Altima
128 (GRACE) column (250 mm x 4.6 mm, 10 μ M), using the conditions as described in [12].
129 Mupirocin was detected at 27.6 min. Pure mupirocin (Sigma) was used as control.

130

131 2.5 PCR for the tailoring enzymes of the mupirocin gene cluster

132 To detect the genes responsible for the maturation of pseudomonic acid A in the five
133 mupirocin producing strains 26 primer sets (Supplementary Table S4) were developed to
134 amplify part of these genes. PCR was done with DreamTaq Green (Thermo Scientific) using
135 the same conditions as for the mupirocin PCR. *P. fluorescens* NCIMB 10586 was used as
136 positive control. For primer set mupU-F/R the PCR was done at 59°C since lower
137 temperatures gave an amplicon of lower size for *Pseudomonas* sp. W2Aug9, W2Jun17 and *P.*
138 *fluorescens* LMG 14677.

139

140 2.6. 16S rRNA and rpoB gene amplification and sequencing

141 The almost complete 16S rRNA gene sequence (position 29 to 1522 in *E. coli*) of the
142 *mmpD*⁺ *Pseudomonas* strains was amplified with primers pA and pH [13]. The PCR mix was
143 prepared using the same conditions as described for the mupirocin PCR with the difference
144 that the PCR was carried out in a final volume of 100 µl and the amount of template was
145 increased to 3 µl. The PCR program used was an initial denaturation of 2 min at 94°C,
146 followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and
147 extension at 72°C for 90 s, followed by an incubation for 10 min at 72°C. The accession
148 numbers of *P. fluorescens* NCIMB 10586, *P. fluorescens* LMG 14677, *Pseudomonas* sp.
149 W2Aug9, *Pseudomonas* sp. W2Jun17, *Pseudomonas* sp. W15Feb34 and *Pseudomonas* sp.
150 B329 are KJ528545, GU198127, KJ528546, KJ528547, EU681017, KJ528548, respectively.
151 The housekeeping gene *rpoB* was amplified using primers LAPS and LAPS27 [14] using the
152 same conditions as for the mupirocin PCR, except that the elongation time was increased to 1
153 min 20 s. The obtained PCR fragments were purified and sequenced at Beckman Coulter
154 Genomics (UK) using amplification and internal primers (for 16S rRNA).

155

156 2.7. Phylogenetic analysis

157 Phylogenetic analysis based on almost complete 16S rRNA gene sequences and partial
158 *rpoB* genes were performed using CLUSTALX and MEGA v5.0 [15]. The neighbor-joining
159 method was used with the Jukes-Cantor model and topological robustness was evaluated by
160 bootstrap analysis based on 1.000 replicates. The 16S rRNA gene sequences of the type
161 strains and the *rpoB* gene of *P. aeruginosa* LMG 1242^T were taken from GenBank.

162

163 2.8. Antibiotic and HCN production

164 Detection of the genes responsible for the production of hydrogen cyanide (HCN) [16]
165 and antibiotics was done by PCR using gene-specific primers. The antibiotics of interest are

166 2,4-diacetylphloroglucinol [17], pyrrolnitrin [17] and phenazine-1-carboxylic acid [18]. The
167 same conditions as for the mupirocin PCR were used except that the elongation time was
168 adapted to the size of the fragment. *P. protegens* Pf-5 was used as positive control.

169

170 2.9. Protease production

171 Protease activity was detected as clearing zones on skim milk agar plates [19].

172

173 2.10. Pyoverdine production

174 Pyoverdine production was verified by measuring the OD at 405nm of the culture
175 supernatant of the cells grown in CAA medium for 48 h at 28°C, 200 RPM. The pyoverdine
176 was semi-purified as described in [20] and identified through a combination of mass analysis
177 and IEF [20].

178

179 2.11. Production and identification of lipopeptides

180 Surfactant production was verified through the drop collapse assay. Therefore
181 bacterial cells were grown for 24h and 10 µl droplets of the culture or the culture supernatant
182 were spotted on parafilm. A flat droplet was indicative of lipopeptide production. Hemolytic
183 activity was detected as a lysis zone surrounding colonies grown for 3 days at 28°C on blood
184 agar.

185 Lipopeptides were analyzed from supernatant samples with a UPLC (Acquity H-class,
186 Waters s.a., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD
187 mass analyzer) on a ACQUITY UPLC® BEH C₁₈ 1.7 µm column. Ten µl was injected and
188 elution was performed at 40°C with a constant flow rate of 0.6 ml min⁻¹ using a gradient of
189 acetonitrile in water both acidified with 0.1% formic acid as follows: 0.5 min at 0%, from 0%
190 to 15% in 2 min, from 15% to 95% in 5 min and maintained at 95% for 1.8 min. Compounds

191 were detected as protonated molecular ions detected in electrospray positive ion mode (scan
192 in the mass range m/z 950-1250) by setting SQD parameters as follows: source temperature
193 130°C; desolvation temperature 300°C, and desolvation/cone nitrogen flow: 1000/50 l h⁻¹. For
194 optimal detection, the cone voltage was set at 80V. The same conditions were used for
195 analysis of lipopeptide in source-fragmentation but the cone voltage was increased to 120V.

196

197 2.12. *In vitro* antagonism assays against *S. aureus*

198 The *in vitro* antagonism of the mupirocin producing *Pseudomonas* strains against *S.*
199 *aureus* 383, a clinical strain isolated from infected femoral pin [21] was tested on 853 agar,
200 blood agar, MH agar, GCA and GCA amended with 100 µM Fe. Five µl of OD₆₀₀ = 0.5 of the
201 *Pseudomonas* strain was inoculated in the center of the plate. The plates were incubated at
202 28°C and after 2 days the cells were killed using chloroform vapors for 25 min. After
203 evaporation of the chloroform a 6 ml overlay of soft agar (0.7% agar) of 853 with 5 x 10⁶
204 cells of *S. aureus* 383, was overlaid on the plate which was subsequently incubated at 37°C.
205 Clear zones of inhibition were measured the next day. All antagonism assays were performed
206 at least two times with 3 replicas.

207

208 2.13. *Transposon mutagenesis and screening for a hemolytic-negative mutant of* 209 *Pseudomonas sp. W2Aug9*

210 The suicide plasmid pUT, which harbors the transposon mini-Tn5 $phoA3$ (Gm^R) [22]
211 was used to generate transposon insertions in the chromosome of *Pseudomonas sp. W2Aug9*.
212 Mid-log phase cultures of *E. coli* SM10 (λ pir), the host of pUT-mini-Tn5 $phoA3$, was mixed
213 with strain W2Aug9 in a 1:1 ratio. The *Pseudomonas* strain was kept at 37°C for 1 h just
214 before mixing with *E. coli* in order to inactivate its restriction system. After overnight
215 incubation on 853 at 28°C, Tn5 insertions were selected on CAA supplemented with 25 µg

216 ml⁻¹ Cm and 200 µg ml⁻¹ Gm. A small bank of 468 transconjugants was established to screen
217 for mutants showing complete loss of hemolytic activity by inoculating with a toothpick
218 mutants on a blood agar plate followed by incubation for 2 days at 28°C. Hemolytic-negative
219 candidates were subsequently verified for protease production.

220

221 *2.14. Transposon mutagenesis and screening for an antagonism-negative mutant of*
222 *10586ΔAT2*

223 An antagonism-negative mutant of the mupirocin-negative strain 10586ΔAT2 of *P.*
224 *fluorescens* NCIMB 10586 was obtained through Tn5 mutagenesis on 10586ΔAT2 using
225 mini-Tn5*phoA3* as described in 2.13, with the modification that the transconjugants were
226 plated on a lower Gm concentration (50 µg ml⁻¹). A library of 2000 candidates was screened
227 for complete loss of *in vitro* antagonism against *S. aureus* 383. Therefore the candidates were
228 grown overnight at 28°C in medium 853 in microtiter plates whereby every two wells were
229 inoculated, alternating between an inoculated and not inoculated well. The cultures were
230 replicated on 853 medium and incubated for 16h at 28°C. An overlay of *S. aureus* 383 was
231 prepared as explained in paragraph 2.12.

232

233 *2.15. Transposon mutagenesis and screening for a pyoverdine-negative mutant of P.*
234 *fluorescens NCIMB 10586*

235 A pyoverdine-negative mutant of *P. fluorescens* sp. NCIMB 10586 was obtained
236 through Tn5 mutagenesis using mini-Tn5*phoA3* as described in 2.13. Tn5 insertions were
237 selected on CAA supplemented with 50 µg ml⁻¹ Gm and 25 µg ml⁻¹ Cm. A bank of 576
238 transconjugants was screened for complete loss of fluorescence in the iron-limiting media
239 CAA.

240

241 2.16. Molecular characterization of the *Tn5* mutants

242 The chromosomal DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen),
243 digested with *Pst*I or *Sal*I (Fermentas) and self-ligated. The DNA flanking the mini-*Tn5**phoA3*
244 was isolated and sequenced as described earlier [23].

245

246 3. Results

247 3.1. Screening for mupirocin producing strains

248 578 *Pseudomonas* strains were screened by PCR for presence of part of the *mmpD*
249 gene fragment. Five strains (0.9% of the collection) gave an amplicon. Three of these strains
250 (W2Aug9, W2Jun17 and W15Feb34) were from the Woluwe River collection [8]. In addition
251 the *mmpD* gene fragment was amplified from *P. fluorescens* LMG 14677 and *Pseudomonas*
252 sp. B329, a grass rhizosphere isolate. Sequencing of the *mmpD* fragments of the 5 candidates
253 and alignment with the gene of *P. fluorescens* NCIMB 10586 revealed that *Pseudomonas* sp.
254 W15Feb34 and B329 have identical sequences to NCIMB 10586, W2Aug9 has a C→T
255 transition at position 255 of the amplified fragment and *P. fluorescens* LMG 14677, W2Jun17
256 and W2Aug9 an A→G transversion at position 489. These differences do not alter the amino
257 acid sequence.

258 Purification of mupirocin from these strains and analysis by HPLC confirmed that they
259 all produce the antibiotic. Mass analysis of the obtained peaks identified pseudomonic acid A
260 and B for all the strains (Supplementary Fig. S2). In contrast to what has been reported
261 previously [6] whereby pseudomonic acid B represented only a minor fraction of the mixture
262 (8%), higher levels of pseudomonic acid B were found (relative abundance between 29 and
263 61%) in all producing strains including *P. fluorescens* NCIMB 10586. This is probably due to
264 differences in growth conditions and/or media. PCR with primers specific for the tailoring
265 genes of the mupirocin gene cluster (results not shown) showed that all the strains share the

266 same genes for the maturation of pseudomonic acid A, including the quorum
267 sensing/quenching system encoded in the gene cluster.

268 The amount of mupirocin produced in the culture supernatant was between 1-15 $\mu\text{g/ml}$
269 depending of the producing strain; *P. fluorescens* LMG 14677 and W15Feb34 produced
270 similar amounts, 6.7 and 5.8 $\mu\text{g/ml}$ respectively, as *P. fluorescens* NCIMB 10586 (8.3 $\mu\text{g/ml}$).
271 B329 produced about half of the amount (4.5 $\mu\text{g/ml}$) of NCIMB 10586. Relatively low
272 concentrations were detected for W2Jun17 which produced only about 1/10 of the amount
273 observed for NCIMB 10586 (1.0 $\mu\text{g/ml}$). W2Aug9 showed the highest level of mupirocin
274 production, producing twice as much as NCIMB 10586 (15.2 $\mu\text{g/ml}$). These values are in the
275 same range as those reported for strains D7 and G11 (1.8-3.5 $\mu\text{g/ml}$) [7]. The differences in
276 mupirocin production between the strains were not reflected in their degree of *in vitro*
277 antagonism against *S. aureus* which were similar. The antagonism of NCIMB 10586 and
278 W2Jun17 showed the lowest level (24.8 ± 0.5 mm and 24.4 ± 0.5 mm, respectively), followed
279 by LMG 14677 (25.7 ± 1.2 mm), W15Feb34 (26.7 ± 1.0 mm) and W2Aug9 (27.3 ± 1.3 mm).
280 The largest inhibition zone was observed for B329 (29.0 ± 0.5 mm). The strains showing the
281 lowest level of antagonism were the strains that do not produce lipopeptides of the
282 massetolide group, these results indicate that production of massetolide might slightly
283 increase *in vitro* antagonism.

284

285 3.2. Identification of mupirocin producing strains

286 Alignment of the 16S rRNA sequences showed that all the mupirocin producing
287 strains had identical sequences. Inclusion of the 16S rRNA genes of strain D7 and G11 [7] in
288 the alignment showed that both strains had also the same 16S rRNA sequence (after
289 truncating the 5' and 3'-end by maximum 14 nucleotides to remove evident sequencing
290 errors) showing that in fact all mupirocin producing strains belong to the same species.

291 To identify the strains a phylogenetic tree was constructed based on the 16S rRNA
292 nucleotide sequence of the selected representative strain *P. fluorescens* NCIMB 10586 and a
293 selection of *Pseudomonas* type strains from the *P. fluorescens* group. *P. fluorescens* NCIMB
294 10586 belongs to the *P. fluorescens* group and the closest type strain to strain NCIMB 10586
295 is *P. azotoformans* IAM 1603 with a bootstrap value of 53% (Fig. 1.A.). The closest relatives
296 were *P. cedrina* subsp. *cedrina* CFML 96-198^T, *P. cedrina* subsp. *fulgida* DSM 14938^T, *P.*
297 *libanensis* CIP 105460^T and *P. synxantha* IAM 12356^T (Fig. 1.A.).

298 The intraspecific diversity of the mupirocin producing strains was assessed on the
299 basis of the sequence of the housekeeping gene *rpoB*. A tree was constructed using the same
300 methodology as for 16S rRNA. The strains formed a clear separate cluster and a unique *rpoB*
301 sequence was found for each strain (Fig. 1.B.).

303 3.3. Production of antimicrobial metabolites and protease

304 The six mupirocin producing strains were screened for additional well-known
305 antimicrobial metabolites and protease. They were screened by PCR for HCN and the
306 antibiotics 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and phenazine-1-carboxylic
307 acid. In the conditions used no amplification was obtained for any of these compounds. All
308 the mupirocin strains produced protease and had similar clear zones around the colony. They
309 all produced the siderophore pyoverdine. Pyoverdines are made of three distinct structural
310 parts: a small peptide chain of 6 to 14 L- and D-amino acids, linked to a yellow-green
311 chromophore group and to a small dicarboxylic acid [24]. Mass analysis showed that all
312 strains produced the same pyoverdine PYO₁₃₅₂₅, with a peptide chain of 7 amino acids with
313 sequence Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser), the parentheses indicate a cyclic
314 structure [25]. This pyoverdine is relatively common and is produced by many strains of the
315 *P. fluorescens* group (Matthijs S., unpublished results).

316

317 *3.4. Several mupirocin producing strains produce lipopeptides of the massetolide group*

318 The positive drop collapse test observed for three of the six strains, namely

319 *Pseudomonas* sp. W2Aug9, *Pseudomonas* sp. B329, and *Pseudomonas* sp. W15Feb34

320 suggested that those isolates may produce compounds with surface tension reducing activity.

321 In addition, together with *P. fluorescens* LMG 14677, they displayed hemolytic activity when

322 tested on red sheep blood cells. Strain W2Aug9 had the largest activity followed by

323 *Pseudomonas* sp. B329, *P. fluorescens* LMG 14677 and *Pseudomonas* sp. W15Feb34. Those

324 phenotypes prompted us to check for possible production of surfactants by these strains.

325 UPLC-MS analysis of culture supernatants revealed that strains W2Aug9, B329, LMG 14677

326 and W15Feb34 but not NCIMB 10586 and W2Jun17 secreted cyclic lipopeptides with major

327 peaks observed at m/z 1126.7, 1140.7, 1154.7, 1168.7 for molecular ions (Fig. 2,

328 Supplementary Fig. S3). These molecular ions correspond to the massetolide group of

329 *Pseudomonas* lipopeptides and similar masses and retention times were observed by injecting330 purified massetolides from *P. fluorescens* SS101 [26, 27, 28]. Further analysis of product ions

331 yielded upon in-source fragmentation of those molecular species confirmed that they

332 correspond to massetolides A/D, L and F/viscosin with either C₁₀ or C₁₂ fatty acid moieties.

333 Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the

334 fatty acid. The occurrence of several derivatives of the lipopeptide reflects some flexibility in

335 amino acid selection and activation by the A domains of the massetolide synthetases.

336 When comparing the level of *in vitro* antagonism of the mupirocin producing strains337 on the different media against *S. aureus* it was observed that strains producing lipopeptides

338 generally showed a weak, but reproducible, larger zone of inhibition in the antagonism against

339 *S. aureus* than strains not producing lipopeptides. To verify whether there was a synergistic

340 effect a hemolytic-negative mutant was created in strain W2Aug9 which showed the largest

341 hemolytic activity. Therefore a Tn5 bank of W2Aug9 was screened for complete loss of
342 hemolytic activity on blood agar. In total 14 hemolytic-negative candidates were obtained. All
343 the candidates were negative in a drop collapse test showing loss of surfactant production.
344 Mutant W2Aug9-F1 was selected from these mutants because it was the only strain that
345 produced similar clear zones on skim milk plates as the wild type. Sequencing and BlastX
346 analysis of the Tn5 flanking region of mutant W2Aug9-F1 showed that it had an insertion into
347 a sequence which showed the highest similarity to *massA* of *Pseudomonas fluorescens* SS101
348 (92% identity at aa level of a 633 bp fragment). The non-ribosomal peptide synthetase
349 enzyme MassA is responsible for the biosynthesis of the first 2 amino acids of the cyclic
350 lipopeptide massetolide [27]. In strain *P. fluorescens* SS101 the *massA* gene is organized in a
351 separate cluster from *massB* and *massC*.

352 When tested for *in vitro* antagonism against *S. aureus* the massetolide-negative mutant
353 showed a similar level of antagonism as the wild type *Pseudomonas* sp. W2Aug9 on all media
354 tested (results not shown) therefore ruling out a synergistic role for the lipopeptides in the *in*
355 *vitro* antagonism against *Staphylococcus*.

356

357 3.5. Loss of mupirocin results in pyoverdine-dependent *in vitro* antagonism

358 The mupirocin producing strains were strongly antagonistic against *S. aureus* 383 on
359 the rich media tested (853, MH and blood agar). The level of *in vitro* antagonism of the six
360 strains was comparable (data not shown) and results are detailed only for *P. fluorescens*
361 NCIMB 10586 (Fig. 3 and Fig. 4A-D-G). The largest inhibition zone (radius of the zone) for
362 NCIMB 10586 was observed on 853 (23.3 ± 1.3 mm) (Fig. 4A), followed by MH (20.2 ± 1.5
363 mm) (Fig. 4D) and blood agar (16.3 ± 0.5 mm) (Fig. 4G). The mupirocin-negative mutant
364 10586 Δ AT2 (Fig. 3), completely lost its ability to inhibit *S. aureus* on blood agar (Fig. 4H).

365 On 853 and MH medium the antagonism was strongly reduced (7.5 ± 1.3 mm) and (5.2 ± 0.8
366 mm), respectively (Fig. 4E and B).

367 To identify the nature of the compound responsible for the *in vitro* antagonism of the
368 mupirocin-negative mutant 10586 Δ AT2 on 853 (Fig. 4B) a Tn5 mutagenesis was carried out
369 and the library was screened for transconjugants that completely had lost the antagonism.
370 Several mutants were obtained and molecular characterization showed that in all these
371 mutants the Tn5 insertions were in the NRPS genes of pyoverdine. As expected, pyoverdine
372 was no longer produced in the mutants, strongly suggesting that pyoverdine is responsible for
373 the antagonism of the mupirocin-negative mutant on medium 853. Mutant 10586 Δ AT2-
374 10H10 was selected, this mutant had a Tn5 insertion in the middle of the *pvdI* gene and the
375 translated sequence of the flanking region of the transposon showed the highest similarity to
376 PvdI of *P. synxantha* BG33R (98% identity at aa level of a 800 bp fragment). The *in vitro*
377 antagonism of this mutant was tested on 853, MH and blood agar (Fig. 3). On blood agar the
378 antagonism was completely lost (Fig. 4I), on 853 (Fig. 4C) and MH agar (Fig. 4F) an
379 inhibition zone was still observed but it was almost completely covered with *S. aureus*. Since
380 pyoverdine was responsible for the observed *in vitro* antagonism the antagonism was also
381 tested on the low iron media GCA and GCA amended with excess iron. On GCA and
382 GCA+Fe medium the wild type showed a clear inhibition zone (Fig. 4J and 4M). The
383 mupirocin-negative mutant showed a clear inhibition zone on GCA (Fig. 4K), this antagonism
384 was the largest observed on all the media tested for this mutant (Fig. 3). With the addition of
385 iron the antagonism was completely lost confirming that an iron regulated compound is being
386 produced (Fig. 4N). The double mupirocin/pyoverdine-negative mutant 10586 Δ dAT2-10H10
387 (Fig. 3) showed an inhibition zone into which *S. aureus* was growing (Fig. 4L) on GCA
388 medium, as for the mupirocin-negative mutant the antagonism was completely lost in the
389 presence of iron (Fig. 4O).

390 To look at the effect of the loss of only pyoverdine on the antagonism against *S.*
391 *aureus*, a pyoverdine-negative mutant, 10586-3H3, was obtained through Tn5 mutagenesis of
392 the wild type NCIMB 10586. This mutant had an insertion into a gene of which the translated
393 sequence had the highest similarity to PvdD of *P. synxantha* BG33R (98% identity at aa level
394 of a 748 bp fragment). The *pvdD* mutant was not affected in the *in vitro* antagonism against *S.*
395 *aureus* on any of the media tested (Fig. 3). The growth inhibition effect due to pyoverdine
396 appeared to be masked by mupirocin for this strain.

397

398 4. Conclusion

399 The polyketide mupirocin is a valuable antibiotic, with a current therapeutic use. Until
400 now it has only been produced from *P. fluorescens* NCIMB 10586 where its synthesis is
401 encoded by a 65-kb long region comprising 35 genes [12, 29]. In this work, five new
402 mupirocin producing strains were identified during the screening of a phylogenetically diverse
403 collection of 578 *Pseudomonas* strains and isolates. Together with the two reported mupirocin
404 producing strains D7 and G11 [7], they all belong to the same species as *P. fluorescens*
405 NCIMB 10586. Accordingly, mupirocin production seems to be restricted to a limited number
406 of isolates belonging to one single species which is different from the currently published
407 *Pseudomonas* type strains.

408 One explanation for the apparent unique phylogenetic affiliation of the mupirocin
409 producing strains would result from the methodology used to detect mupirocin producing
410 isolates. Indeed, when screening for a specific gene by PCR, candidates may be missed
411 because one or both primers do not anneal due to sequence divergence at the primer annealing
412 site. Yet, the mupirocin producing strains D7 and G11 were isolated on the basis of the
413 antimicrobial effect of their supernatant on Gram-positive bacteria and not by PCR. The fact

414 that mupirocin producing strains isolated using 2 different approaches all belong to the same
415 species supports that mupirocin is probably produced by a restricted group of strains.

416 The capacity to produce mupirocin could well have been acquired by horizontal gene
417 transfer in a recent ancestor of the species. Indeed, analysis of the flanking region of the
418 mupirocin gene cluster suggests that the cluster may be an insertion integrated by
419 recombination at tRNA genes as observed for a number of phage and mobile elements [12].
420 Interestingly, the antibiotic thiomarinol produced by the marine bacterium
421 *Pseudoalteromonas* sp. SANK 73390 is a hybrid of two components: pseudomonic acid and
422 pyrrothine [30; 31]. Both compounds are encoded by 2 different clusters on plasmid pTML1
423 [30].

424 Mupirocin has a broad spectrum activity and the acquisition of the mupirocin gene
425 cluster may significantly increase fitness and competitiveness of the species. Yet as a
426 consequence of the small number of mupirocin producing strains some uncertainty remains
427 with respect to the true ecology of the species and its way of life.

428 In our collection and in the study of [7], mupirocin producing strains were
429 predominantly isolated from water and soil environments. Only one strain, *Pseudomonas* sp.
430 B329, was isolated from the rhizosphere of grass. Interestingly, this was the only strain of the
431 six mupirocin producing strains which showed motility under microscope (*results not shown*).
432 Yet, rhizosphere strains are underrepresented in the collection screened; this could explain
433 why only one rhizosphere strain was picked up during the screening.

434 Given the strong antimicrobial activity of mupirocin it would be interesting to test
435 these strains as biocontrol agents against bacterial disease. The ability of some of the
436 mupirocin producing strains to produce lipopeptides of the massetolide group, which have
437 surfactant and antimicrobial properties, could potentially even make the strains active against
438 fungal diseases [32].

439

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444

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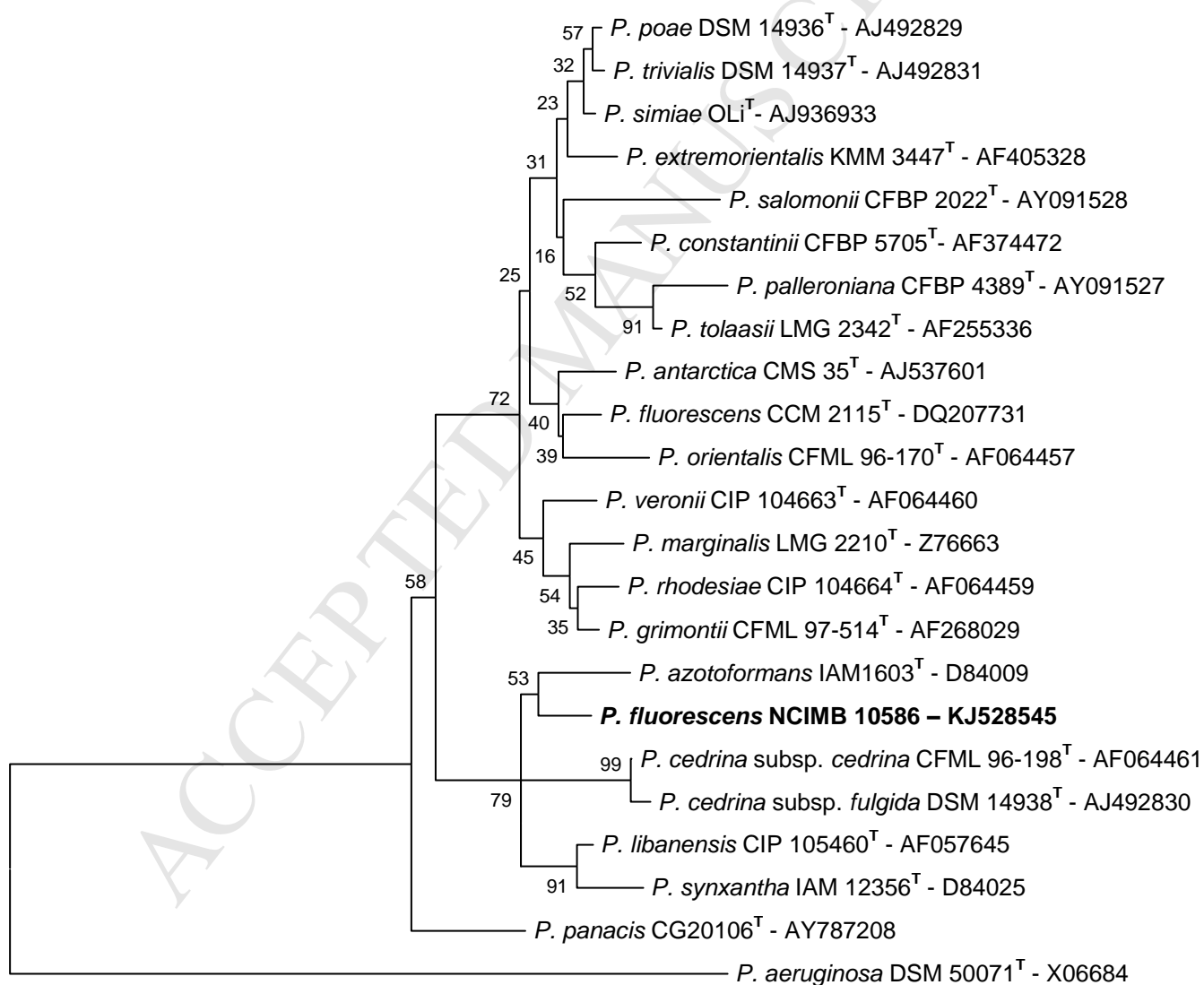
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Table 1. List of strains used in this study.

Strain	Relevant genotype/characteristics	Reference
<i>Pseudomonas fluorescens</i> NCIMB 10586	Mupirocin producing strain isolated from soil, Hampstead Heath, London, U.K.	[1]
10586 Δ AT2	Mupirocin-negative deletion (MmpC Δ 337-548) mutant of <i>P. fluorescens</i> NCIMB 10586	[12]
10586-3H3	Pyoverdine-negative Tn5 mutant of <i>P. fluorescens</i> NCIMB 10586 with a Tn5 insertion into the <i>pvdD</i> gene, Gm ^R	This study
10586 Δ AT2-10H10	Pyoverdine-negative mutant of 10586 Δ AT2 with a Tn5 insertion into the <i>pvdI</i> gene, Gm ^R	This study
<i>Pseudomonas fluorescens</i> LMG 14677	Mupirocin producing strain	[33]
<i>Pseudomonas</i> sp. W2Aug9	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
W2Aug9-F1	Massetolide-negative Tn5 mutant of <i>Pseudomonas</i> sp. W2Aug9 with a Tn5 insertion into <i>massA</i> , Gm ^R	This study
<i>Pseudomonas</i> sp. B329	Mupirocin producing strain isolated from rhizosphere of grass, France	Bodilis J., France
<i>Pseudomonas</i> sp. W2Jun17	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
<i>Pseudomonas</i> sp. W15Feb34	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
<i>Staphylococcus aureus</i> 383	Clinical strain isolated from infected femoral pin, mupirocin-sensitive	[21]

Fig. 1.A. Phylogenetic relationships among 21 *Pseudomonas* type strains of the *P. fluorescens* group and *P. fluorescens* NCIMB 10586 based on 16S rRNA sequences (the accession number of the 16S rRNA sequences are indicated after the strain name). *P. aeruginosa* LMG 1242^T was used as outgroup to root the tree. The tree was constructed using the NJ method. Number at nodes represents levels (%) of bootstrap support from 1000 resampled datasets. The bar represents 0.01 substitutions per nucleotide position.

Fig. 1.A.



0.001

Fig. 1.B. Phylogenetic relationships among the mupirocin producing strains based on *rpoB* gene sequences (the accession number of the *rpoB* gene sequences are indicated after the strain name). *P. aeruginosa* LMG 1242^T was used as outgroup to root the tree. The strains with hemolytic activity are indicated by a black dot. The tree was constructed using the NJ method. Number at nodes represents levels (%) of bootstrap support from 1000 resampled datasets. The bar represents 0.01 substitutions per nucleotide position.

Fig. 1.B.

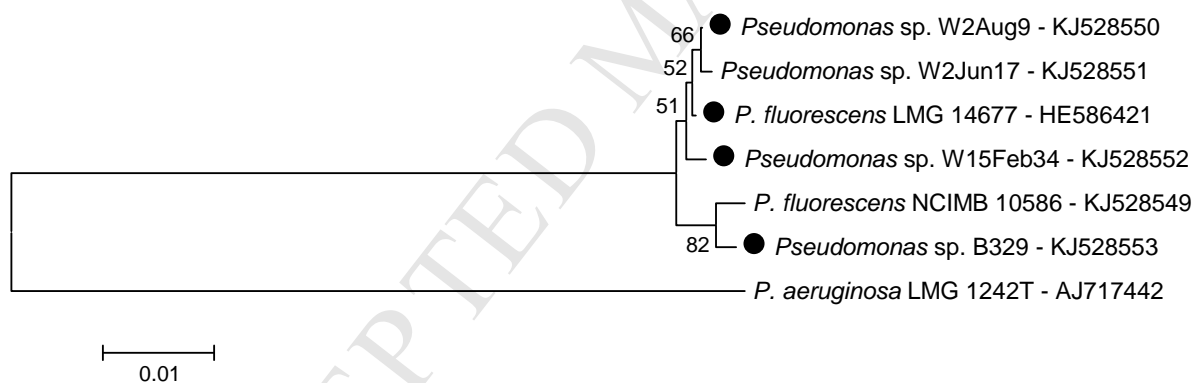


Fig 2. UPLC-MS analysis of culture supernatants of *Pseudomonas* sp. W2Aug9. Strain W2Aug9 secretes cyclic lipopeptides with major peaks observed at m/z 1126.7, 1140.7, 1154.7 and 1168.7 for molecular ions. Further analysis of product ions yielded upon in-source fragmentation of those molecular species confirmed that they correspond to massetolides A/D, L and F/viscosin with either C_{10} or C_{12} fatty acid moieties. Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the fatty acid.

Fig. 2.

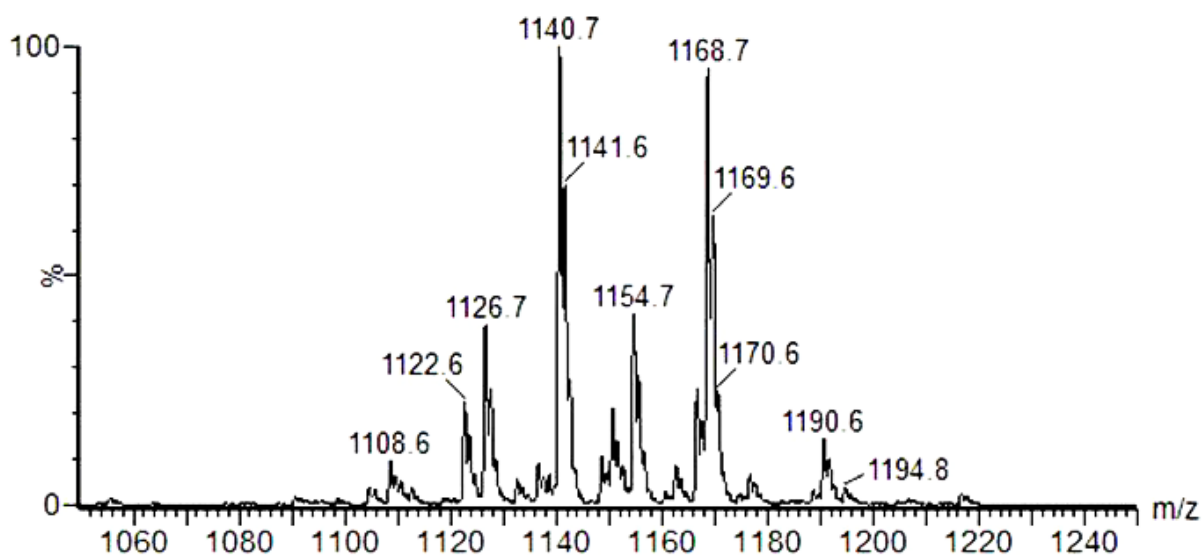


Fig. 3. The *in vitro* antagonism of the wild type *P. fluorescens* NCIMB 10586, the mupirocin-negative mutant 10586 Δ AT2, the pyoverdine-negative mutant 10586-3H3 and the double mupirocin/pyoverdine-negative mutant 10586 Δ AT2-10H10. The bars represent the inhibition zone (mm) observed against *S. aureus* 383. For the double mutant the color of the bar is presented as a gradient since inhibition zones were observed which were partially covered with bacteria.

Fig. 3.

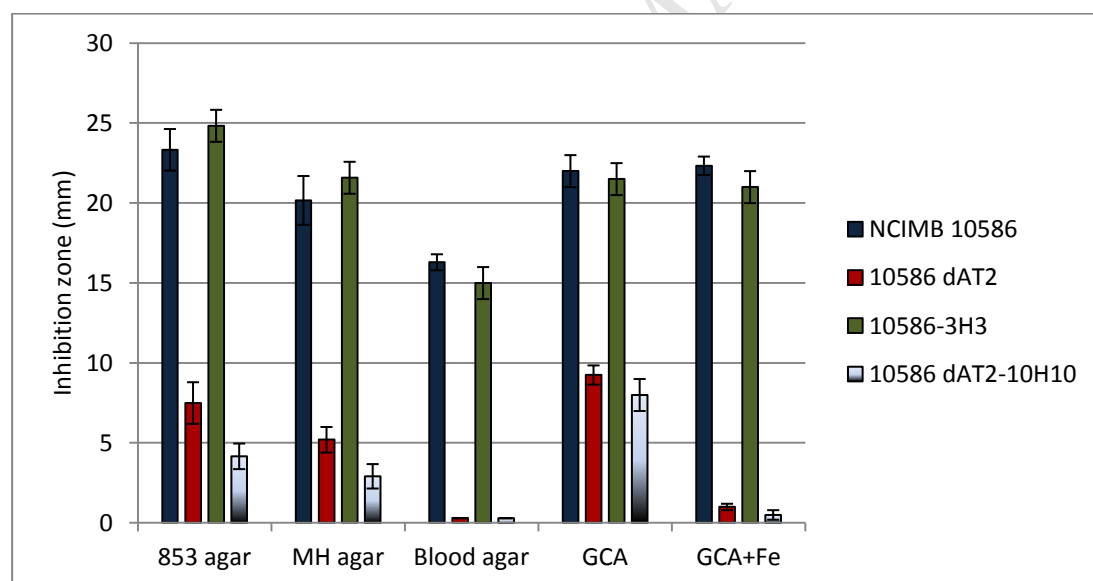
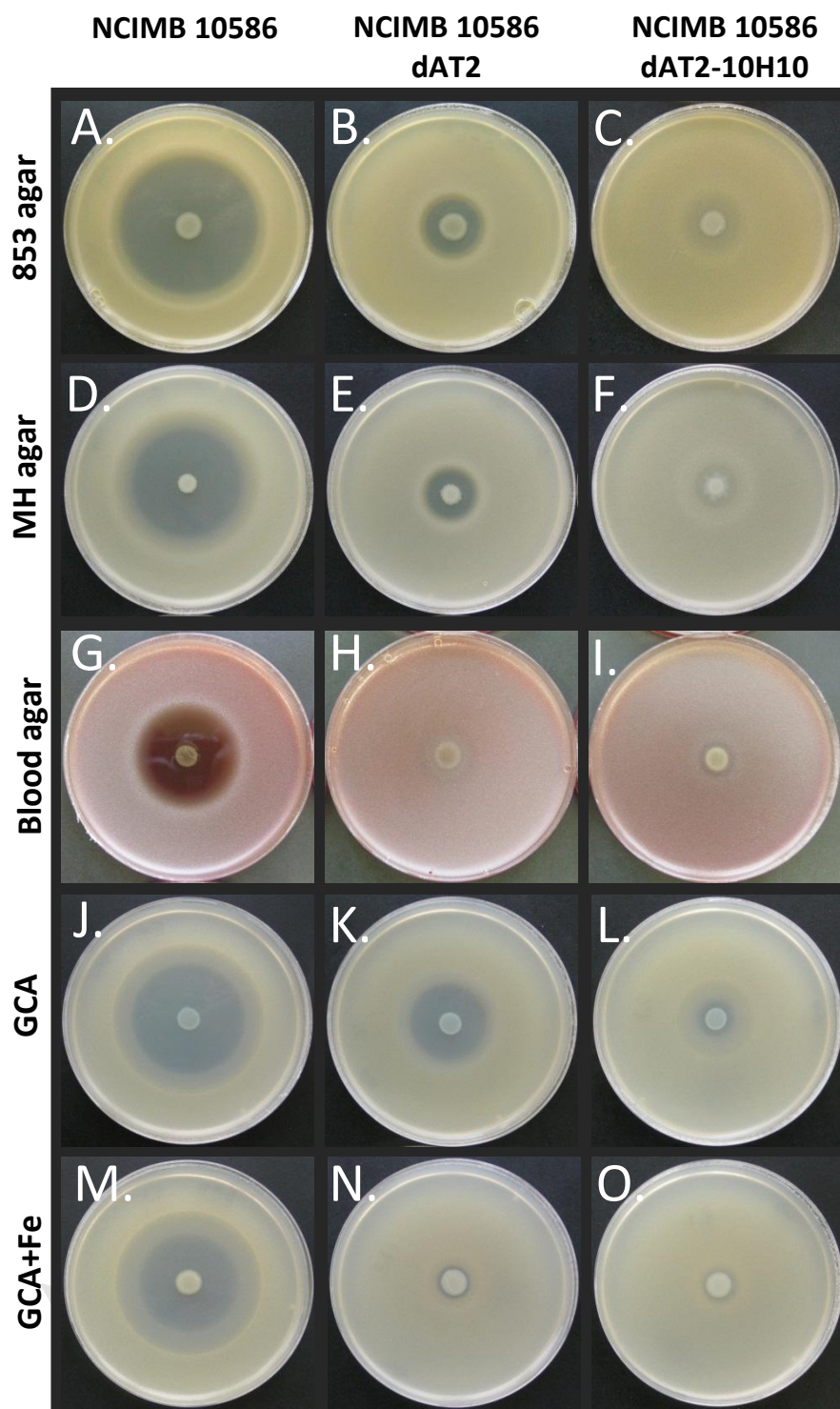


Fig. 4. Comparison of the *in vitro* antagonism of *P. fluorescens* NCIMB 10586 (first column), the mupirocin-negative mutant 10586 Δ AT2 (second column) and the double mupirocin/pyoverdine-negative mutant 10586 Δ AT2-10H10 (last column) against *S. aureus* 383 on 853 agar (A-C), MH agar (D-F), blood agar (G-I), GCA (J-L) and GCA+Fe medium (M-O).

Fig. 4.



Supplementary Table S1. List of *Pseudomonas* type strains obtained from ATCC (American Type Culture Collection, US), BCCM/LMG Culture Collection (Belgium), CCUG (Culture Collection, University of Göteborg, Sweden), CFBP (French Collection of Plant associated bacteria, France), CFML (Collection de la Faculté de Médecine de Lille, France), CIP (Collection de l'Institut Pasteur, France) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

Species	Strain	Biological origin	Geographic origin
<i>P. abietaniphila</i>	CIP 106708 ^T	Aerated lagoon of bleached kraft pulp mill effluent	Kamploops British Columbia Canada
<i>P. aeruginosa</i>	ATCC 10145 ^T		
<i>P. agarici</i>	CFBP 2063 ^T	Mushroom (<i>Agaricus bisporus</i>)	New Zealand
<i>P. alcaliphila</i>	CCUG 136 ^T	Seawater	Near the coast of Rumoi, Hokkaido, Japan
<i>P. amygdali</i>	DSM 7298 ^T	Almond, <i>Prunus dulcis</i>	Greece
<i>P. anguilliseptica</i>	CCUG 35503 ^T	Pond-cultured eels (<i>Anguilla japonica</i>)	Japan
<i>P. antarctica</i>	CIP 108466 ^T	Cyanobacterial mat	Pond L3 Wright Valley Adam's glacier stream 1 Antarctica
<i>P. argentinensis</i>	CIP 108775 ^T	<i>Chloris ciliata</i> , rhizosphere	Cordoba, Argentina
<i>P. arsenicoxydans</i>	CCUG 58201 ^T	Sediment	Chile, Atacama Desert, Camarones Valley
<i>P. asplenii</i>	ATCC 23835 ^T	<i>Asplenium nidus</i>	
<i>P. avellanae</i>	CIP 105176 ^T	Hazelnut (<i>Corylus avellanae</i>)	Greece
<i>P. azotifigens</i>	DSM 17556 ^T	Compost pile	Japan:Okinawa, Nakijin
<i>P. azotoformans</i>	DSM 18862 ^T	Paddies	Japan
<i>P. baetica</i>	LMG 25716 ^T	Diseased <i>Dicologlossa cuneata</i>	
<i>P. balearica</i>	DSM 6083 ^T	Wastewater treatment plant	Mallorca, Spain
<i>P. bauzanensis</i>	LMG 26048 ^T	Soil contaminated with hydrocarbon and heavy metal from an industrial site	Italy, South Tyrol, Bozen
<i>P. benzenivorans</i>	DSM 8628 ^T	Soil and groundwater contaminated with chlorobenzene	USA, Gulf Coast

<i>P. brassicacearum</i> subsp. <i>brassicacearum</i>	DBK11 ^T	Rhizoplane of <i>Brassica napus</i>	Dieulouard, France
<i>P. brassicacearum</i> subsp. <i>neaurantiaca</i>	CIP 109457 ^T	Rhizosphere of hemp plants	Ukraine, near Kiev
<i>P. brenneri</i>	DSM 15294 ^T	French natural mineral water after bottling	Lille, France
<i>P. caricapapayae</i>	CFBP 3204 ^T	<i>Carica papaya</i>	Brazil
<i>P. cedrina</i> subsp. <i>cedrina</i>	LMG 23661 ^T	Spring water	Lebanon
<i>P. cedrina</i> subsp. <i>fulgida</i>	DSM 14938 ^T	Phyllosphere of grasses	Paulinenaue, Germany
<i>P. chlororaphis</i> subsp. <i>aurantiaca</i>	ATCC 33663 ^T		
<i>P. chlororaphis</i> subsp. <i>aureofaciens</i>	LMG 1245 ^T	Maas River clay suspended in kerosene for three weeks	Netherlands
<i>P. chlororaphis</i> subsp. <i>chlororaphis</i>	ATCC 9446 ^T	Plate contaminant	
<i>P. cichorii</i>	CFBP 2101 ^T	<i>Cichorium endivia</i>	Germany
<i>P. citronellolis</i>	CFBP 5585 ^T	Soil under pine trees	
<i>P. composti</i>	DSM 25648 ^T	Mixed vegetables and animal waste compost	
<i>P. congelans</i>	CFBP 7019 ^T	Phyllosphere of grasses	Germany
<i>P. costantinii</i>	CFBP 5705 ^T	<i>Agaricus bisporus</i>	Finland
<i>P. corrugata</i>	LMG 2172 ^T	<i>Lycopersicon esculentum</i> (tomato) pith necrosis	United Kingdom
<i>P. cremoricolorata</i>	DSM 17059 ^T	<i>Oryza sativa</i>	Japan
<i>P. cuatrociénegasensis</i>	LMG 24676 ^T	Water (evaporating lagoon)	Cuatro Ciénegas, Coahuila state, Mexico
<i>P. deceptionensis</i>	LMG 25555 ^T	Marine sediment	Deception Island, South Shetland Islands, Antarctica
<i>P. delhiensis</i>	DSM 18900 ^T	Soil sample contaminated by polycyclic aromatic compounds of fly ash dumping site	India
<i>P. duriflava</i>	DSM 21419 ^T	Desert soil	Xinjiang Province, China
<i>P. entomophila</i>	L48 ^T	<i>Drosophila</i>	Calvaire, Guadeloupe, France
<i>P. extremaustralis</i>	DSM 17835 ^T	Temporary water pond	Danco Coast, Cierva Point, Antarctica
<i>P. extremorientalis</i>	CCUG 51517 ^T	Drinking water reservoir	Vladivostock City, Russia
<i>P. ficuserectae</i>	CCUG 32779 ^T	<i>Ficus erectae</i>	

<i>P. flavescens</i>	CFBP 5586 ^T	Canker tissue of <i>Juglans regia</i>	California
<i>P. fluorescens</i>	ATCC 13525 ^T	Pre-filter tanks	UK
<i>P. fragi</i>	ATCC 4973 ^T		
<i>P. fulva</i>	DSM 17717 ^T	Rice paddy	Japan
<i>P. fuscovaginae</i>	CFBP 2801 ^T	<i>Oriza sativa</i>	
<i>P. gessardii</i>	CFML 95-251 ^T	Mineral water	France
<i>P. graminis</i>	CCUG 51504 ^T	Surface plant material from a mixed meadow	Germany
<i>P. grimontii</i>	DSM 17515 ^T	Mineral water	France
<i>P. guariconensis</i>	LMG 27394 ^T		
<i>P. guineae</i>	CFBP 7180 ^T	Soil	Deception Island (South Shetland Islands), Antarctica
<i>P. helmanticensis</i>	LMG 28168 ^T	Forest soil	Salamanca, Spain
<i>P. japonica</i>	CCUG 59367 ^T	Activated sludge from sewage treatment plant	Adachi-ku, Odaii, Tokyo, Japan
<i>P. jessenii</i>	CFML 95-307 ^T	Mineral water	France
<i>P. jinjuensis</i>	DSM 16612 ^T	Agricultural soil	Korea
<i>P. kilonensis</i>	DSM 13647 ^T	Agricultural soil	Germany
<i>P. knackmussii</i>	DSM 6978 ^T	Sewage plant	Germany
<i>P. koreensis</i>	CCUG 51519 ^T	Agricultural soil	Korea
<i>P. kuykendallii</i>	LMG 26364 ^T	Bioreactor that degrade the herbicide hexazinone, inoculated with soil	Wiggins, Colorado, USA
<i>P. libanensis</i>	CFML 96-195 ^T	Spring water	Lebanon
<i>P. lini</i>	D-LE411J ^T	Bulk and rhizospheric soil	Dijon, France
<i>P. lundensis</i>	DSM 6252 ^T	Prepacked beef	Sweden
<i>P. lurida</i>	CCUG 54630 ^T	Phyllosphere of grasses	Germany
<i>P. lutea</i>	DSM 17257 ^T	Soil	Spain
<i>P. luteola</i>	CIP 102995 ^T	Human wound	
<i>P. mandelii</i>	CFML 95-303 ^T	Mineral water	France
<i>P. marginalis</i>	ATCC 10844 ^T	<i>Cichorium intybus</i> imported from Belgium	USA
<i>P. marincola</i>	LMG 24752 ^T	Deep-sea brittle star	Fiji, at a depth of 480 m
<i>P. mediterranea</i>	CFBP 5447 ^T	Pith necrosis on tomato plant	Italy
<i>P. mendocina</i>	ATCC 25411 ^T	Soil, enrichment with ethanol	

<i>P. mohnii</i>	CCUG 53115 ^T	Sequencing batch reactor treating paper mill effluent, enrichment with isopimaric acid	Canada
<i>P. monteilii</i>	DSM 14164 ^T	Human bronchial aspirate	France
<i>P. moorei</i>	DSM 12647 ^T	Soil samples	
<i>P. moraviensis</i>	DSM 16007 ^T	Soil besides highway	Czech Republic
<i>P. mosselii</i>	ATCC BAA-99 ^T	Medical specimen	France
<i>P. mucidolens</i>	CCUG 1424 ^T	Musty egg	
<i>P. nitroreducens</i>	ATCC 33634 ^T	Oil-brine	Japan
<i>P. oleovorans</i> subsp. <i>oleovorans</i>	CFBP 5589 ^T	Cutting fluid	
<i>P. oleovorans</i> subsp. <i>lubricantis</i>	DSM 21016 ^T	Contaminated metalworking fluids	Houghton, Michigan, USA
<i>P. orientalis</i>	DSM 17489 ^T	Spring water	Lebanon
<i>P. otitidis</i>	DSM 17224 ^T	Ear of patient with acute Otitis externa	USA
<i>P. palleroniana</i>	CFBP 4389 ^T	<i>Oryza sativa</i>	Cameroon
<i>P. panacis</i>	DSM 18529 ^T	Rusty root lesions of Korean ginseng	South Korea
<i>P. panipatensis</i>	LMG 24738 ^T	Oil contaminated soil	Panipat Oil Refinery, India
<i>P. parafulva</i>	DSM 117004 ^T	<i>Oryza sativa</i>	Japan
<i>P. peli</i>	CIP 109374 ^T	Antarctic green alga <i>Pyramimonas gelidicola</i>	Antarctica
<i>P. plecoglossicida</i>	CIP 106493 ^T	Diseased ayu, <i>Plecoglossus altevelis</i>	Japan
<i>P. poae</i>	CFBP 6764 ^T	Grasses, phyllospere	Paulinenaue, Brandenburg, Germany
<i>P. pohangensis</i>	DSM 17875 ^T	Sea shore sand	Korea
<i>P. prosekii</i>	LMG 26867 ^T	Rock biofilm	James Ross Island, Waterfall, west side of Lachmen Crags Antarctica
<i>P. protegens</i>	CHA0 ^T	Soil suppressing black root rot of tobacco (<i>Nicotiana glutinosa</i>)	Switzerland
<i>P. proteolytica</i>	CIP 108464 ^T	Cyanobacterial mat samples	Antarctica
<i>P. putida</i>	CFBP 2066 ^T	Soil, lactate enrichment	United States
<i>P. reinekei</i>	CCUG 53116 ^T	Aerobic zone of Elbe sediment enriched with 4-chlorosalicylate	Germany
<i>P. resinovorans</i>	CCUG 2473 ^T	Soil	France, Vienne

<i>P. rhodesiae</i>	ATCC 17764 ^T	Natural mineral water	France
<i>P. sabulinigri</i>	DSM 23971 ^T	Black sand originating from black volcanic basalt rock	Jeju Island, Soesoggak, Korea
<i>P. salomonii</i>	CFBP 2022 ^T	<i>Allium sativum</i>	France
<i>P. saponiphila</i>	DSM 9751 ^T		Michigan, USA
<i>P. savastanoi</i>	CIP 103721 ^T	<i>Olea europaea</i>	Yugoslavia
<i>P. segetis</i>	CCUG 54777 ^T	Soil	Korea
<i>P. simiae</i>	DSM 18861 ^T	Lung of female monkey (<i>Callithrix geoffroyi</i>) with pneumonitis and pneumonia	Spain
<i>P. straminea</i>	CCUG 12539 ^T	Japanese unhulled rice	Japan
<i>P. stutzeri</i>	ATCC 17588 ^T	Spinal fluid	
<i>P. synxantha</i>	DSM 18928 ^T	Cream	Iowa, USA
<i>P. syringae</i> subsp. <i>syringae</i>	ATCC 19310 ^T	<i>Syringa vulgaris</i>	United Kingdom
<i>P. taetrolens</i>	CCUG 560 ^T	Musty egg	
<i>P. taiwanensis</i>	DSM 21245 ^T	Soil	Tamkang University, Taipei, Taiwan
<i>P. thivervalensis</i>	CFBP 5754 ^T	Rhizoplane of <i>Brassica napus</i>	Sexy-les-Bois, France
<i>P. tolaasii</i>	CFBP 2068 ^T	<i>Agaricus bisporus</i>	United Kingdom
<i>P. trivialis</i>	CFBP 6765 ^T	Grasses, phyllospere	Paulinenaue, Brandenburg, Germany
<i>P. umsongensis</i>	CIP 108618 ^T	Agricultural soil	Korea
<i>P. vancouverensis</i>	ATCC 700688 ^T	Forest soil	Canada
<i>P. veronii</i>	CFML 92-134 ^T	Mineral water	France
<i>P. viridiflava</i>	ATCC 13223 ^T	Dwarf or runner bean	Switzerland
<i>P. vranovensis</i>	DSM 16006 ^T	Soil besides highway	Czech Republic
<i>P. xiamenensis</i>	DSM 22326 ^T	Activated sludge sample collected at Qianpu sewage treatment plant	Fujian, Xiamen, China

Supplementary Table S2. List of *Pseudomonas* non type strains obtained from ATCC (American Type Culture Collection, US), BCCM/LMG Culture Collection (Belgium), CFBP (French Collection of Plant associated bacteria, France), CFML (Collection de la Faculté de Médecine de Lille, France), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

Strain	Species	Biological origin	Geographic origin
ATCC 17400	<i>P. fluorescens</i>	Egg yolk	
ATCC 39167	<i>P. putida</i>		
ATCC 43928	<i>P. chlororaphis</i>	Agricultural field	Denmark
CFBP 2123	<i>P. fluorescens</i>	Tap water	Netherlands
CFBP 2124	<i>P. fluorescens</i>	Lactate-enriched water	
CFBP 2125	<i>P. fluorescens</i>	Enrichment with naphthalene	
CFBP 2129	<i>P. fluorescens</i>	Hydrocarbon enrichment	Berkeley, United States
CFBP 2131	<i>P. fluorescens</i>	Soil	
CFBP 2392		Rhizosphere of <i>Phaseolus vulgaris</i>	France
CFBP 2461	<i>P. putida</i>	Soil	France
CFBP 3140	<i>P. putida</i>		
CFBP 3142	<i>P. putida</i>	Soil	
CFBP 3143	<i>P. putida</i>	Soil	
CFBP 3150	<i>P. fluorescens</i>	Soil	
CFBP 3153	<i>P. marginalis</i> pv. <i>marginalis</i>	Pleural fluid	
CFBP 3155	<i>P. chlororaphis</i>	Farm soil	Peoria, USA
CFBP 4628	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Maine et Loire Angers, France
CFBP 4966	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Guadeloupe, France
CFBP 4967	<i>P. fluorescens</i>	<i>Lycopersicon esculentum</i>	Guadeloupe, France
CFBP 4970	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Guadeloupe, France
CFBP 4971	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Guadeloupe, France
CFBP 4976	<i>Pseudomonas</i> sp.	Tabac	Maine et Loire Angers, France
CFBP 5891	<i>P. putida</i>	<i>Zea mays</i>	Maine et Loire, France
CFBP 5898	<i>P. putida</i>	<i>Zea mays</i>	Maine et Loire, France
CFBP 5913	<i>P. putida</i>	<i>Zea mays</i>	Maine et Loire, France

CFBP 5921	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Guadeloupe, France
CFBP 5930	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Angers, France
CFBP 5933	<i>P. putida</i>	<i>Lycopersicon esculentum</i>	Angers, France
CFML 90-33	<i>P. putida</i>		
CFML 90-40	<i>P. putida</i>		
CFML 90-42	<i>P. putida</i>		
CFML 90-44	<i>P. putida</i>		
CFML 90-51	<i>P. putida</i>	Clinical	
CFML 90-136	<i>P. putida</i>		
CFML 95-275	<i>Pseudomonas</i> sp.		
CFML 96-132	<i>Pseudomonas</i> sp.	Spring water (Kadicha)	Lebanon
CFML 96-188	<i>Pseudomonas</i> sp.		
CFML 96-312	<i>Pseudomonas</i> sp.		
CFML 96-318	<i>Pseudomonas</i> sp.		
CFML 96-319	<i>Pseudomonas</i> sp.		
DSM 3602	<i>P. stutzeri</i>	Black earth soil	Gatton, Queensland, Australia
DSM 50106	<i>P. fluorescens</i>	Seawater	
LMG 1244	<i>P. fluorescens</i>	Polluted seawater	Dresund, Denmark
LMG 14576	<i>P. fluorescens</i>	Well water	
LMG 14677	<i>P. fluorescens</i>		
LMG 2189	<i>P. fluorescens</i>		
LMG 5831	<i>P. fluorescens</i>	Polluted natural stream	South Copenhagen, Denmark
LMG 5848	<i>P. fluorescens</i>	<i>Cichorium intybus</i> , leaves showing blackening of vascular system	United Kingdom

Supplementary Table S3. List of *Pseudomonas* strains and isolates obtained from different laboratories and their biological and geographic origin.

<i>Pseudomonas</i> strains and isolates	Biological and geographic origin	Reference
LUBF-155, LUBF-159, LUBF-169, LUBF-188, LUBF-1102, LUBF-1104, LUBF-1107, LUBF-1112, LUBF-1212, LUBF-1410, LUBF-1615, LUBF-1715	Spoilage of <i>Sparus aurata</i> stored under various conditions	[1]
SWPA 6, SWPA 40A, SWPA 40B, SWPA 36A, SWPA 36B, SWPA 36C, SWPA 10.20A, SWPA 10.20B, SWPA 10.20C, 115 thio, 120, LS2-01, 545, 415, 250A, 250B, 250C, 250D, SWPA0004-10, SW4, SW5, SW6, SW7, SW33, SW11, SW16, SW29, SWPA 115 25-F, SWPA 820 18-F, SWPA 2404 25-T	Seawater, North Sea (few km before the coast, 15m depth)	Jean-Paul Pirnay, Belgium (unpublished)
TKM iso1, TKM iso2, TKC, LKA, TKD1, 4T, 5TWPS, 3TWPS, TW1, 2T	Lake Tanganyika	Jean-Paul Pirnay, Belgium (unpublished)
Br232, Br252	Unfiltered tap water, Burn Centre, Queen Astrid Military Hospital, Brussels, Belgium	Jean-Paul Pirnay, Belgium (unpublished)
Br996	Water tank of a ship	Jean-Paul Pirnay, Belgium (unpublished)
Mex8A, Mex8B1, Mex8C1, Mex8D1	Water of a cenote, Loltun, Mexico	Jean-Paul Pirnay, Belgium (unpublished)
K-SR5, K-SR6, K-SR7, K-SR8, K-SR9, K-SR10, K-SR11, K-SR21, K-SR33	Rhizosphere <i>Salsola vermiculata</i> , Bèchar, Algeria	Khadidja Chafi, Algeria (unpublished)
MFY0	Raw milk	[2]
MFY30, MFY31, MFY32, MFY33, MFY52, MFY76, MFY80,	Bulk soil	[2]

MFY81, MFY122, MFY140, MFY152, MFY160, MFY138,
MFY143, MFY146, MFY220, MFY338, MFY245

OE28.3	Rhizosphere	[2]
MFY59, MFY63,	Urine, Pitié Salpêtrière Hospital, France	[2]
MFY57, MFY68, MFY163	Blood, Pitié Salpêtrière Hospital, France	[2]
MFY161	Blood/urine, Charles Nicolle Hospital, France	[2]
MFY78, MFY79	Dun River, France	[2]
MFY70	Suppuration, Pitié Salpêtrière Hospital, France	[2]
MFY65	Infected sinus	[2]
MFY61	Articular infection	[2]
MFY162	Sputtum, Charles Nicolle Hospital, France	[2]
MFY69	Gastric fluid, Pitié Salpêtrière Hospital, France	[2]
MFY71	Clinical	[2]
R2f, B13, B125, B136, B163, B215, B329	Rhizosphere	Josselin Bodilis, France (unpublished)
R-35697, R-35701, R-35703, R-35705, R-35706, R-35709, R- 35710, R-35717, R-35719, R-35702, R-35711, R-35721, R- 35723, R-35724, R-35708, R-35700, R-35720, R-35707, R- 35725	Milk, Belgium	[3]

PD4, PD5, PD11, PD13, PD15, PD22, PD28, PD31	Arable soil used for potato production, New Brunswick, Canada	[4]
<i>P. putida</i> BTP1, <i>P. fluorescens</i> BTP2, BTP7', BTP9, BTP14, PP, <i>P. putida</i> PutC		Collection obtained from Marc Ongena, Belgium
<i>P. putida</i> Irc204	Soil, Belgium	IRMW
<i>P. fluorescens</i> E211, <i>P. kilonensis/brassicacearum</i> G11, <i>P. fluorescens</i> G27	Rhizosphere of <i>Echinochloa crus-galli</i> / <i>Galium mollugo</i>	[5]
<i>P. putida</i> WCS358, WCS365, <i>P. fluorescens</i> WCS374		
<i>Pseudomonas</i> sp. B10, <i>P. fluorescens</i> G153, <i>P. fluorescens</i> G166, <i>P. fluorescens</i> G173, <i>P. chlororaphis</i> D-TR133, <i>P. putida</i> G4R, <i>P. fluorescens</i> 99-13, <i>P. fluorescens</i> PL7, <i>P. fluorescens</i> PL8, <i>P. fluorescens</i> PL9, <i>Pseudomonas</i> sp. D46, <i>Pseudomonas</i> sp. D47, <i>P. fluorescens</i> Pflii, <i>P. fluorescens</i> Pfl12, <i>P. fluorescens</i> PflW, <i>Pseudomonas</i> sp. F360, <i>P. fluorescens</i> 9AW, <i>P. fluorescens</i> 1547, SB8.3, <i>Pseudomonas</i> sp. 2908, <i>P. fluorescens</i> 1.3, <i>P. fluorescens</i> 18.1, HR6, <i>P. monteilii</i> Lille 1, <i>P. fluorescens</i> 51W, <i>P. rhodesiae</i> Lille 25, Gwose, <i>Pseudomonas</i> sp. A214, <i>P. fluorescens</i> A225, <i>P. putida</i> Thai, <i>P. aeruginosa</i> R', <i>Pseudomonas</i> sp. 6-10, LBSA1, IB3, 7SR1, ML35, Malar, <i>P. aeruginosa</i> Pa6, Lille 17, <i>Pseudomonas</i> sp., <i>Pseudomonas</i> sp. F317, <i>P. fuscovaginae</i> G17, <i>Pseudomonas</i> sp. G400		Collection obtained from Jean-Marie Meyer, France
<i>P. putida</i> GM 10090 (G297), <i>P. putida</i> GM 11799.1 (G309), <i>P. putida</i> GM 12220 (G314)	Plant rhizosphere	[6]
<i>P. putida</i> GS4 (GS12059), <i>P. putida</i> GS35 (GS12082), <i>P. putida</i> GS37 (GS12064)	Plant rhizosphere (banana tree)	[7]

SR11, SR14, SR18, SR28, SR44, SR45, SR54, SR56, SR64, SR66, SR67, SR68, SR70	Metal-contaminated sediment	[8]
<i>P. brassicacearum</i> R1-4	Rhizosphere oilseed rape	[9]
<i>P. thivervalensis</i> DR5, <i>P. brassicacearum</i> BGCR2-9(1)	Bacterial endophyte from field-grown <i>Solanum nigrum</i>	[10]
AF76	Rhizosphere ground nut (<i>Arachis hypogaea</i> L.), India	[11]

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Supplementary Table S4. Primers and their sequences used to amplify part of the tailoring genes of the mupirocin gene cluster.

Primer	Sequence 5'-3'	Size amplicon	Primer	Sequence 5'-3'	Size amplicon
mupZ-F	ATGAATCGCACCTGCATGGC		mupL-F	ATGCAACTGATCACGCACGA	
mupZ-R	GAAGTGCAGGCITCATAAACG	324 bp	mupL-R	ATCCACGTCTGCCTCACCCCT	936 bp
TEB-F	TGACGCTGCTCAATCACGCT		mupM-F	ATGAGTACGGAAGGAAGTGG	
TEB-R	ACCTGCITGAGGGACACCAC	440 bp	mupM-R	GCGGATGAACCAGGACTCTA	1221 bp
mmpC-F	GGGTCGCAATACCGTCACAT		mupO-F	GACATCGTGGGAAAGAGAGG	
mmpC-R	CGTTGAGCGATTTTCGACAAT	2140 bp	mupO-R	GTAGGAGATGATGGCGTGGIT	775 bp
MetD3-F	ATGITCCCCGAGGGTTCCAT		mupP-F	GTGAGCAGGTCCGAACCTG	
MetD3-R	GCTCAGCTCGITCAACAACA	470 bp	mupP-R	TCGGTAAAGGGCAITGAAGTG	699 bp
mupC-F	AAGGTCGTCGGGITCACTGG		mupQ-F	ATGAGAGAGGAACGTAATTGG	
mupC-R	CGGTAAGTGGCTTCCGACAA	531 bp	mupQ-R	CCTTGAAATCITCCAACCCT	1279 bp
macpA-F	ATGAACCCTGAAAGGCGGAA		mupS-F	ATGACTGATGCAGTTTCTGACG	
macpA-R	GITGACGGCACTCATCCAIT	222 bp	mupS-R	TGCGGGTCAAGCCATCC	535 bp
mupD-F	ATGCGTAGGCAGGTAGTCGT		mupT-F	ITGGTAGAGCATCCGCCC	
mupD-R	GGTGAGATGITGCCCGITGA	708 bp	mupT-R	TCACGTACCGCACTCCAGCC	351 bp
mupE-F	GGCGCTTCAACTGGTTGATA		mupU-F	ATGGAACAGITGACCCCTGG	
mupE-R	CCGAAAGAGCCATGAATGTG	864 bp	mupU-R	GCGACACTACCGCCACIT	1336 bp
mupF-F	TCAGGGTGCTGTCGGGGATA		mupV-F	AAAGCTCTATGTGACCGGCG	
mupF-R	GGCAGCCATATTGTGGAAAA	969 bp	mupV-R	GCTGGAITGGCGGCTCT	1630 bp
mupG-F	ATGGCAAGCTCTGACACGCA		mupW-F	GCAAATTGATCGAGCACGTC	
mupG-R	ACTGCCGTGCGGGTTGATAT	918 bp	mupW-R	GGCGTGCTCATAGGTGTCG	1208 bp
mupH-F	ATGACGAGGCAAGTGGGTAT		mupX-F	TGGTCAGCGCGTTCCACTTT	
mupH-R	CCCAGCAGGAIAGACTCGTA	1121 bp	mupX-R	AAGAGTGAGGCGGGCTACG	1510 bp
mupJ-F	ATGAACITCCAGGCCACCGA		mupI-F	TAGCATTGACTGCGTCCAGG	
mupJ-R	TGGCTGACATAGCGGTGGAT	740 bp	mupI-R	GAACATATCACCACGITATCTGGA	555 bp
mupK-F	GGACAGTGTCATCGATITTCAGG		mupR-F	TGCITGAAGACAITCTGATG	
mupK-R	CCAACCTCATCGCCAACCTCC	581 bp	mupR-R	GCCACCCACITCAGACA	550 bp

Supplementary Table S5. The interspecies similarity values for the *rpoB* gene of the mupirocin producing strains.

Species	1	2	3	4	5	6
1. <i>P. fluorescens</i> NCIMB 10586	100.0					
2. <i>Pseudomonas</i> sp. W2Aug9	99.13	100.0				
3. <i>Pseudomonas</i> sp. W2Jun17	99.02	99.89	100.0			
4. <i>Pseudomonas</i> sp. W15Feb34	99.02	99.67	99.57	100.0		
5. <i>Pseudomonas</i> sp. B329	99.57	99.35	99.24	99.24	100.0	
6. <i>P. fluorescens</i> LMG 14677	99.89	99.89	99.79	99.79	99.24	100.0

Supplementary Fig. S1. Part of the *mmpD* gene of *P. fluorescens* NCIMB 10586 that aligns with the complementary sequence of the PltBf and PltBr primers. The nucleotides that mismatch are indicated in red. There was only 1 internal nucleotide mismatch with the forward primer and 3 mismatches of 4 nucleotides (thereby discarding the nucleotide at the 5'end) with the reverse primer.

mmpD NCIMB 10586: 5' -CGGATCATGGACCCCCAGC-3'

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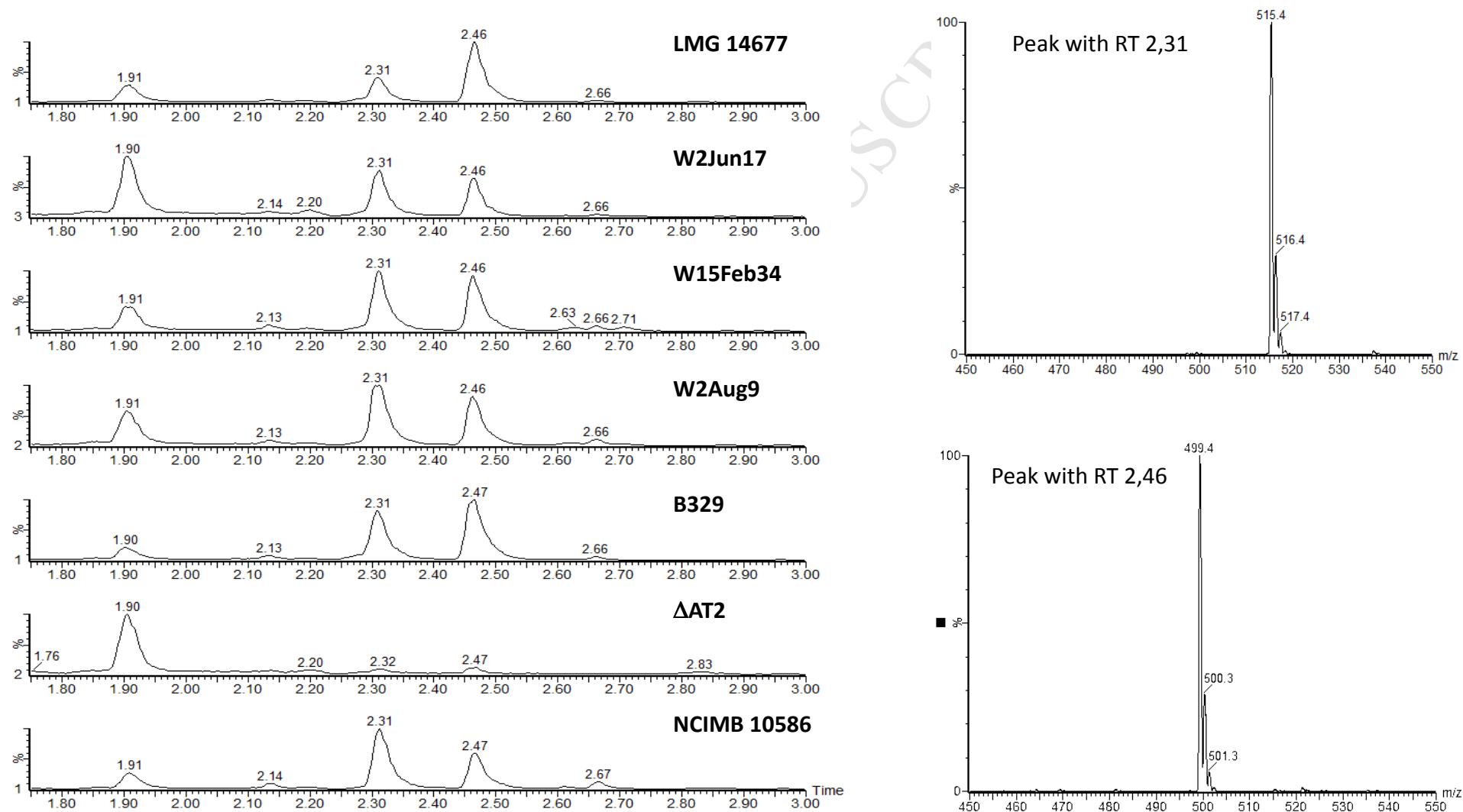
PltBf primer: 5' -CGGAGCATGGACCCCCAGC-3'

mmpD NCIMB 10586: 5' -ATGGCCGATATTGGATTTGGCCGAG-3'

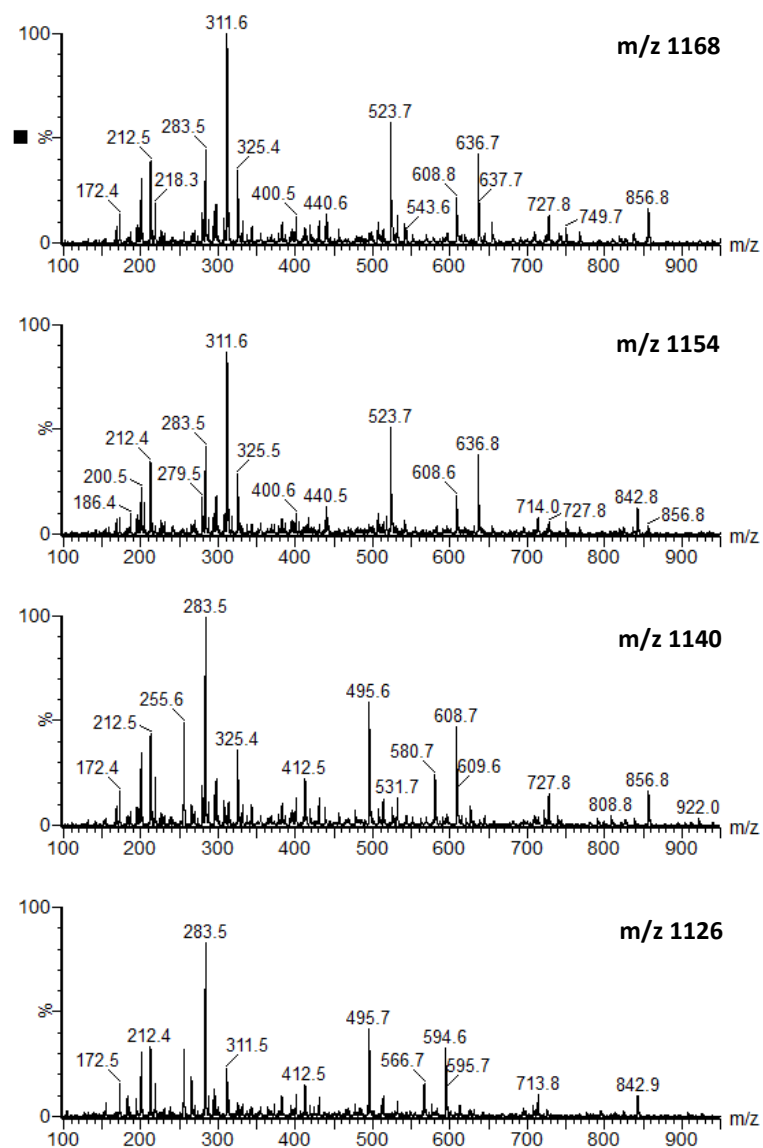
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PltBr primer: 5' -GTGCCCAGATATTGGTCTTGACCGAG-3'

Supplementary Fig. S2. Identification of main peaks in supernatants of the reference strain *P. fluorescens* NCIMB 10586, the mupirocin-negative mutant Δ AT2 and the mupirocin producing strains *P. fluorescens* LMG 14677 and *Pseudomonas* sp. W2Jun17, W15Feb34, W2Aug9 and B329. The peak with retention time (RT) 2.31 is pseudomonic acid B, the peak with RT 2.46 is pseudomonic acid A.



Supplementary Fig. S3. Fragmentation patterns obtained for the main lipopeptide molecular ions in *Pseudomonas* sp. W2Aug9.



Representative ions (m/z)	Fragment species
283,6	C10FA-Leu
412,6	C10FA-Leu-Glu
495,6 (-H ₂ O); 513,6 (-2H ₂ O)	C10FA-Leu-Glu-Thr
594,6 (-H ₂ O); 566,6 (-2H ₂ O)	C10FA-Leu-Glu-Thr-Val
608,6 (-H ₂ O); 580,6 (-2H ₂ O)	C10FA-Leu-Glu-Thr-Ile/Leu
186 (204, +H ₂ O)	Ser-Val
200 (218, +H ₂ O)	Ser-Ile/Leu
299 (313, +H ₂ O)	Leu-Ser-Val
313 (331, +H ₂ O)	Leu-Ser-Ile/Leu
386 (404, +H ₂ O)	Ser-Leu-Ser-Val
400 (418, +H ₂ O)	Ser-Leu-Ser-Ile/Leu
499 (517, +H ₂ O)	Leu-Ser-Leu-Ser-Val
513 (531, +H ₂ O)	Leu-Ser-Leu-Ser-Ile/Leu
612 (630, +H ₂ O)	Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu
695 (713, +H ₂ O)	Thr-Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu
824 (842, +H ₂ O)	Glu-Thr-Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu

Ion assignment:

m/z 1126 peak 1: C₁₀FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L
 m/z 1126 peak 2: C₁₀FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin
 m/z 1140 peak 1: C₁₀FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D
 m/z 1154 peak 1: C₁₂FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L
 m/z 1154 peak 2: C₁₂FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin
 m/z 1168 peak 1: C₁₂FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D