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# Antimicrobial properties of Pseudomonas strains producing the antibiotic mupirocin

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## Accepted Manuscript

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1	Antimicrobial properties of <i>Pseudomonas</i> strains producing the antibiotic mupirocin
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#### 21 Abstract

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

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

37

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

40

#### 41 **1. Introduction**

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

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

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

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

73

#### 74 2. Material and Methods

#### 75 2.1. Bacterial strains

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

83

#### 84 2.2. *Growth conditions*

The *Pseudomonas* strains (Table 1) were routinely grown at 28°C on an in house medium, medium 853, composed of 10 g l<sup>-1</sup> bacto tryptone, 5 g l<sup>-1</sup> yeast extract, 5 g l<sup>-1</sup> NaCl, 1 g l<sup>-1</sup> glucose, 0.7 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> and 0.3 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. *Escherichia coli* SM10 ( $\lambda$ pir) was grown in 853 at 37°C. Gentamicin (Gm) was used at a concentration of 50 µg ml<sup>-1</sup> (*P. fluorescens* NCIMB 10586) or 200 µg ml<sup>-1</sup> (*Pseudomonas* sp. W2Aug9), chloramphenicol (Cm) was used at a concentration of 25 µg ml<sup>-1</sup>. Antagonism tests were performed on 4 different media;

91 medium 853, blood agar (BBL<sup>TM</sup> 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 FeCl<sub>3</sub> to a final concentration of 94 100  $\mu$ M (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

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

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

124

#### 125 2.4. Mupirocin production

Mupirocin was purified from the culture supernatant as described in [12]. The
presence of the antibiotic was determined by HPLC with a reverse-phased C18 Altima
(GRACE) column (250 mm x 4.6 mm, 10 µM), using the conditions as described in [12].
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

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

139

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

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

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<sup>T</sup> 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

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 $\mu$ l 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 <sup>®</sup> BEH $C_{18}$ 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 <sup>-1</sup> 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

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

196

407

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

The *in vitro* antagonism of the mupirocin producing *Pseudomonas* strains against S. 198 aureus 383, a clinical strain isolated from infected femoral pin [21] was tested on 853 agar, 199 blood agar, MH agar, GCA and GCA amended with 100  $\mu$ M Fe. Five  $\mu$ l of OD<sub>600</sub> = 0.5 of the 200 Pseudomonas strain was inoculated in the center of the plate. The plates were incubated at 201 28°C and after 2 days the cells were killed using chloroform vapors for 25 min. After 202 evaporation of the chloroform a 6 ml overlay of soft agar (0.7% agar) of 853 with 5 x  $10^6$ 203 cells of S. aureus 383, was overlaid on the plate which was subsequently incubated at 37°C. 204 Clear zones of inhibition were measured the next day. All antagonism assays were performed 205 at least two times with 3 replicas. 206

207

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

The suicide plasmid pUT, which harbors the transposon mini-Tn*5phoA3* (Gm<sup>R</sup>) [22] was used to generate transposon insertions in the chromosome of *Pseudomonas* sp. W2Aug9. Mid-log phase cultures of *E. coli* SM10 ( $\lambda$ pir), the host of pUT-mini-Tn*5phoA3*, was mixed with strain W2Aug9 in a 1:1 ratio. The *Pseudomonas* strain was kept at 37°C for 1 h just before mixing with *E. coli* in order to inactivate its restriction system. After overnight 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

An antagonism-negative mutant of the mupirocin-negative strain 10586 $\Delta$ AT2 of P. 223 fluorescens NCIMB 10586 was obtained through Tn5 mutagenesis on 10586AAT2 using 224 mini-Tn5phoA3 as described in 2.13, with the modification that the transconjugants were 225 plated on a lower Gm concentration (50 µg ml<sup>-1</sup>). A library of 2000 candidates was screened 226 for complete loss of *in vitro* antagonism against S. aureus 383. Therefore the candidates were 227 228 grown overnight at 28°C in medium 853 in microtiter plates whereby every two wells were inoculated, alternating between an inoculated and not inoculated well. The cultures were 229 replicated on 853 medium and incubated for 16h at 28°C. An overlay of S. aureus 383 was 230 prepared as explained in paragraph 2.12. 231

232

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

A pyoverdine-negative mutant of *P. fluorescens* sp. NCIMB 10586 was obtained through Tn5 mutagenesis using mini-Tn5*phoA3* as described in 2.13. Tn5 insertions were selected on CAA supplemented with 50  $\mu$ g ml<sup>-1</sup> Gm and 25  $\mu$ g ml<sup>-1</sup> Cm. A bank of 576 transconjugants was screened for complete loss of fluorescence in the iron-limiting media CAA.

240

241 2.16. Molecular characterization of the Tn5 mutants

The chromosomal DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen), digested with *Pst*I or *Sal*I (Fermentas) and self-ligated. The DNA flanking the mini-Tn*5phoA3* was isolated and sequenced as described earlier [23].

245

#### 246 **3. Results**

247 3.1. Screening for mupirocin producing strains

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

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

same genes for the maturation of pseudomonic acid A, including the quorumsensing/quenching system encoded in the gene cluster.

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

284

#### 285 *3.2. Identification of mupirocin producing strains*

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

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

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

302

#### 303 *3.3. Production of antimicrobial metabolites and protease*

The six mupirocin producing strains were screened for additional well-known 304 antimicrobial metabolites and protease. They were screened by PCR for HCN and the 305 antibiotics 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and phenazine-1-carboxylic 306 acid. In the conditions used no amplification was obtained for any of these compounds. All 307 the mupirocin strains produced protease and had similar clear zones around the colony. They 308 all produced the siderophore pyoverdine. Pyoverdines are made of three distinct structural 309 parts: a small peptide chain of 6 to 14 L- and D-amino acids, linked to a yellow-green 310 chromophore group and to a small dicarboxylic acid [24]. Mass analysis showed that all 311 strains produced the same pyoverdine  $PYO_{13525}$ , with a peptide chain of 7 amino acids with 312 sequence Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser), the parentheses indicate a cyclic 313 structure [25]. This pyoverdine is relatively common and is produced by many strains of the 314 P. fluorescens group (Matthijs S., unpublished results). 315

316

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

The positive drop collapse test observed for three of the six strains, namely 318 Pseudomonas sp. W2Aug9, Pseudomonas sp. B329, and Pseudomonas sp. W15Feb34 319 suggested that those isolates may produce compounds with surface tension reducing activity. 320 In addition, together with *P. fluorescens* LMG 14677, they displayed hemolytic activity when 321 tested on red sheep blood cells. Strain W2Aug9 had the largest activity followed by 322 Pseudomonas sp. B329, P. fluorescens LMG 14677 and Pseudomonas sp. W15Feb34. Those 323 phenotypes prompted us to check for possible production of surfactants by these strains. 324 UPLC-MS analysis of culture supernatants revealed that strains W2Aug9, B329, LMG 14677 325 and W15Feb34 but not NCIMB 10586 and W2Jun17 secreted cyclic lipopeptides with major 326 peaks observed at m/z 1126.7, 1140.7, 1154.7, 1168.7 for molecular ions (Fig. 2, 327 328 Supplementary Fig. S3). These molecular ions correspond to the massetolide group of Pseudomonas lipopeptides and similar masses and retention times were observed by injecting 329 330 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 correspond to massetolides A/D, L and F/viscosin with either  $C_{10}$  or  $C_{12}$  fatty acid moieties. 332 Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the 333 fatty acid. The occurrence of several derivatives of the lipopeptide reflects some flexibility in 334 amino acid selection and activation by the A domains of the massetolide synthetases. 335

When comparing the level of *in vitro* antagonism of the mupirocin producing strains on the different media against *S. aureus* it was observed that strains producing lipopeptides generally showed a weak, but reproducible, larger zone of inhibition in the antagonism against *S. aureus* then strains not producing lipopeptides. To verify whether there was a synergistic effect a hemolytic-negative mutant was created in strain W2Aug9 which showed the largest

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

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

356

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

The mupirocin producing strains were strongly antagonistic against *S. aureus* 383 on the rich media tested (853, MH and blood agar). The level of *in vitro* antagonism of the six strains was comparable (data not shown) and results are detailed only for *P. fluorescens* NCIMB 10586 (Fig. 3 and Fig. 4A-D-G). The largest inhibition zone (radius of the zone) for NCIMB 10586 was observed on 853 (23.3  $\pm$  1.3 mm) (Fig. 4A), followed by MH (20.2  $\pm$  1.5 mm) (Fig. 4D) and blood agar (16.3  $\pm$  0.5 mm) (Fig. 4G). The mupirocin-negative mutant 10586 $\Delta$ 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  $\pm$  1.3 mm) and (5.2  $\pm$  0.8 366 mm), respectively (Fig. 4E and B).

To identify the nature of the compound responsible for the *in vitro* antagonism of the 367 mupirocin-negative mutant 10586 $\Delta$ AT2 on 853 (Fig. 4B) a Tn5 mutagenesis was carried out 368 and the library was screened for transconjugants that completely had lost the antagonism. 369 370 Several mutants were obtained and molecular characterization showed that in all these mutants the Tn5 insertions were in the NRPS genes of pyoverdine. As expected, pyoverdine 371 was no longer produced in the mutants, strongly suggesting that pyoverdine is responsible for 372 the antagonism of the mupirocin-negative mutant on medium 853. Mutant 10586AAT2-373 10H10 was selected, this mutant had a Tn5 insertion in the middle of the *pvdI* gene and the 374 translated sequence of the flanking region of the transposon showed the highest similarity to 375 PvdI of *P. synxantha* BG33R (98% identity at aa level of a 800 bp fragment). The *in vitro* 376 antagonism of this mutant was tested on 853, MH and blood agar (Fig. 3). On blood agar the 377 antagonism was completely lost (Fig. 4I), on 853 (Fig. 4C) and MH agar (Fig. 4F) an 378 inhibition zone was still observed but it was almost completely covered with S. aureus. Since 379 pyoverdine was responsible for the observed in vitro antagonism the antagonism was also 380 tested on the low iron media GCA and GCA amended with excess iron. On GCA and 381 GCA+Fe medium the wild type showed a clear inhibition zone (Fig. 4J and 4M). The 382 mupirocin-negative mutant showed a clear inhibition zone on GCA (Fig. 4K), this antagonism 383 was the largest observed on all the media tested for this mutant (Fig. 3). With the addition of 384 iron the antagonism was completely lost confirming that an iron regulated compound is being 385 produced (Fig. 4N). The double mupirocin/pyoverdine-negative mutant 10586\dAT2-10H10 386 (Fig. 3) showed an inhibition zone into which S. aureus was growing (Fig. 4L) on GCA 387 medium, as for the mupirocin-negative mutant the antagonism was completely lost in the 388 389 presence of iron (Fig. 4O).

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

397

#### 398 4. Conclusion

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

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

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

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

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.

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

Given the strong antimicrobial activity of mupirocin it would be interesting to test these strains as biocontrol agents against bacterial disease. The ability of some of the mupirocin producing strains to produce lipopeptides of the massetolide group, which have surfactant and antimicrobial properties, could potentially even make the strains active against fungal diseases [32]. 439

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## **Table 1.** List of strains used in this study.

Strain	<b>Relevant genotype/characteristics</b>	Reference
Pseudomonas fluorescens NCIMB 10586	Mupirocin producing strain isolated from soil, Hampstead Heath, London, U.K.	[1]
10586AAT2	Mupirocin-negative deletion (MmpC $\Delta$ 337-548) mutant of <i>P</i> . <i>fluorescens</i> NCIMB 10586	[12]
10586-3H3	Pyoverdine-negative Tn5 mutant of <i>P</i> . fluorescens NCIMB 10586 with a Tn5 insertion into the $pvdD$ gene, Gm <sup>R</sup>	This study
10586ΔAT2-10H10	Pyoverdine-negative mutant of $10586\Delta AT2$ with a Tn5 insertion into the <i>pvdI</i> gene, Gm <sup>R</sup>	This study
Pseudomonas fluorescens LMG 14677	Mupirocin producing strain	[33]
Pseudomonas 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 <sup>R</sup>	This study
Pseudomonas sp. B329	Mupirocin producing strain isolated from rhizosphere of grass, France	Bodilis J., France
Pseudomonas sp. W2Jun17	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
Pseudomonas sp. W15Feb34	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
Staphylococcus aureus 383	Clinical strain isolated from infected femoral pin, mupirocin-sensitive	[21]

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**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<sup>T</sup> 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.





**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 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<sub>10</sub> or C<sub>12</sub> fatty acid moieties. Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the fatty acid.





Fig. 3. The *in vitro* antagonism of the wild type *P. fluorescens* NCIMB 10586, the mupirocinnegative mutant  $10586\Delta AT2$ , the pyoverdine-negative mutant 10586-3H3 and the double mupirocin/pyoverdine-negative mutant  $10586\Delta AT2-10H10$ . 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. 4.** Comparison of the *in vitro* antagonism of *P. fluorescens* NCIMB 10586 (first column), the mupirocin-negative mutant  $10586\Delta AT2$  (second column) and the double mupirocin/pyoverdine-negative mutant  $10586\Delta AT2$ -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).





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

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P. brassicacearum subsp.	DBK11 <sup>T</sup>	Rhizoplane of Brassica napus	Dieulouard, France
brassicacearum			
P. brassicacearum subsp.	CIP 109457 <sup>1</sup>	Rhizophere of hemp plants	Ukraine, near Kiev
neoaurantiaca	T		
P. brenneri	DSM 15294 <sup>1</sup>	French natural mineral water after bottling	Lille, France
P. caricapapayae	CFBP 3204 <sup>T</sup>	Carica papaya	Brazil
P. cedrina subsp. cedrina	$LMG 23661^{T}_{-}$	Spring water	Lebanon
P. cedrina subsp. fulgida	DSM 14938 <sup>T</sup>	Phyllosphere of grasses	Paulinenaue, Germany
P. chlororaphis subsp. aurantiaca	ATCC 33663 <sup>T</sup>		
P. chlororaphis subsp. aureofaciens	LMG 1245 <sup>T</sup>	Maas River clay suspended in kerosene for	Netherlands
		three weeks	
P. chlororaphis subsp. chlororaphis	ATCC 9446 <sup>T</sup>	Plate contaminant	
P. cichorii	CFBP 2101 <sup>T</sup>	Cichorium endivia	Germany
P. citronellolis	CFBP 5585 <sup>T</sup>	Soil under pine trees	-
P. composti	DSM 25648 <sup>T</sup>	Mixed vegetables and animal waste compost	
P. congelans	CFBP 7019 <sup>T</sup>	Phyllosphere of grasses	Germany
P. costantinii	CFBP 5705 <sup>T</sup>	Agaricus bisporus	Finland
P. corrugata	LMG 2172 <sup>T</sup>	Lycopersicon esculentum (tomato) pith	United Kingdom
	DOM 17050T	necrosis	I
P. cremoricolorata	DSM 17059	Oryza sativa	Japan
P. cuatrocienegasensis	LMG 24676	water (evaporating lagoon)	Mexico
P. deceptionensis	LMG 25555 <sup>T</sup>	Marine sediment	Deception Island, South Shetland
Ĩ			Islands, Antarctica
P. delhiensis	DSM 18900 <sup>T</sup>	Soil sample contaminated by polycylic	India
P duriflava	DSM 21/10 <sup>T</sup>	Desert soil	Vinijang Province China
D antomonhila		Drosonhila	Calvaira Guadaloupa Erança
<ul> <li><i>P</i> axtromaustralis</li> </ul>	DSM 17835 <sup>T</sup>	Temporary water pond	Dance Coast Cierva Point
1. exitemuusituus	DOM 1/033	remporary water pond	Antarctica
P. extremorientalis	CCUG 51517 <sup>T</sup>	Drinking water reservoir	Vladivostock City, Russia
P. ficuserectae	CCUG 32779 <sup>T</sup>	Ficus erectae	-

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

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P. mohnii		CCUG 53115 <sup>T</sup>	Sequencing batch reactor treating paper mill	Canada
		т	effluent, enrichment with isopimaric acid	
P. monteilii		DSM 14164 <sup>1</sup>	Human bronchial aspirate	France
P. moorei		DSM 12647 <sup>1</sup>	Soil samples	
P. moraviensis		DSM 16007 <sup>1</sup>	Soil besides highway	Czech Republic
P. mosselii		ATCC BAA-	Medical specimen	France
		99 <sup>1</sup>		
P. mucidolens		CCUG 1424 <sup>1</sup>	Musty egg	
P. nitroreducens		ATCC 33634 <sup>T</sup>	Oil-brine	Japan
<i>P. oleovorans</i> sub	osp. <i>oleovorans</i>	CFBP 5589 <sup>T</sup>	Cutting fluid	
<i>P. oleovorans</i> sub	osp. <i>lubricantis</i>	DSM 21016 <sup>1</sup>	Contaminated metalworking fluids	Houghton, Michigan, USA
P. orientalis		DSM 17489 <sup>T</sup>	Spring water	Lebanon
P. otitidis		DSM $17224^{T}$	Ear of patient with acute Otitis externa	USA
P. palleroniana		CFBP $4389^{T}$	Oryza sativa	Cameroon
P. panacis		DSM 18529 <sup>T</sup>	Rusty root lesions of Korean ginseng	South Korea
P. panipatensis		LMG 24738 <sup>T</sup>	Oil contaminated soil	Panipat Oil Refinery, India
P. parafulva		DSM 117004 <sup>T</sup>	Oryza sativa	Japan
P. peli		CIP 109374 <sup>T</sup>	Antarctic green alga Pyramimonas gelidicola	Antarctica
P. plecoglossicid	а	CIP 106493 <sup>T</sup>	Diseased ayu, Plecoglossus altevelis	Japan
P. poae		CFBP 6764 <sup>T</sup>	Grasses, phyllopshere	Paulinenaue, Brandenburg,
-				Germany
P. pohangensis		DSM 17875 <sup>T</sup>	Sea shore sand	Korea
P. prosekii		LMG 26867 <sup>T</sup>	Rock biofilm	James Ross Island, Waterfall, west
			) *	side of Lachmen Crags Antarctica
P. protegens		CHA0 <sup>T</sup>	Soil suppressing black root rot of tobacco	Switzerland
			(Nicotiana glutinosa)	
P. proteolytica		CIP 108464 <sup>T</sup>	Cyanobacterial mat samples	Antarctica
P. putida		CFBP 2066 <sup>T</sup>	Soil, lactate enrichment	United States
P. reinekei		CCUG 53116 <sup>T</sup>	Aerobic zone of Elbe sediment enriched with	Germany
			4-chlorosalicylate	-
P. resinovorans		CCUG 2473 <sup>T</sup>	Soil	France, Vienne

P. rhodesiae	ATCC 17764 <sup>T</sup>	Natural mineral water	France
P. sabulinigri	DSM 23971 <sup>T</sup>	Black sand originating from black volcanic basalt rock	Jeju Island, Soesgoggak, Korea
P. salomonii	CFBP 2022 <sup>T</sup>	Allium sativum	France
P. saponiphila	DSM 9751 <sup>T</sup>	O Y	Michigan, USA
P. savastanoi	CIP 103721 <sup>T</sup>	Olea europaea	Yugoslavia
P. segetis	CCUG 54777 <sup>T</sup>	Soil	Korea
P. simiae	DSM 18861 <sup>T</sup>	Lung of female monkey (Callithrix geoffroyi)	Spain
		with pneumonitis and pneumonia	-
P. straminea	CCUG 12539 <sup>T</sup>	Japanese unhulled rice	Japan
P. stutzeri	ATCC 17588 <sup>T</sup>	Spinal fluid	
P. synxantha	DSM 18928 <sup>T</sup>	Cream	Iowa, USA
P. syringae subsp. syringae	ATCC 19310 <sup>T</sup>	Syringa vulgaris	United Kingdom
P. taetrolens	CCUG 560 <sup>T</sup>	Musty egg	
P. taiwanensis	$DSM 21245^{T}$	Soil	Tamkang University, Taipei, Taiwan
P. thivervalensis	CFBP 5754 <sup>T</sup>	Rhizoplane of Brassica napus	Sexy-les-Bois, France
P. tolaasii	CFBP $2068^{T}_{-}$	Agaricus bisporus	United Kingdom
P. trivialis	CFBP 6765 <sup>T</sup>	Grasses, phyllopshere	Paulinenaue, Brandenburg,
	T		Germany
P. umsongensis	CIP 108618 <sup>T</sup>	Agricultural soil	Korea
P. vancouverensis	ATCC	Forest soil	Canada
	700688 <sup>1</sup>		
P. veronii	CFML 92-134 <sup>T</sup>	Mineral water	France
P. viridiflava	ATCC 13223 <sup>T</sup>	Dwarf or runner bean	Switzerland
P. vranovensis	DSM 16006 <sup>T</sup>	Soil besides highway	Czech Republic
P. xiamenensis	DSM 22326 <sup>T</sup>	Activated sludge sample collected at Qianpu	Fujian, Xiamen, China
		sewage treatment plant	
	Y		

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

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P. putida
P. putida
Pseudomonas sp.
P. stutzeri
P. fluorescens

Lycopersicon esculentum Lycopersicon esculentum Lycopersicon esculentum

Clinical

Spring water (Kadicha)

Black earth soil Seawater Polluted seawater Well water

Polluted natural stream *Cichorium intybus*, leaves showing blackening of vascular system Guadeloupe, France Angers, France Angers, France

Lebanon

Gatton, Queensland, Australia

Dresund, Denmark

South Copenhagen, Denmark United Kingdom **Supplementary Table S3.** List of *Pseudomonas* strains and isolates obtained from different laboratories and their biological and geographic origin.

Pseudomonas 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 Salsola vermiculata, 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,	[2]
MFY78, MFY79	Dun River, France	[2]
MFY70	Suppuration, Pitié Salpêtrière Hospital,	[2]
MFY65	Infected sinus	[2]
MFY61	Articular infection	[2]
MFY162	Sputtum, Charles Nicolle Hospital,	[2]
MFY69	France Gastric fluid, Pitié Salpêtrière Hospital,	[2]
MFY71	Clinical	[2]
R2f, B13, B125, B136, B163, B215, B329	Rhizosphere	Josselin Bodilis, France
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	(unpublished) [3]

#### PD4, PD5, PD11, PD13, PD15, PD22, PD28, PD31

*P. putida* BTP1, *P. fluorescens* BTP2, BTP7', BTP9, BTP14, PP, *P. putida* PutC

P. putida Irc204

*P. fluorescens* E211, *P. kilonensis/brassicacearum* G11, *P. fluorescens* G27

P. putida WCS358, WCS365, P. fluorescens WCS374

Pseudomonas sp. B10, P. fluorescens G153, P. fluorescens G166, P. fluorescens G173, P. chlororaphis D-TR133, P. putida G4R, P. fluorescens 99-13, P. fluorescens PL7, P. fluorescens PL8, P. fluorescens PL9, Pseudomonas sp. D46, Pseudomonas sp. D47, P. fluorescens Pflii, P. fluorescens Pf112, P. fluorescens PflW, Pseudomonas sp. F360, P. fluorescens 9AW, P. fluorescens 1547, SB8.3, Pseudomonas sp. 2908, P. fluorescens 1.3, P. fluorescens 18.1, HR6, P. monteilii Lille 1, P. fluorescens 51W, P. rhodesiae Lille 25, Gwose, Pseudomonas sp. A214, P. fluorescens A225, P. putida Thai, P. aeruginosa R', Pseudomonas sp. 6-10, LBSA1, IB3, 7SR1, ML35, Malar, P. aeruginosa Pa6, Lille 17, Pseudomonas sp., Pseudomonas sp. G400

Arable soil used for potato production, New Brunswick, Canada

Soil, Belgium

Rhizosphere of *Echinochloa crus*galli/Galium mollugo . . . . .

Collection obtained from Marc Ongena, Belgium

IRMW

[4]

[5]

Collection obtained from Jean-Marie Meyer, France

*P. putida* GM 10090 (G297), *P. putida* GM 11799.1 (G309), *P.* Plant rhizosphere *putida* GM 12220 (G314)

*P. putida* GS4 (GS12059), *P. putida* GS35 (GS12082), *P. putida* GS37 (GS12064)

Plant rhizosphere (banana tree)

[7]

[6]

10

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

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- [11] Matthijs S, Budzikiewicz H, Schäfer M, Wathelet B, Cornelis P, Ornicorrugatin, a new siderophore from *Pseudomonas fluorescens* AF76, Z Naturforsch 63c (2008) 8-12.

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Primer	Sequence 5'-3'	Size amplicon	Primer	Sequence 5'-3'	Size amplicon
mupZ-F	ATGAATCGCACCTGCATGGC		mupL-F	ATGCAACTGATCACGCACGA	
mupZ-R	GAACTGCGGCITCATAAACG	324 bp	mupL-R	ATCCACGTCTGCCTCACCCT	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	CGTTGAGCGATTTCGACAAT	2140 bp	mupO-R	GTAGGAGATGATGGCGTGGIT	775 bp
MetD3-F	ATGITCCCCGAGGGTTCCAT		mupP-F	GTGAGCAGGTCCGAACTTG	
MetD3-R	GCTCAGCTCGITCAACAACA	470 bp	mupP-R	TCGGTAAAGGGCAITGAAGTG	699 bp
mupC-F	AAGGTCGTCGGGITCACTGG		mupQ-F	ATGAGAGAGGAACGTAATTGG	
mupC-R	CGGTAAGTGGCTTCGGACAA	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	TCAGGGTGCTGTCGGGGGATA		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	CCCAGCAGGAITGACTCGTA	1121 bp	mupX-R	AAGAGTGAGGCGGGCTACG	1510 bp
mupJ-F	ATGAACITCCAGGCCACCGA		mupl-F	TAGCATTGACTGCGTCCAGG	
mupJ-R	TGGCTGACATAGCGGTGGAT	740 bp	mupl-R	GAACATATCACCACGITATCTGGA	555 bp
mupK-F	GGACAGTGTCATCGATITTCAGG		mupR-F	TGCITGAAGACAITCTGATG	
mupK-R	CCAACTCCATCGCCAACTCC	581 bp	mupR-R	GCCACCCACITCAGACA	550 bp

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

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

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

**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'

 PltBf primer:
 5'-CGGAGCATGGACCCCCAGC-3'

mmpD NCIMB 10586:

5'-ATGGCCGATATTGGATTTGGCCGAG-3

CEP .

PltBr primer: 5'-GTGCCCGATATTGGTCTTGACCGAG-







595.7

600

713.8

700

842.9

900

800

⊢m/z

412.5

400

500

0

100

200

300

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 <sub>2</sub> O); 513,6 (-2H <sub>2</sub> O)	C10FA-Leu-Glu-Thr
594,6 (-H <sub>2</sub> O); 566,6 (-2H <sub>2</sub> O)	C10FA-Leu-Glu-Thr-Val
608,6 (-H <sub>2</sub> O); 580,6 (-2H <sub>2</sub> O)	C10FA-Leu-Glu-Thr-Ile/Leu
186 (204 - 111 - 0)	
$180(204, \pm \Pi_2 0)$	
200 (218, +H <sub>2</sub> O)	Ser-IIe/Leu
299 (313, +H <sub>2</sub> O)	Leu-Ser-Val
313 (331, +H <sub>2</sub> O)	Leu-Ser-Ile/Leu
386 (404, +H <sub>2</sub> O)	Ser-Leu-Ser-Val
400 (418, +H <sub>2</sub> O)	Ser-Leu-Ser-Ile/Leu
499 (517, +H <sub>2</sub> O)	Leu-Ser-Leu-Ser-Val
513 (531, +H <sub>2</sub> O)	Leu-Ser-Leu-Ser-Ile/Leu
612 (630, +H <sub>2</sub> O)	Val/lle/Leu-Leu-Ser-Leu-Ser-Val/lle/Leu
695 (713, +H <sub>2</sub> O)	Thr-Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu
$874(842 + H_{2}O)$	Glu-Thr-Val/IIe/Leu-Leu-Ser-Leu-Ser-Val/IIe/Leu

#### Ion assignement:

m/z 1126 peak 1:C<sub>10</sub>FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L m/z 1126 peak 2: C<sub>10</sub>FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin m/z 1140 peak 1:C<sub>10</sub>FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D m/z 1154 peak 1: C<sub>12</sub>FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L m/z 1154 peak 2: C<sub>12</sub>FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin m/z 1168 peak 1: C<sub>12</sub>FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D