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Instructions for use

1 Flexible exportation mechanisms of arthrofactin in *Pseudomonas* sp. MIS38

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4 *Running Title*: Arthrofactin exporter

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19

20 **Abbreviations:** aa, amino acids; ArfD, periplasmic protein; ArfE, ATP-binding cassette;

21 bp, base pairs; kb, kilobase pairs; Glb, glibenclamide; Ovd, sodium ortho-vanadate NBD,

22 nucleotide binding domain(s); NRPS, nonribosomal peptide synthetase; TMD,

23 transmembrane domain(s); TMS, transmembrane segment(s)

24   **ABSTRACT**

25   **Aims:**   To obtain further insights into transportation mechanisms of a most effective

26   biosurfactant, arthrofactin in *Pseudomonas* sp. MIS38.

27   **Methods and Results:**   A cluster genes *arfA/B/C* encodes an arthrofactin synthetase

28   complex (ArfA/B/C).   Downstream of the *arfA/B/C* lie genes encoding a putative

29   periplasmic protein (ArfD, 362 aa) and a putative ATP-binding cassette transporter (ArfE,

30   651 aa), namely *arfD* and *arfE,* respectively.   The *arfA/B/C*, *arfD*, and *arfE* form an

31   operon suggesting their functional connection.   Gene knockout mutants ArfD:Km,

32   ArfE:Km, ArfD:Tc/ArfE:Km, and gene overexpression strains MIS38(pME6032_*arfD/E*)

33   and ArfE:Km(pME6032_*arfD/E*) were prepared and analyzed for arthrofactin production

34   profiles.   It was found that the production levels of arthrofactin were temporally reduced

35   in the mutants or increased in the gene overexpression strains, but they eventually

36   became similar level to that of MIS38.   Addition of ABC transporter inhibitors,

37   glibenclamide and sodium ortho-vanadate dramatically reduced the production levels of

38   arthrofactin.   This excludes a possibility that arthrofactin is exported by diffusion with the

39   aid of its own high surfactant activity.

40   **Conclusions:**   ArfD/E is not an exclusive but a primary exporter of arthrofactin during

41   early growth stage.   Reduction in the arthrofactin productivity of *arfD* and *arfE* knockout

42   mutants was eventually rescued by another ABC transporter system.   Effects of *arfD* and

43   *arfE* overexpression were evident only for one-day cultivation.   Multiple ATP dependent

44   active transporter systems are responsible for the production of arthrofactin.

45   **Significance and impact of the study:**   *Pseudomonas* bacteria are characterized to be

46   endued with multiple exporter and efflux systems for secondary metabolites including

47   antibiotics, plant toxins, and biosurfactants.   The present work demonstrates

48   exceptionally flexible and highly controlled transportation mechanisms of a most

49   effective lipopeptide biosurfactant, arthrofactin in *Pseudomonas* sp. MIS38.   Because

50   lipopeptide biosurfactants are known to enhance efficacy of bioactive compounds and

51   *arfA/B/C/D/E* orthologous genes are also found in plant pathogenic *P. fluorescens* and *P.*

52   *syringae* strains, the knowledge would also contribute to develop a technology

53   controlling   plant   diseases.

<u>INTRODUCTION</u>

55   Exportation of many nonribosomal peptides and polyketides requires ABC (ATP-

56 binding cassette) transporter system that couples the transport with ATP hydrolysis. The

57 ABC transporter proteins are generally composed of two hydrophobic transmembrane

58 domains (TMD) and hydrophilic nucleotide-binding domains (NBD) bound or fused to

59 the cytosolic face of the TMD (Biemans-Oldehinkel *et al.* 2006). TMD are hydrophobic

60 parts that create a channel through which the substrate passes during translocation. They

61 are composed of bundles of α-helices that transverse the cytoplasmic membrane several

62 times in a zig-zag fashion. NBD are the engines of ABC transporters that power substrate

63 translocation by ATP hydrolysis and commonly have a set of ATP binding motifs,

64 Walker A and B.

65   An ABC transporter system usually requires two accessory envelope proteins for

66 its full function. One is a membrane fusion protein (or periplasmic protein) which

67 consists of a short N-terminal hydrophobic domain anchoring it to the inner membrane, a

68 large hydrophilic domain located at the periplasm, and a C-terminal domain with a

69 possible β-sheet structure which could interact with the outer membrane protein (Dinh *et*

70 *al.* 1994). Another accessory component is an outer membrane TolC family protein,

71 which transports the efflux substrates to the culture medium (Wandersman and

72 Delepelaire, 2004). These whole components are also required for the type I protein

73 secretion system in most of the gram-negative bacteria (Binet *et al.* 1997).

74   *Pseudomonas* sp. MIS38 produces a cyclic lipopeptide, named as arthrofactin,

75 which is one of the most effective biosurfactants (Morikawa *et al.* 1993). Arthrofactin

76 synthetase genes (*arfA/B/C*) encode a multimodular nonribosomal peptide synthetase

77 (NRPS; ArfA/B/C) whose unique architecture has been reported recently (Roongsawang

78 *et al.* 2003; 2005). ArfA, ArfB, and ArfC proteins consist of two, four, and five modules,

79 where each module contains a set of condensation (C), adenylation (A), and thiolation (T)

80 domains. Seven of the eleven modules incorporate D-form amino acids, where in this

81 case, dual condensation/epimerization (C/E) domains are present in the place of authentic

82 C domains (Balibar *et al.* 2005). At the C-terminal end of the last module of ArfC, two

83 thioesterase (TE) domains are present that are responsible for cyclization and release of

84 the product peptide from the enzyme (Roongsawang *et al.* 2007). The present work

85 focuses on the function of further downstream two genes, encoding a putative periplasmic

86 protein (ArfD) and a putative ABC transporter protein (ArfE).

87   There are reports that ABC transporter genes are clustered along with synthetase

88 genes of secondary metabolites to be exported out from the cells (Méndez and Salas

89 2001). The knowledge prompted us to examine how these two genes, embedded in the

90 arthrofactin synthetase gene cluster, play a role in the exportation of arthrofactin by

91 MIS38 cells. A series of experimental results suggested that ArfD/E is not an exclusive

92 transporter system for arthrofactin and that exportation of arthrofactin is exceptionally

93 flexible enough to regain normal production levels in the transporter gene knockout

94 mutants and the gene overexpression strains.

95

96 **MATERIALS AND METHODS**

97 **Bacterial strains, plasmids and culture conditions**

98   The bacterial strains and plasmids used in this study are listed in Table 1.

99 *Pseudomonas* sp. MIS38 and its derivatives were grown in Luria-Bertani (LB) broth (per

100 liter: 10g tryptone, 5g yeast extract and 5g NaCl, pH 7.3) at 30ºC for arthrofactin

101 production. *E.coli* DH5α was grown at 37ºC while *E.coli* SM10λpir was grown at 30ºC

102 in LB broth. SOC medium was used for cultivation after electroporation (Sambrook and

103 Russel 2001). Antibiotics were used at the following concentrations (mg l^{-1}): kanamycin

104 (Km) 35, chloramphenicol (Cm) 34 and tetracycline (Tc) 40 for *Pseudomonas* sp. MIS38;

105 ampicillin (Ap) 50, Tc 25, and Km 35 for *E. coli*.

106

107 **General molecular biological methods**

108   DNA manipulations were according to standard protocols (Sambrook and Russell

109 2001) unless described in details. Chromosomal DNA of MIS38 and mutant strains were

110 prepared by Marmur method (Marmur 1961). DNA fragments were recovered from

111 agarose gel by QIAquick Gel Extraction Kit (Qiagen) and plasmids were extracted using

112 QIAprep Spin Miniprep Kit (Qiagen). DNA from phage λ-S9 and λ-S12 were extracted

113 using Lambda Maxi Kit (Qiagen). Cohesive ends of gene fragments were occasionally

114 filled using Takara DNA Blunting Kit (Takara Bio) while ligation was performed using

115 Takara Ligation Kit ver. 2.1 (Takara Bio). DNA sequencing was performed by ABI

116 Prism 3100 Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing kit

117 (Applied Biosystems).

118   Standard PCR was performed for 30 cycles using PTC-100 Programmable

119 Thermal Controller (MJ Research) and *Ex-Taq* DNA polymerase (Takara Bio) or KOD

120 *Plus* DNA polymerase (Toyobo). Oligonucleotides for PCR primers were synthesized by

121 Hokkaido System Science. Primers used in this study were listed in Table 2. The

122 nucleotide sequences were analyzed by GENETYX software (GENETYX) and BLAST

123 programs (http://www.ncbi.nlm.nih.gov). Amino acid sequences were analyzed by

124 ClustalW program (http://clustalw.genome.jp).

125

126 **Reverse transcription polymerase chain reaction (RT-PCR)**

127 RT-PCR experiment was performed as follows. Total RNA was extracted from

128 the culture of MIS38 according to manufacturer's protocols using RNAeasy Mini Kit

129 (Qiagen). One microgram of DNase-treated RNA was used to synthesize first-strand

130 cDNA in 20 µl volume with random primers and Reverse Transcriptase System

131 (Promega). After completion of reverse transcription, 1 µl of products was used for PCR

132 amplification of DNA fragments using each specific primer and KOD *Plus* DNA

133 polymerase (Toyobo).

134

135 **Prediction of transmembrane segments (TMS) of ArfE**

136 TMS of ArfE were predicted using the Membrane Protein Explorer (MPex)

137 programme by Jayasinghe *et al.* (2001) at http://blanco.biomol.uci.edu/mpex. This

138 sliding-window hydrophobicity analysis of amino acid sequences of membrane proteins

139 is useful to identify putative transmembrane parts.

140

141 **Gene cloning of a putative periplasmic protein, ArfD and insertion of kanamycin**

142 **resistant gene cassette for gene knockout experiment**

143 Nucleotide sequences of the gene encoding putative periplasmic protein (ArfD) in

144 MIS38 were obtained by Roongsawang *et al.* (2003) (AB107223). Km resistant gene

145 cassette (*kan*) was inserted in the structurally important N-terminal β4 region to construct

146 ArfD:Km. Primers MFP/*Xba*I (f) and 708r(MFP) were used to obtain 700bp fragment at

147 the N-terminal part of ArfD while primers MFP/*Xba*I(r) and 736f(MFP) were used to

148 obtain 1.3kb fragment at the C-terminal part of ArfD. Both 708r(MFP) and 736f(MFP)

149 primers had an additional *Kpn*I site, for ligation purposes between the 700 bp and 1.3 kb

150 fragments. The ligation product yielded *arfDΔN-β4*. DNA sequencing was performed in

151 order to confirm introduction of the deletion without unexpected mutation. On the other

152 hand, 1.2 kb fragment of *kan* was obtained by PCR using pSMC32 and primers

153 pSMC32/*Kpn*I(f) and pSMC32/*Kpn*I(r). It was then cloned into *Kpn*I gap of *arfDΔN-β4*

154 in pGEMT, creating pArfD:Km. After that pArfD:Km was digested with *Xba*I and

155 subcloned into suicide vector pCVD442, producing pArfD:Km442 and transferred into

156 *E.coli* SM10λpir by electroporation. After that, conjugation between MIS38 and

157 SM10λpir(pArfD:Km442) was carried out (Roongsawang *et al.* 2007). Transconjugants

158 were selected on a plate containing both Km (35 mgl⁻¹) and Cm (34 mg l⁻¹). MIS38 was

159 originally resistant to Cm, and Cm inhibited the growth of donor *E.coli* cells. In order to

160 verify successful construction of the mutant gene in MIS38 chromosome, chromosomal

161 DNA from mutants were extracted using InstaGene Matrix (BioRad) and used for PCR

162 amplification with a primer set Fl(MFP)ORF5-7521f and Fl(MFP)ABC-493r. A mutant

163 strain ArfD:Km was thus obtained and used for further studies.

164

165 **Gene cloning of ABC transporter, ArfE and insertion of *kan* for gene knockout**

166 **experiment**

167 The first 120 bp gene encoding N-terminal part of a putative ABC transporter

168 gene had been previously obtained (AB107223). Rest of the complete gene, *arfD/E* was

169 obtained in this study from a genomic library of phage λ-S12 (Roongsawang *et al.* 2003).

170 In order to construct a gene knockout mutant strain ArfE:Km, *kan* cassette was inserted in

171 the *Sma*I site located between linker peptide and Walker B region of ArfE. The 2 kb

172 fragment including *arfE* was amplified by a set of primers ABC/*Xba*I-563f and

173 ABC/*Xba*I-2583r. Then, it was cloned into *Xba*I site of pGEMT-vector, producing

174 pArfETv. The 1.2 kb *kan* was prepared by PCR using pSMC32/*Sma*I(f) and

175 pSMC32/*Sma*I(r). This fragment was then cloned into pArfETv at a newly introduced

176 *Sma*I site in *arfE*, followed by transfer of the resulting 3.2 kb *Xba*I fragment into

177 pCVD442. The following procedures were according to above described. Selection of

178 positive mutants was confirmed using a primer set, ABC/*Xba*I-518f and ABC/*Xba*I-2628r.

179 A mutant strain ArfE:Km was thus obtained and used for further studies.

180

181 **Construction of a double mutant strain ArfD:Tc/ArfE:Km**

182 The 1.4 kb tetracycline resistant gene cassette (*tetA*) was obtained by PCR with a

183 primer set TetA-2(f) and TetA-2(r), and pME6032 as a template. This DNA fragment

184 was cloned into pUC18 at *Sma*I site to construct pTetA32. Then, *Xba*I fragment

185 containing *tetA* was blunt ended and inserted into the blunt ended *Kpn*I site in *arfDΔN-β4*,

186 creating pArfD:Tc. Then, pArfD:Tc442 was obtained by cloning *arfDΔN-β4::tetA into*

187 *Xba*I site of pCVD442. After that, conjugation between strain ArfE:Km and *E. coli*

188 SM10λpir harboring pArfD:Tc442 was carried out. Selection of mutants was performed

189 on the LB agar plate containing both Tc (40 mgl^{-1}) and Cm (34 mgl^{-1}). Then, genomic

190 DNA from the candidate strain was extracted for PCR template, and a set of primers

191 Fl(MFP)ORF57521f and Fl(MFP)ABC493r was used to verify introduction of *tetA* in

192   *arfD*. Another primer set, MfpABC1503f and MfpABC5661r was used to detect the

193   presence of both the *tetA* and *kan* resistant gene cassette in the double mutant, strain

194   ArfD:Tc/ArfE:Km. Double crossover event occurred during the conjugation process

195   without sucrose treatment.

196

197   **Construction of a gene expression plasmid for *arfD* and *arfE***

198   The 3,222 bp gene encoding both *arfD* and *arfE* was amplified using primer set

199   *Sac*IPPP-ABC79f and *Kpn*IPPP-ABC3300r. It was then cloned into the *Sac*I and *Kpn*I

200   site of an expression vector pME6032 to construct pME6032_*arfD/E*, and transformed

201   *E.coli* DH5α. After confirming the correct sequence of the gene fragment, this plasmid

202   or pME6032 vector only was then electrotransferred into wild type MIS38 and strain

203   ArfE:Km. Electroporation was carried out using Electro Gene Transfer Equipment

204   (GTE-10, Shimadzu) with 0.1 cm electrode distance cuvette at a pulse condition of 12.5

205      $kVcm^{-1}$, 35 µF for 3 msec. After applying the electric pulse, the cells were cultured in

206   SOC medium at 30ºC for 2 hr, 150 rpm shaking before spreading on LB agar plate

207   supplemented with 40 mgl^{-1} Tc. The plates were incubated at 30ºC overnight. The

208   obtained strains MIS38(pME6032), MIS38(pME6032_*arfD/E*), ArfE:Km(pME6032) and

209   ArfE:Km(pME6032_*arfD/E*) were used for further analysis.

210

211   **Southern hybridization**

212   Southern hybridization was carried out according to Lim *et al*. (2007) in order to

213   verify successful gene disruption at a single locus of MIS38 chromosome.

214

Analysis of arthrofactin production

215   **Analysis of arthrofactin production**

216   Analysis of arthrofactin production was carried out according to Roongsawang *et*

217   *al.* (2007). Briefly, arthrofactin was precipitated from culture supernatant by addition of

218   diluted HCl to pH 2 followed by centrifuge (10,000 g for 20 min). Hydrophobic fraction

219   of the precipitates containing arthrofactin was extracted by methanol. Samples were then

220   separated by reverse-phase HPLC using Cosmosil 5C18 AR column (4.6 x 150 mm,

221   Nacalai). Detection of compounds was performed either by UV detector (HP1100,

222   Agilent Technologies) or ESI-mass spectrometer (LCQ, Thermo Scientific). The amount

223   of arthrofactin was calculated from the area of peaks recorded by UV detector.

224

225   **ABC transporter inhibitors and their effect on arthrofactin production**

226   It was shown that sodium ortho-vanadate (Ovd) effectively inhibit the activity of

227   an ABC transporter MacA/B in *E.coli* (Tikhonova *et al.* 2007). It has been reported that

228   the Walker A motif in NBD is involved in the Ovd binding for inhibition of ATPase

229   activity (Pezza *et al.* 2002). Glibenclamide (Glb), a sulphonylurea also has been shown

230   to inhibit the activities of various ABC transporters (Serrano-Martin *et al.* 2006). Strain

231   ArfE:Km was grown in LB broth for 14 h until the early stationary phase ($OD_{600}\sim2.2$),

232   after which 0.1 mM or 0.25 mM of Glb (Sigma), or 2 mM sodium Ovd (Wako Pure

233   Chemicals) was added to the culture medium, and further cultivated for 1 and 2 d. Then,

234   the production of arthrofactin was analyzed.

235

236   **Nucleotide and amino acid sequence accession numbers**

237   Nucleotide sequences for the gene encoding putative periplasmic protein (ArfD),

238 putative ABC transporter (ArfE) were registered in the GenBank under accession number

239 AB286215. Amino acid sequences of each protein were submitted under accession

240 number BAC67537 (ArfD) and BAF40423 (ArfE).

241

242 **RESULTS**

243 **Analysis of a putative periplasmic protein gene, *arfD* and an ABC transporter gene,**

244 ***arfE* located downstream of arthrofactin synthetase genes**

245   A putative periplasmic protein gene, *arfD*, was previously found 127 bp

246 downstream of *arfC* (orf5 in AB107223). BLASTP analysis of ArfD showed high

247 homology with members of the periplasmic protein component of membrane transporters

248 such as HlyD from *P. fluorescens* PfO-1 (YP_347946), macrolide ABC efflux type

249 carrier MacA from *P. fluorescens* Pf-5 (YP_259255), *P. syringae* pv. *tomato* DC3000

250 (AAO56330), and *E. coli* K12 (P75830) with 91, 87, 75, and 46% identities, respectively.

251 Each protein represents a three-β-strand hammerhead-shaped structure plus an N-terminal

252 fourth strand, β4 (Athappilly and Hendrickson 1995). It was previously shown that

253 lipoyl/biotinyl proteins and these periplasmic protein components share a common fold

254 known as a flattened β-barrel (Johnson and Church 1999).

    A putative ABC transporter gene, *arfE,* was found just 3 bp downstream of *arfD*.

255

256 It was consisted of 1,953 bp nucleotide sequences encoding a 651 aa protein, ArfE. N-

257 terminal half of ArfE contained a NBD fold which is characterized by two short

258 conserved sequence motifs, named as Walker A (GASGSGKS) and Walker B (VILAD).

259 Another conserved sequence motif called linker peptide (C-loop), LSGGQQQRVS, was

260   also found before Walker B. This linker peptide is the signature of ABC transporter

261   family proteins (Schneider and Hunke 1998).

262   Located at the C-terminal half of this protein contains five putative TMS (Fig. 1),

263   which probably form a TMD. This structural feature suggested that ArfE belongs to an

264   ABC transporter protein group that possesses NBD and TMD in a single polypeptide

265   (Biemans-Oldehinkel *et al.* 2006). BLASTP search revealed homology of ArfE with

266   orthologous proteins from various Gram-negative bacteria, such as hypothetical protein

267   PflO1_2215 in *P. fluorescens* PfO-1 (YP_347947), macrolide ABC efflux proteins MacB

268   in *P. fluorescens* Pf-5 (YP_259256), *P. syringae* pv. *tomato* DC3000 (Q881Q1) and *E.*

269   *coli* K12 (P75831) at 92, 84, 80 and 53% identities, respectively.

270

271   **Polycistronic transcription of the genes**

272   RT-PCR experiment showed that each spacer region between *arfA, arfB, arfC,*

273   *arfD,* and *arfE* was normally amplified (Fig. 2). This result demonstrates that

274   arthrofactin synthetase genes (*arfA/B/C*), together with exporter genes *arfD* and *arfE*

275   were co-transcribed in a single mRNA, sharing the same promoter for gene expression.

276   This operon structure indicates functionally close connection of each gene. ArfD

277   probably constitutes an ABC-transporter system with ArfE. It should be noted that we

278   could not find a gene encoding an outer membrane protein component such as OprM or

279   TolC homologue in the downstream region of *arfE*.

280

281   **Production of arthrofactin by mutant strains**

282   Successful construction of the mutants at a single locus of the chromosome was

283   verified by both PCR and Southern hybridization experiments. Arthrofactin production

284   was analyzed at 6, 9, 12 and 18 h of cultivation in LB broth, where the growth curves

285   (OD_{600}) of the mutants and MIS38 were almost completely fitted. There was a reduction

286   in arthrofactin production by mutant strains ArfD:Km, ArfE:Km, and ArfD:Tc/ArfE:Km,

287   which was similarly reduced to 50% and 70% of strain MIS38 at 6 and 9 h respectively

288   (Table 3). However, after 12 h, there was no significant difference between the

289   production levels of these mutants and strain MIS38. These results suggested that

290   arthrofactin was dominantly exported by ArfD/E transporter but in the mutant strains

291   eventually capable of being exported by another compatible transport system.

292   Furthermore, RT-PCR experiment indicated that expression level of arthrofactin

293   synthetase gene was similar for strains MIS38 and ArfD:Tc/ArfE:Km (Fig. 3).

294

295   **Overexpression of *arfD/E* in strains MIS38 and ArfE:Km**

296   Strains MIS38 and ArfE:Km were transformed by either pME6032_*arfD/E* or

297   pME6032 vector only. The amount of extracellular arthrofactin after 1 d cultivation was

298   increased by 52% in MIS38(pME6032_*arfD/E*) when compared with MIS38(pME6032)

299   (Table 4).   Similar effect was observed for ArfE:Km (pME6032_*arfD/E*) where an

300   increase level was 54%. These results indicate that *arfD* and *arfE* contribute to effective

301   exportation of arthrofactin.   However, after 2 d, production levels of arthrofactin were

302   indistinguishable   between   MIS38(pME6032_*arfD/E*)   (94%)   or

303   ArfE:Km(pME6032_*arfD/E*) (98%) and vector controls (100%).   This result again

304 demonstrates that ArfD/E is not essential exporter system for arthrofactin production in

305 the late growth stage.

306

307 **Effect of ABC transporter inhibitors on arthrofactin production**

308 It is known that arthrofactin carries strong surfactant activities, therefore there is a

309 possibility that arthrofactin was passively exported by diffusion through cellular

310 membranes in the ArfD and ArfE mutant strains. Another possibility is that resistance-

311 nodulation-cell division (RND) antiporter efflux systems are involved in the arthrofactin

312 exportation. In order to examine these possibilities, several inhibitors for general ABC

313 transporters were tested for arthrofactin production.

314 It was first confirmed that the colony forming units (cfu) were not seriously

315 affected by treatment of Glb and Ovd at tested concentrations, suggesting that cellular

316 metabolisms functioned normally over the time (Fig. 4). There was significant reduction

317 of arthrofactin production by ArfE:Km in the presence of 0.1 mM Glb (73%), 0.25 mM

318 Glb (85%), and 0.25 mM Glb + 2 mM Ovd (87%) in 1 d (Fig. 4). Inhibitory effect of 2

319 mM Ovd was small in 1 d (26%) but it became obvious after 2 d (94%). These results

320 allowed us to conclude that exportation of arthrofactin is highly dependent on ABC

321 transporters that require the energy derived from ATP hydrolysis.

322

323 **<u>DISCUSSION</u>**

324 Genes encoding functionally connected enzymes often form a cluster or an operon

325 structure in the bacterial chromosome. This seems to fit the case for a NRPS and the

326 product transporter genes. *P. syringae* pv. syringae strain B301D-R produces two

327 lipodepsipeptide phytotoxins, syringomycin (Syr) and syringopeptin (Syp), and whose

328 synthetase gene clusters are adjacently located with opposite direction. Secretion of these

329 phytotoxins requires two transporter systems, known as SyrD, a protein homologous to

330 membrane proteins of the ABC transporter family, and PseABC, a tripartite transporter

331 system homologous to RND efflux system. *syrD* and *pseABC* are located just

332 downstream of Syr and Syp synthetase genes, respectively. A mutation in *syrD* has been

333 shown to lead almost completely loss of both Syr and Syp production (Quigley *et al.*

334 1993; Grgurina *et al.* 1996). Moreover, transcription level of *syrB,* the synthetase gene,

335 was reduced to 60% (in 2d) and 35% (in 4d) by *syrD* mutation. Then, it was concluded

336 that SyrD is required for full expression of *syrB*. Interestingly, there is no periplasmic

337 and outer membrane protein counterpart gene in the *syr* cluster. On the other hand, *pseC*

338 mutant strain showed mild reduction in Syr production by 41% at 72h and Syp

339 production by 67% at 48h compared to wild type strain B301D-R. There is no report that

340 production levels of Syr and Syp were restored later by another transporter in the *syrD* or

341 *pseABC* mutants.

342   Pyoluteorin is a chlorinated polyketide antibiotic secreted by *P. fluorescens* Pf-5.

343 Brodhagen *et al.* (2005) showed that *pltI* (encoding a periplasmic protein) and *pltJ*

344 (encoding an ABC transporter) mutant strains displayed low pyoluteorin production (23-

345 30% of wild type strain) at 48h and did not accumulate proportionately more of the

346 pyoluteorin intracellularly.   Interestingly, transcription of *pltI* and *pltJ* was enhanced by

347 exogenous pyoluteorin. These are known as reciprocal regulation mechanisms to prevent

348 intracellular accumulation of the product. In the case of pyoluteorin production by

349 *Pseudomonas* sp. M18, the gene disruption of corresponding periplasmic protein and

350   ABC transporter protein both led to a non-detectable production of this antibiotic (Huang

351   *et al.* 2006). We also observed that disruption of the gene encoding ABC transporter for

352   pyoverdine (*pvdE38-ABC*) dramatically reduced production level of pyoverdine, 9% of

353   MIS38 at 72 h (unpublished result). In another study of Gram positive *Bacillus subtilis*

354   168 by Tsuge *et al.* (2001), disruption of *yerP*, an RND efflux protein gene homologue

355   resulted in 6-fold reduction of a lipopeptide biosurfactant surfactin production.

356   In contrast to above information, observation results were very different in each

357   mutant of exporter genes for arthrofactin. Although polar effect of *arfD* mutation on the

358   expression of *arfE* cannot be ruled out, our experimental results suggest that effective

359   exportation only in the early stage of production requires active ArfD/E, whose genes

360   constitute an operon with *arfA/B/C* synthetase genes. The distance between *arfC* and

361   *arfD* was relatively large, 127 bp, for an operon structure. However, there is an example

362   that the distance between *sypB* and *sypC* is 423 bp in syringopeptin synthetase gene

363   operon (AF286216). Gene disruption of *arfD/E* still allowed normal transcription level

364   of *arfA/B/C* (Fig. 3) and did not lead to dramatic loss of arthrofactin production,

365   supporting assumption that there is another transport system that is functionally flexible

366   enough to export arthrofactin. On the other hand, overexpression of *arfD* and *arfE* led to

367   an increase of 50% arthrofactin production in 1 d, indicating that these genes are indeed

368   important for exportation, but the production level was not different after 2 d between the

369   overproducers and MIS38. Exportation may not be a rate limiting step for the production

370   of arthrofactin at this time. In summary, the production level of arthrofactin was highly

371   controlled at a proper level. Although we tried to detect intracellular arthrofactin by

372   sonication and methanol extraction, little accumulation of arthrofactin was observed in

373 mutants and overproducing strains as well as MIS38. This result suggests that production

374 of arthrofactin is coupled with translocation. Because MIS38 was isolated from a highly

375 hydrophobic oil field, production of the strong biosurfactant may be essential to survive

376 and keep preferred habitats.

377 *E. coli* have two notable systems involved in the resistance of macrolide

378 antibiotics, i.e. the AcrAB-TolC and MacAB-TolC system. AcrAB belongs to the RND

379 efflux pumps whereas MacAB belongs to the ABC transporters superfamily (Zgurskaya

380 and Nikaido 2000; Kobayashi *et al.* 2001). Both these protein constitute a complete

381 transporter system with one of multifunctional outer membrane proteins, TolC, for the

382 translocation of substrate antibiotics from periplasm to the extracellular space.

383 Overexpression of *macAB* in *E. coli* KAM3 (*ΔmacAB*) similarly enhanced resistance to

384 erythromycin, clarithromycin, and oleandomycin by 8-fold, demonstrating functionality

385 of the gene products with multidrug efflux (Kobayashi *et al.* 2001). In *P. aeruginosa*,

386 four RND multidrug efflux systems including MexAB-OprM, MexCD-OprJ, MexEF-

387 OrpN, and MexXY-OrpM are known (Poole 2001). It is not clear whether ArfD/E

388 recruits an outer membrane protein component like OprM/J/N or TolC to form an active

389 exporter apparatus for arthrofactin. There are significantly high similarities between

390 ArfD/E and putative MacA/B homologues from *Pseudomonas* strains such as *P.*

391 *fluorescens* PfO-1 (YP_347946/YP_347947), *P. fluorescens* Pf-5 (YP_259255/

392 YP_259256), *P. putida* (ABW17378/ABW17379), and *P. syringae* pv tomato (Q881Q2/

393 Q881Q1). All these sets of ABC transporter system lack outer membrane protein

394 components in the gene cluster. This is the first report that characterizes this group

395 exporter for nonribosomal peptides in *Pseudomonas* cells. When we look into the

396 genome sequence of *P. fluorescens* PfO-1, there are five TolC like outer membrane

397 protein components of putative ABC transporters (YP_348413, YP_347194, YP_346224,

398 YP_345867, YP_345894) and thirteen putative outer membrane protein components that

399 would constitute tripartite RND efflux systems including NodT and OprN. Further study

400 on these candidate proteins is necessary to clarify the whole view of exceptionally

401 flexible arthrofactin-family nonribosomal peptide exporter system in *Pseudomonas* cells.

402

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412 **<u>REFERENCES</u>**

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502

502   Legend of Figures

503

504   Figure 1. Hydropathy plot of ArfE. Abbreviations are NBD, nucleotide binding domain;

505   TMD, transmembrane domain; TMS, transmembrane segment; A, Walker A; B, Walker

506   B; C, C-loop.

507

508   Figure 2. RT-PCR of gene gaps using respective primers sets. Total RNA was prepared

509   from 12 h culture in this experiment. Four sets of primers were designed for amplifying

510   ca. 300 bp DNA fragments at each inter-gene locus, gapArfA/B(f) and gapArfA/B(r) (gap

511   between *arf*A and *arf*B); gapArfB/C(f) and gapArfB/C(r) (gap between *arf*B and *arf*C);

512   and gapArfC/D(f) and gapArfC/D(r) (gap between *arf*C and *arf*D); gapArfD/E(f) and

513   gapArfD/E (r) (gap between *arf*D and *arf*E). DNA bands were all confirmed to be single

514   from a side view of the gel.

515

516   Figure 3. RT-PCR showing expression of *arf*A in wild type strain MIS38 (lanes 1, 3, 5)

517   and a double mutant strain ArfD:Tc/ArfE:Km (lanes2, 4, 6).   A set of primers for

518   amplifying a part of *arf*A, arfA(594f) and arfA(863r), were used in this experiment.

519

520   Figure 4. Arthrofactin production of ArfE:Km in the absence and presence of their

521   respective ABC inhibitors in 1 and 2 d. Glb, Ovd, and cfu are glibenclamide, sodium

522   ortho-vanadate, and colony forming units, respectively.   Standard deviations were

523   calculated from two independent experiments.

524

525

525   Table 1. Strains and plasmids used in this study

Strains and plasmids	Genotype/ relevant characteristics	Reference
Strains		
Escherichia coli		
DH5α	supE44 ΔlacU169(ϕ*lacZ* ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Sambrook and Russell, 2001
SM10λpir	*thi thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu Km	Donnenberg and Kaper, 1991
Pseudomonas sp.		
MIS38 (wild type)	Ap^r Cm^r, isolated from oil spill	Morikawa *et al.*, 1993
ArfD:Km	Ap^r Cm^r Km^r	This study
ArfE:Km	Ap^r Cm^r Km^r	This study
ArfD:Tc/ArfE:Km	Ap^r Cm^r Km^r Tc^r	This study
MIS38 (pME6032_*arfD/E*)	Ap^r Cm^r Tc^r	This study
ArfE:Km (pME6032_*arfD/E*)	Ap^r Cm^r Km^r Tc^r	This study
Plasmids		
pGEMT	Cloning vector, Ap^r	Promega
pUC18	Cloning vector, Ap^r	Sambrook and Russell, 2001
pSMC32	Source of *kan* resistant gene cassette, Km^r	Bartolome *et al.* 1991
pCVD442	R6K*ori*, *mob* RP4 *bla*, *sac*B	Donnenberg and Kaper 1991
pKm/*Kpn*I	pGEMT (*Kpn*I site) containing *kan*, Km^r	This study
pKm/*Sma*I	pGEMT (*Sma*I site) containing *kan*, Km^r	This study
pME6032	Shuttle vector between *Pseudomonas* and *E. coli* containing *lacI^q-Ptac* fragment for gene expression; source of *tetA* gene cassette, Tc^r	Heeb *et al.* 2002
pTetA32	pUC18 (*Sma*I) containing 1.4 kb *tetA*, Tc^r	This study
pArfDΔN-β4	pGEMT (*Xba*I site) containing *arfDΔN-β4*	This study
pArfETv	pGEMT (*Xba*I site) containing *arfE*	This study
pArfD:Km	pGEMT (*Xba*I site) containing *arfDΔ4::kan*, Km^r	This study
pArfE:Km	pGEMT (*Xba*I site) containing *arfE::kan*, Km^r	This study
pArfD:Km442	pCVD442 (*Xba*I site) containing *arfDΔ4::kan*, Km^r	This study
pArfE:Km442	pCVD442 (*Xba*I site) containing *arfE::kan*, Km^r	This study
pArfD:Tc	pGEMT (*Xba*I site) containing *arfDΔ4::tetA*, Tc^r	This study
pArfD:Tc442	pCVD442 (*Xba*I site) containing arfDΔ*β4::tetA*, Tc^r	This study
pME6032_*arfD/E*	pME6032 containing *arfD* and *arfE*	This study

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528 Table 2. Primers used in this study

Primer name	Sequences
MFP/*Xba*I (f)	5' CTAG<u>TCTAGA</u>ACCTTCGCCAACGCCGAC 3'
MFP/*Xba*I (r)	5' CTAG<u>TCTAGA</u>GAGCTGACTCGGACGGTG 3'
708r (MFP)	5' CGG<u>GGTACC</u>CTTCTTCACCTTGTCGCCAAC 3'
736f (MFP)	5' CGG<u>GGTACC</u>CTGGTGCTGCAGAACACC 3'
pSMC32/*Kpn*I(f)	5' CGC<u>GGTACC</u>GTTTTATGGACAGCAAGCGA 3'
pSMC32/*Kpn*I (r)	5' CGC<u>GGTACC</u>CGTCAGTAGCTGAACAGGA 3'
pSMC32/*Sma*I(f)	5' TCC<u>CCCGGG</u>GTTTTATGGACAGCAAGCGA 3'
pSMC32/*Sma*I (r)	5' TCC<u>CCCGGG</u>CCGTCAGTAGCTGAACAGGA 3'
TetA-2(f)	5' GCTGTCGTCAGACCGTCTACG 3'
TetA-2(r)	5' CTAGCTAGT<u>TCTAGA</u>GCGGCC 3'
Fl(MFP)ORF5-7521f	5' CGCTGGGCATCGATCCTG 3'
Fl(MFP)ABC-493r	5' CACCGCCATTCATCAAGGC 3'
ABC/*Xba*I-563f	5' CTAG<u>TCTAGA</u>AGTTGGCGCCGATCCTGCTG 3'
ABC/*Xba*I-2583r	5' CTAG<u>TCTAGA</u>GCAGCATCAATTGGGTGACG 3'
ABC/*Xba*I-518f	5' CTAG<u>TCTAGA</u>TGGTCGGCATCGTCACCCAG 3'
ABC/*Xba*I-2628r	5' CTAG<u>TCTAGA</u>CGCTGTCGAGGTTGTTGGTG 3'
MfpABC1503f	5' CTGTACACGGTGCAGGCAC 3'
MfpABC5661r	5'GTTCGTCGGCGAGAATCAC 3'
gapArfA/B(f)	5' GCGCAGCAAGTGTTGATCCCG 3'
gapArfA/B(r)	5' GTTGAAGGCGGATCGCATGGG 3'
gapArfB/C(f)	5' GCTGCGTCAGGAAGGCATGGAAG 3'
gapArfB/C(r)	5' CGACCTGACCGTGCTTGCTG 3'
gapArfC/D(f)	5' CGATTCTCAAGGCGCCCAACG 3'
gapArfC/D(r)	5' CGATATCCGAGCGTTCGACCG 3'
gapArfD/E(f)	5' GTGCGGGTGCTCGATGCCAAG 3'
gapArfD/E(r)	5' CGGCAGTGGCGTAATCGAGGC 3'
arfA(594f)	5' TCAAGCGTCGCCGCGTTATG 3'
arfA(863r)	5' CCAACCACCCATTCGTCACG 3'
*Sac*IPPP-ABC79f	5' CCG<u>GAGCTC</u>AAGTTGCGCAAAGTCGGTATG 3'
*Kpn*IPPP-ABC3300r	5'GGC<u>GGTACC</u>GTCATCGCTGGCAAGCCAGCT 3'

529

530

530   Table 3. Relative percentage (%) of arthrofactin production between wild type MIS38

531   and its mutant strains

Time (h)	MIS38	ArfD:Km	ArfE:Km	ArfD:Tc/ ArfE:Km
6	100	51	58	49
9	100	73	70	68
12	100	98	97	105
18	100	108	98	94

532   Each score is an average of independent duplicate experiments.

533

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535

536   Table 4. Relative percentage (%) of arthrofactin productivity between wild type MIS38

537   and gene overexpression mutants.

Time (d)	MIS38 (pME6032)	MIS38 (pME6032_*arfD/E*)	ArfE:Km (pME6032)	ArfE:Km (pME6032_*arfD/E*)
1	100	152	100	154
2	100	94	100	98

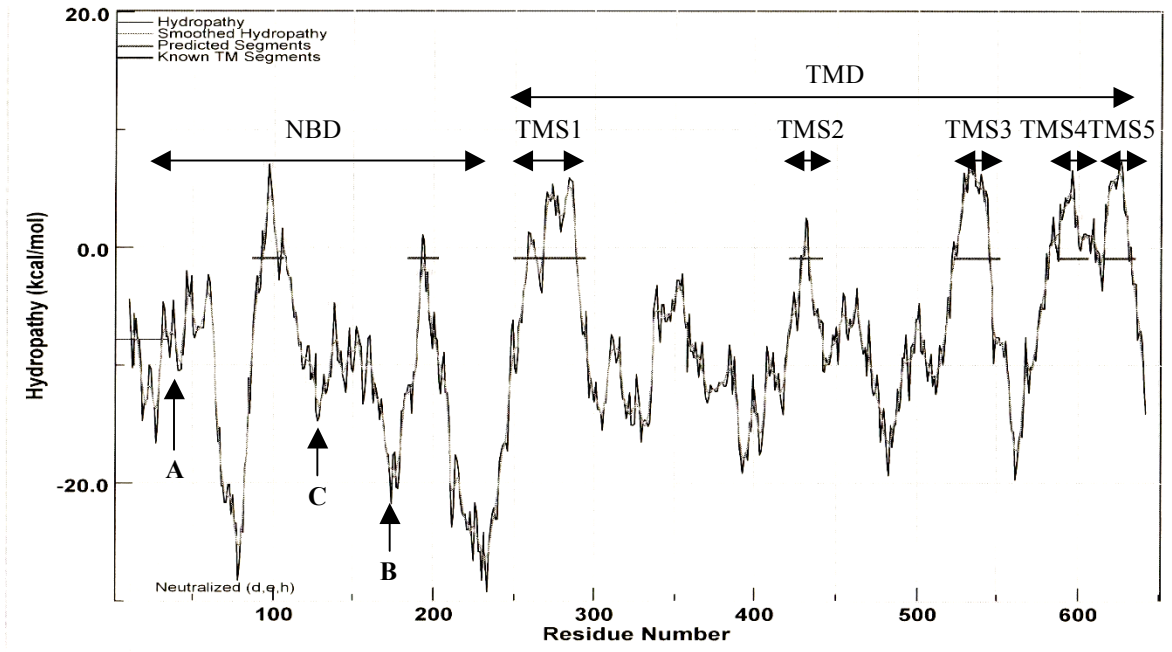538   Each score is an average of independent duplicate experiments.

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Figure 1. Hydropathy plot of ArfE. Abbreviations are NBD, nucleotide binding domain;

TMD, transmembrane domain; TMS, transmembrane segment; A, Walker A; B, Walker
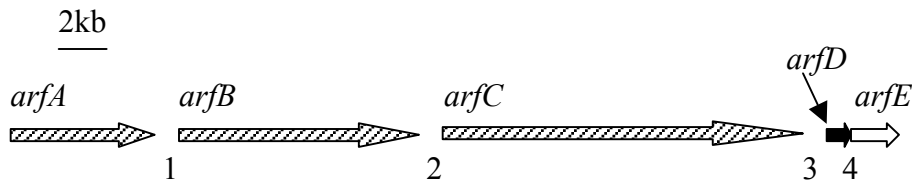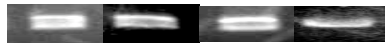
B; C, C-loop.

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548   2kb

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550   *arfA*   *arfB*   *arfC*   *arfD*   *arfE*

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552   1   2   3 4

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555   Lane   1   2   3   4

556 Figure 2. RT-PCR of gene gaps using respective primers sets. Total RNA was prepared

557 from 12 h culture in this experiment. Four sets of primers were designed for amplifying

558 ca. 300 bp DNA fragments at each inter-gene locus, gapArfA/B(f) and gapArfA/B(r) (gap

559 between *arf*A and *arf*B); gapArfB/C(f) and gapArfB/C(r) (gap between *arf*B and *arf*C);

560 and gapArfC/D(f) and gapArfC/D(r) (gap between *arf*C and *arf*D); gapArfD/E(f) and

561 gapArfD/E (r) (gap between *arf*D and *arf*E). DNA bands were all confirmed to be single

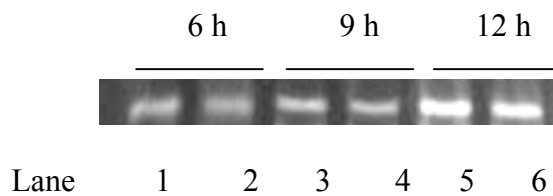562 from a side view of the gel.

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565   6 h   9 h   12 h

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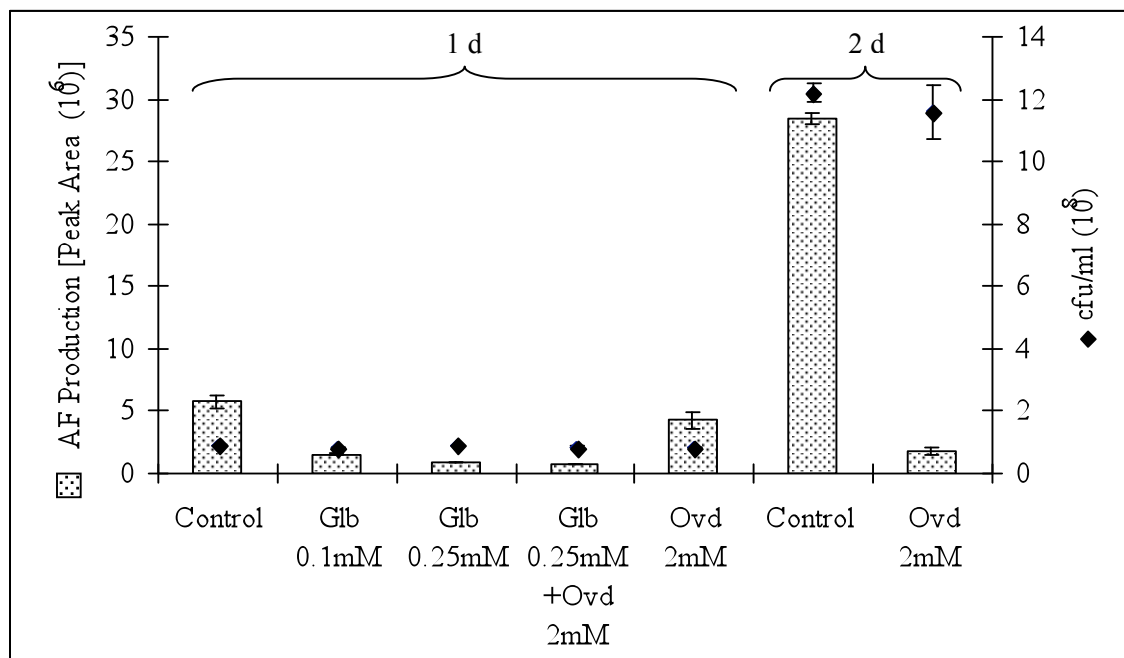
568

569   Lane   1   2   3   4   5   6

570 Figure 3. RT-PCR showing expression of *arfA* in wild type strain MIS38 (lanes 1, 3, 5)

571 and a double mutant strain ArfD:Tc/ArfE:Km (lanes 2, 4, 6).   A set of primers for

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