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Title	Flexible exportation mechanisms of arthrofactin in <i>Pseudomonas</i> sp. MIS38
Author(s)	Lim, S. P.; Roongsawang, N.; Washio, K. et al.
Citation	Journal of Applied Microbiology, 107(1), 157-166 https://doi.org/10.1111/j.1365-2672.2009.04189.x
Issue Date	2009-07
Doc URL	https://hdl.handle.net/2115/43176
Rights	The definitive version is available at www.blackwell-synergy.com
Type	journal article
File Information	JAM107-1_157-166.pdf



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

2 JAM-2008-1744

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4 *Running Title:* Arthrofactin exporter

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17 **Keywords:** biosurfactant, nonribosomal peptide, arthrofactin, ABC transporter, inhibitor,

18 *Pseudomonas*

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 endowed 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.

54 **INTRODUCTION**

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 30°C for arthrofactin
101 production. *E.coli* DH5 α was grown at 37°C while *E.coli* SM10 λ pir was grown at 30°C
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 RNeasy Mini Kit
129 (Qiagen). One microgram of DNase-treated RNA was used to synthesize first-strand
130 cDNA in 20 μ l volume with random primers and Reverse Transcriptase System
131 (Promega). After completion of reverse transcription, 1 μ l 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 β 4 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 *arfD* Δ *N*- β 4. 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 *arfD* Δ *N*- β 4
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* SM10 λ pir by electroporation. After that, conjugation between MIS38 and
157 SM10 λ pir(pArfD:Km442) was carried out (Roongsawang *et al.* 2007). Transconjugants
158 were selected on a plate containing both Km (35 mg l⁻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 *SmaI* 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/*XbaI*-563f and
173 ABC/*XbaI*-2583r. Then, it was cloned into *XbaI* site of pGEMT-vector, producing
174 pArfETv. The 1.2 kb *kan* was prepared by PCR using pSMC32/*SmaI*(f) and
175 pSMC32/*SmaI*(r). This fragment was then cloned into pArfETv at a newly introduced
176 *SmaI* site in *arfE*, followed by transfer of the resulting 3.2 kb *XbaI* fragment into
177 pCVD442. The following procedures were according to above described. Selection of
178 positive mutants was confirmed using a primer set, ABC/*XbaI*-518f and ABC/*XbaI*-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 *SmaI* site to construct pTetA32. Then, *XbaI* fragment
185 containing *tetA* was blunt ended and inserted into the blunt ended *KpnI* site in *arfD* Δ N- β 4,
186 creating pArfD:Tc. Then, pArfD:Tc442 was obtained by cloning *arfD* Δ N- β 4::*tetA* into
187 *XbaI* site of pCVD442. After that, conjugation between strain ArfE:Km and *E. coli*
188 SM10 λ pir harboring pArfD:Tc442 was carried out. Selection of mutants was performed
189 on the LB agar plate containing both Tc (40 mg l⁻¹) and Cm (34 mg l⁻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 *SacI*PPP-ABC79f and *KpnI*PPP-ABC3300r. It was then cloned into the *SacI* and *KpnI*
200 site of an expression vector pME6032 to construct pME6032_*arfD/E*, and transformed
201 *E.coli* DH5 α . 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 μ F for 3 msec. After applying the electric pulse, the cells were cultured in
206 SOC medium at 30°C for 2 hr, 150 rpm shaking before spreading on LB agar plate
207 supplemented with 40 mg l⁻¹ Tc. The plates were incubated at 30°C 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

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 ($OD_{600} \sim 2.2$),
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, β 4 (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).

255 A putative ABC transporter gene, *arfE*, was found just 3 bp downstream of *arfD*.
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 (OD₆₀₀) 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 **DISCUSSION**

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 ($\Delta macAB$) 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 arthrfactin-family nonribosomal peptide exporter system in *Pseudomonas* cells.

402

403 **Acknowledgements**

404 *E. coli* SM10λpir, pSMC32, pCVD442 and pME6032 were kindly donated by Dr.
405 D. A. Hogan (Dartmouth Medical School, NH) and Dr. Dieter Haas (Université de
406 Lausanne, Switzerland).

407 S.P. L acknowledges her Ph.D. fellowship from MEXT (no. 040318). This work
408 was supported by a Grant-in-Aid for Scientific Research for Exploratory Research from
409 Ministry of Education, Culture, Sports, Science and Technology (MEXT) (no. 17510171,
410 19380189), Institute for Fermentation, Osaka (IFO), and New Energy and Industrial
411 Technology Development Organization (NEDO).

412

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499 periplasmic lipoprotein AcrA and the inner-membrane-associated multidrug efflux
500 pump AcrB from *Escherichia coli*. *J Bacteriol* **182**, 4264-4267.

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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
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511 between *arfA* and *arfB*); gapArfB/C(f) and gapArfB/C(r) (gap between *arfB* and *arfC*);
512 and gapArfC/D(f) and gapArfC/D(r) (gap between *arfC* and *arfD*); gapArfD/E(f) and
513 gapArfD/E (r) (gap between *arfD* and *arfE*). 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 *arfA* 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
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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		
<i>Escherichia coli</i>		
DH5 α	supE44 Δ lacU169(ϕ lacZ Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook and Russell, 2001
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km</i>	Donnenberg and Kaper, 1991
<i>Pseudomonas</i> sp.		
MIS38 (wild type)	Ap ^r Cm ^r , isolated from oil spill	Morikawa <i>et al.</i> , 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	Ap ^r Cm ^r Tc ^r	This study
(pME6032_arfD/E)		
ArfE:Km	Ap ^r Cm ^r Km ^r Tc ^r	This study
(pME6032_arfD/E)		
Plasmids		
pGEMT	Cloning vector, Ap ^r	Promega
pUC18	Cloning vector, Ap ^r	Sambrook and Russell, 2001
pSMC32	Source of <i>kan</i> resistant gene cassette, Km ^r	Bartolome <i>et al.</i> 1991
pCVD442	R6Kori, <i>mob</i> RP4 <i>bla</i> , <i>sacB</i>	Donnenberg and Kaper 1991
pKm/ <i>KpnI</i>	pGEMT (<i>KpnI</i> site) containing <i>kan</i> , Km ^r	This study
pKm/ <i>SmaI</i>	pGEMT (<i>SmaI</i> site) containing <i>kan</i> , Km ^r	This study
pME6032	Shuttle vector between <i>Pseudomonas</i> and <i>E. coli</i> containing <i>lacI^q-Ptac</i> fragment for gene expression; source of <i>tetA</i> gene cassette, Tc ^r	Heeb <i>et al.</i> 2002
pTetA32	pUC18 (<i>SmaI</i>) containing 1.4 kb <i>tetA</i> , Tc ^r	This study
pArfD Δ N- β 4	pGEMT (<i>XbaI</i> site) containing <i>arfD</i> Δ N- β 4	This study
pArfETv	pGEMT (<i>XbaI</i> site) containing <i>arfE</i>	This study
pArfD:Km	pGEMT (<i>XbaI</i> site) containing <i>arfD</i> Δ β 4:: <i>kan</i> , Km ^r	This study
pArfE:Km	pGEMT (<i>XbaI</i> site) containing <i>arfE</i> :: <i>kan</i> , Km ^r	This study
pArfD:Km442	pCVD442 (<i>XbaI</i> site) containing <i>arfD</i> Δ β 4:: <i>kan</i> , Km ^r	This study
pArfE:Km442	pCVD442 (<i>XbaI</i> site) containing <i>arfE</i> :: <i>kan</i> , Km ^r	This study
pArfD:Tc	pGEMT (<i>XbaI</i> site) containing <i>arfD</i> Δ β 4:: <i>tetA</i> , Tc ^r	This study
pArfD:Tc442	pCVD442 (<i>XbaI</i> site) containing <i>arfD</i> Δ β 4:: <i>tetA</i> , Tc ^r	This study
pME6032_arfD/E	pME6032 containing <i>arfD</i> and <i>arfE</i>	This study

526

527

528

528 Table 2. Primers used in this study

Primer name	Sequences
MFP/ <i>Xba</i> I (f)	5' CTAGTCTAGAACCTTCGCCAACGCCGAC 3'
MFP/ <i>Xba</i> I (r)	5' CTAGTCTAGAGAGCTGACTCGGACGGTG 3'
708r (MFP)	5' CGGGGTACCCTTCTCACCTTGTCGCCAAC 3'
736f (MFP)	5' CGGGGTACCCTGGTGCTGCAGAACACC 3'
pSMC32/ <i>Kpn</i> I(f)	5' CGCGGTACCGTTTTATGGACAGCAAGCGA 3'
pSMC32/ <i>Kpn</i> I (r)	5' CGCGGTACCCCGTCAGTAGCTGAACAGGA 3'
pSMC32/ <i>Sma</i> I(f)	5' TCCCCCGGGTTTTATGGACAGCAAGCGA 3'
pSMC32/ <i>Sma</i> I (r)	5' TCCCCCGGGCCGTCAGTAGCTGAACAGGA 3'
TetA-2(f)	5' GCTGTCGTCAGACCGTCTACG 3'
TetA-2(r)	5' CTAGCTAGTTCTAGAGCGGCC 3'
Fl(MFP)ORF5-7521f	5' CGCTGGGCATCGATCCTG 3'
Fl(MFP)ABC-493r	5' CACCGCCATTCATCAAGGC 3'
ABC/ <i>Xba</i> I-563f	5' CTAGTCTAGAAAGTTGGCGCCGATCCTGCTG 3'
ABC/ <i>Xba</i> I-2583r	5' CTAGTCTAGAGCAGCATCAATTGGGTGACG 3'
ABC/ <i>Xba</i> I-518f	5' CTAGTCTAGATGGTCGGCATCGTCACCCAG 3'
ABC/ <i>Xba</i> I-2628r	5' CTAGTCTAGACGCTGTCGAGGTTGTTGGTG 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' CGACCTGACCGTGTCTGCTG 3'
gapArfC/D(f)	5' CGATTCTCAAGGCGCCCAACG 3'
gapArfC/D(r)	5' CGATATCCGAGCGTTCGACCG 3'
gapArfD/E(f)	5' GTGCGGGTGCTCGATGCCAAG 3'
gapArfD/E(r)	5' CGGCAGTGCGTAATCGAGGC 3'
arfA(594f)	5' TCAAGCGTCGCCGCTTATG 3'
arfA(863r)	5' CCAACCACCCATTCGTCACG 3'
<i>Sac</i> I PPP-ABC79f	5' CCGGAGCTCAAGTTGCGCAAAGTCGGTATG 3'
<i>Kpn</i> I PPP-ABC3300r	5' GGCGGTACCGTCATCGCTGGCAAGCCAGCT 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

534

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.

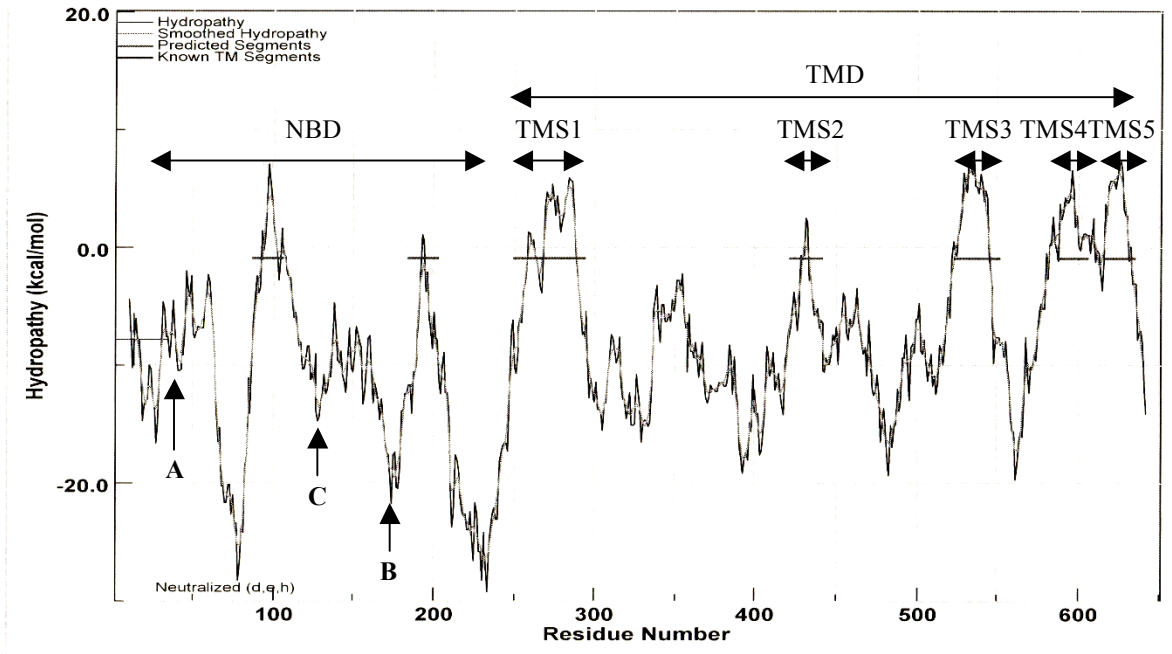
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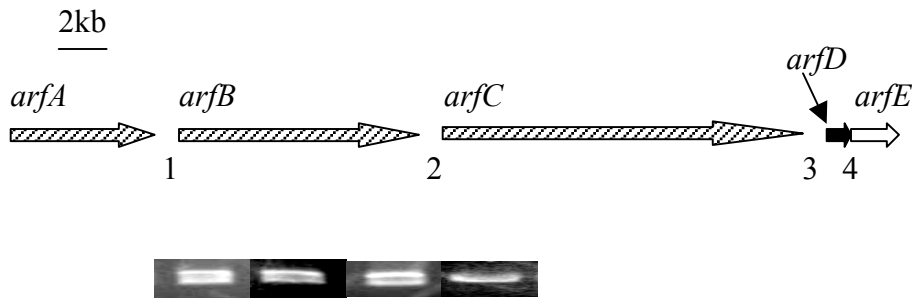
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Lane 1 2 3 4

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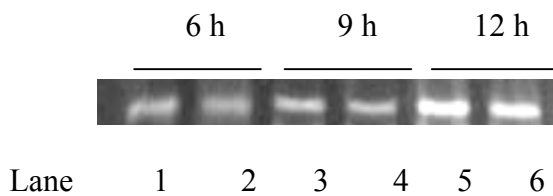
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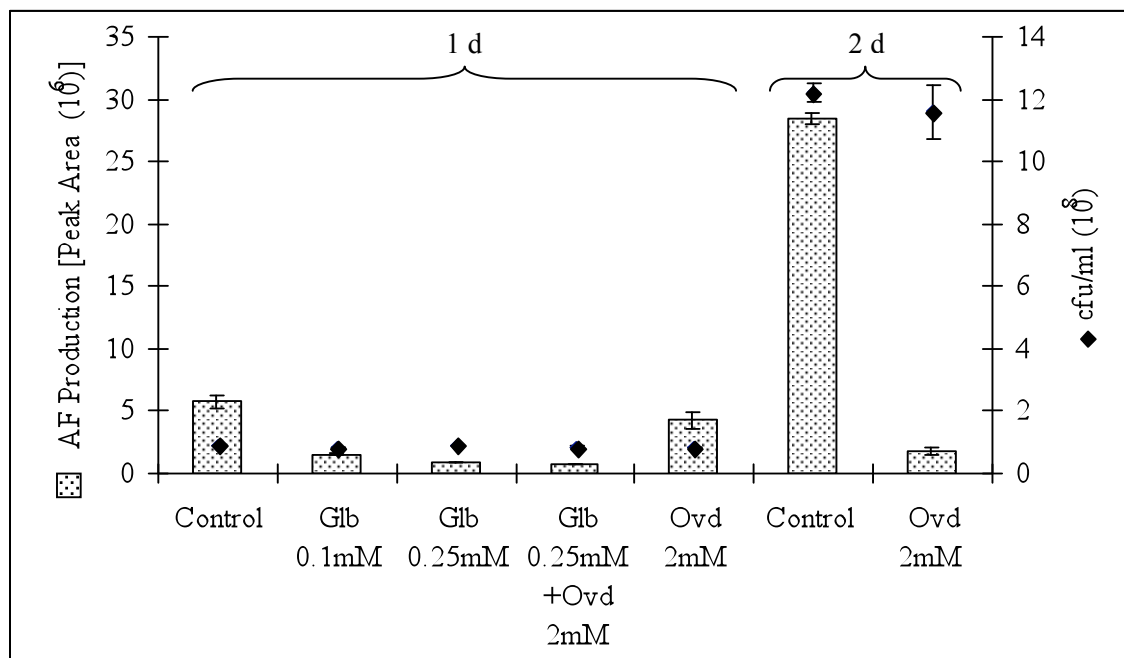
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