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

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Running Title: Arthrofactin exporter

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Abbreviations: aa, amino acids; ArfD, periplasmic protein; ArfE, ATP-binding cassette; bp, base pairs; kb, kilobase pairs; Glb, glibenclamide; Ovd, sodium ortho-vanadate NBD, nucleotide binding domain(s); NRPS, nonribosomal peptide synthetase; TMD, transmembrane domain(s); TMS, transmembrane segment(s)

ABSTRACT

Aims: To obtain further insights into transportation mechanisms of a most effective biosurfactant, arthrofactin in *Pseudomonas* sp. MIS38.

Methods and Results: A cluster genes *arfA/B/C* encodes an arthrofactin synthetase complex (ArfA/B/C). Downstream of the *arfA/B/C* lie genes encoding a putative periplasmic protein (ArfD, 362 aa) and a putative ATP-binding cassette transporter (ArfE, 651 aa), namely *arfD* and *arfE,* respectively. The *arfA/B/C*, *arfD*, and *arfE* form an operon suggesting their functional connection. Gene knockout mutants ArfD:Km, ArfE:Km, ArfD:Tc/ArfE:Km, and gene overexpression strains MIS38(pME6032_*arfD/E*) and ArfE:Km(pME6032_*arfD/E*) were prepared and analyzed for arthrofactin production profiles. It was found that the production levels of arthrofactin were temporally reduced in the mutants or increased in the gene overexpression strains, but they eventually became similar level to that of MIS38. Addition of ABC transporter inhibitors, glibenclamide and sodium ortho-vanadate dramatically reduced the production levels of arthrofactin. This excludes a possibility that arthrofactin is exported by diffusion with the aid of its own high surfactant activity.

Conclusions: ArfD/E is not an exclusive but a primary exporter of arthrofactin during early growth stage. Reduction in the arthrofactin productivity of *arfD* and *arfE* knockout mutants was eventually rescued by another ABC transporter system. Effects of *arfD* and *arfE* overexpression were evident only for one-day cultivation. Multiple ATP dependent active transporter systems are responsible for the production of arthrofactin.

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

INTRODUCTION

Exportation of many nonribosomal peptides and polyketides requires ABC (ATP-binding cassette) transporter system that couples the transport with ATP hydrolysis. The ABC transporter proteins are generally composed of two hydrophobic transmembrane domains (TMD) and hydrophilic nucleotide-binding domains (NBD) bound or fused to the cytosolic face of the TMD (Biemans-Oldehinkel *et al.* 2006). TMD are hydrophobic parts that create a channel through which the substrate passes during translocation. They are composed of bundles of α-helices that transverse the cytoplasmic membrane several times in a zig-zag fashion. NBD are the engines of ABC transporters that power substrate translocation by ATP hydrolysis and commonly have a set of ATP binding motifs, Walker A and B.

An ABC transporter system usually requires two accessory envelope proteins for its full function. One is a membrane fusion protein (or periplasmic protein) which consists of a short N-terminal hydrophobic domain anchoring it to the inner membrane, a large hydrophilic domain located at the periplasm, and a C-terminal domain with a possible β-sheet structure which could interact with the outer membrane protein (Dinh *et al.* 1994). Another accessory component is an outer membrane TolC family protein, which transports the efflux substrates to the culture medium (Wandersman and Delepelaire, 2004). These whole components are also required for the type I protein secretion system in most of the gram-negative bacteria (Binet *et al.* 1997).

Pseudomonas sp. MIS38 produces a cyclic lipopeptide, named as arthrofactin, which is one of the most effective biosurfactants (Morikawa *et al.* 1993). Arthrofactin synthetase genes (*arfA/B/C*) encode a multimodular nonribosomal peptide synthetase

(NRPS; ArfA/B/C) whose unique architecture has been reported recently (Roongsawang *et al.* 2003; 2005). ArfA, ArfB, and ArfC proteins consist of two, four, and five modules, where each module contains a set of condensation (C), adenylation (A), and thiolation (T) domains. Seven of the eleven modules incorporate D-form amino acids, where in this case, dual condensation/epimerization (C/E) domains are present in the place of authentic C domains (Balibar *et al.* 2005). At the C-terminal end of the last module of ArfC, two thioesterase (TE) domains are present that are responsible for cyclization and release of the product peptide from the enzyme (Roongsawang *et al.* 2007). The present work focuses on the function of further downstream two genes, encoding a putative periplasmic protein (ArfD) and a putative ABC transporter protein (ArfE).

There are reports that ABC transporter genes are clustered along with synthetase genes of secondary metabolites to be exported out from the cells (Méndez and Salas 2001). The knowledge prompted us to examine how these two genes, embedded in the arthrofactin synthetase gene cluster, play a role in the exportation of arthrofactin by MIS38 cells. A series of experimental results suggested that ArfD/E is not an exclusive transporter system for arthrofactin and that exportation of arthrofactin is exceptionally flexible enough to regain normal production levels in the transporter gene knockout mutants and the gene overexpression strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* sp. MIS38 and its derivatives were grown in Luria-Bertani (LB) broth (per

liter: 10g tryptone, 5g yeast extract and 5g NaCl, pH 7.3) at 30ºC for arthrofactin production. *E.coli* DH5α was grown at 37ºC while *E.coli* SM10λpir was grown at 30ºC in LB broth. SOC medium was used for cultivation after electroporation (Sambrook and Russel 2001). Antibiotics were used at the following concentrations (mg l^{-1}): kanamycin (Km) 35, chloramphenicol (Cm) 34 and tetracycline (Tc) 40 for *Pseudomonas* sp. MIS38; ampicillin (Ap) 50, Tc 25, and Km 35 for *E. coli*.

General molecular biological methods

DNA manipulations were according to standard protocols (Sambrook and Russell 2001) unless described in details. Chromosomal DNA of MIS38 and mutant strains were prepared by Marmur method (Marmur 1961). DNA fragments were recovered from agarose gel by QIAquick Gel Extraction Kit (Qiagen) and plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen). DNA from phage λ-S9 and λ-S12 were extracted using Lambda Maxi Kit (Qiagen). Cohesive ends of gene fragments were occasionally filled using Takara DNA Blunting Kit (Takara Bio) while ligation was performed using Takara Ligation Kit ver. 2.1 (Takara Bio). DNA sequencing was performed by ABI Prism 3100 Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Standard PCR was performed for 30 cycles using PTC-100 Programmable Thermal Controller (MJ Research) and *Ex-Taq* DNA polymerase (Takara Bio) or KOD *Plus* DNA polymerase (Toyobo). Oligonucleotides for PCR primers were synthesized by Hokkaido System Science. Primers used in this study were listed in Table 2. The nucleotide sequences were analyzed by GENETYX software (GENETYX) and BLAST

programs (http://www.ncbi.nlm.nih.gov). Amino acid sequences were analyzed by ClustalW program (http://clustalw.genome.jp).

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR experiment was performed as follows. Total RNA was extracted from the culture of MIS38 according to manufacturer's protocols using RNAeasy Mini Kit (Qiagen). One microgram of DNase-treated RNA was used to synthesize first-strand cDNA in 20 μl volume with random primers and Reverse Transcriptase System (Promega). After completion of reverse transcription, 1 μl of products was used for PCR amplification of DNA fragments using each specific primer and KOD *Plus* DNA polymerase (Toyobo).

Prediction of transmembrane segments (TMS) of ArfE

TMS of ArfE were predicted using the Membrane Protein Explorer (MPex) programme by Jayasinghe *et al.* (2001) at http://blanco.biomol.uci.edu/mpex. This sliding-window hydrophobicity analysis of amino acid sequences of membrane proteins is useful to identify putative transmembrane parts.

Gene cloning of a putative periplasmic protein, ArfD and insertion of kanamycin resistant gene cassette for gene knockout experiment

Nucleotide sequences of the gene encoding putative periplasmic protein (ArfD) in MIS38 were obtained by Roongsawang *et al.* (2003) (AB107223). Km resistant gene cassette (*kan*) was inserted in the structurally important N-terminal β4 region to construct

ArfD:Km. Primers MFP/*Xba*I (f) and 708r(MFP) were used to obtain 700bp fragment at the N-terminal part of ArfD while primers MFP/*Xba*I(r) and 736f(MFP) were used to obtain 1.3kb fragment at the C-terminal part of ArfD. Both 708r(MFP) and 736f(MFP) primers had an additional *Kpn*I site, for ligation purposes between the 700 bp and 1.3 kb fragments. The ligation product yielded *arfD*Δ*N-β4*. DNA sequencing was performed in order to confirm introduction of the deletion without unexpected mutation. On the other hand, 1.2 kb fragment of *kan* was obtained by PCR using pSMC32 and primers pSMC32/*Kpn*I(f) and pSMC32/*Kpn*I(r). It was then cloned into *Kpn*I gap of *arfD*Δ*N-β4* in pGEMT, creating pArfD:Km. After that pArfD:Km was digested with *Xba*I and subcloned into suicide vector pCVD442, producing pArfD:Km442 and transferred into *E.coli* SM10λpir by electroporation. After that, conjugation between MIS38 and SM10λpir(pArfD:Km442) was carried out (Roongsawang *et al.* 2007). Transconjugants were selected on a plate containing both Km (35 mgl^{-1}) and Cm (34 mg l^{-1}). MIS38 was originally resistant to Cm, and Cm inhibited the growth of donor *E.coli* cells. In order to verify successful construction of the mutant gene in MIS38 chromosome, chromosomal DNA from mutants were extracted using InstaGene Matrix (BioRad) and used for PCR amplification with a primer set Fl(MFP)ORF5-7521f and Fl(MFP)ABC-493r. A mutant strain ArfD:Km was thus obtained and used for further studies.

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

The first 120 bp gene encoding N-terminal part of a putative ABC transporter gene had been previously obtained (AB107223). Rest of the complete gene, *arfD/E* was

obtained in this study from a genomic library of phage λ-S12 (Roongsawang *et al.* 2003). In order to construct a gene knockout mutant strain ArfE:Km, *kan* cassette was inserted in the *Sma*I site located between linker peptide and Walker B region of ArfE. The 2 kb fragment including *arfE* was amplified by a set of primers ABC/*Xba*I-563f and ABC/*Xba*I-2583r. Then, it was cloned into *Xba*I site of pGEMT-vector, producing pArfETv. The 1.2 kb *kan* was prepared by PCR using pSMC32/*Sma*I(f) and pSMC32/*Sma*I(r). This fragment was then cloned into pArfETv at a newly introduced *Sma*I site in *arfE*, followed by transfer of the resulting 3.2 kb *Xba*I fragment into pCVD442. The following procedures were according to above described. Selection of positive mutants was confirmed using a primer set, ABC/*Xba*I-518f and ABC/*Xba*I-2628r. A mutant strain ArfE:Km was thus obtained and used for further studies.

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

The 1.4 kb tetracycline resistant gene cassette (*tetA*) was obtained by PCR with a primer set TetA-2(f) and TetA-2(r), and pME6032 as a template. This DNA fragment was cloned into pUC18 at *Sma*I site to construct pTetA32. Then, *Xba*I fragment containing *tetA* was blunt ended and inserted into the blunt ended *Kpn*I site in *arfD*Δ*N-β4*, creating pArfD:Tc. Then, pArfD:Tc442 was obtained by cloning *arfD*Δ*N-β4::tetA into* *Xba*I site of pCVD442. After that, conjugation between strain ArfE:Km and *E. coli* SM10λpir harboring pArfD:Tc442 was carried out. Selection of mutants was performed on the LB agar plate containing both Tc (40 mgl^{-1}) and Cm (34 mgl^{-1}). Then, genomic DNA from the candidate strain was extracted for PCR template, and a set of primers Fl(MFP)ORF57521f and Fl(MFP)ABC493r was used to verify introduction of *tetA* in

arfD. Another primer set, MfpABC1503f and MfpABC5661r was used to detect the presence of both the *tetA* and *kan* resistant gene cassette in the double mutant, strain ArfD:Tc/ArfE:Km. Double crossover event occurred during the conjugation process without sucrose treatment.

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

The 3,222 bp gene encoding both *arfD* and *arfE* was amplified using primer set *Sac*IPPP-ABC79f and *Kpn*IPPP-ABC3300r. It was then cloned into the *Sac*I and *Kpn*I site of an expression vector pME6032 to construct pME6032_*arfD/E*, and transformed *E.coli* DH5α. After confirming the correct sequence of the gene fragment, this plasmid or pME6032 vector only was then electrotransferred into wild type MIS38 and strain ArfE:Km. Electroporation was carried out using Electro Gene Transfer Equipment (GTE-10, Shimadzu) with 0.1 cm electrode distance cuvette at a pulse condition of 12.5 $kVcm^{-1}$, 35 μF for 3 msec. After applying the electric pulse, the cells were cultured in SOC medium at 30ºC for 2 hr, 150 rpm shaking before spreading on LB agar plate supplemented with 40 mgl^{-1} Tc. The plates were incubated at 30ºC overnight. The obtained strains MIS38(pME6032), MIS38(pME6032_*arfD/E*), ArfE:Km(pME6032) and ArfE:Km(pME6032_*arfD/E*) were used for further analysis.

Southern hybridization

Southern hybridization was carried out according to Lim *et al*. (2007) in order to verify successful gene disruption at a single locus of MIS38 chromosome.

Analysis of arthrofactin production

Analysis of arthrofactin production was carried out according to Roongsawang *et al.* (2007). Briefly, arthrofactin was precipitated from culture supernatant by addition of diluted HCl to pH 2 followed by centrifuge (10,000 g for 20 min). Hydrophobic fraction of the precipitates containing arthrofactin was extracted by methanol. Samples were then separated by reverse-phase HPLC using Cosmosil 5C18 AR column (4.6 x 150 mm, Nacalai). Detection of compounds was performed either by UV detector (HP1100, Agilent Technologies) or ESI-mass spectrometer (LCQ, Thermo Scientific). The amount of arthrofactin was calculated from the area of peaks recorded by UV detector.

ABC transporter inhibitors and their effect on arthrofactin production

It was shown that sodium ortho-vanadate (Ovd) effectively inhibit the activity of an ABC transporter MacA/B in *E.coli* (Tikhonova *et al.* 2007). It has been reported that the Walker A motif in NBD is involved in the Ovd binding for inhibition of ATPase activity (Pezza *et al.* 2002). Glibenclamide (Glb), a sulphonylurea also has been shown to inhibit the activities of various ABC transporters (Serrano-Martin *et al*. 2006). Strain ArfE:Km was grown in LB broth for 14 h until the early stationary phase (OD_{600}~2.2), after which 0.1 mM or 0.25 mM of Glb (Sigma), or 2 mM sodium Ovd (Wako Pure Chemicals) was added to the culture medium, and further cultivated for 1 and 2 d. Then, the production of arthrofactin was analyzed.

Nucleotide and amino acid sequence accession numbers

Nucleotide sequences for the gene encoding putative periplasmic protein (ArfD), putative ABC transporter (ArfE) were registered in the GenBank under accession number AB286215. Amino acid sequences of each protein were submitted under accession number BAC67537 (ArfD) and BAF40423 (ArfE).

RESULTS

Analysis of a putative periplasmic protein gene, *arfD* and an ABC transporter gene, *arfE* located downstream of arthrofactin synthetase genes

A putative periplasmic protein gene, *arfD*, was previously found 127 bp downstream of *arfC* (orf5 in AB107223). BLASTP analysis of ArfD showed high homology with members of the periplasmic protein component of membrane transporters such as HlyD from *P. fluorescens* PfO-1 (YP_347946), macrolide ABC efflux type carrier MacA from *P. fluorescens* Pf-5 (YP_259255), *P. syringae* pv. *tomato* DC3000 (AAO56330), and *E. coli* K12 (P75830) with 91, 87, 75, and 46% identities, respectively. Each protein represents a three-β-strand hammerhead-shaped structure plus an N-terminal fourth strand, β4 (Athappilly and Hendrickson 1995). It was previously shown that lipoyl/biotinyl proteins and these periplasmic protein components share a common fold known as a flattened β-barrel (Johnson and Church 1999).

A putative ABC transporter gene, *arfE,* was found just 3 bp downstream of *arfD*. It was consisted of 1,953 bp nucleotide sequences encoding a 651 aa protein, ArfE. N-terminal half of ArfE contained a NBD fold which is characterized by two short conserved sequence motifs, named as Walker A (GASGSGKS) and Walker B (VILAD). Another conserved sequence motif called linker peptide (C-loop), LSGGQQQRVS, was

also found before Walker B. This linker peptide is the signature of ABC transporter family proteins (Schneider and Hunke 1998).

Located at the C-terminal half of this protein contains five putative TMS (Fig. 1), which probably form a TMD. This structural feature suggested that ArfE belongs to an ABC transporter protein group that possesses NBD and TMD in a single polypeptide (Biemans-Oldehinkel *et al.* 2006). BLASTP search revealed homology of ArfE with orthologous proteins from various Gram-negative bacteria, such as hypothetical protein PflO1_2215 in *P. fluorescens* PfO-1 (YP_347947), macrolide ABC efflux proteins MacB in *P. fluorescens* Pf-5 (YP_259256), *P. syringae* pv. *tomato* DC3000 (Q881Q1) and *E. coli* K12 (P75831) at 92, 84, 80 and 53% identities, respectively.

Polycistronic transcription of the genes

RT-PCR experiment showed that each spacer region between *arfA, arfB, arfC, arfD,* and *arfE* was normally amplified (Fig. 2). This result demonstrates that arthrofactin synthetase genes (*arfA/B/C*), together with exporter genes *arfD* and *arfE* were co-transcribed in a single mRNA, sharing the same promoter for gene expression. This operon structure indicates functionally close connection of each gene. ArfD probably constitutes an ABC-transporter system with ArfE. It should be noted that we could not find a gene encoding an outer membrane protein component such as OprM or TolC homologue in the downstream region of *arfE*.

Production of arthrofactin by mutant strains

Successful construction of the mutants at a single locus of the chromosome was verified by both PCR and Southern hybridization experiments. Arthrofactin production was analyzed at 6, 9, 12 and 18 h of cultivation in LB broth, where the growth curves (OD_{600}) of the mutants and MIS38 were almost completely fitted. There was a reduction in arthrofactin production by mutant strains ArfD:Km, ArfE:Km, and ArfD:Tc/ArfE:Km, which was similarly reduced to 50% and 70% of strain MIS38 at 6 and 9 h respectively (Table 3). However, after 12 h, there was no significant difference between the production levels of these mutants and strain MIS38. These results suggested that arthrofactin was dominantly exported by ArfD/E transporter but in the mutant strains eventually capable of being exported by another compatible transport system. Furthermore, RT-PCR experiment indicated that expression level of arthrofactin synthetase gene was similar for strains MIS38 and ArfD:Tc/ArfE:Km (Fig. 3).

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

Strains MIS38 and ArfE:Km were transformed by either pME6032_*arfD/E* or pME6032 vector only. The amount of extracellular arthrofactin after 1 d cultivation was increased by 52% in MIS38(pME6032_*arfD/E*) when compared with MIS38(pME6032) (Table 4). Similar effect was observed for ArfE:Km (pME6032_*arfD/E*) where an increase level was 54%. These results indicate that *arfD* and *arfE* contribute to effective exportation of arthrofactin. However, after 2 d, production levels of arthrofactin were indistinguishable between MIS38(pME6032_*arfD/E*) (94%) or 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

ABC transporter protein both led to a non-detectable production of this antibiotic (Huang *et al.* 2006). We also observed that disruption of the gene encoding ABC transporter for pyoverdine (*pvdE38-ABC*) dramatically reduced production level of pyoverdine, 9% of MIS38 at 72 h (unpublished result). In another study of Gram positive *Bacillus subtilis* 168 by Tsuge *et al.* (2001), disruption of *yerP*, an RND efflux protein gene homologue resulted in 6-fold reduction of a lipopeptide biosurfactant surfactin production.

In contrast to above information, observation results were very different in each mutant of exporter genes for arthrofactin. Although polar effect of *arfD* mutation on the expression of *arfE* cannot be ruled out, our experimental results suggest that effective exportation only in the early stage of production requires active ArfD/E, whose genes constitute an operon with *arfA/B/C* synthetase genes. The distance between *arfC* and *arfD* was relatively large, 127 bp, for an operon structure. However, there is an example that the distance between *sypB* and *sypC* is 423 bp in syringopeptin synthetase gene operon (AF286216). Gene disruption of *arfD*/*E* still allowed normal transcription level of *arfA/B/C* (Fig. 3) and did not lead to dramatic loss of arthrofactin production, supporting assumption that there is another transport system that is functionally flexible enough to export arthrofactin. On the other hand, overexpression of *arfD* and *arfE* led to an increase of 50% arthrofactin production in 1 d, indicating that these genes are indeed important for exportation, but the production level was not different after 2 d between the overproducers and MIS38. Exportation may not be a rate limiting step for the production of arthrofactin at this time. In summary, the production level of arthrofactin was highly controlled at a proper level. Although we tried to detect intracellular arthrofactin by 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

genome sequence of *P. fluorescens* PfO-1, there are five TolC like outer membrane protein components of putative ABC transporters (YP_348413, YP_347194, YP_346224, YP_345867, YP_345894) and thirteen putative outer membrane protein components that would constitute tripartite RND efflux systems including NodT and OprN. Further study on these candidate proteins is necessary to clarify the whole view of exceptionally flexible arthrofactin-family nonribosomal peptide exporter system in *Pseudomonas* cells.

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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

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509 from 12 h culture in this experiment. Four sets of primers were designed for amplifying
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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 *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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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 *arfDΔβ4*::*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 *arfDΔβ4*::*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 *arfDΔβ4*::*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’ CTAGTCTAGAACCTTCGCCAACGCCGAC 3’
MFP/*Xba*I (r)	5’ CTAGTCTAGAGAGCTGACTCGGACGGTG 3’
708r (MFP)	5’ CGGGGTACCCTTCTTCACCTTGTCGCCAAC 3’
736f (MFP)	5’ CGGGGTACCCTGGTGCTGCAGAACACC 3’
pSMC32/*Kpn*I(f)	5’ CGCGGTACCGTTTTATGGACAGCAAGCGA 3’
pSMC32/*Kpn*I (r)	5’ CGCGGTACCCCGTCAGTAGCTGAACAGGA 3’
pSMC32/*Sma*I(f)	5’ TCCCCCGGGGTTTTATGGACAGCAAGCGA 3’
pSMC32/*Sma*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/*Xba*I-563f	5’ CTAGTCTAGAAGTTGGCGCCGATCCTGCTG 3’
ABC/*Xba*I-2583r	5’ CTAGTCTAGAGCAGCATCAATTGGGTGACG 3’
ABC/*Xba*I-518f	5’ CTAGTCTAGATGGTCGGCATCGTCACCCAG 3’
ABC/*Xba*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’ 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’ CCGGAGCTCAAGTTGCGCAAAGTCGGTATG 3’
*Kpn*IPPP-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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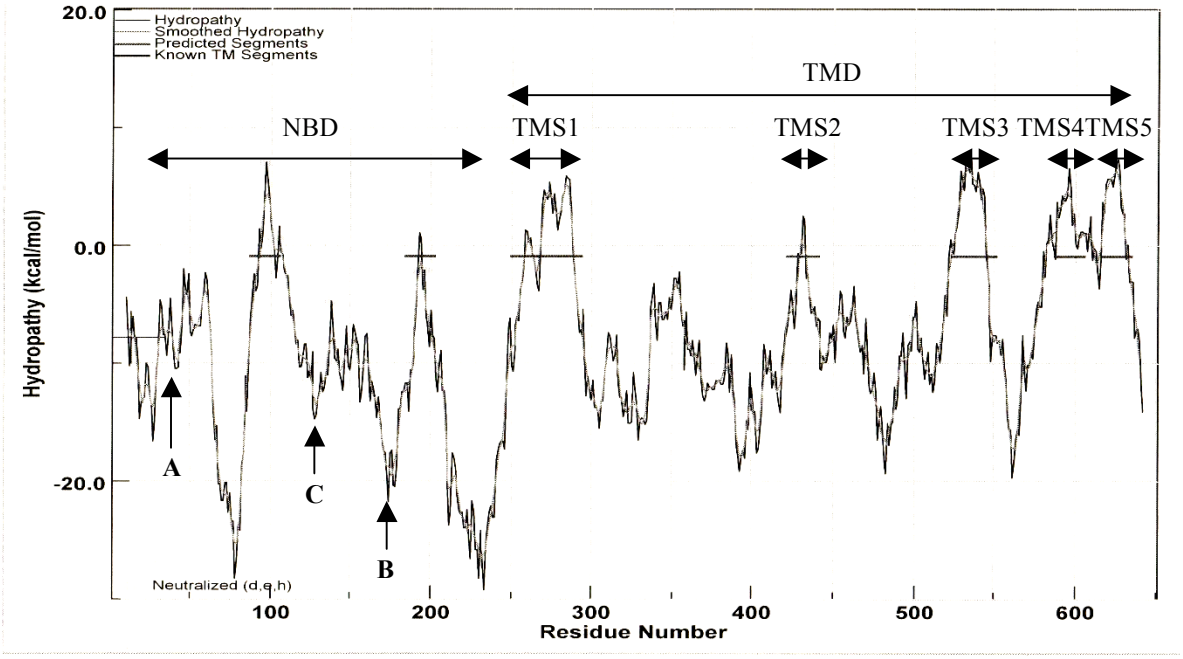

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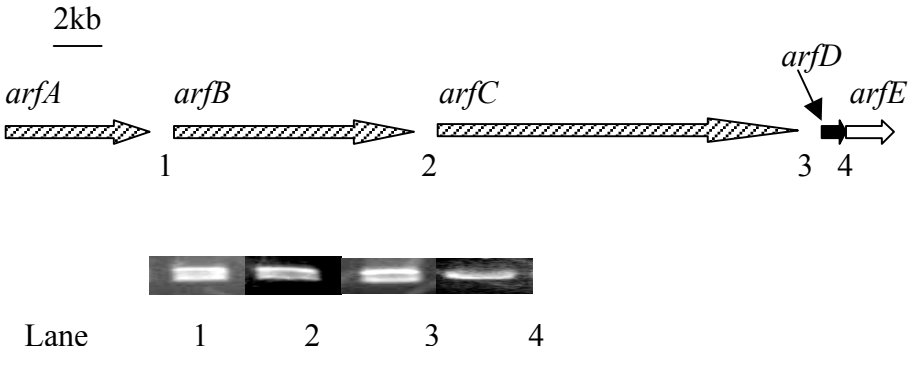

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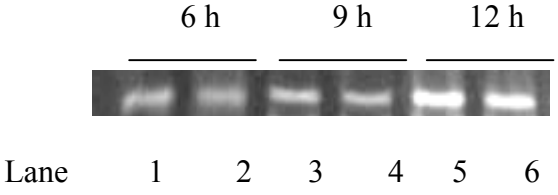

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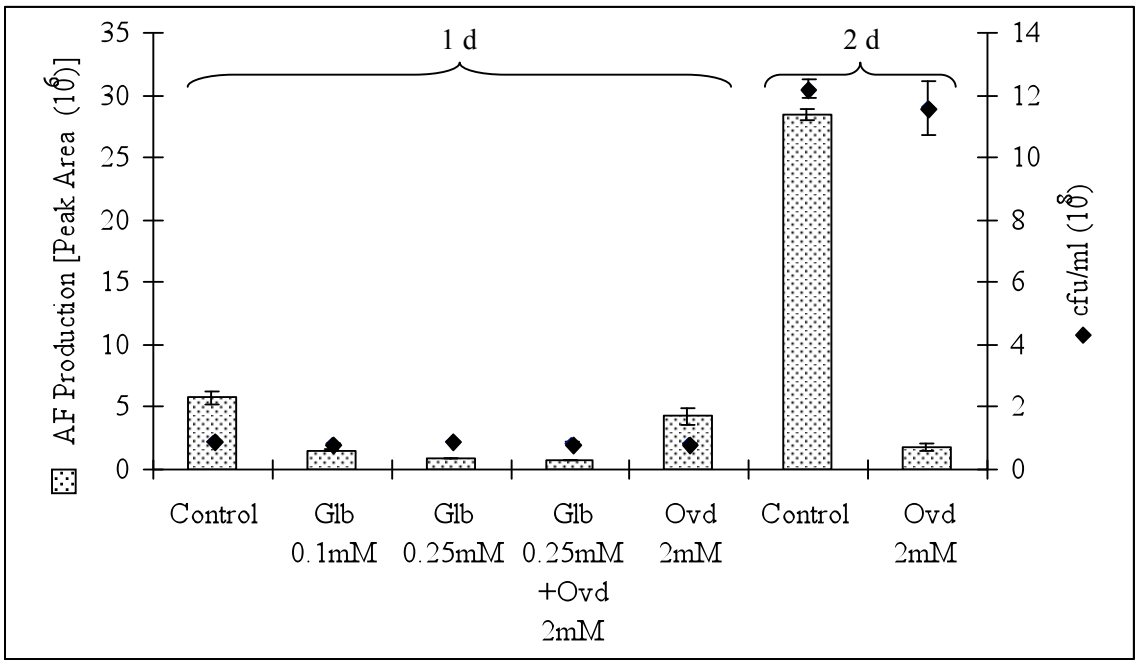

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