

THE FUNCTION OF THE ACR GENES IN PHENAZINE REGULATION AND
TRANSPORT IN THE BIOLOGICAL CONTROL STRAIN *PSEUDOMONAS*
CHLORORAPHIS 30-84

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

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May 2014

Major Subject: Horticulture

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ABSTRACT

The genes *acrA* and *acrB*, first identified in *Escherichia coli* encode components of a Resistance-Nodulation-Cell Division (RND) efflux system shown to be important for resistance to multiple antimicrobials. In *Pseudomonas chlororaphis* 30-84, transcriptional analysis demonstrated that the RpeA/RpeB two-component transduction system differentially regulated two genes immediately adjacent to *rpeA* and *rpeB* annotated as *acrA* and *acrB*. In the present study, I showed that constitutive expression of the 30-84 *acrA/acrB* genes conferred acriflavine resistance to *E. coli* and enhanced acriflavine resistance in 30-84 and also conferred resistance to tetracycline, chloramphenicol, and carbenicillin. Importantly, constitutive expression of *acrA/acrB* enhanced the growth of *E. coli* in the presence of ~300-800 µg of total phenazines extracted from 30-84, demonstrating the function of AcrA and AcrB in phenazine transport. Constitutive overexpression of the *acrAB* operon *in trans* in wild type 30-84 resulted in enhanced phenazine production and transport. Phenazine production by the 30-84 *rpeB* mutant was not rescued by overexpression of *acrA/acrB* unless other RpeA/RpeB controlled traits involved in the regulation of phenazine biosynthesis such as quorum sensing also were restored. Earlier and greater production and diffusion of phenazines significantly increased the capacity of 30-84 to inhibit fungal growth. These data indicated that *acrA/acrB* enhanced RND-type transporter activities and phenazine transport. Based on the findings from this study, a model is proposed to illustrate how

phenazine transport and biosynthesis are related in 30-84 and whether transport may contribute to positive catabolic feedback regulation of phenazine biosynthesis.

ACKNOWLEDGEMENTS

I would like to express my special thanks to my supervisor Dr. Pierson, for her invaluable help during the past school year. I am thankful that I was fortunate enough to meet her in my early youth, who once led me to the academic world of microbiology and offered constant encouragement and insightful counsel to help me strive for the “best-case scenario” of this paper.

And also I would like to thank Dr. Klein and Dr. Volder, for their substantial contribution to my proposal, thesis, and their encouragement on my graduate school life in America.

I appreciate my dear lab members, Dr. Leland Pierson, Dr. Julien Levy, Dr. Dongping Wang, Jun Myoung Yu, Cameron Finley, Robert (RJ) Dorosky, Anna Casto, Paula Luera, Sean Burris, Michael Cook, Kathryn Holkamp, and Alexa Scribner for their time and efforts to offer me precious support about my projects. Dr. Levy, mainly responsible for my molecular experiment techniques, gave me lots of guidance through every step of the experiment and supervised my lab report regularly to train me to be a good scientist. Jun Myoung Yu gave me so many suggestions to my projects and taught me so many knowledge to use all the equipments in lab and always reminded me when I did something wrong. I am very grateful about these warnings especially when I am in startup stage for my research life. He sets a very good example for my future Ph.D study.

Thanks to the Horticulture Department for their summer fellowship and many activities.

I would like to appreciate my beloved families and friends – I am so glad that none of them cares anything about what I am studying on; they only care about me, my health and my happiness.

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1. INTRODUCTION

Pseudomonas chlororaphis 30-84 is a biological control agent that has been studied extensively because it has the capacity to suppress take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis var. tritici* when applied to wheat seeds or seed beds (1). Take-all is a wilt disease of wheat and barley that is one of the most important root diseases of wheat in the world (1).

Pseudomonas chlororaphis 30-84 produces bright orange-colored secondary metabolites known as phenazines that are important for the strain's ability to inhibit pathogens, colonize the plant rhizosphere, and form biofilms (2). Phenazines are compounds with a nitrogen-containing ring structure that differ from one another on the basis of the substitutive groups attached to the aromatic backbone (3). They are redox active compounds that are toxic to a variety of microbes and are capable of altering the environment outside of cells (3).

Previous work demonstrated that, like many secondary metabolites, the production of phenazines by 30-84 is controlled by a complex regulatory network (4). This network includes the PhzI/PhzR quorum sensing system and multiple two component signal transduction (TCST) systems, including RpeA/RpeB (4). Quorum sensing is a strategy used by many Gram negative bacteria to regulate the expression of secondary metabolites according to the density of accumulated bacterial signal molecules (5). In 30-84, the PhzI/PhzR quorum sensing system directly regulates the

transcription of the phenazine biosynthetic operon (6). In contrast, TCSTs serve as a basic mechanism to allow organisms to sense and respond to changes in environmental conditions. These systems typically consist of a membrane-bound histidine kinase sensor (HK) and a cytoplasmic response regulator (RR) and typically control *multiple biosynthetic operons and their regulators* (4). In 30-84, RpeA (a sensor protein) and RpeB (the cognate response regulator) control phenazine biosynthesis via the regulation of Pip, a TetR transcriptional regulator homolog, and the PhzI/PhzR quorum sensing system (4).

I hypothesize that due to the redox activity of these secondary metabolites, *phenazine production and transport are likely to be coordinately regulated*. Coordinated regulation may be needed to enable bacterial cells to protect themselves from potentially damaging redox activity and to insure that phenazines are exported outside the cell, where they exert significant influence on the environment and biological control activities (7-9) (See Literature Review). Support for this hypothesis was garnered recently when it was shown that the RpeA/RpeB TCST controls both the production and accumulation of phenazines in supernatants (4).

The RpeA/RpeB TCST system was originally identified by random mutagenesis (10). A mutant with defects in *rpeA* appeared dark orange on plates, demonstrating that *rpeA* represses phenazine production (e.g. when it was mutated the result was over-expression and heavy diffusion of phenazines into the media). In contrast, a mutation in *rpeB* resulted in reduced phenazine production, suggesting positive control

of phenazines by RpeB (fig. 1). Mutants with defects in both *rpeA* and *rpeB* have the *rpeB* mutant phenotype.

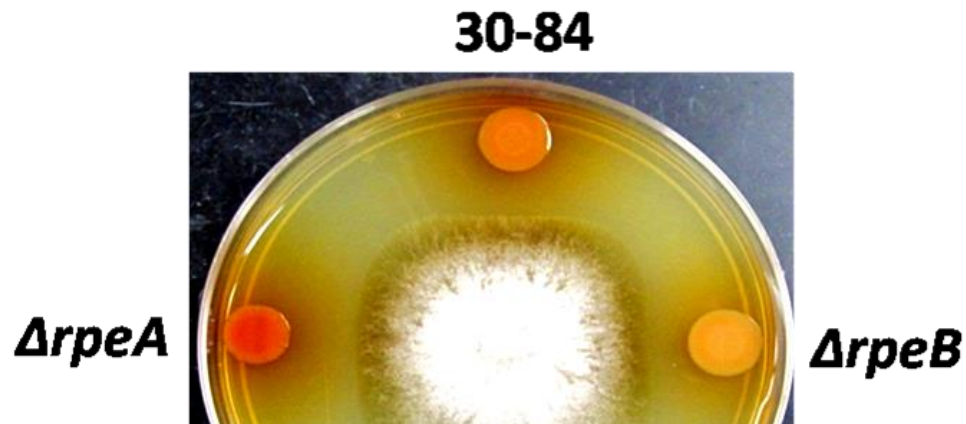


Figure 1. The phenotype of wild type 30-84 and the *rpeA* and *rpeB* mutants on Luria-Bertani (LB) medium. Notice the mutations of *rpeA* and *rpeB* result in more versus less phenazine production and diffusion into the agar medium, respectively, compared to the wild type. Greater phenazine production and diffusion result in greater inhibition of the fungus *Gaeumannomyces graminis*.

In order to gain a better understanding of how RpeA/RpeB control phenazine production, transcriptomic analysis of *rpeA* and *rpeB* mutants compared to the wild type was conducted using RNA-Seq (Pierson, unpublished). Data from the RNA-Seq analysis demonstrated that two genes, annotated as *acrA* and *acrB* and located next to *rpeA* and *rpeB* on the bacterial chromosome (fig. 2), were among the most highly up-regulated in the *rpeA* mutant and the most highly down regulated in the *rpeB* mutant relative to wild type, respectively. The genes *acrA* and *acrB* are annotated as acriflavine resistance genes, (hence the acronym *acr* for acriflavine resistance) (11). Acriflavine, derived from acridine, is a type of antibiotic capable of inhibiting the growth of some fungi and

bacteria (12) and which also has structural similarity to phenazines (fig. 2). The differential regulation of *acrA/acrB* by RpeA and RpeB was confirmed via quantitative real-time PCR (fig. 3) (Pierson, unpublished).

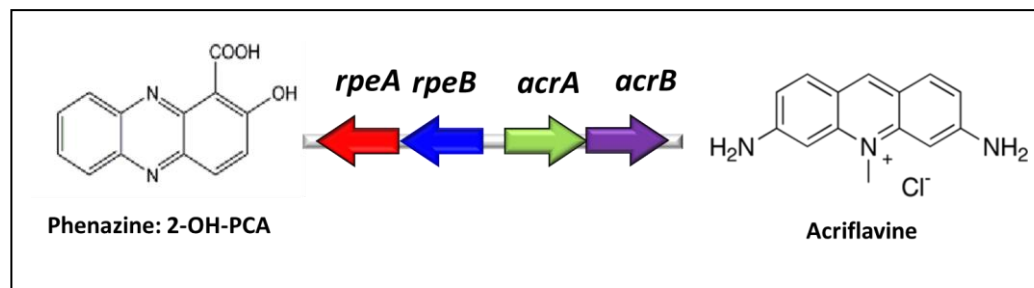


Figure 2. The structures of 2-OH-PCA (2 hydroxy-phenazine carboxylic acid, a phenazine produced by 30-84) and acriflavine. The location of the *acrA/acrB* genes relative to *rpeA/rpeB* on the bacterial chromosome is also shown.

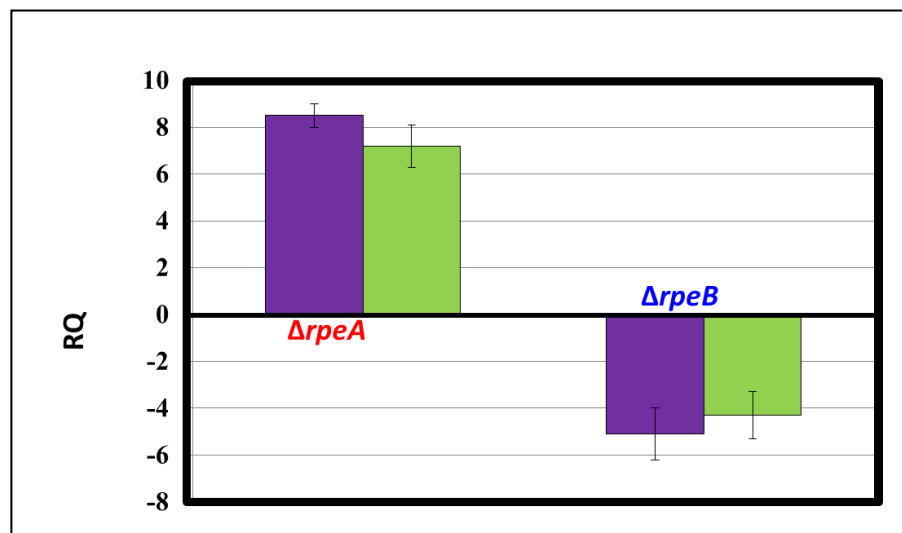


Figure 3. The relative transcript abundances of *acrB* (purple) and *acrA* (green) in the *rpeA* and *rpeB* mutants relative to wild type. Transcript abundance is expressed as a relative quotient (RQ), defined as the ratio of the transcript abundance from the mutant to the wild type, e.g.

$$\frac{\text{transcript abundance mutant}}{\text{transcript abundance wild type}}$$

In *E. coli*, *Pseudomonas*, and other Gram negative bacteria *acrA* and *acrB* encode components of a Resistance-Nodulation-Cell Division (RND) efflux system shown to be important for resistance to antimicrobials, including the antibiotic acriflavine (13) (see Literature Review). Consistent with the hypothesis that the 30-84 *acrA/acrB* genes, which are regulated by the RpeA/RpeB TCST, also encode an RND efflux system, in a preliminary experiment I found that *rpeA* mutants are more resistant to acriflavine than the wild type, whereas *rpeB* mutants are significantly less resistant to acriflavine than either wild type or *rpeA* mutants (fig. 4). Based on similarity in the structure of acriflavine and phenazines and the higher levels of phenazine accumulated in the liquid supernatant of *rpeA* mutants, I hypothesize that the *acrA/acrB* genes also function in *the export of phenazines* outside the cell.

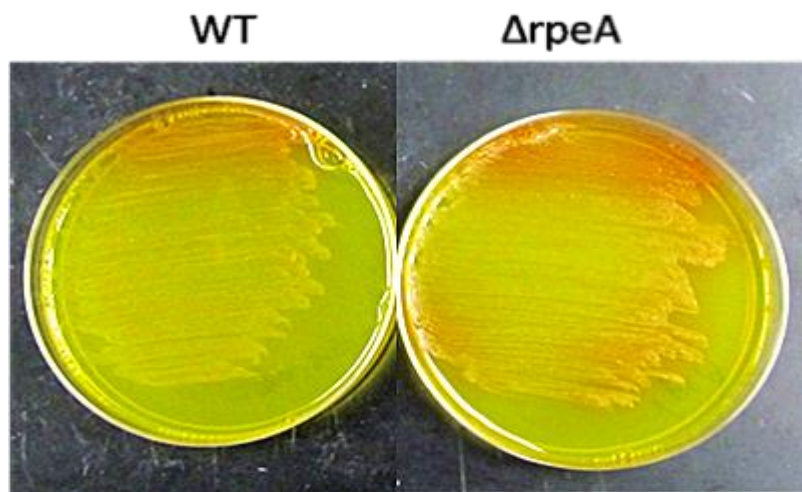


Figure 4. Growth of the wild type and the *rpeA* mutant on LB plates containing 0.01% acriflavine. Notice that the *rpeA* mutant has significantly more growth and is able to produce more phenazine than the wild type in the presence of acriflavine. The *rpeB* mutant is almost completely susceptible to 0.01% acriflavine (data not shown).

In this study, I used a genetic approach consisting of a) constitutive gene expression and b) gene deletion studies to evaluate the hypotheses that the 30-84 *acrA* and *acrB* genes function as an RND type transporter 1) capable of conferring multi-drug resistance and 2) transporting phenazines. For the constitutive gene expression studies, the 30-84 *acrA/acrB* genes were PCR amplified and cloned behind the constitutive promoter *Ptac*, a modified lac operon promoter, in the broad host range vector KT2 (fig. 5A) (14). This vector was then transferred to *E. coli*, wild type 30-84 and various mutant derivatives of 30-84 (e.g. $\Delta rpeA$ and $\Delta rpeB$ mutants, and $\Delta acrA$, once constructed) to evaluate the effect of constitutive expression of the *acrA/acrB* genes on drug resistance and phenazine biosynthesis and transport. For the gene deletion studies, recombination fragments consisting of a kanamycin cassette with its own promoter flanked by a 100 nt arm having homology to *acrA* were generated via PCR using plasmid pUC4K as a template (fig. 5B). The recombination fragment was then transformed into wild type 30-84 and screened for homologous recombination resulting in the replacement of the majority of region of the bacterial chromosome encoding these genes by the KM^R marker (except for the first and last 100 nt). Thus far I have not identified a homologous recombinant. The resulting mutant, once obtained, will be assessed for loss of transport function and more specifically loss or reduced phenazine transport function.

I summarize the current findings by proposing a hypothetical model describing the regulation and function of the *acrA/acrB* genes in phenazine biosynthesis and transport and the biological control activity of *P. chlororaphis* 30-84 (see Discussion).

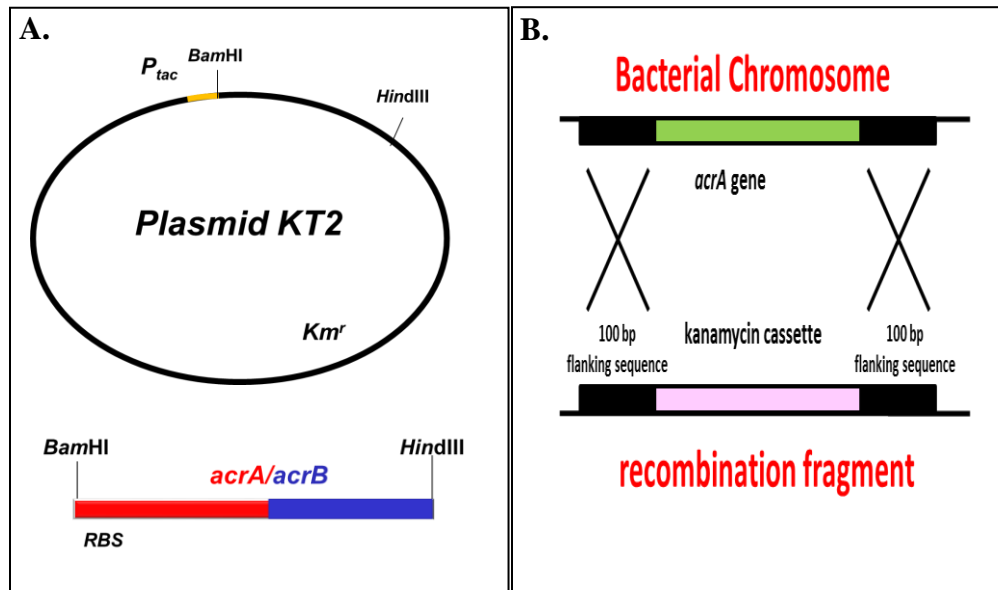


Figure 5. Methods for constitutive gene expression and gene deletion studies. **A.** For the constitutive gene expression studies, the 30-84 *acrA/acrB* genes were PCR amplified and cloned behind the constitutive promoter *P_{tac}* into either plasmid KT2 (*Km^R*) or GT2 (*Gm^R*). This vector was then transferred to *E. coli*, wild type 30-84 and various mutant derivatives of 30-84 resulting in the constitutive expression the *acrA/acrB* genes *in trans*. **B.** For the gene mutation studies, a recombination fragment consisting of a kanamycin cassette with its own promoter flanked by a 100 nt arm having homology to either *acrA* was generated. The recombination fragments were then transformed into wild type 30-84 and screened for homologous recombination, resulting in the replacement of the majority of the coding region *in the bacterial chromosome* by the *KM^R* marker.

2. LITERATURE REVIEW

The association between plants and microorganism can be beneficial, neutral or pathogenic. Among the beneficial soilborne microbes, rhizobacteria have drawn considerable attention because they are more likely to possess characteristics that make them well suited as biological control agents or plant growth promoting rhizobacteria (PGPR) (15). *Pseudomonas* spp. have several traits that enable them to be good PGPR, including a) the ability to grow rapidly and utilize seed and root exudates to better colonize the rhizosphere, and b) the ability to produce a wide spectrum of bioactive compounds such as antibiotics to inhibit plant pathogenic microorganisms (16). Fluorescent pseudomonads that serve as biological control agents produce various secondary metabolites that have been identified as powerful mechanisms of disease control, including phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin. These secondary metabolites are recognized as antibiotics because of their role in plant pathogen inhibition (17-19).

Many bacterial species including members of the genera *Pseudomonas*, *Streptomyces* and *Burkholderia* produce redox-active compounds known as phenazine (20). Phenazines are compounds with a nitrogen-containing ring structure that differ from one another on the basis of the substitutive groups attached to the aromatic backbone (3). Different species often produce one or more different phenazine derivatives and the spectrum of derivatives may differ significantly among different

isolates (3). The production of phenazines has been related to many toxic effects and these effects vary depending on the phenazine derivative(s) produced by the microbe (3). The toxicity of these phenazine compounds depends largely on their redox activity, which determines their ability to generate reactive oxygen species (ROS) (3). For example, the toxicity of pyocyanin (1-hydroxy-*N*-methylphenazine, PCN) produced by the opportunistic human pathogen *Pseudomonas aeruginosa* is primarily the result of the creation of ROS. In this process, PCN undergoes aerobic redox cycling to generate superoxide and hydrogen peroxide. These ROS induce cell injury in other organisms (21). Beyond antibiosis, phenazines also have been shown to serve other roles for the producing organism, including acting as electron shuttles and contributing to biofilm formation/architecture and rhizosphere competence (3).

Plant-associated biological control agents produce a diversity of phenazine compounds that have been demonstrated to be important for biological control activity. For example, phenazine-1-carboxylate (PCA) secreted by *Pseudomonas fluorescens* 2-79 contributes to biocontrol activity against fungal phytopathogens such as *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all diseases of wheat (22). *P. chlororaphis* 30-84, a biocontrol agent also used to control take-all disease, produces three phenazine derivatives, PCA, 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and 2-hydroxy-phenazine (2OHPZ) (1). The bacterium *Pseudomonas chlororaphis* PCL1391 produces phenazine-1-carboxamide, which plays a role in the control of tomato foot and root rot caused by *Fusarium oxysporum* (23).

Given the toxicity of phenazines, a reasonable evolutionary question to address is: What mechanism do bacteria producing the phenazines utilize in order to avoid the toxic influence of these compounds on themselves? A major mechanism bacteria use to protect themselves from the harmful effects of antibiotics is the establishment of a low permeability barrier combined with active outward transport (17). Bacteria often surround themselves with a low permeability barrier so as to decrease the influx of antimicrobials into the cells (9). For example, Gram-positive bacteria have a thick layer of peptidoglycan in their cell wall, which not only provides rigidity to the cell but limits the diffusion of small diffusible molecules. Although Gram-negative bacteria such as *Escherichia coli* do not have as thick a peptidoglycan layer, they protect themselves with a second outer membrane, which is composed of lipopolysaccharide (LPS). The outer membrane and LPS have been shown to make Gram-negative bacteria more resistant than Gram-positive bacteria to lipophilic inhibitors including dyes, detergents, and antibiotics (13). Since *Pseudomonas* species are Gram-negative bacteria, they have this type of architecture and method of protection. These same cells also use efflux systems to transport antimicrobial compounds that are self-produced or present in the environment out of the cell in an energy-dependent way, and thereby become intrinsically resistant to many antibiotics (12).

There are three major types of efflux systems in Gram-negative bacteria, the Major Facilitator (MF) type, the Staphylococcus Multidrug Resistance (SMR) type, and the Resistance, Nodulation, Division (RND) type (24). The acriflavine resistance

AcrAB-TolC complex in *E. coli* belongs to the RND type of efflux system (13). RND efflux systems are referred to as “proton antiporters,” which means they generate and use a proton gradient across the membrane “to power efflux” by “exchanging one H⁺ ion for one drug molecule” (25). RND type efflux systems that confer multi-drug resistance such as the MexAB-OprM complex also have been studied in *Pseudomonas*, especially *P. aeruginosa* (26, 27). A comparison of the RND efflux systems in *E. coli* and *P. aeruginosa* appears below.

Analysis of the AcrAB-TolC complex in *E. coli* revealed that disruption at the *acr* locus caused *E. coli* cells to be “hyper-susceptible to hydrophobic growth inhibitors such as basic dyes, detergents and many antibiotics” (28). Ma *et al.* (28) analyzed the *acr* locus using functional complementation, and subsequent sequence analysis revealed three genes, *acrR*, *acrA*, and *acrB*, expressed on two opposite operons at this locus. This organization is similar to the organization of RND genes in *P. aeruginosa* (fig. 6). Furthermore, a time-course study of the accumulation of acriflavine in *acrAB*⁺ (over-expressing) and *acrAB*⁻ (mutant) strains firmly proved that *acrAB* encodes an efflux pump (29). A model of the AcrAB-TolC complex is shown in fig. 7. In *E. coli*, *acrB* encodes a large protein (e.g. containing 1048 amino acids) with 12 putative transmembrane α -helices that is believed to be associated with the phospholipid bilayer of the inner membrane of the bacterial cell wall (30). The smaller (397 amino acids) AcrA is thought to be a periplasmically located lipoprotein based on its “nasal elongated form, which is consistent with its function in linking or fusing inner and outer

membranes” (30). AcrA has been shown to be required for the function of AcrB in intact cells (30). A third protein, TolC (506 amino acids), is thought to interact with AcrA to form an outer membrane channel and is required for the production of a functional efflux pump (31). The gene encoding TolC is not located in the *acrAB* operon. Ma *et al.* (32) determined that the other gene at the *acr* locus (but divergently transcribed), the gene *acrR*, encodes a transcription regulator with a helix-turn-helix domain at its N-terminus, which serves as a transcription repressor of the *acrAB* operon (32). Subsequent work demonstrated that *acrAB* is also controlled by global regulators such as MarA, SoxS, and Rob (33, 34). In *E. coli*, the *acrEF* operon also encodes an RND efflux pump with slightly different antimicrobial substrate affinity. AcrE and AcrF are 80 and 88% similar to AcrA and AcrB, respectively (32).

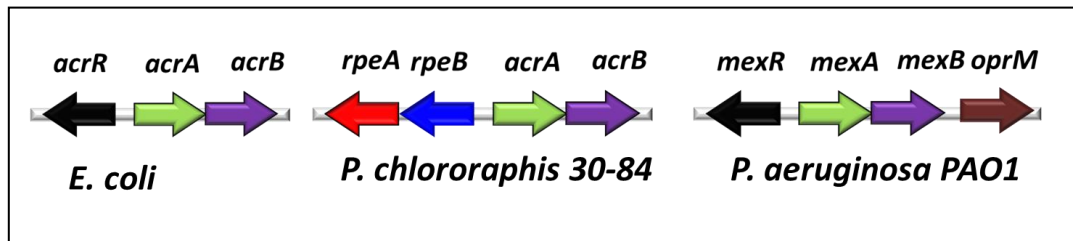


Figure 6. Comparison of the orientation of the *acr* locus in *E. coli*, the *acr* locus in *P. chlororaphis* 30-84, and the *mexAB-oprM* operon in *P. aeruginosa* based on gene annotation.

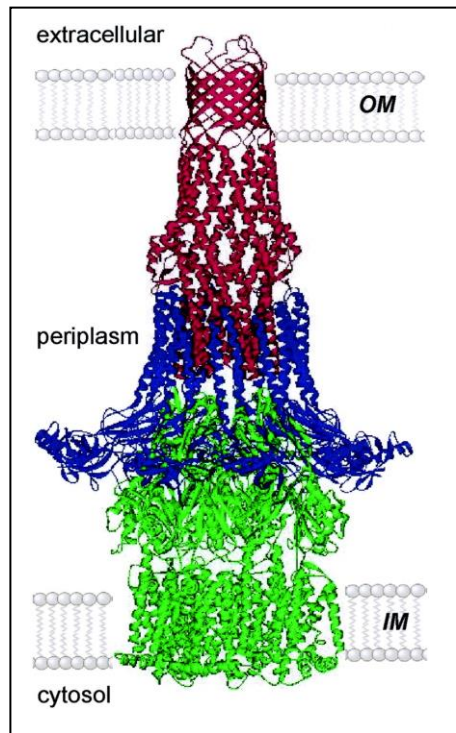


Figure 7. The modeled structure of the AcrAB-TolC complex in *E. coli*, an RND type efflux system, is similar to the MexAB-OprM complex in *P. aeruginosa*. The green protein depicts the inner membrane localized protein corresponding to AcrB in *E. coli* (or by homology, MexB in *P. aeruginosa*). The blue and red proteins depict the periplasmically localized AcrA and the pore forming outer membrane protein TolC in *E. coli* (or by homology, the MexA and OprM proteins in *P. aeruginosa*) respectively (25).

Pseudomonas aeruginosa is an important opportunistic pathogen that can become resistant to antimicrobials during antibiotic therapy (13). In *P. aeruginosa*, four multi-component, multi-drug resistant RNDs have been identified: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (25). These RNDs have the same three-protein structure as described for the AcrAB-TolC RND shown in fig. 7. Of these four RND systems, the MexAB-OprM is most similar to the AcrAB-TolC system of *E. coli*. It was previously reported that MexA has 71% similarity with AcrA, MexB has

83% similarity with AcrB, and OprM has 35% similarity with TolC (25). Comparing the 4 RNDs, MexB, MexD, MexF and MexY are predicted to have high structural homology to the inner membrane protein AcrB. Similarly, structural homology is predicted for the MexA, MexC, MexE, MexX and the periplasmically localized AcrA and for the outer membrane proteins OprM, OprJ, OprN, OprM and TolC (35).

The organization of the genes encoding these RND efflux systems is also similar among species (fig. 6). Typically, the genes are organized as an operon such that the gene encoding the periplasmic localized protein is located adjacent to the gene encoding the inner membrane protein and these are usually co-transcribed (fig. 6). The gene encoding the outer membrane protein is often, but not always located with the other genes; exceptions include *E. coli acrAB* and *tolC* and *P. aeruginosa mexXY* and *oprM* (25). A divergently transcribed regulator gene (typically a repressor) is usually located adjacent to the gene encoding the periplasmic localized protein. (25)

Similar to *E. coli*, the different RND efflux systems vary in their substrate transport affinities, but differences in the regulation of the four RND efflux systems also have been observed (36). For example the MexAB-OprM transporter system is constitutively expressed at high level in *P. aeruginosa* wild type (36, 37). In contrast the MexCD-OprJ efflux system is not expressed in the *P. aeruginosa* wild type (38). However, expression of MexCD-OprJ occurs by mutation of NfxB, a transcriptional repressor. Mutations in the *nfxB* gene result in overexpression of the RND efflux system and increased multidrug resistance *in vivo* (39). MexEF-OprN is regulated by a

transcriptional activator, MexT. The MexT protein appears to be inactive in wild type cells and mutations, which can change the protein to an active state, thus causing increased expression of the MexEF-OprN efflux system (40). Also, increased expression of the mexEF-oprN efflux system and resistance to multiple antibiotics resulted from mutations in the *mexS* gene (41). Unlike other *P. aeruginosa* efflux systems, *mexXY* is induced by its transporting antibiotic substrates, including amino glycosides, tetracycline and erythromycin (36). The expression of MexXY-OprM is repressed by MexZ, a member of the TetR family of transcriptional regulators (42).

Amino acid sequence comparison of the 30-84 *acrA* and *acrB* gene products revealed that they share 44 and 54% amino acid identity with the *E. coli* *acrA* and *acrB* gene products and are predicted to be the same size. Several 30-84 genes (12 genes) are annotated as *tolC*, the best match sharing 47 % amino acid identity with *E. coli* TolC. The 30-84 *acr* locus is similar in organization to the *E. coli* *acr* locus (fig. 6), containing *acrA* and *acrB* in the same order and not *tolC*; the RNA-Seq data indicate that similar to *E. coli*, the 30-84 *acrA* and *acrB* genes are co-transcribed (Pierson, unpublished). However, 30-84 does not have the divergently transcribed transcriptional regulator *acrR*; instead the regulators RpeA/RpeB are co-located and divergently transcribed. The 30-84 *acrA* and *acrB* gene products are most similar to the *P.aeruginosa* PAO1 proteins MexE (36% amino acid identity) and MexF (44% amino acid identify), respectively.

Although the role of RND systems in drug resistance has been well documented, little is known about the mechanisms of phenazine transport or whether an RND system

may be responsible for phenazine transport, as hypothesized in this study. The possible role of an AcrAB-like transporter in phenazine export was hypothesized by Girard *et al.* (43). Working with *Pseudomonas chlororaphis* PCL139, they showed that Pip, a TetR type transcriptional regulator similar to AcrR controls the production of phenazine-1-carboxamide (PCN) via the regulation of the quorum sensing system PhzI/PhzR. They hypothesized that Pip may directly regulate the expression of genes encoding an efflux pump analogous to AcrR. They further speculated that since PCN is similar in structure to *N*-acylhomoserine lactone (*N*-AHL) quorum sensing signals, this efflux pump may also transport AHLs. The transport system was not identified in this study (43). However, it was shown that although the expression of *E. coli* *acrR* is under the regulation of the molecules secreted by their target pumps, the transcription of *pip* was not influenced by *N*-AHL signals or PCN. This finding suggests that if indeed an RND system functions in phenazine transport in their system, Pip may not be the transport regulator or it may not regulate the transport system in a manner analogous to AcrR. Similar to *P. chlororaphis* PCL139, the quorum sensing regulators PhzI and PhzR are regulated by Pip in *P. chlororaphis* 30-84. Based on the interesting speculations of Girard *et al.* (43), I also investigate the role of Pip in the regulation of the *acrAB* operon in 30-84. A model summarizing the phenazine regulon of 30-84 is provided in the Discussion.

3. MATERIALS AND METHODS

3.1 Bacteria, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *P. chlororaphis* 30-84 and mutant derivatives were grown at 28 °C on agar plates or in liquid with shaking at 200 rpm in either Luria-Bertani medium (LB), pigment production medium D (PPMD) or AB minimal medium supplemented with 2% casamino acids (AB) as described previously (4). *Escherichia coli* was grown at 37 °C in LB. Antibiotics were used where appropriate at the following concentrations for *E. coli*: 50 µg/ml kanamycin (KM), 15 µg/ml gentamycin (GM), and 0.001% acriflavine; for *P. chlororaphis*: 50 µg/ml KM, 50 µg/ml GM, 0.025% acriflavine, and 100 µg/ml Rifampin (RIF).

3.2 DNA manipulations and sequence analysis

Plasmid DNA isolation, cloning, restriction enzyme digestion and T4 DNA ligation were performed using standard protocols. Polymerase chain reaction (PCR) was carried out using *Taq* DNA polymerase (either Life Technologies, Carlsbad, CA or Promega, Madison, WE) at 94 °C for 10 min, followed by 30 cycles of 95 °C for 30 sec, the appropriate annealing temperature for 30 sec and 72 °C for 90 sec (or longer depending on gene size), and a final elongation step of 72 °C for 10 min.

Oligonucleotides and primers are listed in Table 2. Amplicons were initially cloned using a TOPO[®] TA cloning system (Life Technologies). DNA sequencing was performed at the DNA Service Center in the Borlaug Center (Texas A&M University, College

Station, TX). Transformation of *E. coli* was carried out using electroporation ready *E. coli* DH5 α according to manufacturer protocols (Life Technologies).

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant characteristics	Reference or source
<i>P.chlororaphis</i>		
30-84	Phz ⁺ Rif ^R wild type	Whistler & Pierson (10)
30-84rpeA	Phz ⁺ Rif ^R Km ^R , rpeA : : km	Wang <i>et al.</i> (4)
30-84rpeB	Phz ⁺ Rif ^R Km ^R , rpeB : : km	Wang <i>et al.</i> (4)
30-84pip	Phz ⁺ Rif ^R Km ^R , pip: : Tn5	Wang <i>et al.</i> (4)
30-84phzR	Phz ⁻ Rif ^R Km ^R , phzR: : Tn5 genomic fusion	Pierson <i>et al.</i> (6)
30-84acrAB	Phz ⁺ Rif ^R Km ^R , harboring <i>Ptac::acrA/acrB</i>	This study
<i>E. coli</i>		
DH5 α	F ⁻ <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)I169 Φ 80 <i>lacZ</i> Δ M15 λ ⁻	Invitrogen Life Technology
HB101	F ⁻ <i>hsdS20</i> (τ_B^- m_B^-) <i>supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5</i> λ ⁻	Invitrogen Life Technology
Plasmids		
pGT2	pPROBE-GT ['] : Gm ^R , GFP-based promoter trap vector containing a promoterless <i>gfp</i> gene	Miller <i>et al.</i> (14)
pKT2	pPROBE-KT ['] : Km ^R , GFP-based promoter trap vector containing a promoterless <i>gfp</i> gene	Miller <i>et al.</i> (14)
pKT2 <i>Ptac</i> :A/B	pKT2 containing the 4.3 kb DNA fragment <i>Ptac::acrA/acrB</i>	This study
pGT2 <i>Ptac</i> :A/B	pGT2 containing the 4.3 kb DNA fragment <i>Ptac::acrA/acrB</i>	This study
pGT2 _{pacrA} :A/B	pGT2 containing the 4.3 kb DNA fragment <i>pacrA::acrA/acrB</i>	This study

Km^R, Ap^R, Gm^R, and Rif^R, kanamycin, ampicillin, gentamicin, and rifampicin resistance, respectively

Table 2. Oligonucleotides for gene amplification, cloning verification, and qPCR.

	Oligonucleotide	Sequence (5'– 3')
1	F-acrABam	GCGGGATCCCAGCATCAGGTCGAGAATCA(<i>Bam</i> H1)
2	R-acrAHind	GCGAAGCTTGACAATGTCGGCGAACTCTT(<i>Hind</i> III)
3	F-acrB	CTGATCGGCTTCAACCTGAC
4	R-acrBHind	GCGAAGCTTTCCAGCCGATGAGAATCTT (<i>Hind</i> III)
5	F-acrA-kan	ATGTCGAAAAACCTGTTTGCGCCGTTCTGCCTGTT ATTGCTGGCAGCCGCGCTGAGCGCTTGC GACCGGGCCTCCACCGCCGAAGAGCAGGCGCCGT TGGCAAAGCCACGTTGTGTCTCAAATC
6	R-acrA-kan	GTCCAGTGGTCTGGGCAAGGGGAAGAGGGCTGTC GTCGATCTGCACCTTTTCGCCCGGACG GGCGTGCTGCAGGCCCTCGACGACGATACGGTTCG CCGGCTCTGATTAGAAAACTCATCG
7	F-acrB-kan	TGCCGCAATTCTTTATCGACCGTCCGGTGTTCGCCT GGGTGGTCGCCCTGTTTATCCTGCTGG CCGGCGCCCTCTCCATCCCGCAGTTGCCGGTGGCG CACAAAGCCACGTTGTGTCTCAAATC
8	R-acrB-kan	CGACGCTCACCGCTTTTTTGCCGTTGCGCCTGACC AGCCTCATCACCACCACGAAGAACAC CGGCACGAATACCACCGCCAGGGTCGCGGTGATC ATGCCTCTGATTAGAAAACTCATCG
9	F-acrA	ATGTCGAAAAACCTGTTTGC
10	R-acrA	GTCCAGTGGTCTGGGCAAG
11	F-acrB	TGCCGCAATTCTTTATCGAC
12	R-acrB	CGACGCTCACCGCTTTTT
13	F-TOPO	GTAAAACGACGGCCAG
14	R-TOPO	CAGGAAACAGCTATGAC

Briefly, 3 μ l of electrocompetent cells were diluted in 200 μ l of ice water, mixed with \sim 100 μ g of plasmid DNA, and cooled on ice 30 min. Electroporation was carried out using a Bio-Rad Micro Pulser Electroporator with a cuvette (2 mm gap) according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). Cells were then grown in 250 μ l of S.O.C. Media (Life technologies) for 2 h at 37 $^{\circ}$ C with shaking and then plated on appropriate selection media. Conjugation was carried out via triparental mating as described previously (4). Briefly, the donor (*Escherichia coli DH5 α*), the helper (*E. coli* HB101), and the recipient (30-84 derivatives) were grown separately in overnight cultures. Equal amounts of each were spotted onto sterile nitrocellulose filter on LB agar plates and incubated first at 37 $^{\circ}$ C for 3 h then at 28 $^{\circ}$ C for 48 h. The resulting cells were resuspended in 5 ml sterile deionized water and 100 μ l were spread onto LB plates supplemented with RIF to select against *E. coli* and other antibiotics as appropriate. Nucleic acid and amino acid homology searches were conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>). *P. chlororaphis (aureofaciens)* 30-84 draft genomic sequence is available in GenBank (Genbank NZ_CM001559.1). The *P. chlororaphis* 30-84) biosynthetic operon contains seven conserved biosynthetic genes *phzXYFABCD* (according to the original nomenclature, (44, 45), which correspond to *phzABCDEFG* (according to the *P. fluorescens* nomenclature). Here I use the *P. chlororaphis* nomenclature to conform to the original literature.

3.3 Generation of vectors for the constitutive expression of *acrA/acrB*

The sequences flanking the *acrA* (Pchl3084-3226) and *acrB* (Pchl3084-3227) genes were used to design primers to amplify these genes, including the promoter and ribosome binding site preceding *acrA*. The primer pairs F-*acrABam* and R-*acrAHind* were used to amplify a region of the 30-84 chromosome starting 300 nt upstream of the ATG start and a ~1 kb portion of the *acrB* gene containing a unique *XhoI* site within the gene. The F-*acrB* and R-*acrBHind* primers were used to amplify 80 bp upstream of the unique *XhoI* site in *acrB* and 300 nt downstream of the *acrB* stop codon. By adding appropriate restriction enzyme sequences to the primers, the *acrA* amplicon included *BamHI* and *Hin* II I restriction enzyme sites at the 3' and 5' ends, respectively, and the *acrB* amplicon included a *HindIII* restriction enzyme site at the 5' end. The *acrA* gene was subcloned into the multiple cloning site of pUC19 using *BamHI* and *HindIII*. The *acrB* gene was then subcloned into pUC 19 containing *acrA* using *XhoI* and *HindIII*. The *BamHI-acrA/acrB-HindIII* fragment was then cloned separately into both KT2 and GT2. Prior to insertion of the *acrA/acrB* fragment the *Ptac* promoter was inserted separately into both KT2 and GT2 at the *EcoRI-BamHI* site. The final plasmids were designated pKT2*Ptac:A/B* and pGT2*Ptac:A/B*, respectively. In order to observe the regulation of the *acrA* promoter in the 30-84 *rpeB* mutant, the *BamHI-acrA/acrB-HindIII* fragment was cloned into GT2 without *Ptac* such that gene expression was driven by the *acrA* promoter. This plasmid was designated pKT2*pacrA:A/B*. Plasmids were confirmed by

restriction enzyme analysis and partial sequencing and then conjugated into 30-84 and appropriate mutant derivatives.

3.4 Effect of *acrA/acrB* on resistance to acriflavine and other antimicrobials

E. coli and 30-84 containing either pKT2*Ptac*:A/B or KT2 with no insert were grown with shaking in LB supplemented with 50 µg/ml KM to an optical density of 0.8 at 620 nm. *E. coli* and 30-84 were grown in 37 °C and 28 °C, respectively. Cells were collected via centrifugation and resuspended in fresh medium. These cultures were used to inoculate (1:100) LB liquid medium, supplemented with acriflavine at a concentration of 0.01% for *E. coli* and 0.025% for *P. chlororaphis*, respectively. The OD₆₂₀ were measured after incubation for 14 h.

For the other antimicrobials, overnight cultures were prepared in the same way and used to inoculate broth containing antibiotics at a rate of 1:100. A dilution series (twofold) of each antibiotic was prepared at the following concentrations for *E. coli*: (0.25, 0.50, 1, 2, 4, 8, 16 µg/ml) and 30-84 (2, 4, 8, 16, 32, 64, 128 µg/ml). Cell growth, measured by OD₆₂₀, was assayed after 16 h shaking (200 rpm). Data were reported as the minimal inhibition concentration (MIC), e.g. the lowest concentration at which bacterial growth was negative. An OD₆₂₀ of less than 0.05 was considered to be negative growth. For *E. coli*, antibiotics tested included carbenicillin, chloramphenicol, piperacillin, and tetracycline and for 30-84 included the same antibiotics plus gentamicin, nalidixic acid, and polymixin B. Antibiotics were obtained from Sigma-Aldrich (St. Louis, MO) and

Amresco (Solon, OH). All assays were conducted with 3 replicates and repeated at least twice.

3.5 Effect of *acrA/acrB* on growth and phenazine production and transport

P. chlororaphis 30-84 and derivatives each containing either pGT2*Ptac*:A/B or GT2 with no insert were grown overnight (14 to 18 h) in AB, LB or PPMD broth to late exponential phase and adjusted to an OD₆₂₀ of 1.8. Supernatants were collected after centrifugation (3250 x g) for 15 min. The supernatants were acidified to pH 2 with concentrated HCl. Total phenazines were extracted twice with an equal volume of benzene for 1 h. The benzene phase was collected and evaporated under air. Dried phenazines were dissolved in 0.1 N NaOH and quantified by UV-visible spectroscopy using 0.1 N NaOH as the blank. Phenazine extraction was confirmed via UV visible spectra (200-600 nm). Phenazines were quantified at OD₃₆₇. All assays were conducted with 3 replicates and repeated at least twice.

For analysis of growth curves, treatments were grown and adjusted to the same optical density as above. Fresh cultures were inoculated (1:1000) and measurements were taken every 1 to 3 h during the exponential phase of growth (between 5 h and 10 h). Additional measurements at 25 h and 30 h confirmed that the cultures had entered stationary phase. At each time point, samples were collected and phenazines extracted as above. All assays consisted of three independent replicate cultures and the assay was repeated twice.

3.6 Demonstration that AcrA and AcrB function in phenazine transport in *E. coli*

E. coli containing either pKT2*Ptac::acrA/acrB* or KT2 with no insert were grown overnight with shaking in LB supplemented with 50 µg/ml KM to an optical density of 1.2 at OD₆₂₀. These cultures were then used as inoculum for the growth curves; inoculum was added to fresh LB supplemented with 50 µg/ml KM (1:100 dilution) amended with total phenazines (prepared as above in 0.1N NaOH at a concentration of 800 µg/ml) or without phenazines. The pH was adjusted to ~7 using 0.1 N HCl. Bacterial growth rate (measured as OD₆₂₀) was measured every two hour. All assays consisted of three independent replicate cultures and the assay was repeated twice.

3.7 Effect of *acrA/acrB* on pathogen inhibition

Overnight cultures of 30-84 containing either pKT2*Ptac:A/B* or KT2 with no insert were grown and 15 µl were spotted onto LB plates amended with 0.5 % potato dextrose agar. After 2 days of growth at 28 °C, a 5 mm plug of *G. graminis* was placed on the center of the plates. After 4 days, zones of inhibition defined as the distance between the edge of the bacterial colony and the fungal mycelium, were measured. Measurements were taken on three replicate plates per assay and one representative experiment is presented. The experiment was repeated three times with similar results.

3.8 Generation of the *acrA* mutant

An AcrAB deficient mutant will be generated by replacing most of the coding sequence of *acrA* with a kanamycin resistance cartridge (except for the first and last 100 nt). Because AcrA is required for the formation of a functional AcrAB-TolC RND efflux

system in *E. coli*, it is likely that mutation of *acrA* will completely disrupt the RND system in 30-84. If necessary, I will create a *acrA/acrB* double mutant. Mutations will be created by replacing the target genes in the bacterial chromosome with a recombination fragment containing a kanamycin (Km) cassette flanked at both ends by 100 nts with sequence homology to both ends of the *acrA* gene (see fig. 5). When the recombination fragment is transformed into the bacterial cell, the replacement of the *acrA* gene in the chromosome with the kanamycin cartridge occurs via homologous recombination. As described by Lesic and Rahme (46), the rate of homologous recombination can be improved via the introduction of the RedS plasmid (containing the Red operon from bacteriophage lambda), which causes recombination events to occur at a high frequency; the Red operon is under the control of an arabinose-inducible promoter. Deletions can be confirmed via PCR using combinations of the primers F-*acrA* and R-*acrA*, F-*acrB* and R-*acrB* and internal primer pairs, km1 and km2 of the kanamycin gene (Table 1).

Several steps of the process have been completed. The first step was to make the recombination fragment using PCR with pUC4K as template for the kanamycin resistance gene. The recombination fragment consists of a kanamycin cassette under the control of its own promoter, flanked at each end by 100 nt with sequence homology to *acrA*. The 5' ends of the forward and reverse primers used for the generation of the recombination fragment contained 100 nt with sequence homology to the first 100 bases or the last 100 bases of the *acrA* gene, respectively, whereas the 3' end of the forward and reverse primer contained 20 bases with sequence homology to the start or end of the

kanamycin resistance gene, respectively (fig. 5). The resulting recombination fragment was cloned into the TOPO TA vector (Life Technologies).

The recombination fragment was introduced into *P. chlororaphis* via a two-step process. First, the RedS plasmid (pUCP18-RedS, Table 1) was transformed into *P. chlororaphis* 30-84 wild type via electroporation. Then the recombination fragment was transformed into strain *P. chlororaphis* 30-84 (pUCP18-RedS) via electroporation. For the second transformation, *P. chlororaphis* 30-84 (pUCP18-RedS) was grown in LB broth at 26 °C overnight, then reinoculated into LB liquid medium containing 0.1 % arabinose (1: 100) to insure expression of the Red operon and cultures were grown to a final OD₆₂₀ of 0.6. Cells in exponential phase were collected to make competent cells. Transformants were plated on LB supplemented 50 µg/ml KM.

4. RESULTS

In this study, a genetic approach consisting of constitutive gene expression and gene mutation studies was used to evaluate the hypotheses that the *P. chlororaphis* 30-84 AcrA and AcrB proteins function as an RND type transporter capable of conferring multi-drug resistance and transporting phenazines.

4.1 Constitutive expression of *acrA/acrB* enhanced RND-type transporter activities

For the constitutive gene expression studies, the 30-84 *acrA/acrB* genes were PCR amplified and cloned into either plasmid KT2 or GT2 behind the constitutive promoter *P_{tac}*, a modified *lac* operon promoter. Both plasmids KT2 and GT2, differing only in the antibiotic resistance genes they contain, replicate and are maintained with antibiotic selection in *E. coli* and are maintained without selection in 30-84. The *acrA/acrB* containing plasmid KT2 $P_{tac}::acrA/acrB$ was transformed into *E. coli* via electroporation and tested for acriflavine resistance and then conjugated into 30-84. Bacterial growth (measured as turbidity at OD₆₂₀) after 14 hours growth in acriflavine was used as an indicator of resistance. Consistent with the hypothesis that the 30-84 AcrA and AcrB proteins function as an RND transport system, constitutive expression of the *acrA/acrB* genes conferred acriflavine resistance to *E. coli* (fig. 8A) and enhanced acriflavine resistance in 30-84 (fig. 8B) relative to the wild type strains carrying the plasmid without an insert. Consistent with the observation that transcript abundances of *acrA* and *acrB* are higher in 30-84 *rpeA* mutants and lower in 30-84 *rpeB* mutants, *rpeA*

mutants were more resistant to acriflavine than the wild type. Overexpression of the transport genes in wild type made them as resistant to acriflavine as the 30-84 *rpeA* mutant. As expected the *rpeB* mutant was highly susceptible to acriflavine (fig. 8B).

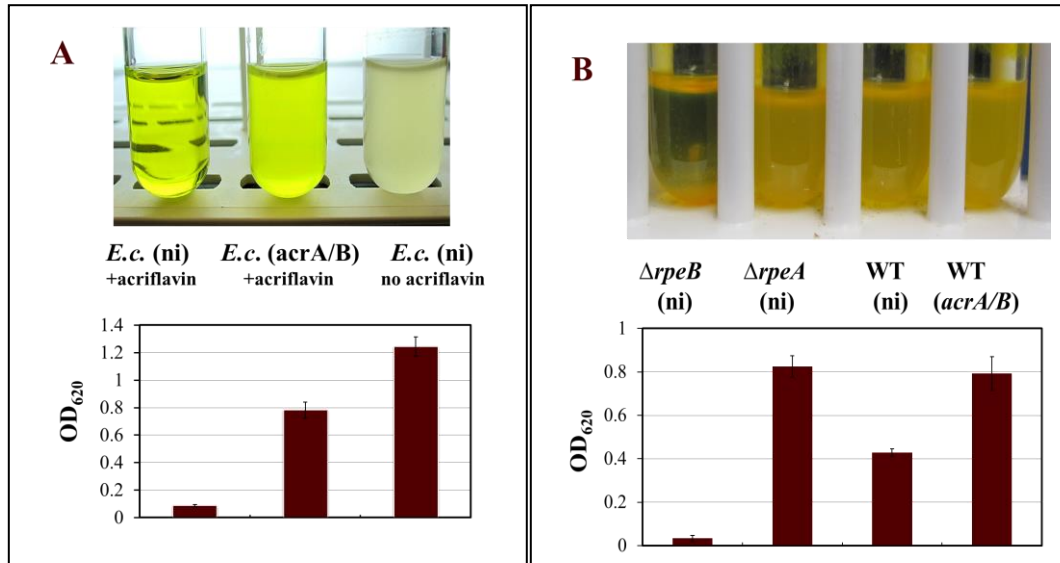


Figure 8. Acriflavine resistance assay. **A.** The assay was conducted using *E. coli* (*E.c.*) containing the KT2 plasmid with no insert (ni) or with *Ptac::acrA/acrB*. Strains were grown 14 h in LB medium with or without 0.01% acriflavine (which colors the media yellow). Bacterial growth (indicated by turbidity) was measured at OD₆₂₀ (data are means and standard errors of 3 reps). **B.** Wild type (WT) *P. chlororaphis* 30-84 containing the plasmid with no insert (ni) or with *acrA/acrB* and derivatives defective in *rpeB* or *rpeA* (each containing the control plasmid) were grown overnight in LB, adjusted to equivalent cell densities, and inoculated into LB + 0.025% acriflavine (the higher concentration colors the media orange). Culture growth at 14 h was measured at OD₆₂₀ (data are means and standard errors of 3 reps).

To determine whether transcriptional repression of the *acrA/acrB* genes in the *rpeB* mutant is responsible for the enhanced sensitivity of this derivative to acriflavine, extra copies of *acrA/acrB* driven by either the constitutive promoter (*Ptac::acrA/acrB*) or the native *acrA* promoter (*PacrA::acrA/acrB*) were introduced into GT2 (fig. 9).

Constitutive expression of *acrA/acrB* resulted in complete restoration of acriflavin resistance in the *rpeB* mutant. However, consistent with the hypothesis that *acrA/acrB* is regulated transcriptionally by RpeB, additional copies of *acrA/acrB* behind the native *acrA* promoter resulted in substantially lower acriflavine resistance.

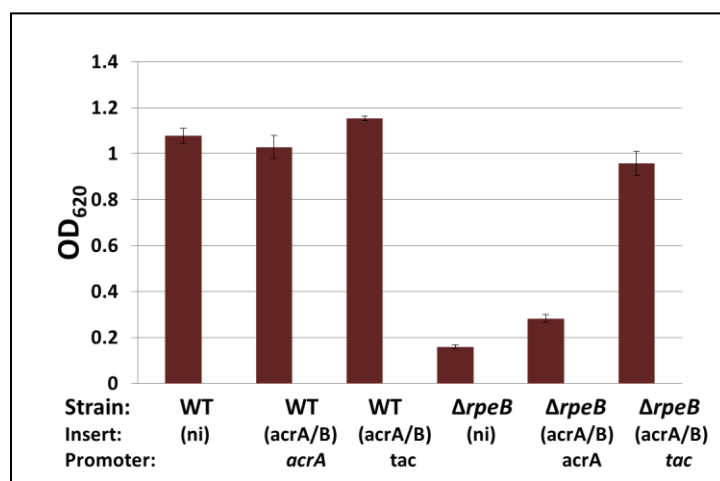


Figure 9. Acriflavine resistance assay demonstrating RpeB control of the *acrA* promoter. Wild type (WT) *P. chlororaphis* 30-84 and the *rpeB* mutant containing either GT2 plasmid with no insert (ni) or with extra copies of *acrA/acrB* driven by either the native *acrA* promoter (*p_{acrA}::acrA/acrB*) or the constitutive promoter (*P_{tac}::acrA/acrB*). Treatments were grown overnight in LB, adjusted to equivalent cell densities, and inoculated into LB + 0.025% acriflavine. Culture growth at 14 h was measured at OD₆₂₀ (data are means and standard errors of 3 reps).

To determine whether genes that regulate phenazine *biosynthesis* also regulate the *acrA/acrB* genes, the *rpeB* mutant (deficient in phenazine biosynthesis and transport) was complemented with the wild type *rpeB* gene (should restore both phenazine biosynthesis and transport) or *pip* (a transcriptional regulator required for phenazine biosynthesis (4) (fig. 10). As reported by Wang *et al.*, complementation of *rpeB* with

either gene restored phenazine production (4). Cell growth in the presence of acriflavine was also measured as a measure of transport activity (OD_{620}). Although complementation of the *rpeB* mutant with wild type *rpeB* restored transport activity, complementation of *rpeB* with *pip* did not. Furthermore mutants defective in *pip* (data not shown) or *phzR* were able to grow in media supplemented with acriflavine as well as wild type (fig. 10).

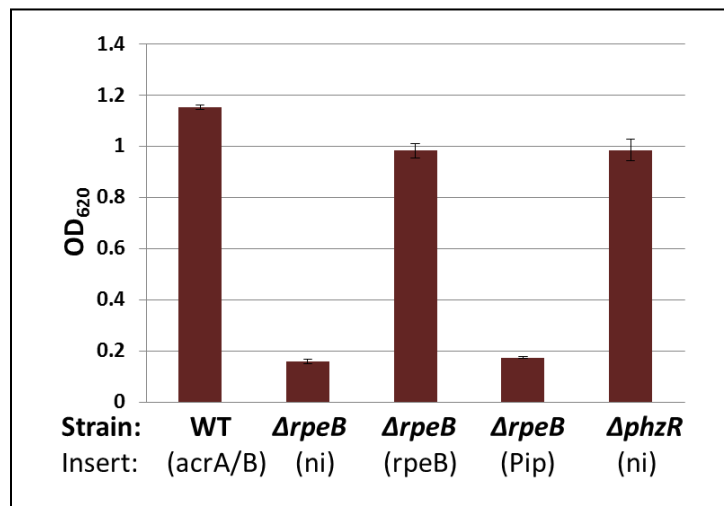


Figure 10. Acriflavine resistance by various 30-84 derivatives. The assay included: wild type (WT) *P. chlororaphis* 30-84 containing the GT2 plasmid with extra copies of *acrA/acrB* driven by the constitutive promoter (*Ptac::acrA/acrB*), the *rpeB* mutant derivative complemented with either the wild type *rpeB*(*Ptac::rpeB*) or *pip*(*Ptac::rpeB*) genes or containing only the control plasmid (GT2 with no insert), and the *phzR* mutant derivative (containing the control plasmid). Treatments were grown overnight in LB, adjusted to equivalent cell densities, and inoculated into LB + 0.025% acriflavine. Culture growth at 14 h was measured at OD_{620} (data are means and standard errors of 3 reps).

Previous reports demonstrated that suppression of RND-type efflux systems such as the MexAB-OprM system resulted in the cells becoming two- to fourfold more susceptible to such antibiotics as aztreonam, tetracycline, carbenicillin, chloramphenicol, and novobiocin (40). In order to determine whether *acrA/acrB* also contributes to multi-drug resistance, *P. chlororaphis* containing either the plasmid KT2 with no insert (ni) or with *Ptac::acrA/acrB* were grown in LB in the presence of increasing (by twofold) concentrations of antimicrobials, as is the standard protocol for assessing the minimum inhibitory concentration (MIC). Cell growth (asOD₆₂₀) was assayed after 16h shaking. An OD₆₂₀ of less than 0.05 was considered to be negative growth. Susceptibility was expressed as the MIC (µg/ml), the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight (16 h) incubation. Constitutive overexpression of *acrA/acrB* in 30-84 resulted in a four-fold increase in resistance to tetracycline and chloramphenicol (Table 3), but not to other antimicrobials such as nalidixic acid, gentamicin, or polymyxin b (data not shown).

Table 3. Antimicrobial susceptibility (MIC).

Antibiotics ($\mu\text{g/ml}$)	30-84 (ni)	30-84 (<i>acrA/B</i>)
Tetracycline	2 ^a	8
Chloramphenicol	16	128
Carbenicillin	2	32

^aThe concentration ($\mu\text{g/ml}$) of antimicrobial that inhibited visible growth following overnight incubation.

4.2 Constitutive expression of *acrA/acrB* enhanced phenazine accumulation in 30-84

Constitutive expression of *acrA/acrB* in wild type 30-84 resulted in increased amounts of phenazine in culture supernatants compared to wild type containing the control plasmid, consistent with *acrA* and *acrB* functioning in phenazine transport (Figs. 11 and 12). To insure that the enhancement in phenazine production due to overexpression of *acrA/acrB* was not simply a result of differential growth, growth curves and phenazine production by 30-84 wild type (WT) containing the plasmid KT2 with no insert (ni) or with *Ptac::acrA/acrB* were compared. Data confirmed that there is no effect of constitutive expression of *acrA/acrB* on bacterial growth rate. However, constitutive over-expression of *acrA/acrB* resulted in greater phenazine production at lower cell densities, compared to the wild type containing the control plasmid (fig. 12).

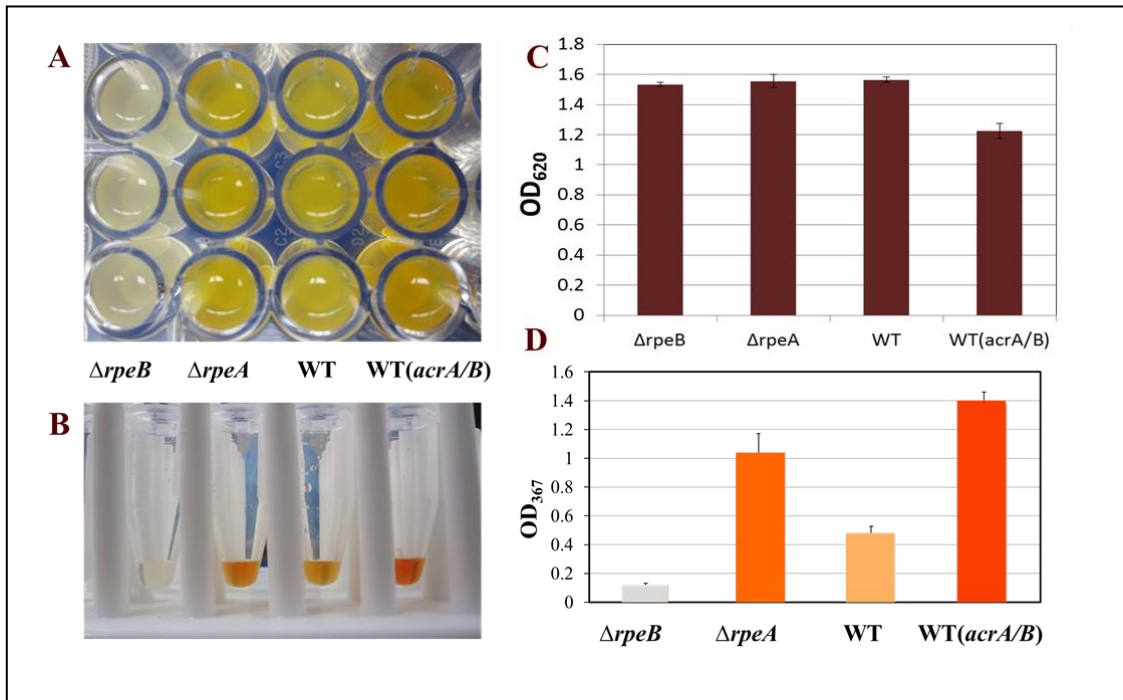


Figure 11. The effect of constitutive expression of *acrA/acrB* on phenazine production in 30-84. **A.** Wild type (WT) *P. chlororaphis* 30-84 containing the KT2 plasmid with no insert (ni) or with *Ptac::acrA/acrB* and derivatives defective in *rpeB* or *rpeA* (each containing the control plasmid) were grown 14 h in PPMD medium. **B.** Bacterial growth was measured at OD₆₂₀ and all cultures exceeded 1.5. **C.** Culture supernatants were extracted for total phenazines (via acidification and benzene). Benzene was evaporated and phenazines were resuspended in 0.1 N NaOH. Phenazine extraction was confirmed via UV visible spectra (200-600 nm). **D.** Phenazines were quantified at OD₃₆₇ (data show means and SE of 3 reps). Data confirm that additional copies of *acrA/acrB* increase phenazine amounts in culture supernatants, consistent with their functioning in phenazine transport.

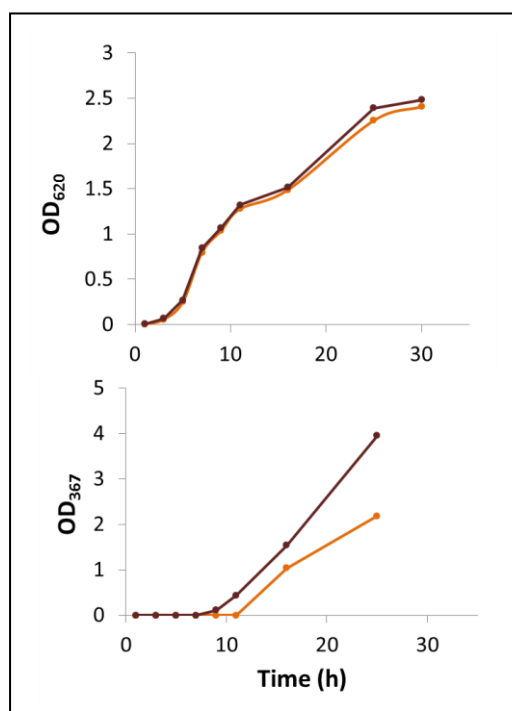


Figure 12. Growth curves and phenazine production by 30-84 wild type (WT) containing the plasmid KT2 with no insert (orange line) or with *Ptac::acrA/acrB* (brown line). Cells were grown in PPMD medium with shaking at 28 °C. Bacterial growth was measured at OD₆₂₀. Culture supernatants were extracted for total phenazines and quantified at OD₃₆₇. Data are the means of 3 replicates. Standard errors for both measurements were quite small (<0.12) and are not shown.

To determine whether transcriptional repression of the *acrA/acrB* genes in the *rpeB* mutant is responsible for the reduction in phenazine production compared to wild type, extra copies of *acrA/acrB* driven by either the constitutive promoter (*Ptac::acrA/acrB*) or the native *acrA* promoter (*PacrA::acrA/acrB*) were introduced onto GT2 (fig. 13). Bacterial strains were grown in PPMD for 16 h to an OD₆₂₀ of 1.3, and the amount of extracted phenazines was measured at OD₃₆₇. As expected the *rpeB* mutant produced very little phenazine during this time period. Expression of the *acrA/acrB*

genes *in trans* in the *rpeB* mutant did not restore phenazine production to wild type levels. This finding is consistent with the hypothesis that *rpeB* regulates both phenazine production and transport, e.g. over-expression of *acrA/acrB* in the *rpeB* mutant would not be expected to restore the wild type phenotype unless other RpeA/RpeB controlled traits involved in the regulation of phenazine biosynthesis such as quorum sensing were also restored. However, the amount of phenazine produced by $\Delta rpeB$ containing *acrA/acrB* (*PacrA::acrA/acrB*) varied by media type (fig. 14).

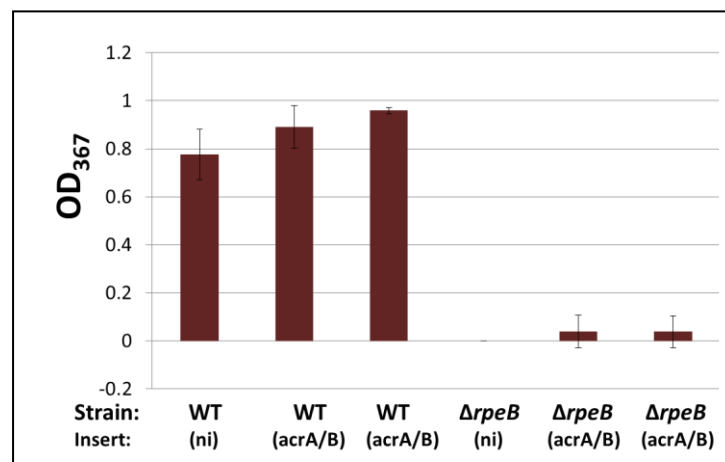


Figure 13. Phenazine production by the 30-84 *rpeB* mutant is not rescued by overexpression of *acrA/acrB*. Wild type (WT) *P. chlororaphis* 30-84 and the *rpeB* mutant containing either the GT2 plasmid with no insert (ni) or with extra copies of *acrA/acrB* driven by either the native *acrA* promoter (*PacrA::acrA/acrB*) or the constitutive promoter (*Ptac::acrA/acrB*). Bacterial strains were grown in PPMD for 16 h at 28 °C with shaking to an OD₆₀₀ of 1.3. Phenazine was extracted in benzene and the amount of phenazine was measured at OD₃₆₇. Data points represent means \pm standard errors of three replicates.

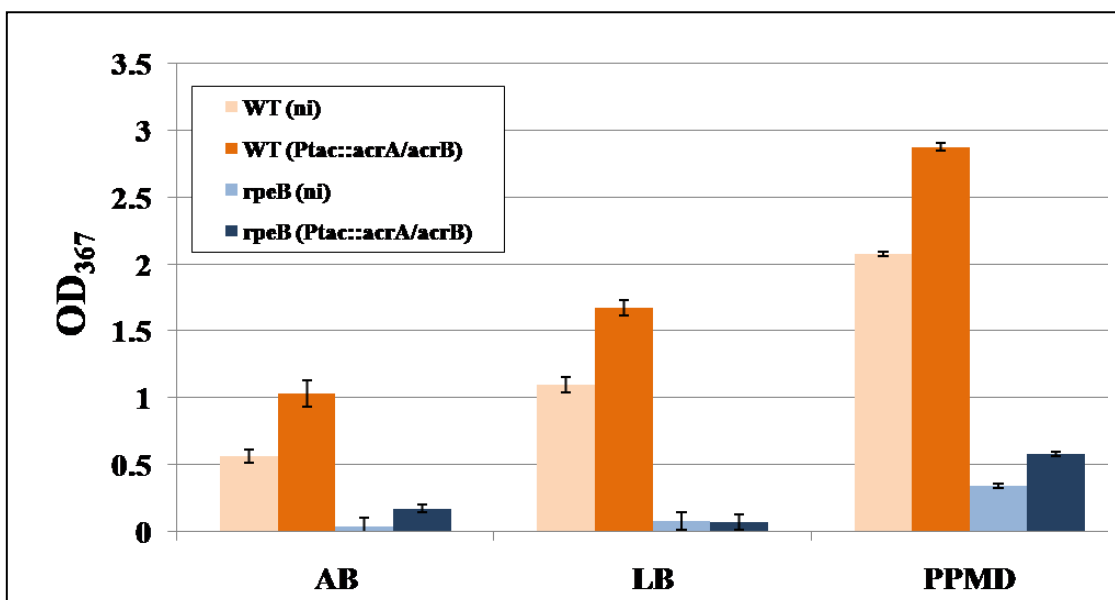


Figure 14. Phenazine production in different media. Phenazine production by the wild type and the *rpeB* mutant containing either the control plasmid (GT2) or constitutively expressing the *acrA/acrB* (e.g. containing the insert *Ptac::acrA/acrB*). For maximal phenazine production bacteria were grown in different media (AB, LB, or PPMD) for 48 h at 28 °C with shaking. Phenazine was extracted in benzene and the amount of phenazine was measured at OD₃₆₇. Data points represent means ±standard errors of three replicates. Similar results were obtained in two independent experiments.

4.3 Demonstration that AcrA and AcrB function in phenazine transport

Total phenazine extracted from 30-84 was inhibitory to the growth of *E. coli* starting at a concentration of ~300 µg/ml (data not shown) and became more inhibitory as the concentration was increased to ~800 µg/ml (fig. 15 A, B). For this assay, the growth of *E.coli* with the plasmid containing *PacrA::acrA/acrB* was compared to the growth of *E. coli* with the control plasmid in LB containing 800 µg/ml phenazine. In the absence *acrA/acrB*, growth was significantly decreased, although after 16 h the growth

of *E. coli* with and without *acrA/acrB* was equivalent. In the absence of phenazines, the *E. coli* with and without *acrA/acrB* grew at the same rate, indicating that the increase in growth in the presence of phenazines was due entirely to the ability to transport phenazines. Importantly, the total phenazine extract contained a mixture of non-hydroxylated and hydroxylated phenazines (e.g., PCA, 2OHPCA, 2OHPhz). In contrast, commercial phenazine (SigmaAldrich) did not inhibit *E. coli* at a concentration of ~300 µg/ml (diluted in DMSO—phenazine was not soluble above this concentration). These data are consistent with previous observations that the phenazines produced by 30-84 can be inhibitory to *E. coli* (Pierson, unpublished). *E. coli* expressing 30-84 *acrA/acrB* were protected from phenazines as indicated by their ability to grow in concentrations of 800 µg/ml as well as *E. coli* in media without phenazines (fig. 15 B, C). The ability to resist inhibition by phenazine demonstrates for the first time that the 30-84 AcrA and AcrB function in their transport. These results suggest that the native *E. coli* TolC was able to facilitate the formation of a complete transport system.

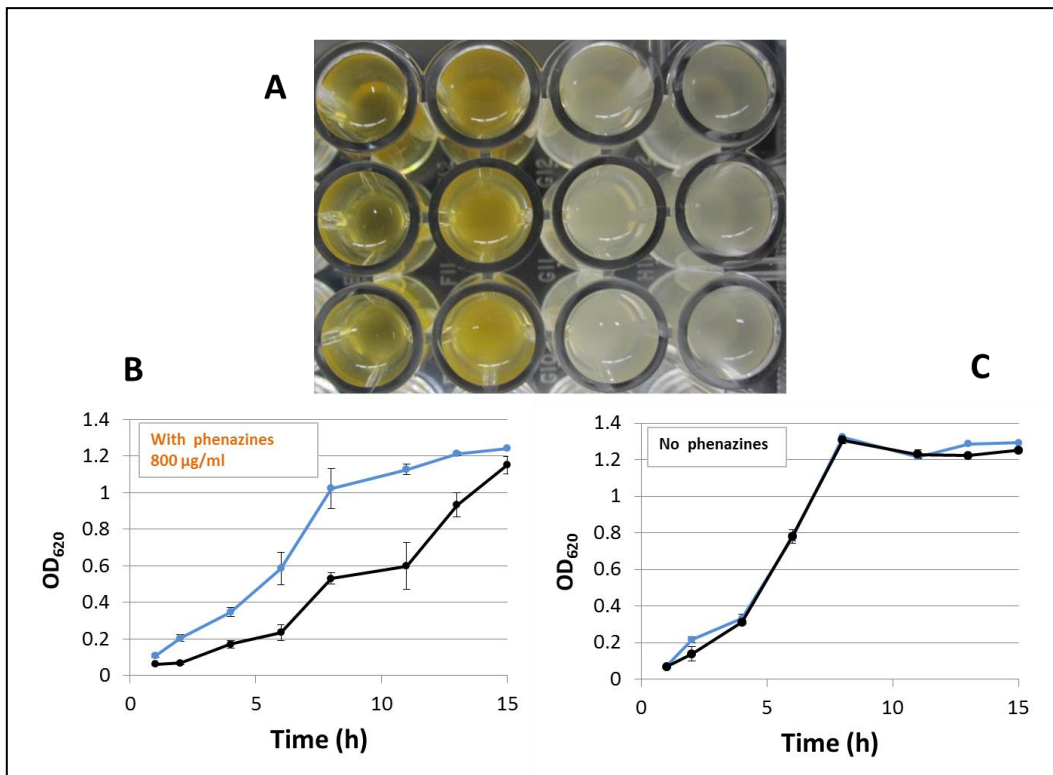


Figure 15. Demonstration that AcrA and AcrB function in the transport of phenazines in *E. coli*. A. Bacterial growth (after 9 h) of *E. coli* with the control plasmid (first column) or the plasmid containing *PacrA::acrA/acrB* (second column) when grown with 800 µg/ml phenazine or without phenazines (third and fourth columns, respectively) as indicated by turbidity. B. Growth curve of *E. coli* with the control plasmid (black line) or the plasmid containing *PacrA::acrA/acrB* (blue line) when grown with 800 µg/ml phenazine. C. Growth curve of *E. coli* with the control plasmid (black line) or the plasmid containing *PacrA::acrA/acrB* (blue line) when grown without phenazine.

4.4 Constitutive expression of *acrA/acrB* in 30-84 results in enhanced pathogen inhibition *in vitro*

The constitutive expression of *acrA/acrB* can have important implications for biological control activity. As expected earlier and greater production and diffusion of phenazines significantly affects the capacity of 30-84 to inhibit fungi under conditions where diffusion is limited such as on agar plates. For example inhibition of the fungus

G. graminis was greater for 30-84 cells containing copies of *acrA/acrB* than for cells containing the control plasmid (Fig 16).

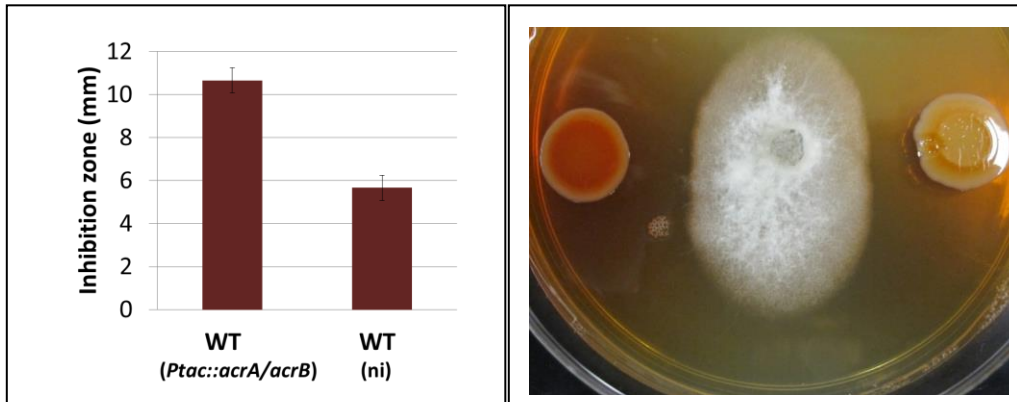


Figure 16. Fungal inhibition assay. Overnight cultures of wild type 30-84 containing either the control plasmid KT2 or the plasmid with *Ptac::acrA/acrB* were spotted onto LB plates amended with 0.5% potato dextrose agar. After 2 days of growth at 28 °C, a 5 mm plug of *G. graminis* was placed in the center of the plates. The zones of inhibition were measured at 4 days post-inoculation. Data represent means±standard errors of three replicates. The experiment was repeated three times with similar results.

4.5 Creation of the *acrA* deletion mutant

Preliminary studies confirm that the recombination fragment is correctly constructed and is now contained within the TOPO TA vector. The fragment has been transferred to electrocompetent 30-84 cells via electroporation. Efforts to obtain double crossovers have been unsuccessful to date. One possibility for this lack of double crossovers is that mutations in phenazine transport may be lethal, however, this possibility has not been confirmed. More likely the amount of sequence homologous to *acrA* is too small and longer homology arms on either side of the kanamycin cartridge are needed to facilitate efficient production of double crossovers.

5. DISCUSSION

In this study I have demonstrated for the first time that the *P. chlororaphis* 30-84 *acrA* and *acrB* genes encode an RND type efflux system that is capable of transporting phenazines and other antimicrobials. This was demonstrated using a constitutive gene expression approach in which a fragment containing the 30-84 genes annotated as *acrA* and *acrB* were cloned behind the constitutive promoter *P_{tac}* and the genes expressed on a very stable plasmid *in trans* in either *E. coli* DH5 α or 30-84. This experiment confirmed that constitutive expression of *acrA/acrB* conferred acriflavine resistance to *E. coli* and increased acriflavine resistance in 30-84. Constitutive overexpression of the genes in 30-84 also increased resistance to other antimicrobials. Importantly, constitutive expression of *acrA/acrB* enhanced the growth of *E. coli* in the presence of ~300-800 μg of total phenazines extracted from 30-84, demonstrating the function of the AcrA and AcrB proteins in phenazine transport.

As in other studies, the present results showed that expression of the periplasmically located protein (AcrA) and the inner membrane protein (AcrB) were sufficient to incorporate efficient acriflavine transport function in *E. coli*. Expression of the third component of the RND system, the outer membrane protein (TolC), was not necessary for efficient transport function (32). These results suggest that the endogenous *E. coli* TolC protein or another outer membrane homolog is sufficient to complement this deficiency. This speculation is supported by the observation that the outer membrane

protein is typically not co-transcribed with the structural genes and often not even located close by on the chromosome. Co-transcription suggests that the gene products likely function together. Previous reports suggest that in the absence of the AcrA and AcrB proteins, the *E. coli* TolC protein may still be found in the outer membrane, indicating it is not specifically a component of the AcrAB efflux system (47).

Furthermore, within the genome there are many genes annotated as encoding outer membrane proteins that might serve as a substitute. The complete lack of acriflavine resistance in *E. coli* DH5 α was somewhat surprising given that the AcrAB-TolC system was first described in *E. coli*. One hypothesis is that *E. coli* DH5 α , a laboratory derivative of K12, lacks either *acrA* or *acrB*, or both, or they are not functional or not expressed. This deficiency was useful for demonstrating that protection from phenazines via the 30-84 AcrAB transport system was not likely due to a native AcrAB system.

The data support the hypothesis that the 30-84 AcrAB system is capable of transporting a diversity of antimicrobials with a spectrum similar to that observed for the AcrAB-TolC system in *E. coli* (28). For example, clear differences in MIC for tetracycline, chloramphenicol and carbenicillin were observed between wild type *P. chlororaphis* transformed with the control plasmid and wild type *P. chlororaphis* constitutively overexpressing *acrA/acrB*. Also similar to the AcrAB-TolC system in *E. coli*, no enhancement in growth due to constitutive overexpression of *acrA/acrB* in the presence of gentamicin and polymixin B was observed. However, in contrast to results with *E. coli*, the 30-84 AcrAB system did not confer resistance to nalidixic acid. It has

been shown that substrate binding to AcrB is largely responsible for substrate affinity (25). This may depend on the partial binding of the substrate to the inner membrane first and then the AcrB protein. A possible explanation for this difference in substrate affinity or transport efficiency between *E. coli* and 30-84 may be that despite the structural similarity of the AcrA and AcrB proteins, there may be differences in the AcrB proteins (or both AcrA and AcrB), particularly at the binding sites (25). Furthermore, it is possible that the 30-84 AcrAB system may have a higher affinity or transport efficiency for phenazines than other RND systems. This hypothesis needs further testing. Future research could include expressing other RND pumps in *E. coli* DH5 α and comparing phenazine transport activity. It is interesting to note that many Gram negative bacteria like *E. coli* and *Pseudomonas* have multiple demonstrated or putative (based on protein homology) RND efflux systems. In spite of high protein homologies, differences in the AcrA and AcrB components may confer differences in substrate affinity. It was previously suggested that “environmental” species are more likely to have more efflux systems, presumably because they are exposed to a greater diversity of antimicrobial substances (48).

Despite the similarity in the organization between the 30-84 *acrA* and *acrB* genes and the homologs in other RND systems, the regulation of these genes in 30-84 is somewhat unique. For many of the RND efflux systems, a transcriptional regulator, typically a repressor is located adjacent to and divergently transcribed from the genes encoding the structural components. For example, in *E. coli*, *acrR* is located upstream of

acrA and *acrB* and this gene represses the expression of *acrA* and *acrB*. Mutations in *acrR* cause *E. coli* to become more resistant to AcrAB-transported antibiotics (29). In *Pseudomonas*, *mexR*, *nfxB*, and *mexZ* are transcriptional regulators (repressors) located upstream of the *mexAB*, *mexCD*, and *mexXY* operons, respectively (36, 39, 41). The operon *mexEF* is regulated by two proteins immediately adjacent to the operon, *mexS*, a transcriptional repressor, and *mexT*, a transcriptional activator via a somewhat complicated interaction (41). In contrast to all of these systems, *rpeB* (the transcriptional regulator) and *rpeA* (a sensor kinase) (composing a two component regulatory system) are immediately adjacent and divergently transcribed from the 30-84 *acrAB* operon.

The presence of a two component regulatory system upstream of the genes encoding the structural proteins has been observed previously in *Acinetobacter baumannii*, where the AdeABC RND efflux system is regulated by the two component system AdeS/AdeR (49). The organization of the locus is the same as is found in 30-84. In this system, mutation in *adeS* (the sensor kinase) causes an increase in susceptibility to antibiotics (e.g. loss of transporter function); the effect mutation in *adeR* (the response regulator) could not be determined (polar mutation). However, substitutions in the AdeS and AdeR proteins resulted in constitutive expression of the efflux system and antibiotic resistance (49). Marchand et al. (49) postulated that the histidine kinase, AdeS, like most histidine kinase proteins, had the capacity to both phosphorylate (kinase activity) and dephosphorylate (phosphorylase activity) the AdeR transcriptional regulator. However net kinase activity typically resulted in activation of the transcriptional regulator.

Mutation of either component resulted in the loss of kinase activity, loss of activation of the transport system, and thus increased susceptibility. Point mutations in the specific receiver domains resulted in loss of phosphorylase activity, constitutive kinase activity, and increased resistance. However, the regulatory situation is slightly different for 30-84, where mutation in *rpeA* causes up-regulation of the *acrAB* operon and mutation in *rpeB* or both genes causes down regulation of the *acrAB* operon (unpublished data). It was postulated that phosphorylated RpeB promotes the expression of *pip* and the quorum sensing genes *phzI/phzR* (4). RpeA, a transmembrane protein is thought to modulate the ratio of phosphorylated and non-phosphorylated RpeB in response to environmental conditions, thus modulating phenazine biosynthesis in response to those same environmental states. According to Wang et al. (4), “the level of active, phosphorylate RpeB is possibly controlled by RpeA or other small phosphor-donors such as acetyl phosphate.” As expected loss of the transcriptional activator RpeB results in loss of phenazine biosynthesis and transport. Interestingly, loss of RpeA results in the loss of the ability to dephosphorylate RpeB and thus loss of the ability to modulate the expression of phenazine biosynthesis and transport in response to environmental conditions.

The present results suggest that the regulation of the biosynthesis and transport of phenazines should be coordinately regulated (Fig 17). Phenazine biosynthesis is regulated directly by the quorum sensing system PhzI/PhzR. These genes are, in turn, regulated in a hierarchical way by Pip and RpeB, respectively, although the need for this

hierarchical regulation is not well understood; it may be related to observations that sub-inhibitory levels of phenazine may serve as a signal that promotes further biosynthesis whereas higher levels may promote a stress response (50). Based on the homology between Pip and AcrR, it was postulated previously that analogous to AcrR, Pip might regulate the expression of genes encoding an efflux pump (43). In the present study it was shown that the *acrAB* operon is transcriptionally regulated by RpeB, but is not controlled by Pip or PhzR. Thus, RpeB links both phenazine biosynthesis and transport. It is interesting to consider why such an elaborate mechanism for linking both functions exists, e.g. why not transcriptionally regulate both the *phz* biosynthetic operon and the *acrAB* operon by quorum sensing? The hypothesis is that AcrA/AcrB function in the transport of a diversity of antimicrobials of which phenazine is just one example. Thus direct regulation of the AcrAB efflux system occurs via a membrane bound sensor kinase capable of modulating transcriptional regulator activity in response to stress. Furthermore, rapid response to environmental stress is likely facilitated by this type of regulation because RpeA has both kinase and phosphorylase activity, e.g. control is modulated by the ratio of active to non-active transcriptional regulator (e.g. phosphorylated to dephosphorylated RpeB) rather than by an increase in the amount of the regulator (e.g. via transcription and translation). A model describing this proposed interconnected regulatory system for the control of phenazine biosynthesis and transport is shown in Figure 17.

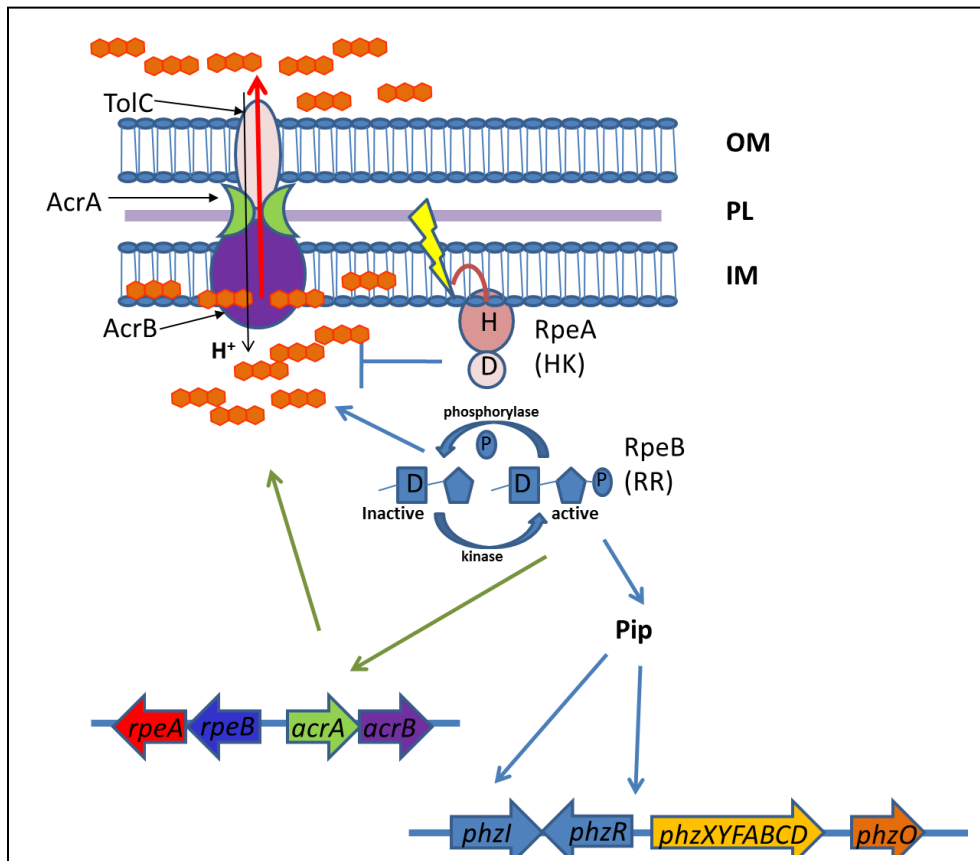


Figure 17. Proposed model of RpeA/RpeB regulation of phenazine biosynthesis and transport. The phenazine locus contains four operons: the phenazine biosynthetic genes *phzXYFABCD* encode enzymes for synthesis of PCA, *phzO* encodes an enzyme responsible for producing the hydrolylated phenazines 2OHPCA and 2OHPHZ, *phzI* encodes a signal synthase responsible for the production of an AHL signal, and *phzR* encodes a transcriptional regulator that dimerizes with the AHL signal to recruit polymerase to the *phzXYFABCD* operon and initiate expression. The quorum sensing system PhzI/PhzR is hierarchically regulated by Pip, a transcriptional regulator, and RpeB. RpeA/RpeB compose a two component signal transduction system. RpeB is a transcriptional regulator that is active when phosphorylated. RpeA is a membrane bound sensor kinase that is capable of kinase and phosphorylase activity in response to environmental stimuli. Active phosphorylated RpeB positively regulates the phenazine biosynthetic regulatory pathway. It also positively regulates the transcription of *acrA* and *acrB* which are co-transcribed. *acrB* encodes an inner membrane protein which together with AcrA (a periplasmically located protein) and TolC (an outer membrane protein), forms a channel that functions like RND type proton antiporters (25). Transported substrates are believed to bind to the inner membrane and then the binding site on AcrB. It is believed that one substrate molecule is transported for every proton.

The study of phenazines is important due to their role in effective biological control activity. The present study was initiated with the hypothesis that the AcrAB efflux system was a phenazine transporter, and if so, it should be coordinately regulated with biosynthesis in order to protect the cells from the toxic effects of phenazines. However, I discovered it is capable of transporting diverse antimicrobials. These results now suggest that the coordination also may be a defense strategy against other microbes that produce antimicrobials. In this scenario, the transport genes that provide resistance are coordinately expressed with the production of phenazines. This might be a competitive mechanism that may contribute to competence in the plant rhizosphere. However this hypothesis needs further testing.

Ongoing work is focused on generating a 30-84 AcrA mutant. The first step will be to complement the mutant to demonstrate that it is correctly constructed. Once a 30-84 AcrA mutant is successfully constructed, it will be used to determine the impact of loss of the AcrAB efflux system on bacterial fitness. Plans include comparing wild type and mutant fitness (measured using bacterial growth curves) in response to various antimicrobials or stresses. It is also important to examine fitness on plants. Experiments would include introducing the wild type and mutant individually and in mixture in sterile and natural soil (e.g. with natural rhizosphere microbes). Finally, it is possible that the AcrAB efflux system could be involved in the transport of plant signals or plant antimicrobials and so plant-microbe interactions might be altered. The role of plant signals might be tested by comparing wild type and mutant chemotactic behavior and the

role of antimicrobials could be tested with growth curve assays or root colonization assays.

6. CONCLUSIONS

This study was initiated based on speculation that *P. chlororaphis* 30-84 genes annotated as homologs to *E. coli* *acrA/acrB* genes, might encode an AcrAB-TolC RND efflux system. My results demonstrate that constitutive expression of *acrA/acrB* conferred acriflavine resistance to *E. coli* and increased acriflavine resistance in 30-84, consistent with these genes encoding an AcrAB-TolC type efflux system.

I tested the hypothesis that similar to the *E. coli* AcrAB-TolC RND efflux system, the 30-84 system would transport a diversity of substrates. Compared to wild type, constitutive overexpression of the *acrA/acrB* genes in 30-84 also increased resistance to some antimicrobials but not others, demonstrating that the 30-84 system could transport multiple substrates, but has limited substrate affinity or transport efficiency.

I tested the hypothesis that this efflux system would be capable of transporting phenazines based on the similarity in the molecular structure of acriflavin and phenazine. Compared to wild type, constitutive expression of *acrA/acrB* facilitated the growth of *E. coli* in the presence of ~300-800 μg of total phenazines extracted from 30-84, demonstrating the function of AcrA and AcrB in phenazine transport.

I tested the hypothesis that if intercellular concentration of phenazine served as a signal to increase phenazine biosynthesis, constitutive expression of the efflux system might result in greater phenazine production and biological control activity. Compared

to wild type, constitutive expression of *acrA/acrB* induced earlier and greater production and diffusion of phenazines and significantly affected the capacity of 30-84 to inhibit fungi growth.

I tested the hypothesis that the RpeA/RpeB two component signal transduction system might be involved in the coordinate regulation of phenazine biosynthesis and transport. The results showed that RpeB controls both phenazine biosynthesis and transport. However the two regulatory pathways are not linked by any regulatory intermediates. Constitutive expression of the *acrA/acrB* genes *in trans* in a *rpeB* mutant did not rescue phenazine production unless other traits, regulated by RpeA/RpeB, that involved the regulation of phenazine biosynthesis such as quorum sensing were also restored. Constitutive expression of *pip* *in trans* did not restore acriflavine resistance and Pip and PhzR mutants were both acriflavine resistant.

Finally, the present results outline a path for future studies that may elucidate the role of the 30-84 AcrAB efflux system in biological control activity, bacterial fitness, rhizosphere competence and competition, and plant-microbial interactions.

REFERENCES

1. **Weller DM, Cook RJ.** 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. *Phytopathology*. **73**: 463-469. doi:10.1094/Phyto-73-463.
2. **Zhang Z, Pierson LS.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol*. **67**: 4305-4315. doi:10.1128/AEM.67.9.4305-4315.2001.
3. **Pierson LS, Pierson EA.** 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl Microbiol Biotechnol*. **86**: 1659-1670. doi: 10.1007/s00253-010-2509-3.
4. **Wang D, Yu JM, Pierson LS, Pierson EA.** 2012. Differential regulation of phenazine biosynthesis by RpeA and RpeB in *Pseudomonas chlororaphis* 30-84. *Microbiology*. **158**: 1745-1757. doi: 10.1099/mic.0.059352-0.
5. **Chancey ST, Wood DW, Pierson EA, Pierson LS.** 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl Environ Microbiol*. **68**: 3308-3314. doi:10.1128/AEM.68.7.3308-3314.2002.
6. **Pierson LS, Keppenne VD, Wood DW.** 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J Bacteriol*. **176**: 3966-3974.
7. **Price-Whelan A, Dietrich LE P, Newman DK.** 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol*. **2**: 71-78. doi:10.1038/nchembio764.
8. **Britigan BE, Roeder TL, Rasmussen GT, Shasby DM, McCormick ML, Cox CD.** 1992. Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas*-associated tissue injury. *J. Clin. Invest*. **90**: 2187-2196. doi: 10.1172/JCI116104.
9. **Van Bambeke F, Balzi E, Tulkens PM.** 2000. Antibiotic efflux pumps. *Biochem*

- Pharmacol. **60**: 457-470. doi: 10.1016/S0006-2952(00)00291-4.
10. **Whistler CA, Pierson LS.** 2003. Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. J. Bacteriol. **185**: 3718-3725. doi: 10.1128/JB.185.13.3718-3725.2003.
 11. **Nakamura H.** 1965. Gene-controlled resistance to acriflavine and other basic dyes in *Escherichia coli*. J. Bacteriol. **90**: 8-14.
 12. **Nikaido H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science. **264**: 382-388. doi: 10.1126/science.8153625.
 13. **Nikaido H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178**: 5853.
 14. **Miller WG, Leveau JHJ, Lindow SE.** 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact. **13**: 1243-1250. doi: 10.1094/MPMI.2000.13.11.1243
 15. **Lugtenberg B, Kamilova F.** 2009. Plant-growth-promoting rhizobacteria. Annu Rev Microbiol. **63**: 541-556. doi: 10.1146/annurev.micro.62.081307.162918.
 16. **Weller DM.** 2007. Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology. **97**: 250-256. doi: 10.1094/PHYTO-97-2-0250.
 17. **Raaijmakers JM, Vlami M, De Souza JT.** 2002. Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek. **81**: 537-547. doi: 10.1023/A:1020501420831.
 18. **Thomashow LS.** 1996. Biological control of plant root pathogens. Curr Opin Biotechnol. **3**: 343-347. doi: 10.1016/S0958-1669(96)80042-5.
 19. **Weller DM.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu Rev Phytopathol. **6**: 379-407. doi: 10.1146/annurev.py.26.090188.002115.
 20. **Gibson J, Sood A, Hogan DA.** 2009. *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. Appl Environ Microbiol. **75**: 504-513. doi: 10.1128/AEM.01037-08.
 21. **Reszka KJ, Xiong Y, Sallans L, Pasula R, Olakanmi O, Hassett DJ, Britigan BE.**

2012. Inactivation of the potent *Pseudomonas aeruginosa* cytotoxin pyocyanin by airway peroxidases and nitrite. *Am J Physiol Lung Cell Mol Physiol*. **302**: 1044-1056. doi: 10.1152/ajplung.00172.2011.
22. **Thomashow LS, Weller DM, Bonsall RF, Pierson LS.** 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Appl Environ Microbiol*. **56**: 908-912.
23. **Chin-A-Woeng TFC, Bloemberg GV, Mulders IHM, Dekkers LC, Lugtenberg BJ.** 2000. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant Microbe Interact*. **13**: 1340-1345. doi: 10.1094/MPMI.2000.13.12.1340
24. **Lewis K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. *Trends Biochem Sci*. **19**: 119-123. doi: 10.1016/0968-0004(94)90204-6.
25. **Piddock LJV.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev*. **19**: 382-402. doi: 10.1128/CMR.19.2.382-402.2006
26. **Akama H, Kanemaki M, Yoshimura M, Tsukihara T, Kashiwagi T, Yoneyama H, Narita S, Nakagawa A, Nakae T.** 2004. Crystal structure of the drug discharge outer membrane protein, OprM, of *Pseudomonas aeruginosa* dual modes of membrane anchoring and occluded cavity end. *J Biol Chem*. **279**: 52816-52819. doi: 10.1074/jbc.C400445200
27. **Akama H, Matsuura T, Kashiwagi S, Yoneyama H, Narita S, Tsukihara T, Nakagawa A, Nakae T.** 2004. Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. *J Biol Chem*. **279**: 25939-25942. doi: 10.1074/jbc.C400164200
28. **Ma D, Cook DN, Hearst JE, Nikaido H.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. *Trends Microbiol*. **2**: 489-493. doi: 10.1016/0966-842X(94)90654-8.
29. **Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Mol Microbio*. **19**: 101-112. doi: 10.1046/j.1365-2958.1996.357881.x

30. **Zgurskaya HI, Nikaido H.** 1999. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc Natl Acad Sci.* **96**: 7190-7195. doi: 10.1073/pnas.96.13.7190
31. **Fralick JA.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *J Bacteriol.* **178**: 5803-5805.
32. **Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J Bacteriol.* **175**: 6299-6313.
33. **Skarstad K, Thöny B, Hwang D S, Kornberg A.** 1993. A novel binding protein of the origin of the *Escherichia coli* chromosome. *J Biol Chem.* **268**: 5365-5370.
34. **Cohen S P, Hächler H, Levy S B.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *J Bacteriol.* **175**: 1484-1492.
35. **Magnet S, Courvalin P, Lambert T.** 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother.* **45**: 3375-3380. doi: 10.1128/AAC.45.12.3375-3380.2001.
36. **Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* **44**: 3322-3327. doi:10.1128/AAC.44.12.3322-3327.2000
37. **Poole K, Krebs K, McNally C, Neshat S.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol.* **175**: 7363-7372.
38. **Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP.** 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother.* **45**: 428-432. doi: 10.1128/AAC.45.2.428-432.2001.
39. **Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat**

- S, Yamagishi J, Li XZ, Nishino T.** 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol Microbio. **21**: 713-725. doi: 10.1046/j.1365-2958.1996.281397.x.
40. **Maseda H, Uwate M, Nakae T.** 2010. Transcriptional regulation of the *mexEF-oprN* multidrug efflux pump operon by MexT and an unidentified repressor in *nfxC*-type mutant of *Pseudomonas aeruginosa*. FEMS Microbiol Lett. **311**: 36-43. doi: 10.1111/j.1574-6968.2010.02063.x.
41. **Uwate M, Ichise Y, Shirai A, Omasa T, Nakae T, Maseda H.** 2013. Two routes of MexS-MexT-mediated regulation of MexEF-OprN and MexAB-OprM efflux pump expression in *Pseudomonas aeruginosa*. Microbiol Immunol. **57**: 263-272. doi: 10.1111/1348-0421.12032.
42. **Matsuo Y, Eda S, Gotoh N, Yoshihara E, Nakae T.** 2004. MexZ-mediated regulation of *mexXY* multidrug efflux pump expression in *Pseudomonas aeruginosa* by binding on the *mexZ-mexX* intergenic DNA. FEMS Microbiol Lett. **238**: 23-28. doi: 10.1016/j.femsle.2004.07.010.
43. **Girard G, Barends S, Rigali S, Van Rij ET, Lugtenberg BJ, Bloemberg GV.** 2006. Pip, a novel activator of phenazine biosynthesis in *Pseudomonas chlororaphis* PCL1391. J Bacteriol. **188**: 8283-8293. doi: 10.1128/JB.00893-06.
44. **Pierson LS, Gaffney T, Lam S, Gong F.** 1995. Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30–84. FEMS Microbiol Lett. **134**: 299-307. doi: 10.1111/j.1574-6968.1995.tb07954.x.
45. **Mavrodi DV, Blankenfeldt W, Thomashow LS.** 2006. Phenazine Compounds in Fluorescent *Pseudomonas* Spp. Biosynthesis and Regulation*. Annu Rev Phytopathol. **44**: 417-445. doi: 10.1146/annurev.phyto.44.013106.145710.
46. **Lesic B, Rahme LG.** 2008. Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa*. BMC Mol Biol. **9**: 20. doi: 10.1186/1471-2199-9-20.
47. **Aono R, Tsukagoshi N, Yamamoto M.** 1998. Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12. J Bacteriol. **180**: 938-944.

48. **Piddock LJV.** 2006. Multidrug-resistance efflux pumps? not just for resistance . Nat Rev Microbiol. 4: 629-636. doi: 10.1038/nrmicro1464.
49. **Marchand I, Damier-Piolle L, Courvalin P, Lambert T.** 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob Agents Chemother. **48**: 3298-3304. doi: 10.1128/AAC.48.9.3298-3304.2004.
50. **Girard G, Rigali S.** 2011. Role of the phenazine-inducing protein Pip in stress resistance of *Pseudomonas chlororaphis*. Microbiology. **157**: 398-407. doi: 0.1099/mic.0.043075-0.