Bacterial Multidrug Efflux Pumps Serve Other Functions

This clinically important form of drug resistance is merely "incidental" to the other activities of these efflux pumps

Keith Poole

Antibiotic efflux is one of several resistance mechanisms that are found in bacterial pathogens. Members of the resistance-nodulation-division (RND) family of antimicrobial exporters are particular noteworthy owing to the breadth of antimicrobials that these multidrug pumps accommodate. Found generally in gram-negative bacteria, where they are highly conserved, RND family members are almost always chromosomally encoded. RND pumps typically function as tripartite systems, with the cytoplasmic membrane (CM) RND component working with an outer membrane-spanning channel-forming protein, the outer membrane factor, and a periplasmic component, the membrane fusion protein, that links the membrane components (Fig. 1).

Summary

- The resistance-nodulation-division (RND) family of antimicrobial efflux pumps is commonly found in gram-negative bacteria and almost always chromosomally encoded.
- Multiple RND pumps occur in the same organism where they often exhibit complex patterns of regulation that is independent of antibiotic exposure, consistent with antibiotics not being their natural substrates.
- Ribosome disruption, the presence of reactive oxygen species, bile salts, other membrane-damaging agents, or other stresses can trigger expression of genes encoding RND efflux pumps in some microbes.
- RND pumps influence pathogenesis and can sometimes function as virulence determinants.

Although RND pumps accommodate antibiotics and thus undermine the clinical effectiveness of these agents, antibiotics typically do not induce RND-type efflux systems. Instead, other processes that are unrelated to antibiotics influence RND pump expression, revealing complex regulatory patterns that are incompatible with these pumps playing a simple role in drug efflux and resistance. In general, however, the natural substrates of these efflux systems remain unidentified.

Challenges in Defining Natural Functions of Efflux Pumps

Because so much attention has focused on the role of RND family and similar efflux systems in discharging antibiotics from bacterial cells, researchers at first uncovered few insights into the natural functions of these systems. Although researchers have, for instance, isolated many mutants that either lack or show elevated pump production, their focus on drug resistance as an indirect measure of efflux gene expression has usually yielded regulatory mutations that promote constitutive pump expression. These mutations tend to occur in local regulatory genes, providing little to no insight into the environmental or cellular circumstances that recruit these efflux systems. Possibly, under circumstances where the natural efflux substrate is produced and stimulates efflux gene expression, preferential export of the natural substrate might preclude antibiotic export. As such, mutations that stimulate efflux gene expression via production of the natural substrate would not yield antibiotic

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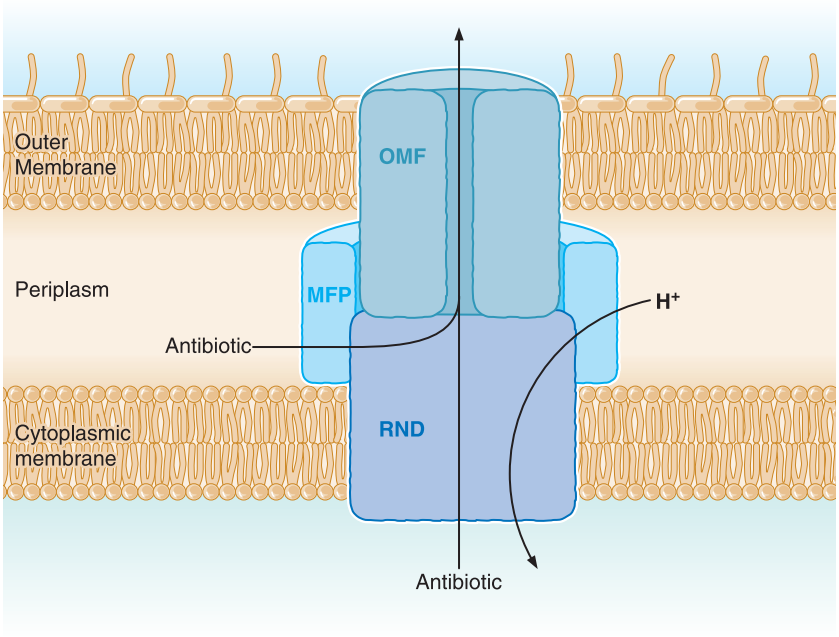
FIGURE 1



Model of RND pump with antibiotic export (from periplasm or cytoplasm) coupled to proton import. The resistance-nodulation-division (RND), membrane fusion protein (MFP) and outer membrane factor (OMF) components are highlighted.

resistance, and efflux screens based on antibiotic resistance would miss such mutants. Efflux screens based on gene expression may prove more useful for elucidating the natural function of these systems.

Perhaps the earliest sign of a nonantibiotic, natural substrate for an RND family multidrug pump came in a study by Robert Helling and colleagues at the University of Michigan in Ann Arbor. These researchers discovered that certain mutants of *Escherichia coli* with defects in nucleotide or amino acid biosynthetic pathways show elevated expression of the *acrAB* genes encoding the AcrAB-TolC components of the major multidrug efflux system of this organism. Helling and his collaborators proposed that metabolites that accumulated because of pathway blockages caused by these mutations are substrates of the efflux pump, which monitors metabolite levels and prevents them rising to toxic levels.

Antibiotic-Independent Phenotypes of Pump-Producing *P. aeruginosa* mutants

There are clear indications that many RND family multidrug efflux systems are not intended as determinants of antimicrobial resistance, such as the observed pleiotropic effects of RND pump loss or overproduction on bacteria independent of antibiotics. For example, DNA microarray studies carried out in collaboration with Charles Dean of Novartis Institutes for Biomedical Research (NIBR), Cambridge, Mass., reveal a myriad of changes in gene expression in mutants hyperexpressing or lacking the MexAB-OprM efflux system in *Pseudomonas aeruginosa*. These effects cannot be related to the export of antimicrobials and so must reflect the impact of pump activity on cell physiology, presumably as a result of the export of one or more cell-associated substrates.

Further support for RND pumps as other than resistance determinants comes from the observed in vivo selection of pump-producing mutants in the absence of antibiotics. Mex pump-producing *P. aeruginosa* mutants have, for example, been recovered from experimental infections of mice not exposed to antibiotics. The *mtrCDE* genes encoding a multidrug efflux system in *Neisseria meningitidis* are similarly up-regulated during experimental intracellular infection of human cells—again, a response to a nonantibiotic stimulus.

Efflux Pumps as Components of Bacterial Stress Responses Affecting Translation

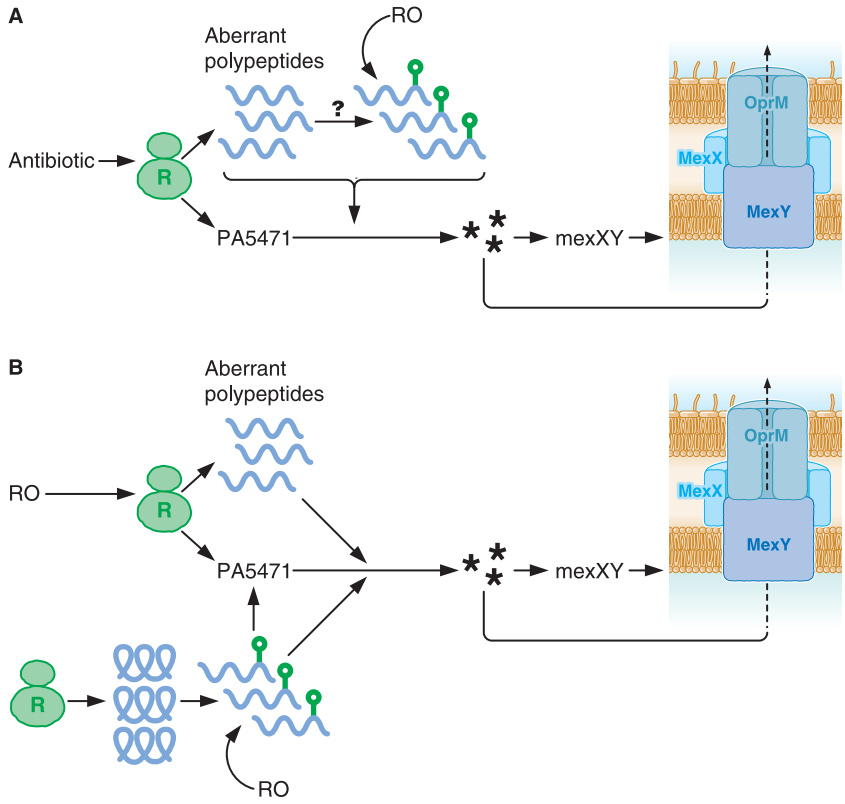
The MexXY-OprM multidrug efflux system is a major determinant of aminoglycoside resistance in clinical strains of *P. aeruginosa*, particularly those isolated from the lungs of chronically infected individuals with cystic fibrosis (CF). A unique feature of this efflux system is its induction by many of the antimicrobials that it exports. However, only those antibiotics that interact with or disrupt ribosomes stimulate MexXY production, suggesting that ribosome stress and the consequences of that stress are signals for MexXY recruitment.

MexXY production in response to ribosome-disrupting antibiotics is dependent upon the product of a gene of unknown function, PA5471, which is also inducible by ribosome-disrupting agents. PA5471 expression off a plas-

mid is sufficient to drive *mexXY* expression in the absence of antibiotic exposure, indicating that PA5471 mediates antibiotic stimulation of *mexXY*. Maximal antibiotic-inducible *mexXY* expression does, however, require both antibiotics and PA5471.

Ribosome-targeting antibiotics compromise the translation of messenger RNA into proteins, leading to misreading of those messages and/or their premature termination to produce aberrant and potentially disruptive polypeptides. These polypeptides may be the actual signals for PA5471/*mexXY* induction (Fig. 2A), with PA5471/MexXY functioning in their removal. Mutations in the *fmt* gene encoding the enzyme responsible for adding formyl groups to methione residues that initiate translation also show high-level expression of PA5471 and *mexXY*, according to Dean and colleagues at NIBR in Cambridge, Mass. Here again, defects in protein translation are linked to MexXY recruitment in *P. aeruginosa*.

FIGURE 2



Proposed model for antibiotic and reactive oxygen (RO) simulation of *mexXY* efflux gene expression in *P. aeruginosa*. (A) Ribosome (R) disruption by antibiotics that target this structure causes mistranslation and production of aberrant polypeptides as well as induction of PA5471. These aberrant polypeptides will be prone to oxidative modification by RO that is produced naturally during aerobic growth of bacteria and apparently flags them for destruction and removal, their accumulation likely to compromise cell health. The aberrant polypeptides and/or their oxidatively modified counterparts (w), in conjunction with PA5471 (possibly as substrates for PA5471 whose activity remains unidentified) generate the natural substrate (*) of MexXY-OprM, which induces *mexXY* expression and provides for MexXY-OprM-mediated export of *. (B) RO generated endogenously as a result of aerobic growth or produced exogenously by immune effector cells (i.e., during infection) may interfere with proper ribosome function (due to oxidative damage of ribosomal components), yielding aberrant polypeptides, or damage normal polypeptides following synthesis by the ribosome. In the former instance, RO also induces PA5471 expression that, in conjunction with the aberrant polypeptides yields the natural MexXY-OprM substrate (*) as in (A). Oxidatively damaged polypeptides (w) might themselves promote PA5471 expression and together with PA5471 yield the natural MexXY-OprM substrate for export.

Efflux Pumps as Components of Oxidative and Nitrosative Stress Responses

Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are produced naturally during aerobic growth and denitrification (reduction of nitrate to ammonia or elemental nitrogen), respectively, and by host immune cells during infection. Referred to generally as reactive oxygen (RO) (H_2O_2) and reactive nitrogen (RN) (NO), these compounds have significant antimicrobial activity, exerting their effects by damaging several types of bacterial macromolecules, including proteins, lipids, and DNA.

DNA microarray studies reveal that the MexEF-OprN efflux system of *P. aeruginosa* is up-regulated in response to both of these "stressors." Moreover, mutants lacking an oxidoreductase (MexS) that may play a role in resistance to RO and/or RN also hyperexpress MexEF-OprN. One possibility, then, is that this efflux system exports cellular constituents damaged by RO/RN.

Exposure to H_2O_2 also promotes expression of the PA5471 gene implicated in drug-inducible *mexXY* expression. MexXY production, too, is enhanced upon exposure to this RO compound, and like antibiotic stimulation of efflux gene expression, this is PA5471-dependent. Possibly, then, *mexXY* induction by RO is a response to RO-mediated damage. In linking RO and antibiotic inducibility of PA5471 and *mexXY*, it is

worth noting that aberrant polypeptides such as those produced during exposure to ribosome-disrupting antimicrobials are prone to cell-mediated oxidation that targets these polypeptides for destruction and/or removal. Sam Dukan and colleagues at Göteborg University in Göteborg, Sweden, have shown, for example, that disruption of ribosomes via mutation or treatment with the aminoglycoside antibiotic streptomycin caused *E. coli* cells to produce aberrant proteins that were prone to oxidation. Exposure of bacterial cells to oxidative stress, in the form of certain antiseptics or human immune effector cells that release RO compounds, will also produce oxidation of bacterial proteins and polypeptides, possibly targeting them for removal via the same mechanism that handles antibiotic-induced aberrant polypeptides.

The common recruitment of PA5471 and MexXY by ribosome-targeting antibiotics and RO (Fig. 2B) is consistent with PA5471/MexXY contributing to a natural bacterial process for removing abnormal proteins that accumulate in response to environmental stresses. Abnormal polypeptides and proteins also tend to accumulate in nongrowing senescent bacteria, owing to reduced translational fidelity in such organisms, and these proteins are also prone to cell-mediated oxidative damage and disposal. Whether this involves PA5471 and MexXY remains to be seen.

Efflux Pumps as Components of Membrane Stress Responses

Bile salts are membrane-perturbing, detergent-like molecules that are abundant in the guts of mammals and birds. Gut commensal and pathogenic bacteria such as *E. coli*, *Salmonella enterica* serovar Typhimurium, *Campylobacter jejuni*, and *Vibrio cholerae* resist the potentially lethal action of such bile salts, relying in part on the same RND family multidrug pumps that confer antibiotic resistance (see table in online version of this article). These pumps are induced by bile salts, consistent with bile resistance being their primary function, but their observed export of bile indicates that they deal only with the source of the stress, not its consequences.

A variety of additional membrane-damaging agents, including antiseptics, detergents, solvents, and cationic peptides, can induce the MexCD-OprJ multidrug efflux system in *P. aeruginosa*. This induction depends on the envelope stress sigma factor AlgU, a homologue of the well-characterized RpoE sigma factor of *E. coli* that coordinates the expression of numerous genes in response to envelope stress in this organism. MexCD-OprJ may function in the export of membrane constituents liberated or damaged as a consequence of the action of these agents or may export lipid constituents that need to be replaced as the membrane is modified to adapt to the presence of these MDAs (Fig. 3). Alternatively, or additionally, MexCD-OprJ may be involved in the normal process of membrane turnover, not simply as part of an adaptive response to environmental stresses but to deal with membrane damage that may occur naturally during bacterial growth.

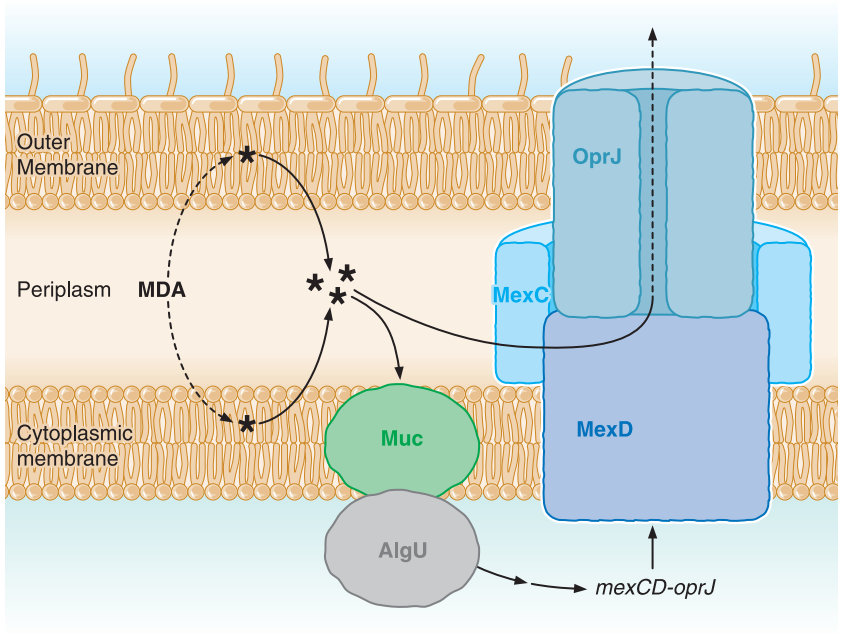
Efflux Pumps and Bacterial Cell-Cell Communication

Extracellular signaling molecules called autoinducers (AIs) mediate communication within bacterial populations, coordinating gene expression within a population in response to environmental factors and cell density. The term quorum sensing (QS) is used to describe this form of cell density-dependent gene expression, reflecting the need for sufficient bacteria (i.e., a quorum) to trigger gene expression. This is achieved by AIs that are produced by a population reaching a critical threshold concentration and subsequently re-entering cells, where they stimulate target gene expression. The first observations that AIs were substrates for RND family multidrug pumps were made in studies of *P. aeruginosa*, where the MexAB-OprM efflux system implicated in intrinsic and acquired multidrug resistance was shown to accommodate AIs called N-acyl homoserine lactones (HSLs). RND pumps that export HSLs have also been discovered in *Burkholderia pseudomallei*, where the BpeAB-OprB pump that is implicated in intrinsic multidrug resistance is required for HSL secretion.

The RND pump MexHI-OpmD is also linked to AI production in *P. aeruginosa*—mutants lacking this pump show defects in AI production—although apparently not because this pump exports de novo synthesized AIs. Indeed, export of de novo-synthesized AI may not be a function of the *P. aeruginosa* Mex pumps since they can also pump already-exported AIs, effec-

tively preventing their re-entry into cells and, so, blocking their subsequent stimulation of AI- or QS-dependent genes. For example, mutants of *P. aeruginosa* that overproduce MexAB-OprM or a second RND pump, MexEF-OprN, produce less than the usual amount of AI in part because they exclude extracellular AIs, the genes for AI synthesis being AI dependent. In this regard, AI export is unlikely to be an intended function of the RND pumps but rather a secondary effect of broad-based efflux activity that reflects their natural but as yet undefined function.

FIGURE 3



Proposed model for the AlgU-dependent induction of the *P. aeruginosa mexCD-oprJ* efflux operon by membrane-damaging agents (MDA). Membrane disruption by MDAs such as detergents, ethanol, solvents, biocides, and cationic antimicrobial peptides leads to release and/or damage of outer membrane and/or cytoplasmic membrane lipid constituents (*) whose replacement is essential for proper membrane function. These released or damaged constituents signal the CM-associated Muc proteins that control the activity of AlgU, a sigma factor that positively regulates (directly or indirectly) *mexCD-oprJ* expression. MexCD-OprJ is then responsible for export of the damaged/released membrane constituents.

Efflux Pumps as Virulence Determinants

For *P. aeruginosa* at least, QS and efflux are linked. Thus, with QS having an important role in expression of virulence determinants, many of which are AI-dependent, it is hardly surprising that RND family pumps are linked to pathogenesis in this microorganism (see table online). For example, mutants of *P. aeruginosa* hyperexpressing MexAB-OprM or MexEF-OprM, both of which show defects in QS (reduced AI synthesis), are attenuated for virulence. *P. aeruginosa* mutants hyperexpressing MexCD-OprJ or MexEF-OprN also show reduced expression of type III secretion systems associated with export of virulence factors implicated in pathogenesis, although the nature of the link between efflux and type III secretion remains poorly defined. Mutants of *P. aeruginosa* lacking MexHI-OpmD, which are similarly defective with respect to QS production of AIs, are also attenuated for virulence.

While a mutant lacking MexAB-OprM shows reduced invasion of kidney cell monolayers and is attenuated for virulence in animals, the invasion defect is not reversed by adding HSL, suggesting that the impact of MexAB-OprM loss on virulence in this instance is not explainable by QS. Overproduction of an RND-type multidrug efflux system, SmeDEF, in *Stenotrophomonas maltophilia* is also linked to reduced virulence.

Given the importance of bile efflux/resistance for bacterial survival in the gut, bile-accommodating RND pump homologues of the AcrAB multidrug efflux system of *E. col* have also been implicated in the pathogenesis of organisms such as *S. enterica* and *C. jejuni*. Pump-deficient mutants of these organisms, for example, are compromised for gut colonization and/or persistence. Though they are bile-inducible and expressed in vivo in animal models and infected patients, the AcrAB-like pumps of *V. cholerae* do not appear to be essential for virulence.

The MtrCDE multidrug efflux system of *N. meningitidis* has also been linked to virulence by virtue of its role in protection against host antimicrobial compounds. Specifically, the pump is linked to resistance to cationic antimicrobial peptides (CAPs), components of innate immunity, although it is not clear that this is a result of CAP export by this pump.

In certain plant pathogenic organisms such as *Agrobacterium tumefaciens*, *Erwinia* sp., and *Ralstonia solanacearum*, RND family efflux sys-

Table 1. RND type multidrug efflux systems of *P. aeruginosa*

Efflux system	Regulator(s)[a]	Substrate(s)
MexAB-OprM	MexR, NalC, NalD	β-lactams, fluoroquinolones, tetracycline, macrolides, chloramphenicol, biocides
MexCD-OprJ	NfxB	β-lactams, fluoroquinolones, tetracycline, macrolides, chloramphenicol, biocides
MexEF-OprN	MexT	Fluoroquinolones, chloramphenicol, biocides
MexXY-OprM	MexZ	Aminoglycosides, β-lactams, fluoroquinolones, tetracycline, macrolides, chloramphenicol
MexJK-OprM/OpmH[a]	MexL	Fluoroquinolones, tetracycline, macrolides, biocides
MexHI-OpmD	—[b]	Fluoroquinolones
MexVW-OprM	—	Fluoroquinolones, tetracycline, macrolides, chloramphenicol
MexPQ-OpmE	—	Fluoroquinolones, tetracycline, macrolides, chloramphenicol
MexMN-OprM	—	Chloramphenicol

[a]MexJK can operate with OprM (for antibiotics) or OpmH (for the biocide triclosan) as the OMF component. [b]None identified. No putative regulatory genes linked to these efflux genes in the chromosome.

tem that accommodate antibiotics in vitro also play a crucial role in virulence by protecting cells from isoflavanoid plant antimicrobial defense compounds. These isoflavanoids typically induce efflux gene expression during infection, and mutant bacteria lacking these pumps show reduced or impaired virulence. Moreover, phenolics such as salicylic acid that are produced by infected plants to stimulate plant antibacterial defense mechanisms also upregulate the RND type multidrug efflux systems that protect *Erwinia chrysanthemi* from plant antimicrobials.

In at least two instances, RND pumps function directly in pathogenesis, being portals for export of virulence factors themselves. RND-pump encoding genes are linked to an operon involved in the synthesis of the phytotoxin toxoflavin in *Burkholderia glumae* and are necessary for virulence, which is consistent with a role in toxoflavin export.

In *Pseudomonas syringae,* too, an RND efflux operon, *pseABC,* whose expression in *E. coli* promotes antibiotic resistance, is linked to export of a plant-damaging phytotoxin that is produced during infection. PseABC mutants, which are less than half as virulent as wild-type cells, remain equally resistant to antibiotics. One explanation is that preferential export of phytotoxin in *P. syringae* precludes export of antibiotics. Similarly, the MbsA transporter implicated in lipid A/LPS export in *E. coli* accommodates and provides resistance to antibiotics such as erythromycin only when expressed in an LPS-free organism, possibly because the drugs face no competition for efflux via MsbA in such organisms.

Multiple RND-Type Multidrug Efflux Systems Occur in Individual Organisms

Many gram-negative bacteria possess more than one RND pump family member, each of which may export many of the same antimicrobials. For instance, *P. aeruginosa* has more than 10 RND family transporters, of which 9 accommodate multiple, often the same, antibiotics (Table 1). These efflux systems also appear to be independently regulated in *P. aeruginosa* (Table 1). As with the redundancy with respect to antimicrobial substrate specificity, this is inconsistent with an intended function in antibiotic export.

The complexity of RND pump regulation in *P. aeruginosa* further belies a simple antibiotic resistance function. The MexAB-OprM pump, for example, is expressed constitutively at moderate levels in culture, shows growth phase and QS control, and is regulated by at least three repressors, MexR, NalC, and NalD, that control *mexAB-oprM* expression.

Clinical Significance of Multidrug Efflux Systems

Despite drug efflux not being the natural function of RND pumps, these pumps enable many pathogens to resist antimicrobial drugs and thus compromise treatment of infectious diseases. As such, elucidating the "natural" functions of these efflux systems, how they are regulated and what environmental circumstances promote their expression will be critical in predicting when and where in a clinical setting they might be recruited in pathogenic bacteria and how they compromise antimicrobial chemotherapy.

The demonstration, for example, that RO stimulates expression of PA5471 and *mexXY*, and that MexXY is an important determinant of

aminoglycoside resistance in *P. aeruginosa* is significant since environmental RO may promote MexXY-OprM-mediated aminoglycoside resistance. Indeed, this organism encounters substantial RO in the lungs of CF patients, and MexXY-mediated aminoglycoside resistance is disproportionately represented among strains of *P. aeruginosa* recovered from CF lung infections. Moreover, Eric Smith and colleagues at the University of Washington have shown that most *P. aeruginosa* strains recovered from CF lung infections harbor mutations in the *mexZ* gene encoding a repressor of *mexXY* and, so, are likely to express this efflux system. In this instance, environmental conditions at the site of infection may be promoting efflux gene expression, rendering the pathogen resistant to antimicrobials.

SUGGESTED READING

Brown, D. G., J. K. Swanson, and C. Allen. 2007. Two host-induced *Ralstonia solanacearum* genes, *acrA* and *dinF*, encode multidrug efflux pumps and contribute to bacterial wilt virulence. Appl. Environ. Microbiol. **73**:2777–2786.

Chan, Y. Y., H. S. Bian, T. M. Tan, M. E. Mattmann, G. D. Geske, J. Igarashi, T. Hatano, H. Suga, H. E. Blackwell, and K. L. Chua. 2007. Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. J. Bacteriol. **189**:4320–4324.

Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J. Exp. Med. **196**:109–118.

Kang, H., and D. C. Gross. 2005. Characterization of a resistance-nodulation-cell division transporter system associated with the *syr-syp* genomic island of *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. **71**:5056–5065.

Lin, J., C. Cagliero, B. Guo, Y. W. Barton, M. C. Maurel, S. Payot, and Q. Zhang. 2005. Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. J. Bacteriol. **187**:7417–7424.

Morita, Y., M. L. Sobel, and K. Poole. 2006. Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product. J. Bacteriol. **188**:1847–1855.

Muller, J. F., A. M. Stevens, J. Craig, and N. G. Love. 2007. Transcriptome analysis reveals that multidrug efflux genes are upregulated to protect *Pseudomonas aeruginosa* from pentachlorophenol stress. Appl. Environ. Microbiol. **73**:4550–4558.

Piddock, L. J. 2006. Multidrug-resistance efflux pumps—not just for resistance. Nature Rev. Microbiol. **4**:629–636.

Poole, K. 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann. Med. **39**:162–176.

Sobel, M. L., K. Poole, and S. Neshat. 2005. Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J. Bacteriol. **187**:1246–1253.