



Genome-Wide Transposon Screen of a *Pseudomonas syringae* *mexB* Mutant Reveals the Substrates of Efflux Transporters

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ABSTRACT Bacteria express numerous efflux transporters that confer resistance to diverse toxicants present in their environment. Due to a high level of functional redundancy of these transporters, it is difficult to identify those that are of most importance in conferring resistance to specific compounds. The *resistance_nodulation_division* (RND) protein family is one such example of redundant transporters that are widespread among Gram-negative bacteria. Within this family, the MexAB-OprM protein complex is highly expressed and conserved among *Pseudomonas* species. We exposed barcoded transposon mutant libraries in isogenic wild-type and $\Delta mexB$ backgrounds in *P. syringae* B728a to diverse toxic compounds *in vitro* to identify mutants with increased susceptibility to these compounds. Mutants with mutations in genes encoding both known and novel redundant transporters but with partially overlapping substrate specificities were observed in a $\Delta mexB$ background. Psyr_0228, an uncharacterized member of the major facilitator superfamily of transporters, preferentially contributes to tolerance of acridine orange and acriflavine. Another transporter located in the inner membrane, Psyr_0541, contributes to tolerance of acriflavine and berberine. The presence of multiple redundant, genomically encoded efflux transporters appears to enable bacterial strains to tolerate a diversity of environmental toxins. This genome-wide screen performed in a hypersusceptible mutant strain revealed numerous transporters that would otherwise be dispensable under these conditions. Bacterial strains such as *P. syringae* that likely encounter diverse toxins in their environment, such as in association with many different plant species, probably benefit from possessing multiple redundant transporters that enable versatility with respect to toleration of novel toxicants.

IMPORTANCE Bacteria use protein pumps to remove toxic compounds from the cell interior, enabling survival in diverse environments. These protein pumps can be highly redundant, making their targeted examination difficult. In this study, we exposed mutant populations of *Pseudomonas syringae* to diverse toxicants to identify pumps that contributed to survival in those conditions. In parallel, we examined pump redundancy by testing mutants of a population lacking the primary efflux transporter responsible for toxin tolerance. We identified partial substrate overlap for redundant transporters, as well as several pumps that appeared more substrate specific. For bacteria that are found in diverse environments, having multiple, partially redundant efflux pumps likely allows flexibility in habitat colonization.

KEYWORDS endophytes, epiphytes, fitness

Bacteria, like all living organisms, must tolerate a variety of potentially harmful, chemically diverse molecules present in the environment. While many of these compounds can be degraded to prevent their accumulation to harmful levels within the cell, a common response to the presence of toxins is exportation of such toxins. Bacterial efflux transporters can function both to achieve stress tolerance and to

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contribute to virulence by secreting toxins or effectors of various kinds (1, 2). A given bacterial species commonly possesses a wide variety of efflux transporters, and it is presumed that they differ in the specificity of toxins that they export. Broad-specificity transporters actively remove toxins from the cell, and their substrates can include heavy metals, solvents, dyes, detergents, and antibiotics, as well as certain host-derived products (3–6). Promiscuous transporters in that category include the multidrug-resistant (MDR) efflux transporters that remove a wide range of structurally diverse chemical compounds from the cell interior (7). While genes encoding these exporters can be found on plasmids, pathogenic and nonpathogenic bacteria have comparable numbers of chromosomally encoded MDR systems (8). A high level of redundancy of transporters exists within a given strain, where they may share many of the same substrates (9, 10). These observations raise the issue of why so many transporters are present in a given bacterium, given the broad substrate range of these MDR pumps.

MDR efflux transporters are structurally diverse, being found in at least five distinct protein families: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, the ATP-binding cassette (ABC) superfamily, and the resistance-nodulation-division (RND) family (11, 12). Many of these transporter classes contain both substrate-specific transporters and less-specific MDR-type efflux pumps (8). For example, the characterized MFS transporters in *Escherichia coli* range from highly specific sugar-proton symporters such as LacY and XylE to multidrug efflux transporters such as EmrD (13). Our understanding of efflux-mediated resistance to toxins and antibiotics would benefit from identifying both the MDR transporters within the larger collection of genomic transporters for a given organism and the specific substrates for those transporters.

Substrate redundancy poses a challenge for the study of MDR transporters, as alternative transporters can mask mutations that disrupt the function of a given pump under investigation (9). The large number of potential MDR transporters in a given strain also makes their investigation by analysis of targeted gene deletion mutants laborious. An example of one of the few such intensive studies that have been performed is that of Sulavik et al. (14), who tested the susceptibility of *E. coli* strains with null mutations in seven known and nine predicted efflux genes against 35 toxicants. That study, and others (15, 16), identified the RND transporter AcrAB-TolC as the major determinant of intrinsic toxicant resistance in *E. coli*. Homologs of AcrAB-TolC are common in Gram-negative bacteria and have been shown to contribute to the virulence of plant pathogens as diverse as *Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xylella fastidiosa* (17–20). Interestingly, inhibition of efflux pumps by known chemical inhibitors can increase the antimicrobial activity of compounds produced by plants (21–23) and might be considered a plant disease resistance trait.

P. syringae pv. *syringae* is a plant pathogen that is commonly found both in association with plants and in the water cycle (24). Those are habitats in which it might be expected to encounter a variety of toxic compounds. *P. syringae* pv. *syringae* strain B728a (B728a) was originally isolated from bean (*Phaseolus vulgaris*) (25) and is exposed to the antimicrobial compounds phaseollin and coumestrol, which accumulate in bean leaves in response to inoculation with both compatible and incompatible *P. syringae* strains (26). This species also can interact with many other plant species and thus is exposed to a variety of preformed inhibitors on plant surfaces as well as to induced phytoalexins and other toxic compounds found within diseased tissues. It also can be a transient soil colonist, being associated with dead plant material, and is likely exposed to the myriad of inhibitory compounds typically found in soils. MexAB-OprM is the best-characterized AcrAB-TolC homolog present in *Pseudomonas* species. While this protein complex has been proposed to secrete the iron-chelating molecule pyoverdine (27), it also contributes significantly to antibiotic resistance (11). In strain B728a, genes in this operon are generally expressed at much higher levels than genes encoding other RND transporters, both in culture and in cells on the leaf surface as well as in the apoplast (28). However, in *P. aeruginosa*, expression of *mexAB-oprM* is inversely corre-

lated with expression of the related RND transporter operons *mexEF-oprN* and *mexCD-oprJ* (29). This suggests that regulation of the overall repertoire of efflux transporters is tightly controlled and that alterations in one or more can lead to compensatory changes in the others.

To examine the role of MexAB-OprM homologs and other potential MDR transporters present in strain B728a, we interrogated the fitness of a large library of randomly barcoded *mariner* transposon mutants in culture media containing diverse antimicrobial compounds. Random barcode-transposon insertion sequencing (RB-TnSeq) is a modification of transposon insertion sequencing where each transposon insertion is tagged with a unique 20-nucleotide barcode (30). As transposon insertions are mapped only once for a given library, this reduces the effort required for the use of that library for analysis of fitness contributions of genes under multiple conditions. Changes in relative barcode abundances over time are used as a proxy for the relative fitness contribution of a given gene under a given condition. This method can be used to associate bacterial genes with their importance to fitness under different growth conditions and has been used to improve genome annotations for diverse bacterial species (31). Of particular importance for this current study are the ease and scale associated with the use of this method to correlate genes encoding transporter proteins with their likely substrates. Here, we used RB-TnSeq to identify likely substrates for B728a efflux transporters, with a particular focus on complementary RND homologs of MexAB-OprM.

RESULTS

Creation of a barcoded transposon library in *P. syringae* B728a Δ mexB. To test the role of redundant RND efflux proteins *in vitro*, we created a barcoded *mariner* transposon library in an unmarked B728a Δ mexB mutant. Comparisons of gene fitness in the mutant library with that of the barcoded transposon library in wild-type (WT) strain B728a (32) allowed us to directly test the fitness contributions of B728a transporters in both genetic backgrounds, enabling the complementarity of other transporters with MexAB-OprM to be quantified. The Δ mexB transposon library contains 237,285 unique insertion strains at a median density of 16 central insertion strains per gene and insertion density and distribution levels similar to those of the WT library (see Table S1 in the supplemental material; see also Fig. S1 in the supplemental material). We hypothesized that insertional mutants in efflux genes in the Δ mexB genotype would be less fit than mutants in the WT genotype, particularly when exposed to toxic substrates of MexAB-OprM. The B728a genome encodes 668 predicted transport proteins (33); here, we primarily focused on the RND transporters but also investigated members of the other protein families that typically encode MDR efflux transporters. The B728a genome encodes 16 RND transporters, including MexAB-OprM (33).

The *mexB* deletion completely eliminates the activity of MexAB-OprM, including inactivation of OprM. To determine if the deletion of *mexB* resulted in a polar mutation, we expressed *mexAB* and *mexAB-oprM* under the control of the native promoter in the Δ mexB mutant strain. While the Δ mexB strain containing the plasmid expressing the entire operon was able to tolerate acriflavine, berberine, and phloretin at or above WT concentrations, the Δ mexB strain expressing only *mexAB* was not (see Table 2; see also Fig. S2). This indicates that the Δ mexB transposon library does not produce a functional OprM.

Testing the fitness of mutants in the WT and Δ mexB transposon libraries *in vitro*. Both mutant libraries were grown in the rich culture medium King's B (KB) with different antimicrobial compounds. For the compounds where the MIC was known for the B728a WT and Δ mexB strains (19), we used $1/4$ the MIC. We successfully assayed fitness for 16 unique antimicrobial compounds (Table S2). Many of these compounds were most soluble in DMSO (dimethyl sulfoxide), and the controls contained the concentrations of DMSO used in these assays. For each gene, fitness was calculated as the \log_2 ratio of barcode abundance following growth in a given condition relative to barcode abundance measured initially at time 0. As expected, insertions in the majority

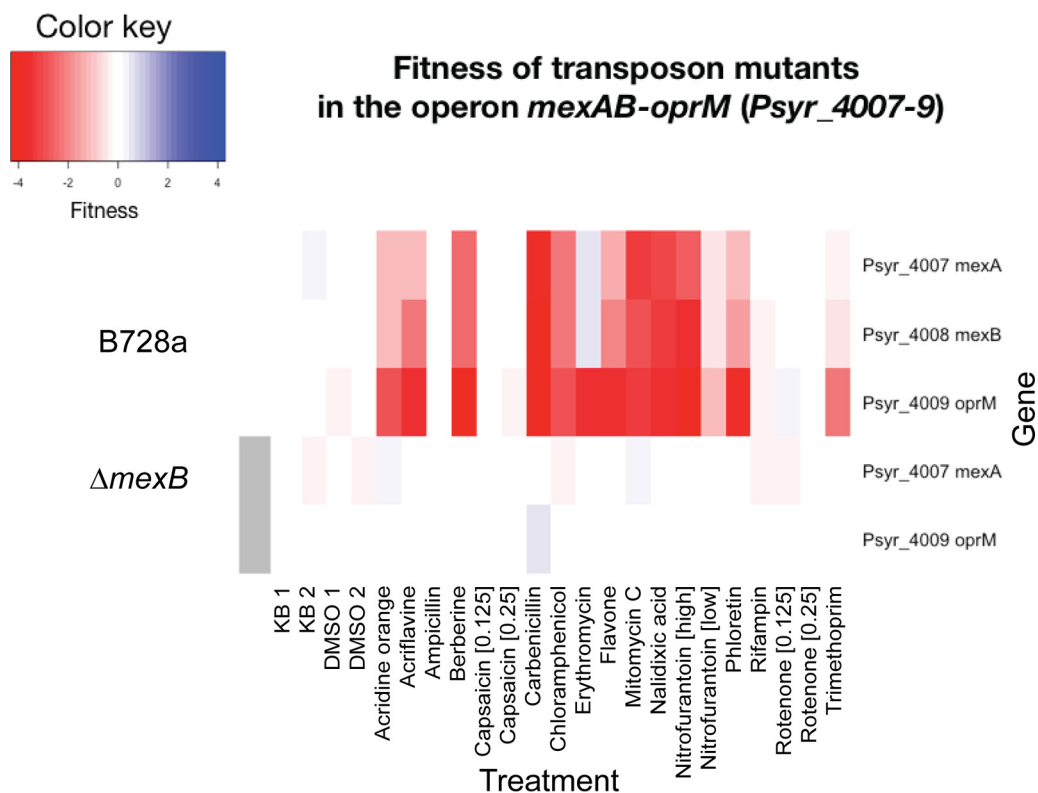


FIG 1 Fitness of transposon insertional mutants in the operon *mexAB-oprM* (*Psysr_4007-9*) both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as \log_2 change in relative insertion strain barcode abundance for a given gene.

of genes did not contribute to fitness as measured by relative barcode abundance in the population, and thus the fitness values for most genes were close to 0. A mutant with a fitness value of -1 is approximately 50% less abundant than the typical strain in the library under that experimental condition.

Identification of MexAB-OprM substrates. For many substrates, the dominant activity of MexAB-OprM seen in other species was expected to mask any requirement for complementary transporters. We therefore hypothesized that the $\Delta mexB$ deletion library would unmask the contributions of such alternative transporters to tolerance of various toxins. The majority of antimicrobial compounds tested here were substrates of the MexAB-OprM transporter and have known MICs (19). Other previously uninvestigated compounds included capsaicin, flavone, rifampin, and rotenone.

For the majority of the antimicrobial compounds tested, disruption of any gene in the MexAB-OprM operon resulted in decreased fitness relative to insertions in the average gene in the WT strain (Fig. 1). No fitness change was observed for insertional strains in this operon when cells were exposed to capsaicin, rifampin, or rotenone. There was also no fitness change seen under conditions of exposure to ampicillin, a previously reported substrate (19). For erythromycin, only transposon insertions in *oprM* decreased mutant fitness. Flavone is a likely a substrate of this complex due to the large decreases in fitness comparable to that seen upon exposure to known substrates that accompanied disruptions across the *mexAB-oprM* operon. Using this method, we would expect insertional mutations in *mexA* and *oprM* to be neutral in the $\Delta mexB$ genetic background. All strains in the $\Delta mexB$ transposon library indeed were unable to assemble a complete MexAB-OprM complex, since insertions elsewhere in the *mexAB-oprM* operon did not alter fitness. For all antimicrobial compounds tested, *mexA* and *oprM* are dispensable in the $\Delta mexB$ library, with fitness values close to 0 (Fig. 1).

Transporters homologous to MexAB contribute to resistance to diverse toxicants. To identify the efflux transporters that made the largest contribution to toler-

TABLE 1 RND operons homologous to *mexAB-oprM* that likely contribute to multidrug resistance^a

Locus	Gene	Description	B728a	$\Delta mexB$
Psyr_4007	<i>mexA</i>	Secretion protein HlyD	10	0
Psyr_4008	<i>mexB</i>	Hydrophobe/amphiphile efflux-1 HAE1	10	NA
Psyr_4009	<i>oprM</i>	RND efflux system, outer membrane lipoprotein, NodT	13	NA
Psyr_2967	<i>mexE</i>	Secretion protein HlyD	1	9
Psyr_2968	<i>mexF</i>	Hydrophobe/amphiphile efflux-1 HAE1	1	8
Psyr_2969	<i>oprN</i>	RND efflux system, outer membrane lipoprotein, NodT	0	6
Psyr_2482	<i>muxA</i>	Secretion protein HlyD	1	8
Psyr_2483	<i>muxB</i>	Acriflavin resistance protein	1	5
Psyr_2484	<i>muxC</i>	Acriflavin resistance protein	1	5
Psyr_2485	<i>opmB</i>	RND efflux system, outer membrane lipoprotein, NodT	1	6
Psyr_2283	<i>mexC</i>	Secretion protein HlyD	4	2
Psyr_2282	<i>mexD</i>	Hydrophobe/amphiphile efflux-1 HAE1 (no outer membrane protein in operon)	4	2
Psyr_0344		Secretion protein HlyD	0	1
Psyr_0345		Secretion protein HlyD	0	1
Psyr_0346	<i>mexK</i>	Acriflavin resistance protein (no outer membrane protein in operon)	0	1
Psyr_2193		Secretion protein HlyD	0	0
Psyr_2194		Acriflavin resistance protein (no outer membrane protein in operon)	0	0
Psyr_2864		RND efflux system, outer membrane lipoprotein, NodT	0	0
Psyr_2865		Hydrophobe/amphiphile efflux-1 HAE1	0	0
Psyr_2866		Secretion protein HlyD	1	0
Psyr_3130		Secretion protein HlyD	0	0
Psyr_3131		Secretion protein HlyD	0	0
Psyr_3132		Acriflavin resistance protein (no outer membrane protein in operon)	0	0

^aThe number of experimental conditions where a significant contribution to fitness was seen (fitness value less than -1) is shown for mutants in a WT or $\Delta mexB$ background. A total of 23 treatments were examined, including 2 KB controls, 2 DMSO controls, and 16 unique compounds (3 at two concentrations). NA, not applicable.

ance of various toxicants, we calculated the number of compounds in which genes encoding transporters or transporter components had a fitness value of less than -1 . RND transporters generally made a larger contribution to toxin tolerance in the $\Delta mexB$ library than in the WT, but some were important in both backgrounds (Table 1). Due to our experimental design focusing on known MexAB-OprM substrates, *mexA* and *mexB* were required for competitive fitness in the presence of 10 compounds. The outer membrane transporter OprM is likely shared with additional efflux pumps and so is required for tolerance of an additional three compounds. In the $\Delta mexB$ deletion background, *mexA* is dispensable.

In addition to MexAB, four homologous RND transporters contributed substantially to tolerance of several toxicants (Table 1). The *mexEF-oprN* operon, however, was required for tolerance of these compounds only in the absence of MexB (Fig. 2). MexEF-OprN is likely redundant with MexAB-OprM for the substrates acridine orange, acriflavine, berberine, chloramphenicol, flavone, nalidixic acid, nitrofurantoin, and phloretin. The lack of a phenotype for mutants in the WT background is consistent with the likely subsidiary role of this transporter for these shared substrates and therefore with masking by the more highly expressed MexAB-OprM under these conditions.

Psyr_2483-5 is likely redundant with MexAB-OprM with respect to the substrates acriflavine, berberine, carbenicillin, chloramphenicol, erythromycin, and phloretin, with decreased fitness for disruptions in the $\Delta mexB$ genetic background (Fig. 3). Psyr_2483-5 independently contributes to resistance to rifampin. This apparent operon encodes an unnamed RND transporter that is most similar to the MuxABC-OpmB complex in other pseudomonads, such as *P. aeruginosa* PAO1 (Table S3).

MexCD (Psyr_2282-3) was required for full competitive fitness in the presence of acriflavine, berberine, erythromycin, and nalidixic acid (Fig. S3). Importantly, these phenotypes could be seen in both the WT and $\Delta mexB$ backgrounds. The negative fitness values for insertional mutants in this operon were, however, greater in the WT background, although this may have been at least partially due to the higher toxicant concentrations used in testing the mutants in the WT background than were used with the $\Delta mexB$ library. This operon does not encode an outer membrane protein, and a gene for such a required component is likely located elsewhere in the genome.

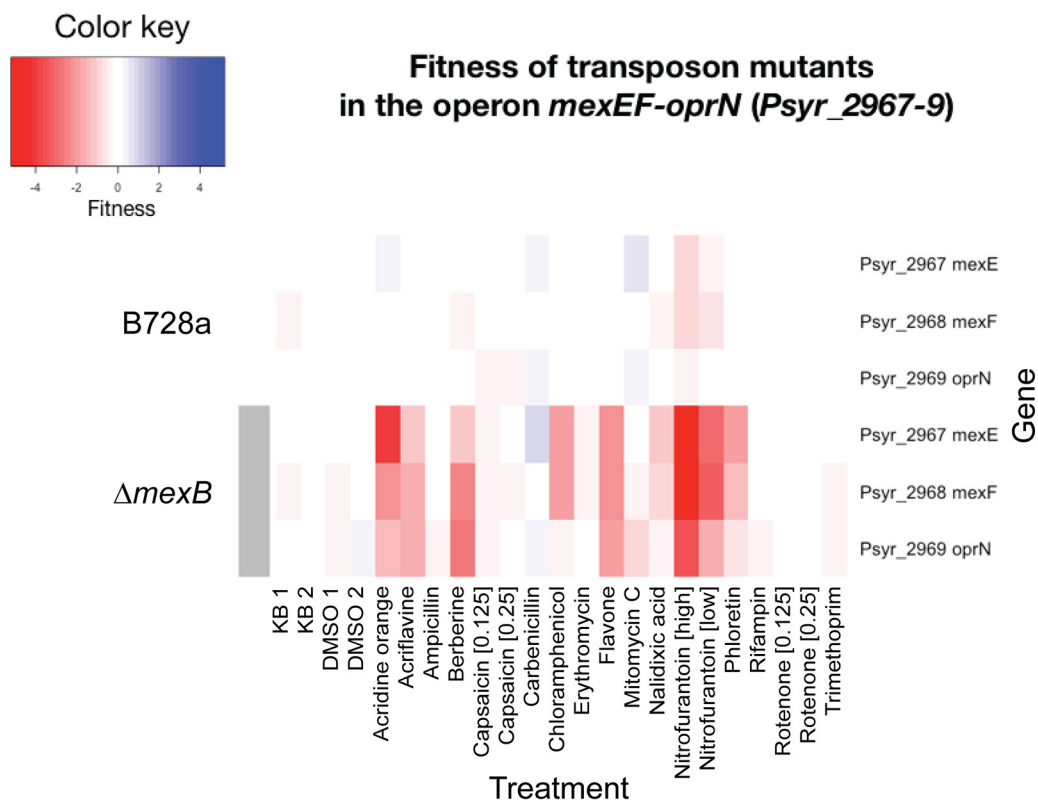


FIG 2 Fitness of transposon insertional mutants in the operon *mexEF-oprN* (*Psysr_2967-9*) both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as \log_2 change in relative insertion strain barcode abundance for a given gene.

Among the compounds tested here, *Psysr_0344-6* contributed to tolerance of phloretin only in the $\Delta mexB$ background (Fig. 4). Interestingly, disruption of any gene in this operon resulted in a mutant strain that was more fit than mutants of other genes under conditions of exposure to acriflavine, berberine, or nalidixic acid, but only if *mexB* was also absent. This operon likely requires an unknown outer membrane protein located elsewhere in the genome.

Inner membrane transporters appear more substrate specific. The same computational analysis was used to interrogate the MFS and other inner membrane transporters to identify those with fitness contributions in the presence of various toxic compounds in either or both genetic backgrounds. Among 68 MFS transporters examined, only *Psysr_0228* contributed to tolerance of any tested compound. *Psysr_0228* contributed to competitive fitness in both acriflavine and acridine orange (Fig. 5). Interestingly, this gene contributed to acriflavine resistance even in a WT background, indicating that its role was independent of *mexB*. In contrast, this gene contributed to acridine orange resistance only in the $\Delta mexB$ genotype. Disruption of the gene encoding the SMR transporter *Psysr_0541* strongly decreased fitness in berberine and mildly decreased fitness in acriflavine, independently of the *mexB* genotype (Fig. 6). In the $\Delta mexB$ strain, disruption of *Psysr_0541* resulted in mild susceptibility to carbenicillin (Fig. 6). For those substrates where fitness was dependent on the *mexB* genotype, it is not clear if these inner membrane transporters contribute to toxin tolerance requiring the entire MexAB-OprM complex or just the OprM outer membrane protein.

Several ABC transporters contribute to growth in rich media. Most genes annotated as encoding ABC transporter subunits were putative amino acid or carbohydrate transporters. Under the conditions tested, several ABC transporters were required for competitive fitness in the rich medium controls. For example, insertions in *Psysr_0917-8*, encoding the polysaccharide permease ABC transporter RfbAB-2, strongly

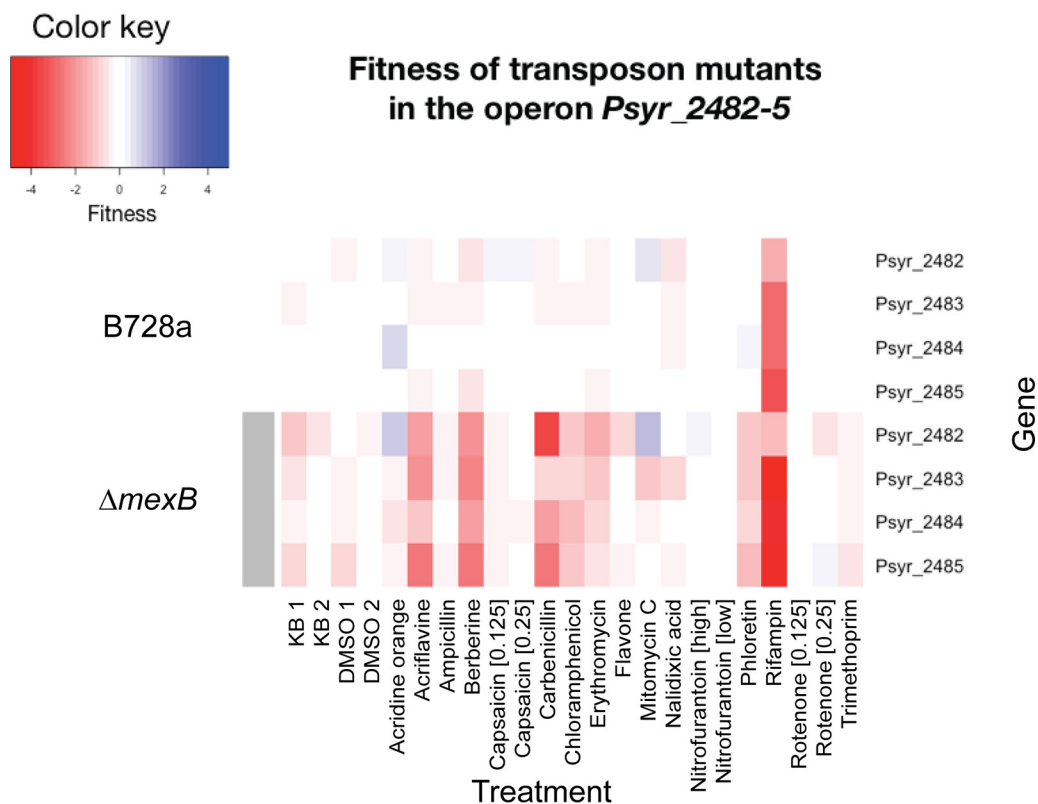


FIG 3 Fitness of transposon insertional mutants in the operon *muxABC-oprM* (*Psyr_2482-5*) both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as \log_2 change in relative insertion strain barcode abundance for a given gene.

decreased fitness under all conditions, including the controls containing only KB medium, in both the WT and $\Delta mexB$ genotypes (Fig. S4a). RfbAB-2 appears nearly essential for growth in KB. Genes encoding a putative peptide ABC transporter, *Psyr_1754-9*, were required for competitive fitness under all conditions, including in the KB controls, but only in a $\Delta mexB$ mutant background (Fig. S4b).

Overlapping substrate specificities between transporters. The MDR efflux transporters tested here displayed a range of apparent substrates, with various degrees of overlap of MexAB-OprM (Fig. 7). Compounds such as acriflavine, berberine, and phloretin are probable substrates of multiple RND transporters. MexCD was required for tolerance of fewer compounds tested than MexAB, MexEF, or MuxABC. As hypothesized, inner membrane transporters *Psyr_0228*, *Psyr_4519*, and *Psyr_0541* contributed to resistance to only a few toxicants. However, for the compounds examined here, no other inner membrane transporters contributed to competitive fitness.

Assays to test predicted mutant phenotypes. Using RB-TnSeq, the fitness contributions of the various transporter genes were examined at only one or two concentrations of a particular toxicant. The lower fitness values seen for various mutants suggested a hypersensitivity to that compound. To confirm that disruption of these transporters reduced the concentration of the toxicant at which any growth could occur, we constructed targeted deletion mutants of the substrate binding proteins MexF, MuxB, and MexK (*Psyr_0346*) in both the WT and $\Delta mexB$ genotypes. We used broth serial dilution tests and zone of inhibition (ZOI) assays to examine the antibiotic susceptibility profiles of these strains (Table 2; see also Fig. S5). The measured MIC values were generally higher in the KB rich medium than in the M9 minimal medium. However, as fitness assays for the transposon libraries were conducted in a rich medium we aimed to test susceptibility phenotypes under the same condition. The $\Delta mexB$ $\Delta mexF$ double mutant was more susceptible to berberine than the WT and either

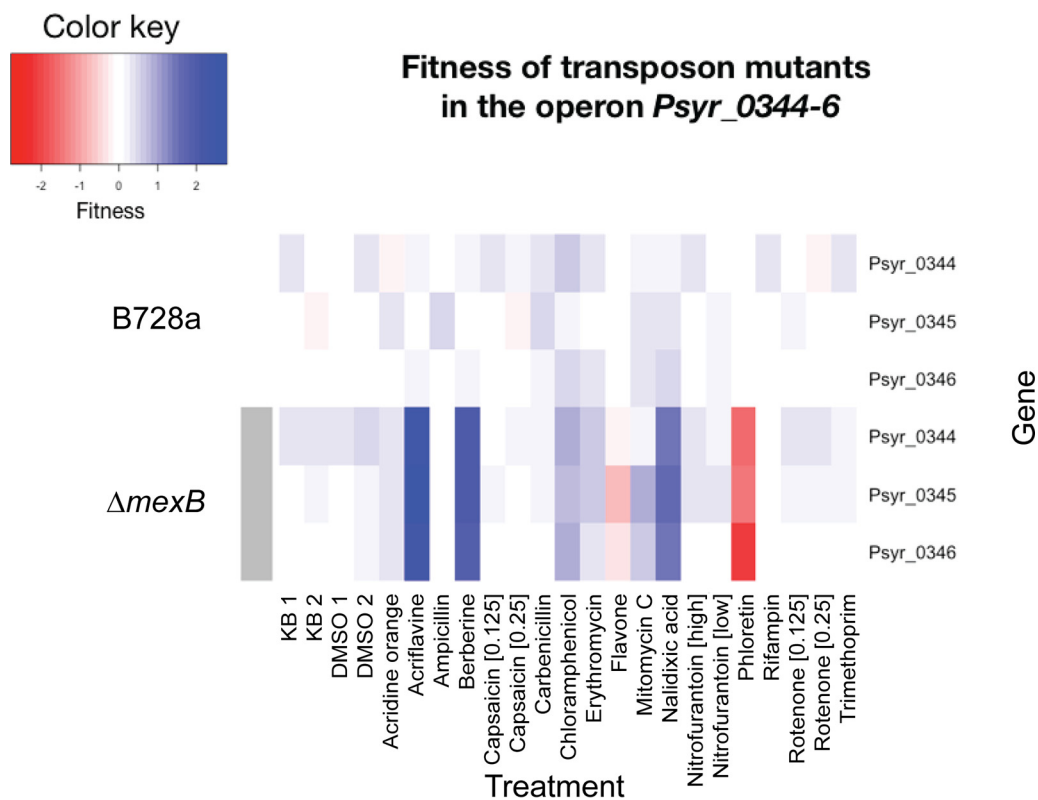


FIG 4 Fitness of transposon insertional mutants in the operon *Psyr_0344-6* both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as \log_2 change in relative insertion strain barcode abundance for a given gene.

single-deletion strain (Table 2; see also Fig. S5a). Similarly, the $\Delta mexB \Delta muxB$ double mutant was more sensitive to berberine, phloretin, and rifampin than the WT and either single-deletion strain (Table 2; see also Fig. S5b). While double mutants in a $\Delta mexB$ mutant background were more susceptible to acriflavin than the $\Delta mexB$ mutant itself, as evidenced by a larger zone of inhibition in the more sensitive ZOI assay and as predicted by the RB-TnSeq data, the extreme sensitivity of the $\Delta mexB$ mutant to acriflavin apparently obscured differences in the sensitivities of the double mutants as assessed in MIC assays. As suggested by the positive fitness values observed in mixture studies, the $\Delta mexB \Delta mexK$ double mutant exhibited decreased susceptibility to acriflavin and berberine in comparison to the $\Delta mexB$ mutant (Table 2; see also Fig. S5c). In most cases, there was a direct relationship between the change in the MIC of a particular compound for a mutant and the fitness value for that mutant exposed to that chemical (Fig. S6).

DISCUSSION

Bacteria encounter and must tolerate diverse and potentially toxic molecules in the various habitats that they occupy. While efflux transporters are generally thought to play a major role in the tolerance of toxins, it is still unclear why bacterial genomes encode such a large number of MDR transporters. With high expression levels and wide substrate ranges, RND efflux transporters such as AcrAB-TolC and MexAB-OprM are essential for environmental survival and colonization of eukaryotic hosts (34). However, for the same reasons, these transporters are thought to mask the contributions to resistance of homologous RND transporters (14). Prompting our investigation in *P. syringae*, studies have shown that expression of multiple alternative transporters is increased when these primary RND pumps are disrupted (29, 35). Our studies of these alternative and redundant pumps have benefited from the availability of a method that

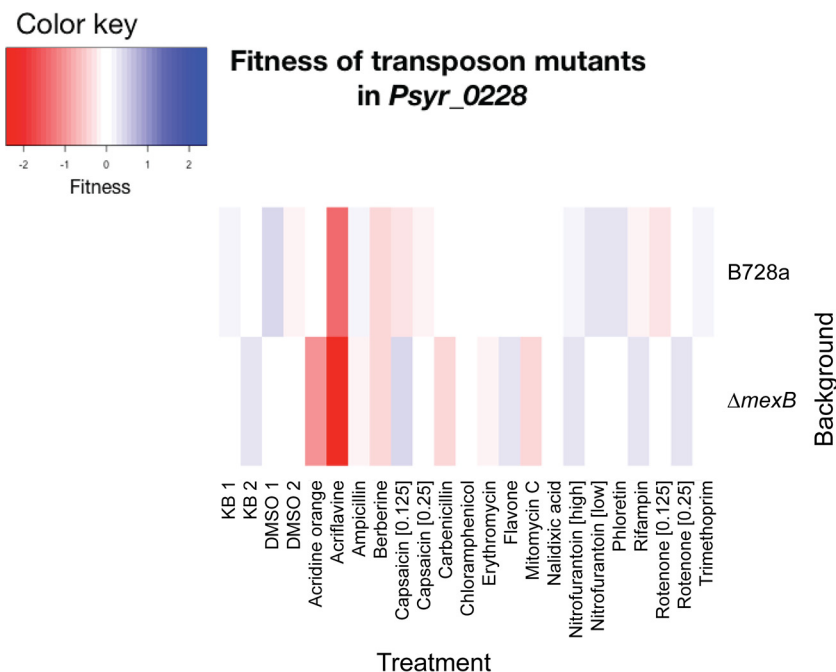


FIG 5 Fitness of transposon insertional mutants in the major facilitator superfamily transporter gene *Psyr_0228* both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as the log₂ change in relative insertion strain barcode abundance for a given gene.

has previously been successfully employed that relies on the use of hypersusceptible pump mutants to unmask redundant transporters (36–38).

Strain B728a contains a diverse array of transporters, many with likely roles in multidrug resistance (33). The MexAB-OprM complex has been shown to be important

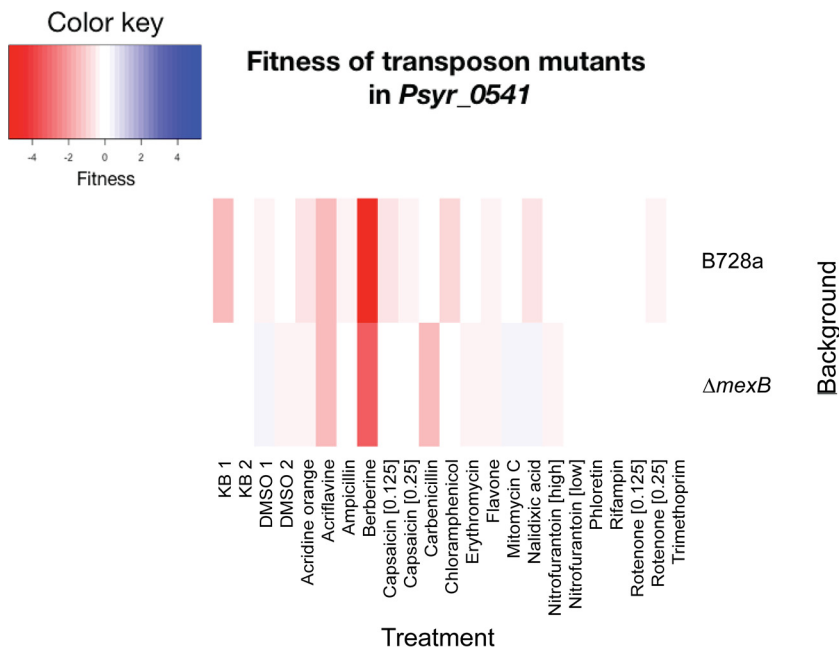


FIG 6 Fitness of transposon insertional mutants in the SMR gene *Psyr_0541* both in a WT background and in cells in which *mexAB-oprM* has been disrupted. “KB” and “DMSO” represent the media and media plus solvent control, respectively. Fitness was calculated as the log₂ change in relative insertion strain barcode abundance for a given gene.

Pump Type Gene/Operon	RND					MFS	SMR
	MexAB	MexCD	MexEF	0344-6	MuxABC	0228	0541
Acridine orange	Blue		Blue			Blue	
Acriflavine	Blue			Red	Blue		
Ampicillin	?						
Berberine	Blue			Red	Blue		Blue
Capsaicin							
Carbenicillin	Blue				Blue		
Chloramphenicol	Blue			Red			
Erythromycin	?	Blue			Blue		
Flavone			Blue				
Mitomycin C	Blue						
Nalidixic acid		Blue		Red			
Nitrofurantoin			Blue				
Phloretin	Blue				Blue		
Rifampin					Blue		
Rotenone							
Trimethoprim	Blue						

FIG 7 Likely substrates of *P. syringae* B728a multidrug resistance RND transporters, MFS transporter Psyr_0228, and SMR pump Psyr_0541. Blue cells indicate the compound is an apparent substrate of that transporter, with insertional mutants of a given gene having decreased fitness (fitness value less than -1) in the WT and/or the $\Delta mexB$ genetic backgrounds. Red cells indicate increased fitness of insertional mutants in this operon.

for several *P. syringae* strains for tolerance of a variety of antibiotics and other toxins, as well as for virulence during plant colonization (19). However, it has remained a challenge to associate particular transporters with their substrates, especially for host-produced compounds. With few exceptions (17, 39), genes encoding efflux transporters in addition to AcrAB-TolC and MexAB-OprM have not been observed to contribute to fitness during plant colonization (32, 40, 41), likely due to their high level of redundancy. Here, we used a transposon library constructed in a $\Delta mexB$ strain to reveal the role of several partially redundant RND efflux pumps. While several of these pumps appeared to have been completely masked by the role of MexAB-OprM under the conditions used here, some were complementary to MexAB-OprM, contributing incrementally to tolerance of various toxicants.

Among those transporters that played an incremental role in tolerance were *mexEF-oprN* and *muxABC-opmB*. These transporters, however, were important for only a subset of the MexAB-OprM substrates tested. We expected MexEF-OprN to be important in the $\Delta mexB$ genotype for two main reasons. First, previous transcriptional measurements in *P. syringae* strain B728a showed that after *mexAB-oprM*, *mexEF-oprN* is generally the second most highly expressed RND transporter under a variety of conditions in culture and *in planta* (28). Second, overexpression of *mexEF-oprN* in *P. aeruginosa* strains lacking *mexAB-oprM* increased resistance to several antibiotics (10). MuxABC-OpmB is

TABLE 2 Antimicrobial susceptibility of *P. syringae* B728a wild-type, derivative mutant, and complementation strains

Strain	MIC ($\mu\text{g/ml}$) in indicated medium							
	Acriflavine		Berberine		Phloretin		Rifampin	
	KB	M9	KB	M9	KB	M9	KB	M9
<i>P. syringae</i> B728a	30	7.5	1,000	1,000	1,000	1,000	>200	>200
<i>P. syringae</i> $\Delta mexB$	3.75	3.75	125	125	250	62.5	>200	>200
<i>P. syringae</i> $\Delta mexF$	30	7.5	1,000	500	1,000	1,000		
<i>P. syringae</i> $\Delta mexB \Delta mexF$	3.75	3.75	62.5	62.5	250	62.5		
<i>P. syringae</i> $\Delta muxB$	15	7.5	1,000	1,000	>1,000	1,000	>200	>200
<i>P. syringae</i> $\Delta mexB \Delta muxB$	3.75	3.75	62.5	62.5	62.5	62.5	25	25
<i>P. syringae</i> $\Delta mexK$	30	7.5	1,000	500	>1,000	1,000		
<i>P. syringae</i> $\Delta mexB \Delta mexK$	7.5	3.75	250	125	62.5	62.5		
<i>P. syringae</i> B728a pMexAB	30	7.5	500	500	1,000	500		
<i>P. syringae</i> $\Delta mexB$ pMexAB-OprM	>60	30	500	500	1,000	1,000		
<i>P. syringae</i> $\Delta mexB$ pMexAB	3.75	3.75	62.5	62.5	250	62.5		

an unusual RND operon because it is expected to have two inner membrane components. This transporter was characterized in *P. aeruginosa* only recently (42) and is homologous to the *E. coli* complex MdtABCD (also annotated as YegMNOB) (36, 37). Rifampin was not previously known to be a substrate of any Mex pumps (10), but full resistance appears to require the MuxABC-OpmB complex.

Of the eight RND operons likely involved in multidrug resistance, four do not contain genes encoding outer membrane proteins. Transporters such as these likely require the presence of outer membrane proteins, encoded by genes located elsewhere in the genome. The archetypal outer membrane protein TolC and its homologs (such as OprM and OprN in *Pseudomonas* species) can couple to many different transporters and apparently do not play a role in substrate specificity (43). Since the construction of the unmarked $\Delta mexB$ strain also disrupted the *oprM* gene, this may have reduced the functionality of transporters MexCD, Psyr_0344-6, Psyr_2193-4, and Psyr_3130-2 in that mutant if they require OprM for maximum functionality. Combined with the minor roles of these other transporters relative to that of MexAB-OprM, this would potentially explain the lack of a fitness cost of their disruption in both the WT and $\Delta mexB$ genotypes. We should emphasize, however, that nearly all of the compounds tested here were known MexAB-OprM substrates. In such a scenario, it might be expected that MexAB-OprM would play a dominant role in tolerance of such compounds. Very few studies have addressed the breadth of the substrate range for MexAB-OprM beyond that of clinically important antibiotics, and, given the wide variety of other toxic natural products that a bacterial species such as *P. syringae* would encounter in its myriad of habitats, it seems likely that many would be found for which these other efflux transporters might play a dominant role. Indeed, evidence for such a role for Psyr_0228 in the tolerance of acriflavin was seen here. It should prove fruitful to test this hypothesis further by the testing of these transposon libraries for their tolerance of diverse additional compounds. Not only will this provide further evidence of the constraints of MexAB-OprM in the chemical ecology of *P. syringae*, but it will also provide contextual evidence for the retention of the myriad of other efflux pumps in this species that presumably remain under selection, suggesting its importance in at least some settings encountered by this cosmopolitan bacterium. While we observed redundancy in efflux transporter function, seen in the overlapping substrate ranges of several RND transporters, it may simply benefit the cell to have multiple mechanisms of resistance for a given toxicant.

In Gram-negative bacteria, inner membrane transporters such as those in the MFS can function cooperatively with outer membrane transporters (like RND transporters) (9). This has been proposed to synergistically increase overall toxin resistance for the cell (10). Together with the high level of substrate promiscuity for most MDR RND transporters, we reasoned that inner membrane transporters might contribute to substrate specificity. This would prevent unnecessary efflux, attenuating the proton motive force. If this were true, we would expect to observe that inner membrane transporters would each contribute strongly to resistance to only a few toxicants. For Psyr_0228, which contributes to the tolerance of only acridine orange and acriflavine, two molecules that are structurally similar, this appeared to be the case. (Technically, acridine orange and acriflavine represent three molecules, as acriflavine is typically sold as a mixture of the related molecules acriflavine and proflavine). SMR protein Psyr_0541 is homologous to the quaternary ammonium compound resistance protein QacE in *P. fluorescens* SBW25 and QacH in *P. aeruginosa* PA14 (44). Both acriflavine and berberine are quaternary ammonium compounds, and it is noteworthy that Psyr_0541 was found to be necessary for tolerance of both of these compounds. Insertional mutants at this locus were particularly susceptible to berberine. The inner membrane transporters Psyr_0228 and Psyr_0541 appear to share some substrates with MexAB-OprM. More information is required to determine to what extent these transporters function cooperatively, if at all. In addition, it is possible that these transporters interact with molecules that are not substrates of MexAB-OprM, potentially with the assistance of

outer membrane transporters other than OprM. It would be interesting if these protein interactions differed among particular substrates.

Efflux transporters are typically easily detected and annotated computationally due to their sequence homology and transmembrane domains. Here, we show that, using a range of structurally diverse antimicrobial compounds, RB-TnSeq can be used to characterize the substrates of MDR efflux transporters. This method is sensitive because it measures competitive fitness, rather than simple inhibition of growth, a phenotype important to bacteria in complex environments where they face competition with other microbes. RB-TnSeq is also very cost-effective once transposon mutants are initially mapped, making it practical to readily test a large number of conditions and compounds. Characterizing MDR transporter redundancy is essential to our understanding of not just clinical antibiotic resistance but also the role of these abundant proteins in bacterial survival in diverse environments.

Using RB-TnSeq in a hypersusceptible mutant strain allowed us to examine the role of alternative transporters that might not otherwise be active or discernible. Furthermore, plant-produced compounds are often not as toxic to bacteria as common antibiotics produced by bacteria or fungi, and this has been hypothesized to be due to the activity of efflux transporters (21). There is some evidence that plants can produce MDR pump inhibitors in addition to antimicrobial compounds (45), and this reinforces the importance of multi-“drug” resistance transporters in tolerating diverse plant-produced and other naturally occurring antimicrobial molecules. Multiple-component outer membrane transporters are apparently highly redundant, while inner membrane transporters can be more substrate specific. This may help explain the abundance of partially redundant MDR efflux transporters found in many bacteria, especially those strains typically found in environments with exposure to diverse toxic chemicals.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. syringae* pv. *syringae* B728a was originally isolated from a bean leaf (*Phaseolus vulgaris*) in Wisconsin (25). The complete genome sequence for B728a is available on NCBI GenBank under accession number [CP000075.1](#) (46). B728a and derivative mutant strains were grown at 28°C on King’s B (KB) agar or in KB broth (47). *E. coli* strains S17-1, TOP10, and XL1-Blue were grown on LB agar or in LB broth at 37°C. When appropriate, the following antibiotics were used at the indicated concentrations: 100 µg/ml rifampin, 50 µg/ml kanamycin, 15 µg/ml tetracycline, 100 µg/ml spectinomycin, and 40 µg/ml nitrofurantoin. A full list of strains, plasmids, and primers used in this study is contained in Table S4 in the supplemental material.

Complementation of the *mexB* deletion strain. Expression constructs of *mexAB* and *mexAB-OprM* were constructed through PCR amplification of this partial or complete operon, as well as of 1,161 bp of upstream sequence that would include potential promoter regions. These PCR products were ligated into the XbaI and EcoRI restriction enzyme sites of the plasmid p519ngfp (48). The ligation mixture was subsequently transformed into chemically competent *E. coli* XL1-Blue. Plasmids were confirmed to contain the correct insertion sequences by Sanger sequencing and were then electroporated into *E. coli* donor strain S17-1. Plasmids were introduced into B728a WT and B728a Δ *mexB* strains by conjugation on KB overnight and were then selected for 3 days on KB containing kanamycin and nitrofurantoin (*E. coli* counterselection).

Construction of bar-coded transposon libraries. Construction of the B728a WT barcoded transposon insertion library was described previously (32). The Δ *mexB* library was constructed in a similar fashion. For the Δ *mexB* library, we first removed the kanamycin resistance cassette from the B728a Δ *mexB* deletion mutant. The pFLP2 Ω plasmid, containing the Flp recombinase and a spectinomycin resistance cassette (49), was introduced into the Δ *mexB* mutant by conjugation. Exconjugants were selected on spectinomycin. Colonies were screened for the loss of kanamycin resistance by plating, and the loss of the kanamycin cassette in sensitive colonies was confirmed by PCR. The pFLP2 Ω plasmid was cured from the deletion strain by several overnight passages in KB containing rifampin only, and spectinomycin sensitivity was confirmed by plating. The rifampin-resistant Δ *mexB* deletion mutant was used as the recipient for conjugation with the barcoded *mariner* transposon library using the same protocol.

In vitro growth of the library. Aliquots of the transposon libraries that had been stored at –80°C were removed from cold storage, thawed, and inoculated into 25 ml fresh KB with 100 µg/ml kanamycin and grown for approximately 7 h at 28°C with shaking until the culture reached mid-log phase (optical density at 600 nm [OD₆₀₀] of 0.5 to 0.7). For time 0 samples, 1-ml aliquots were pelleted by centrifugation and the pellets were frozen at –20°C until DNA purification. All *in vitro* experiments were performed in 24-well plates containing 1 ml total volume per well. Each compound used was added to KB containing 100 µg/ml kanamycin to reach a final volume of 950 µl. Cell suspensions (50 µl) were added to each well last. Libraries were grown overnight (15 h) at 28°C with shaking. The cells from each well were then pelleted and frozen until DNA purification.

DNA isolation and library preparation. DNA from frozen pellets was isolated using a Qiagen DNeasy blood & tissue kit according to manufacturer's instructions. Cell lysis was performed at 50°C for 10 min per manufacturer instructions. Purified genomic DNA was measured on a NanoDrop device, and 200 ng of total DNA was used as a template for DNA barcode amplification and adapter ligation as established previously (30). For each time 0 sample, two separately purified DNA samples were sequenced as technical replicates.

Sequencing and generation of fitness data. Barcode sequencing, mapping, and analysis to calculate the relative abundances of barcodes were performed using the RB-TnSeq methodology and computation pipeline developed by Wetmore et al. (30; code available at bitbucket.org/berkeleylab/feba/). Briefly, TnSeq was used to map the insertion sites and for association of the DNA barcodes with these insertions. Fitness values for each gene were calculated as the \log_2 ratio of relative barcode abundance following library growth under a given condition divided by the relative abundance in the time 0 sample. Barcode counts were summed between replicate time 0 samples. For analysis, genes were required to have adequate coverage in the time 0 sample, i.e., at least 3 reads per strain and 30 reads per gene (30). The fitness values were calculated based on the "central" transposon insertions only, i.e., those within the central 10% to 90% of a gene. Fitness values were normalized across the genome such that the median gene had a fitness value of 0. All experiments described here passed previously described quality control metrics (30). Experimental fitness values are publicly available at fit.genomics.lbl.gov.

Identification of efflux pumps and assessment of fitness. We focused our initial analysis on genes that are homologous to *mexAB-oprM*. In the Transport Database (membranetransport.org) (33), B728a is annotated as containing 16 RND transporters. We manually curated this list of genes to focus on those operons potentially involved in "multidrug resistance," namely, known *mex* genes or operons containing an "acriflavin resistance" or "hydrophobe/amphiphile efflux-1" and HlyD secretion protein. We filtered out 2 pseudogenes and 6 genes encoding proteins with known or likely unrelated function: protein export proteins SecD and SecF (P syr_1230-1), a syringomycin efflux protein (P syr_2622), and heavy metal efflux pump CzcA (P syr_4803). *P syr_1128* is adjacent to a heavy metal (Cu/Ag) two-component system. *P syr_1701* is a hypothetical protein in the syringolin A biosynthesis gene cluster.

For the remaining eight RND operons, we compared fitness values for all genes within each operon across all experiments that passed quality control in both libraries. We focused on pumps where multiple encoding genes in the same operon contributed strongly to competitive fitness (fitness values of less than -1). As a negative control, the eight genes with expected functions unrelated to drug resistance were confirmed to not be required under any conditions tested (fitness values of less than -0.5 in both libraries). We performed the same analysis using 68 predicted MFS genes (encoding a mixture of potential drug resistance transporters as well as diverse sugar transporters), 210 ABC transporter genes, SMR gene *P syr_0541*, and *norA* (P syr_0073). Heat maps were plotted in R (50) using the *gplots* package, version 3.0.1.1 (51).

Construction of targeted deletion mutants. Deletion strains were constructed using an overlap extension PCR protocol as described previously (52). Briefly, 1-kb DNA fragments upstream and downstream of the genes of interest were amplified along with a kanamycin resistance cassette from pKD13 (53). These three fragments were joined by overlap extension PCR. The resulting fragment was blunt end ligated into the *Sma*I site of pTsacB (54) and transformed into *E. coli* subcloning strain TOP10 or strain XL1-Blue and then *E. coli* conjugation donor strain S17-1. This suicide plasmid was conjugated into B728 on KB overnight and was then selected for 3 days on KB containing kanamycin and nitrofurantoin. Putative double-crossover colonies that were kanamycin resistant and tetracycline sensitive were selected for screening using external primers and further confirmed by PCR and Sanger sequencing.

Drug susceptibility tests. The MICs of drugs for *P. syringae* strains were determined by growth of cells in 2-fold dilutions of test compounds in 96-well plates containing KB or M9 minimal medium supplemented with 0.2% (vol/vol) glycerol to reach a total volume of 200 μ l per well. Bacterial cultures were grown to mid-log phase, diluted to an OD₆₀₀ of 0.3, and washed three times in M9 before inoculation of 20 μ l into each well. A semipermeable sealing membrane (Breathe-Easy) was used to cover the plates, which were maintained at 28°C with shaking. Growth of bacteria was examined by visual inspection after 24 h in KB and 48 h in M9. Three or four replicates were measured for each strain, compound, and medium combination. For zone of inhibition assays, strains were grown overnight on KB agar containing rifampin, resuspended in 10 mM KPO₄, and diluted to an OD₆₀₀ of 0.1. A 200- μ l volume of the cell suspension was spread on each plate. Paper discs (6-mm diameter) were used to absorb the following antibiotics (vehicle): acriflavin (20 μ l of 3 mg/ml H₂O), berberine (30 μ l of 50 mg/ml DMSO), flavone (15 μ l of 20 mg/ml DMSO), and phloretin (30 μ l of 30 mg/ml DMSO). Three replicate discs were used per plate. Plates were incubated overnight at 28°C, and the diameter of the clearance zone was measured. Graphs were plotted in R using the *ggplot2* package version 3.1.1 (55).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02614-19>.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.1 MB.

FIG S4, PDF file, 0.1 MB.

FIG S5, PDF file, 0.1 MB.

FIG S6, PDF file, 0.1 MB.

TABLE S1, PDF file, 0.04 MB.

TABLE S2, PDF file, 0.04 MB.

TABLE S3, PDF file, 0.04 MB.

TABLE S4, PDF file, 0.1 MB.

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