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

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Triclosan is an antiseptic frequently added to items as diverse as soaps, lotions, toothpaste, and many commonly used household fabrics and plastics. Although wild-type *Pseudomonas aeruginosa* expresses the triclosan target enoyl-acyl carrier protein reductase, it is triclosan resistant due to expression of the MexAB-OprM efflux system. Exposure of a susceptible Δ(*mexAB-oprM*) strain to triclosan selected multidrug-resistant bacteria at high frequencies. These bacteria hyperexpressed the MexCD-OprJ efflux system due to mutations in its regulatory gene, *nfxB*. The MICs of several drugs for these mutants were increased up to 500-fold, including the MIC of ciprofloxacin, which was increased 94-fold. Whereas the MexEF-OprN efflux system also participated in triclosan efflux, this antimicrobial was not a substrate for MexXY-OprM.

Pseudomonas aeruginosa is a clinically significant pathogen, particularly in immunocompromised hosts (36). Infections caused by this bacterium are difficult to treat due to its many intrinsic and acquired antibiotic resistances. Intrinsic resistance is mostly attributable to the expression of several multidrug resistance (MDR) efflux systems. The *P. aeruginosa* genome (35) contains structural genes for at least 12 resistance nodulation type efflux systems, of which only 4, i.e., MexAB-OprM (27), MexCD-OprJ (26), MexEF-OprN (13), and MexXY (1, 21, 38), have been characterized. Exposure to selected substrates can select for their upregulated or constitutive expression (13, 14, 26, 38).

2-Hydroxyphenylethers are a class of compounds that exhibit broad-spectrum antimicrobial activity. Triclosan is the most potent and widely used member of this class (2, 5) and is used in hand soaps, lotions, toothpastes, and oral rinses, as well as in fabrics and plastics. It was long thought to act as a nonspecific "biocide" (29), but recent biochemical and genetic studies have shown that triclosan acts on a defined bacterial target in the fatty acid biosynthetic pathway, enoyl-acyl carrier protein (ACP) reductase (FabI) (7, 9, 10, 12, 18, 20) or its homolog InhA in mycobacteria (18). Some bacteria possess triclosan-resistant enoyl-ACP reductase homologs (FabK), and to date *P. aeruginosa* is unique among gram-negative bacteria in that it possesses both triclosan-sensitive and -resistant enzymes (8). Alterations in FabI active-site residues confer resistance to triclosan (9, 10, 20). Of particular concern is that such amino acid changes selected by exposure to triclosan lead to cross-resistance with other antimicrobial agents (9), including clinically used front-line drugs, since some mutations leading

to triclosan resistance in *Mycobacterium smegmatis* also caused resistance to isoniazid (18). Moreover, triclosan is a substrate of a multidrug efflux pump in clinical and laboratory *Escherichia coli* strains (19). We have recently shown that *P. aeruginosa* strain PAO1 is intrinsically resistant to triclosan by virtue of expression of the MexAB-OprM efflux pump (32), and the same is true for all strains of this species tested to date (our unpublished results).

While the contribution of antibiotic exposure to development of MDR due to efflux pump expression has clearly been documented in vitro and in vivo, little is known about antiseptic resistance mechanisms (30) and their possible contribution to MDR. In this paper we present results that triclosan is a substrate for multiple *P. aeruginosa* efflux pumps and that it is capable of selecting not just for mutants resistant to this particular antiseptic but, perhaps more importantly, also for MDR bacteria.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and molecular biology techniques. The bacterial strains used in this study are shown in Table 1. Unless otherwise noted, bacteria were grown at 37°C in Luria-Bertani (LB) medium or on LB agar (31) or in Mueller-Hinton broth (MHB; Difco, Detroit, Mich.). For plasmid maintenance, *P. aeruginosa* media were supplemented with 200 μg of carbenicillin/ml. Unmarked efflux pump-negative mutants were derived using a previously described Flp/*FRT* recombinase technology (11). The sources for the mutant alleles were pPS952 for Δ(*mexAB-oprM*) (32), pPS1008 for Δ(*mexCD-opJ*) (derived by deletion of a 6,138-bp region encompassing three *Cla*I fragments from pKMJ002 [26]), and pPS1128 for Δ(*mexXY*) (derived by deletion of a 2,868-bp DNA fragment encompassing several *Sal*I-*Xho*I fragments from pAMR-1 [38]). The chromosomal deletions were verified by PCR and genomic Southern analyses. Standard molecular biology methods were used (31). Plasmid pKMM128 is pAK1900 (28) expressing *oprM* (16).

Antimicrobial susceptibility testing. MICs were determined by the twofold broth microdilution technique according to National Committee for Clinical Laboratory Standards guidelines (22) or by the E-test system and the protocols provided by the supplier (AB Biodisk, Piscataway, N.J.) (ciprofloxacin and tetracycline only).

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype or characteristic	Source or reference
PAO1	Wild type; produces MexAB-OprM	37
PAO200	Δ(mexAB-oprM)	32
PAO200-2	PAO200 nfxB; overproduces MexCD-OprJ	This study
PAO200-3	PAO200 nfxB; highly overproduces MexCD-OprJ	This study
PAO200-4	PAO200 nfxB; highly overproduces MexCD-OprJ	This study
KG3056	nfxB; overproduces MexCD-OprJ	6
KG2239	PAO1 with Δ(mexR-mexAB-oprM)	16
N103	KG2239 with Δ(mexXY)	16
PAO-7H	Overproduces MexEF-OprN	13
PAO3579	PAO1 with ΔamrR (ΔmexZ)[a]	38
PAO238	PAO200 with Δ(mexCD-oprJ)	This study
PAO253	PAO-7H with Δ(mexAB-oprM)	This study
PAO255	PAO253 with Δ(mexEF-oprN)	This study
PAO267	PAO3579 with Δ(mexAB-oprM)	This study
PAO280	PAO267 with Δ(mexXY)	This study

[a] amrR is identical to mexZ (1).

Selection and characterization of triclosan-resistant mutants. For isolation of triclosan-resistant derivatives of Δ(mexAB-oprM) strain PAO200, cells were grown in LB medium to stationary phase (A_{540}, ~2.6). These cells were plated on *Pseudomonas* isolation agar (PIA; Difco) whose formulation contained 25 μg of triclosan/ml. After an overnight incubation at 37°C, the colonies growing on the PIA plates were counted. For PCR amplification of the *nfxB* coding region from genomic DNA templates, two primers were designed: nfxB-up (5′-ACAATCtAGAAAAACCAACCGGG), which contained a single base mismatch (lowercase t) and which introduced an *Xba*I site (underlined) 27 bp upstream of the *nfxB* start codon, and nfxB-down (5′-CCGGAATTCCTGG GGGAGGTG), which primes to a region centered 236 bp downstream of *nfxB* containing an *Eco*RI site (underlined). PCRs were performed using *Taq* DNA polymerase (Qiagen, Santa Clarita, Calif.). The 828-bp PCR fragments were cloned as *Xba*I-*Eco*RI fragments into pUCP21T (33). Nucleotide sequences were determined by automated sequencing in the University of Colorado at Boulder

sequencing facility. Extensions were primed utilizing the commercially available 24-nucleotide pUC/M13 reverse and forward sequencing primers for sequencing the cloned PCR fragments and the *nfxB*-up primer for the direct sequencing of PCR fragments. Computer-assisted sequence analyses were performed utilizing the SeqEd (Applied Biosystems, Foster City, Calif.) program.

Detection of outer membrane proteins. Cells of various *P. aeruginosa* strains were grown in LB medium to log phase (A_{540}, ~1.0). Samples of cells (1 ml) were harvested, centrifuged, and resuspended in the appropriate volumes of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 5% β-mercaptoethanol) to adjust for differences in cell densities. The resuspended cells were boiled for 4 min, and samples corresponding to ~25 μg of protein were analyzed by electrophoresis on 0.1% SDS–10% PAGE gel (pH 9.2) (15). The electrophoretically separated proteins were electroblotted onto nitrocellulose membranes, and the blots were processed as previously described (34). Hybridizing antibodies were detected using an antimouse antibody conjugated to horseradish peroxidase (HRP), and bound HRP activity was detected by exposure to luminogen substrate and X-ray film, according to the manufacturer's (Amersham, Arlington Heights, Ill.) protocol.

RESULTS AND DISCUSSION

Triclosan is a substrate for multiple MDR efflux pumps. Our previous study (32) indicated that triclosan is a substrate for MexAB-OprM. Since MDR efflux systems export a variety of structurally unrelated substrates (23), we hypothesized that triclosan may be a substrate not only for MexAB-OprM but also for other *P. aeruginosa* efflux pumps. Defined mutants were obtained, and their triclosan susceptibilities were assessed by MIC determinations (Table 2). Triclosan was a substrate for all tripartite efflux pumps analyzed in this study, including MexAB-OprM, MexCD-OprJ, and MexEF-OprN. Deletion mutants defective in these pumps all became triclosan susceptible. Mutant strain PAO267, expressing only

TABLE 2. Antimicrobial susceptibilities of *P. aeruginosa* strains used in this study

Strain (plasmid)	Efflux protein(s) expressed	MIC (μg/ml)[a] of:					
		TRI	TET	CIP	TMP	ERY	GEN
PAO1	MexAB-OprM	>128[b]	16	0.064	512	256	1.6
PAO200	None[c]	24[e]	0.5	0.008	32	8	0.2
PAO200-2	MexCD-OprJ	>128	40	0.375	1,024	1,024	0.2
PAO200-3	MexCD-OprJ	>128	>256	0.75	>1,024	>1,024	0.1
PAO200-4	MexCD-OprJ	>128	>256	0.75	>1,024	>1,024	0.1
PAO238	None	20[e]	0.75	0.006	32	32	0.2
PAO253	MexEF-OprN	>128	6	2	>1,024	16	0.2
PAO255	None	24[e]	0.5	0.012	16	16	0.2
PAO3579	MexXY	>128	16	0.025	512	512	>3.2
PAO267	MexXY	32	0.5	0.016	16	32	0.2
PAO280	None	32	0.19	0.012	16	16	0.2
PAO267(pAK1900)	MexXY	32	0.5	0.012	16	32	0.2
PAO267(pKMM128)	MexXY-OprM	64	48	0.19	128	512	>3.2
PAO280(pAK1900)	None	32	0.25	0.008	16	16	0.2
PAO280(pKMM129)	OprM	64	0.5	0.012	16	32	0.2
KG2239(pAK1900)	MexXY[d]	32	0.5	0.008	16	32	0.2
KG2239(pKMM128)	MexXY-OprM	32	16	0.047	32	256	1.6
N103(pAK1900)	None	32	0.25	0.008	16	16	0.2
N103(pKMM128)	OprM	32	0.5	0.012	16	32	0.2

[a] The MICs of triclosan (TRI), tetracycline (TET), ciprofloxacin (CIP), trimethoprim (TMP), erythromycin (ERY), and gentamicin (GEN) were determined by either the microdilution method (TRI, TMP, ERY, and GEN) or the E-test method (CIP and TET). Values shown represent the averages of at least two experiments. Cells containing pAK1900 and pKMM128 were pregrown in MHB medium with 200 μg of carbenicillin/ml; no carbenicillin was present during incubation with triclosan.
[b] Triclosan is insoluble in aqueous solutions at concentrations >128 μg/ml.
[c] None implies that neither of the hitherto-characterized efflux systems, i.e., MexAB-OprM, MexCD-OprJ, MexEF-OprN, or MexXY, is expressed. The expression status of any other chromosomally encoded efflux systems in these mutants is unknown.
[d] Recent data indicate that MexXY is not expressed at detectable levels unless cells are grown in the presence of certain antibiotics (16).
[e] When determined by the twofold serial dilution method, this value was 32 μg/ml; to obtain the indicated value, cells were grown in MHB containing triclosan increasing in 2-μg/ml increments, starting at 16 μg/ml.

MexXY, was triclosan susceptible and behaved the same as a strain (PAO280) expressing neither of the hitherto-characterized efflux pumps.

Since it has been proposed that MexXY requires OprM for function (1, 16, 21), we considered the possibility that strain PAO267 was not triclosan resistant because it lacks OprM. To test this hypothesis, we electroporated OprM-expressing pKMM128 and its vector control into PAO267 and its Δ(mexXY) derivative, PAO280. Only PAO267 containing pKMM128 effluxed tetracycline, gentamicin, erythromycin, trimethoprim, and ciprofloxacin (Table 2), indicating that it expressed a functional MexXY-OprM system. However, this strain did not efflux triclosan. The observed twofold increase in MIC from 32 μg/ml in the vector control to 64 μg/ml in the OprM-expressing strain was the same as the one observed in strain PAO280 harboring the same plasmids but lacking the MexXY system. We also tested KG2339/pKMM128, a strain known to express a functional MexXY-OprM system (16), and obtained similar results (Table 2). The MICs were slightly higher in the PAO267 background since MexXY expression is constitutive in this strain but inducible in KG2339 (16). These data conclusively demonstrated that triclosan was not a MexXY-OprM substrate.

Triclosan selects for multidrug-resistant *P. aeruginosa*. When susceptible cells of Δ(mexAB-oprM) strain PAO200 were exposed to triclosan, resistant mutants were readily obtained. To assess the frequency with which triclosan-resistant mutants were derived, we plated PAO200 cells on PIA medium and selected spontaneous triclosan-resistant mutants. Such mutants were obtained at a frequency of 10^{-6}. Three randomly picked triclosan-resistant derivatives, PAO200-2 to PAO200-4, were further analyzed, and all of them exhibited an MDR phenotype (Table 2), including resistance to the clinically administered drug ciprofloxacin, whose MIC for two of the three mutants analyzed was increased 94-fold.

Probing whole-cell extracts with anti-OprJ- and anti-OprN-specific monoclonal antibodies revealed that all three triclosan-resistant derivatives of PAO200 hyperexpressed OprJ but not OprN, demonstrating that their MDR phenotype was due to expression of the MexCD-OprJ efflux system (Fig. 1A). Although reference strain KG3056 was previously described as an OprJ type B hyperproducer (6), OprJ production in this strain was only a fraction of its expression in the three triclosan-resistant strains (Fig. 1A).

To genetically verify that the MexCD-OprJ efflux system was expressed in response to exposure of PAO200 to triclosan, we isolated two *mexCD-oprJ* deletion mutants, PAO238 and PAO239. These mutants no longer expressed OprJ (not shown), were triclosan susceptible, and lost their MDR phenotype (Table 2).

Triclosan selects for *nfxB* mutations. Expression of multidrug efflux systems is the result of exposure to antibiotics in both laboratory (6, 13, 26, 28) and clinical settings (39). Exposure of *P. aeruginosa* to norfloxacin selects for mutants which express MexCD-OprJ due to mutations in regulatory gene *nfxB* (6, 24, 26). Nucleotide sequence analysis of the PCR-amplified *nfxB* gene from strain PAO200 and its triclosan-resistant derivatives demonstrated that expression of the MexCD-OprJ efflux system in the triclosan-resistant mutant strains was indeed due to *nfxB* mutations (Fig. 1B). One strain, PAO200-4,
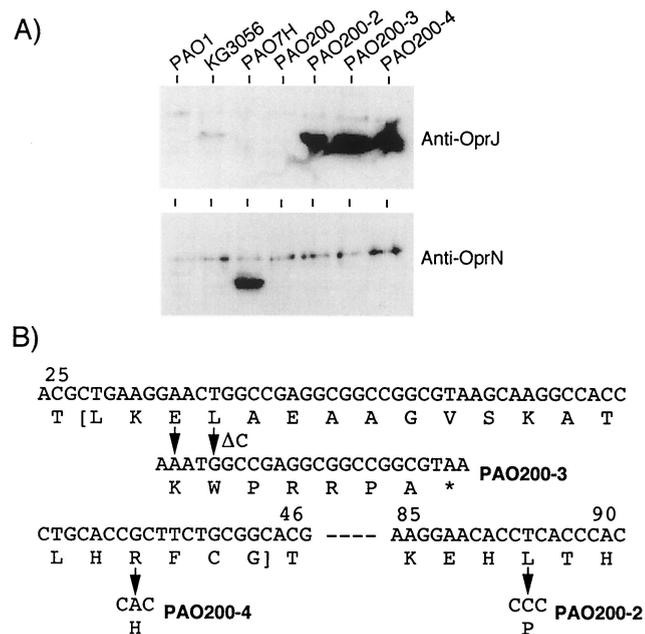

FIG. 1. Western blots of *P. aeruginosa* cell lysates and mutations causing triclosan resistance. (A) Standardized amounts of whole-cell lysates were separated on a 0.1% SDS–10% PAGE gel and electroblotted on nitrocellulose membranes, and the membranes were probed with monoclonal antibodies against OprJ and OprN. The strains analyzed were PAO1 OprM⁺; KG3056 OprJ⁺; PAO7H OprN⁺; PAO200, an OprM null PAO1 mutant (Δ[mexAB-oprM]); and PAO200-2, PAO200-3, and PAO200-4, spontaneous triclosan-resistant *nfxB* derivatives of PAO200. (B) Mutations leading to triclosan resistance. The *nfxB* genes from PAO200 and its three triclosan-resistant derivatives, PAO200-2, PAO200-3, and PAO200-4, were amplified by PCR from genomic DNA templates and sequenced. The *nfxB* sequence from each strain shown is the consensus obtained from six separate sequencing reactions; it was determined in duplicate from two separate clones, as well as in duplicate by directly sequencing the PCR products. Only portions of the *nfxB* sequence are shown, and codons are numbered as previously described (24). Arrows, changes from the PAO200 sequence. Amino acid residues constituting the putative helix-turn-helix DNA binding domain of NfxB are bracketed.

contained a mutation that affected the helix-turn-helix DNA binding domain of NfxB, and strain PAO200-2 contained a mutation elsewhere in *nfxB*. The third strain, PAO200-3, contained two mutations in the helix-turn-helix region, and one of them also caused a frameshift and early termination at codon 35 of *nfxB*. Some of the mutations previously isolated by exposure to norfloxacin affected similar regions of NfxB; an Arg-to-Gly change at amino acid residue 42 caused by norfloxacin (24) corresponded to an Arg-to-His change caused by triclosan. To confirm that triclosan resistance was solely caused by *nfxB* mutations, we transformed a plasmid expressing a wild-type *nfxB* gene into the three mutant strains. In all three transformed strains, the MICs were similar to the ones observed with strain PAO200 (data not shown).

Implications of efflux-mediated triclosan resistance. Our results show that *P. aeruginosa* possesses multiple triclosan resistance mechanisms. These include efflux via the MexAB-OprM, MexCD-OprJ, and MexEF-OprN systems and probably FabI target mutations (12). However, in contrast to that in *E. coli*, where exposure to triclosan readily selects *fabI* mutants and

overproduction of FabI leads to increased triclosan resistance (9, 10, 20), the first line of defense against triclosan in *P. aeruginosa* seems to be efflux and/or other hitherto-unknown resistance mechanisms, e.g., decreased outer membrane permeability (17). Whereas in *P. aeruginosa* overexpression of efflux pumps increased triclosan MICs by more than sixfold, overexpression of the AcrAB pump in *E. coli* increased the MIC only twofold (19). The MexXY system did not efflux triclosan, even in the presence of OprM.

Although possible links of cross-resistance between antiseptics and antibiotics due to efflux have been suggested before (19, 30), our studies demonstrate for the first time that exposure of a clinically significant bacterium to the antiseptic triclosan efficiently can select for MDR derivatives, including high-level resistance to an antipseudomonas drug. Exposures to antibiotics and triclosan select for similar regulatory mutations leading to expression of a multidrug efflux system. Although MexEF-OprN exports triclosan, we have not yet observed MexEF-OprN-expressing triclosan-resistant derivatives when plating either Δ(*mexAB-oprM*) strain PAO200 or Δ(*mexAB-oprM*) Δ(*mexCD-OprJ*) strain PAO238 on triclosan-containing medium. Since we have not systematically searched for MexEF-OprN-expressing derivatives of these strains, we cannot yet explain the apparent lack of such mutants. MDR *P. aeruginosa* is of foremost clinical importance since it is the leading cause of death in many hospital-acquired infections because of its intrinsic resistance to many antibiotics (36). Furthermore, most cystic fibrosis patients succumb to the debilitating effects of chronic *P. aeruginosa* infections due to eventual therapeutic failures caused by MDR-resistant bacteria (25). It has been well established that the massive prescription of antibiotics and their nonregulated and extensive usage are the main causes for the development of extensive antibiotic resistance in bacteria (3, 4). Since antimicrobial agents provide the selective pressure for the development of resistance, the control of antibiotic usage is essential to prevent the development of resistance to antibiotics. Our results raise the notion that widespread and unregulated use of triclosan may promote the selection of MDR bacteria and thus compound antibiotic resistance.

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