Bacterial Resistance to Antisense Peptide-Phosphorodiamidate Morpholino Oligomers

Running Title: Morpholino oligomer resistance

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Peptide phosphorodiamidate morpholino oligomers (PPMO) are synthetic DNA mimics that bind complementary RNA and inhibit bacterial gene expression. \((RFF)_3\text{RXB- AcpP}\) PPMO \((R,\ \text{arginine}; F, \text{phenylalanine}; X, 6\text{-aminohexanoic acid}; B, \beta\text{-alanine})\) is complementary to 11 bases of the essential gene \(acpP\) (encodes acyl carrier protein). The MIC of \((RFF)_3\text{RXB- AcpP}\) was 2.5 \(\mu\text{M}(14 \mu\text{g/ml})\) in \textit{Escherichia coli} W3110. The rate of spontaneous resistance of \textit{E. coli} to \((RFF)_3\text{RXB- AcpP}\) was \(4 \times 10^{-7}\) mutations/cell division. A spontaneous \((RFF)_3\text{RXB-AcpP}-\text{resistant mutant (PR200.1)}\) was isolated. The MIC of \((RFF)_3\text{RXB-AcpP}\) was 40 \(\mu\text{M}(224 \mu\text{g/ml})\) in PR200.1. The MICs of standard antibiotics were identical in PR200.1 and W3110. The sequence of \(acpP\) was identical in PR200.1 and W3110. PR200.1 was also resistant to other PPMOs conjugated to \((RFF)_3\text{RXB}\) or peptides with a similar composition or pattern of cationic and non-polar residues. Genomic sequencing of PR200.1 identified a mutation in \(sbmA\), which encodes an active transport protein. In separate experiments, a \((RFF)_3\text{RXB-AcpP}-\text{resistant isolate (RR3)}\) was selected from a transposome library, and the insertion was mapped to \(sbmA\). Genetic complementation of PR200.1 or RR3 with \(sbmA\) restored susceptibility to \((RFF)_3\text{RXB-AcpP}\). Deletion of \(sbmA\) caused resistance to \((RFF)_3\text{RXB-AcpP}\). We conclude that resistance to \((RFF)_3\text{RXB-AcpP}\) was linked to the peptide and not the PMO, dependent on the composition or repeating pattern of amino acids, and caused by mutations in \(sbmA\). The data further suggest that \((RFF)_3\text{R-XB}\) PPMOs may be transported across the plasma membrane by SbmA.
Introduction

Antibiotic resistance in bacteria continues to be a serious problem. The number of antibiotic-resistant pathogens is increasing, the level of resistance to standard antibiotics is increasing, and the percentage of isolates with resistance to multiple antibiotics has risen dramatically in recent years (3, 37). At the same time, the number of antibiotics that are being developed has decreased significantly, particularly for those targeting Gram-negative bacteria. Most of the new antibiotics that have been approved for use in the United States in the past 40 years are not new classes of antibiotics, but simply chemical derivatives of the same antibiotic classes that were discovered in the mid twentieth century (8). There is an urgent need for new antibiotics particularly for those with novel or innovative strategies of targeting bacterial pathogens that cause serious diseases (3, 22).

Genomics has created an attractive potential for developing innovative strategies that address the problem of antibiotic resistance. Synthetic antisense oligomers, such as peptide nucleic acids (14), phosphorothioates (16), and phosphorodiamidate morpholino oligomers (11, 15), silence expression of bacterial genes. Gene-silencing oligomers decrease expression of reporter genes such as luciferase, activate endogenous genes such as β-galactosidase, and inhibit growth and kill bacteria by targeting essential genes (10). Antisense oligomers targeted to specific, essential bacterial genes reduce infections and increase survival in mouse models of infection (12, 15, 40).

Antisense oligomers require assistance to cross the outer membrane of Gram-negative bacteria because of their molecular weight and polar characteristics. Short amphipathic peptides have been attached to antisense oligomers, and this has greatly improved their entry into Gram-negative bacteria and increased their potency (11, 13, 27).
Membrane-penetrating peptides have diverse sequences, but many are cationic and amphipathic. Previous investigations suggest that a repeated peptide motif with one cationic residue followed by either one or two hydrophobic residues may be an important feature for efficient membrane penetration (39). More recently, we have compared a variety of membrane-penetrating peptides for their abilities to enhance the efficacy of peptide-phosphorodiamidate morpholino oligomers (PPMO), and found differences among peptides that vary in their pattern of alternating cationic and nonpolar residues and their amino acid compositions (27).

Despite the progress that has been made on improving efficacy and potency of antisense oligomers, little is known about bacterial resistance to these compounds. Some naturally-occurring antimicrobial peptides, which have some similar characteristics to the synthetic peptides used to make peptide-oligomers, do not appear to cause resistance in bacteria (38). One report of resistance to an antisense morpholino oligomer found a mutation in the region of a virus genome targeted by the oligomer (28). Resistance to any antibiotic is always an important characteristic to be determined during drug development. Ultimately the frequency of antibiotic resistance will manifest itself in the clinic, and will play a role in its use for any particular indication.

In this report, we characterize spontaneous resistance to a peptide-PMO (PPMO) and compare cross-resistance to other antibiotics, PPMOs with different peptides but the same PMO, and PPMOs with the same peptide but targeted to different genes. Furthermore, the same gene that causes PPMO-resistance is identified in isolates from two independent strategies of selection.
Material and Methods

Bacterial strains. Wild type K-12 *E. coli* W3110 was used for selecting spontaneous mutants that are resistant to the PPMO (RFF)$_3$RXB-AcpP. Spontaneous mutants that are resistant to (RFF)$_3$RXB-AcpP were selected by growth in Mueller-Hinton II broth supplemented with at 8 x MIC of (RFF)$_3$RXB-AcpP. Liquid cultures were grown in either Mueller-Hinton II or LB broth. LB agar was used for growth on solid medium. Transformants with pSE380myc-luc (11) were grown in LB supplemented with 50 µg/ml ampicillin (Sigma-Aldrich, St. Louis, MO).

Oligopeptide transport mutants PA0183 (*opp*), PA0333 (*opp dpp*), PA0410 (*opp tpp*), PA0643 (*opp dpp tpp*), and PA0610 (*opp dpp tpp*), which were derived from parent strain Morse 2034 (*trpE9851 leu 277 F–*IN(*rrnD–rrnE*)) have been described (36) and were gifts from J. W. Payne (University of Wales, Bangor, UK).

In-frame, non-polar knock-out strains *E. coli* JW3496 (*dctA*), JW5730 (*eptA*), and JW0368 (*sbmA*) and their isogenic parent strain BW25113 (2), were provided from the Keio collection by the National BioResource Project (NIG, Japan). The knock-out strains were grown in LB broth with 50 µg/ml kanamycin (Sigma-Aldrich). The IPTG-inducible *sbmA* expression plasmid (which we call pSbmA, from strain b0377) and empty vector control (pNTR-SD) (34) were also provided by the National BioResource Project (NIG, Japan) and grown in LB broth with 20 µg/ml ampicillin.

PPMO. PPMOs were synthesized at AVI BioPharma (Corvallis, Oregon) as described (39). The base sequence of all PPMOs targeted to *acpP* (AcpP) is 5’-CTTCGATAGTG-3’, to *ftsZ* (FtsZ) is 5’-TCCATTGGTTC-3’, and to *luc* (Luc) is 5’-AACGTTGAGIG. Inosine in place of
a guanine in the Luc PPMO was necessary to make the oligomer soluble in aqueous solutions by avoiding guanine quartet structure. The scrambled base sequence control (Scr) is 5’-TCTCAGATGGT-3’.

**Antibiotics.** Antibiotics were purchased from Sigma-Aldrich, except bleomycin (Enzo Life Science, Farmingdale, NY).

**Minimal inhibitory concentration.** Minimal inhibitory concentration was determined by the microdilution method (5) in Mueller-Hinton II broth. For MICs using XL1-Blue MRF’ and RR3, Mueller-Hinton II broth was supplemented with 1% tryptone.

**Luciferase expression.** Spontaneous (RFF)₃RXB-AcpP-resistant mutants were made chemically competent and transformed as described (29) with pSE380myc-Luc (11). Overnight cultures were grown aerobically at 37°C in LB medium plus 50 µg/ml ampicillin (LBA), and then diluted 2 x 10⁻² into LBA with or without various concentrations (8, 20 and 50 µM) of (RFF)₃RXB-Luc or (RFF)₃RXB-Scr, and grown aerobically at 37°C for 7 h. Samples were analyzed for luciferase expression by luminometry as described (11).

**Rate of spontaneous resistance.** The rate of spontaneous resistance to peptide-PMO was measured by the method of Luria and Delbruck (23) as described (33). An overnight culture was diluted to 1 x 10⁴ cfu/ml in LB medium and divided into 20 x 1 ml aliquots. Each aliquot was grown overnight at 37°C with aeration, and then 1 µl or 50 µl of each was spread on 20 agar plates (60 mm x 15 mm) of LB plus 20 µM peptide-PMO. Plates were grown overnight at 37°C with aeration, and colonies were enumerated.

**Screening transposome mutants.** EX-Tn5<R6Kyo/Kan-2>Tnp (Epicentre, Madison, WI) was electroporated into *E. coli* XL1-Blue MRF’, and 3 x 10³ transductants were selected on LB kanamycin (15 µg/ml) plates. The transductants were pooled and stored in
PBS + 15% glycerol at -75°C. The pooled transductants were thawed and 1 x 10^4 cfu was spread on an LB plate that included 20 μM (RFF)_3RXB-AcpP. Insertion mutations from PPMO-resistant mutants were sequenced by rescue cloning as described by the manufacturer (Epicentre). Insertions in sbmA were confirmed using polymerase chain reaction (PCR) as described (18). Briefly, PCR reactions contained chromosomal DNA extracted from bacteria using a commercial kit (DNAeasy, Qiagen, Valencia, CA) and primers (IDT Technologies, Coralville, IA) that flank the insertion site: 5' - GATTGCCGTTATCTTCTGGC and 5' - GCTCAAGGTATGGGTTACTTCC. Thirty PCR reaction cycles were: denature, 95°C, 0 sec; anneal 45°C, 0 sec; extend 72°C, 1 min. PCR reactions were run on a 1605 air thermo-cycler (Idaho Technology, Idaho Falls, ID).

**Sequencing acpP.** The acpP allele from each strain analyzed was amplified by polymerase chain reaction (18) using as template a single bacterial colony picked from a growth plate, Promega Taq polymerase (Madison, WI), and the following primers (Invitrogen, Carlsbad, CA): 5’-AACGTAAAATCGTGGTAAGACC-3’, and 5’-TAACGCCTGTTGGCGTTGATG-3’. The PCR products were gel-purified using Qiagen MinElute PCR purification kit (Valencia, CA), and sequenced using the same primers shown above at the core laboratory of the Center for Genome Research, Oregon State University.

**Genomic sequencing.** Genomic DNA from the W3110 wild type and PR200.1 was generated by standard procedures (1). DNA was sheared by sonication and processed for Illumina high-throughput sequencing as previously described (31, 32). Data analyses to find individual point mutations were carried out as described (32).
Results

Spontaneous mutants resistant to peptide-PMO. Spontaneous resistance was apparent from growth that occasionally occurred in some cultures that included (RFF)$_3$RXB-AcpP (X is 6-aminohexanoic acid and B is β-alanine) at concentrations above the MIC. Growth above the MIC was never observed in cultures that included other AcpP PPMOs with different peptides attached to the same PMO, such as (RX)$_6$B-AcpP or (RXR)$_4$XB-AcpP. The rate of spontaneous resistance to (RFF)$_3$RXB-AcpP was measured and found to be $4 \times 10^{-7}$ mutations/cell generation.

Susceptibility to antibiotics and growth rate. Colonies were isolated from a single liquid culture of W3110 grown with $8 \times$ MIC (20 μM = 112 μg/ml) of (RFF)$_3$RXB-AcpP. One colony (PR200.1) was picked at random and further characterized. PR200.1 was equally susceptible as the parent strain W3110 to each antibiotic tested (MIC = 4 μg/ml, 1.25 μg/ml, 1.25 μg/ml, 0.125 μg/ml, and 10 μg/ml for ampicillin, tetracycline, kanamycin, polymyxin B, and rifampin, respectively). These results indicate that this particular PPMO-resistant isolate was not resistant to antibiotics in general.

Doubling times of PR200.1 and W3110 were identical and no difference in growth rate was observed in liquid or solid media.

Sequences of acpP alleles. The target of the PMO, acpP, was sequenced in PR200.1 and W3110 and found to be identical (data not shown).

MICs of AcpP PPMOs attached to various peptides. MICs for different AcpP PPMOs were measured using PR200.1 and W3110 as indicators (Table 1). All of the AcpP PPMOs tested had the same base sequence, but had different peptides attached. The attached peptides differed not only in their amino acid compositions, but also in the pattern of...
repeating sequences of cationic and nonpolar residues. Repeating patterns of amino acids, often including cationic and non-polar residues, are important features of membrane-penetrating peptides (17, 41, 42). Four of the AcpP PPMOs, including (RFF)₃RXB-AcpP, had peptides with a repeating amino acid motif of cationic-nonpolar-nonpolar (C-N-N), and one of these was composed of D- instead of the usual L-amino acids. One AcpP PPMO was conjugated to (RX)₆B, which has a repeating motif of cationic-nonpolar (C-N). Two other AcpP PPMOs were conjugated to peptides with a repeating motif of cationic-nonpolar-cationic (C-N-C): (RXR)₄XB or (RFR)₄XB. Another AcpP PMO was conjugated to RTRTRFLRRTXB, which does not conform to any of the other repeat patterns. All of these PPMOs with various peptides attached to the same AcpP PMO have been previously characterized and found to be effective in inhibiting growth of *E. coli* (27).

The results show that PR200.1 was resistant to every AcpP PMO tested with the C-N-N peptide motif, but was fully susceptible to (RX)₆B-AcpP PMO and (RXR)₄XB-AcpP PMO (Table 1). However, PR200.1 was resistant to (RFR)₄XB-AcpP, which shares the C-N-C motif with (RXR)₄XB-AcpP, but like (RFF)₃RXB-AcpP contains phenylalanine instead of 6-aminohexaonoi acid. Compared to the susceptible parent strain W3110, resistance to (RFF)₃RXB-AcpP in PR200.1 increased the MIC 16-fold. PR200.1 was also resistant to the D-isomeric form of (RFF)₃RXB. The MIC of RTRTRFLRRTXB-AcpP, which lacks a repeating amino acid motif but includes one phenylalanine, increased only 2-fold using PR200.1 compared to W3110 as indicator. The MICs of scrambled-base sequence PPMOs composed with each of the same peptides used for the AcpP PPMOs were undetectable (>80 μM) in every case.
(RFF)3RXB-PPMOs targeted to various genes. PR200.1 was tested for susceptibility to two PPMOs, each with the (RFF)3 peptide motif, but different base sequences. One PPMO is complementary to ftsZ, which is an essential gene involved in cell division. The other PPMO is targeted to a luciferase reporter gene (luc).

Exponential cultures were grown for 18 h with (RFF)3RXB-FtsZ, which is targeted to ftsZ, or a scrambled (Scr) base sequence control. Samples of each culture were then plated, and viable cells counted. PR200.1 grew to normal cell density, whereas the viable cell count of the parent strain W3110 was reduced by over 2 orders of magnitude in the presence of (RFF)3RXB-FtsZ (Figure 1). The Scr had no effect on growth of either W3110 or PR200.1.

In other experiments, exponential cultures of W3110 and PR200.1 were grown for 7 h with various concentrations of (RFF)3RXB-Luc, which is targeted to a luciferase reporter gene, or the scrambled base control (RFF)3RXB-Scr. A plasmid that expresses luciferase had been transferred into PR200.1 prior to the experiment. Samples of each culture were then analyzed by luminometry for luciferase activity. The results show that (RFF)3RXB-Luc did not inhibit luciferase in PR200.1 at any of the 3 concentrations tested (Figure 2). In comparison, W3110 showed inhibition of luciferase that was proportional to the concentration of PPMO added. The scrambled sequence control did not inhibit luciferase in either strain. There were no differences in the growth (optical density) of any of the cultures (data not shown).

Peptide transport mutants. The above results indicated that resistance to PPMOs is linked to the peptide moiety. We hypothesized that PPMO-resistance could be caused by a mutation in one of three known oligopeptide transporters. To test this, the MIC was measured using various strains with mutations in one, two, or all three oligopeptide transporters (Table
The results show that (RFF)₃RXB-AcpP had the same MIC in PR200.1 as the parent (non-mutant) strain. (RFF)₃RXB-Scr scrambled control showed no detectable MIC (>160 μM).

**Genomic sequencing.** The genomes of PR200.1 and its parent strain W3110 were sequence and compared. The results indicated that a total of 3 genes had mutations in PR200.1 compared to W3110: dctA, eptA, and sbmA. In dctA, there were 2 transition mutations at bases 3958154 (T→A) and 395153 (A→G), both of which are in codon 396, that caused a missense from ile to ala. In eptA, there was one transversion mutation at base 4339795 (T→A) that affected codon 259 and caused a missense from ser to thr. In sbmA, there was one transversion mutation at base 396121 (T→G) that changed codon 87 (ser to ala). No deletions or insertions were detected in any gene.

**Characterize deletion mutants.** In frame knock-out mutants of dctA, eptA, and sbmA were tested for susceptibility to (RFF)₃RXB-AcpP and compared to the parent strain (BW25113). The MIC of (RFF)₃RXB-AcpP was the same (2 μM = 11 μg/ml) using either the dctA⁻ or eptA⁻ knock-out strains or the parent strain. The MIC using the sbmA⁻ strain was 32 μM (179 μg/ml).

The MIC of (RXR)₄XB-AcpP was measured using sbmA⁻ strain as indicator, and found to be 2 μM (11 μg/ml), the same as its isogenic parent strain.

**Complementation with pSbmA.** PR200.1 was genetically complemented with an IPTG-inducible expression plasmid that encodes sbmA (pSbma), or its empty control. The complemented strain was grown with IPTG and used to measure the MIC of (RFF)₃RXB-AcpP. The MIC was 1 μM and 32 μM in the induced, sbmA⁺ complemented strain and the empty vector control strain, respectively.
Transposome mutants. *E. coli* XL1-Blue MRF’ was mutagenized with the transposome EZ-Tn5 and a library of $1 \times 10^4$ mutants was spread on selection plates that included 20 μM (RFF)$_3$R-AcpP. Two colonies grew on the selection plate, and the mutated gene in each was sequenced. The sequences of both isolates indicated that the transposome had inserted into the exact same position in *sbmA* in each isolate, suggesting that the two colonies were clones. The isolates were named RR3.

RR3 was characterized by measuring the MICs of various standard antibiotics (Table 3). All standard antibiotics tested had the same MIC using either XL1-Blue MRF’ or RR3 as indicator, including two peptide antibiotics colistin and polymyxin B. However, RR3 was about 4-fold resistant to each of the peptide antibiotics bleomycin and phleomycin. PR200.1 was also 4-fold resistant to bleomycin (MIC = 5.6 μM [8 μg/ml]) and phleomycin (MIC = 5.2 μM [8 μg/ml]) compared to W3110 (bleomycin MIC = 1.4 μM [2 μg/ml]; phleomycin MIC = 1.3 μM [2 μg/ml]).

The MICs of (RFF)$_3$R-AcpP and (RXR)$_4$XB-AcpP were measured using RR3 or XL1-Blue MRF’ as indicators. The results show that RR3 was 32-fold resistant to (RFF)$_3$RXB-AcpP, and 8-fold resistant to (RXR)$_4$XB-AcpP (Table 3). RR3 was also resistant to the PPMO made with D-amino acids ([D-(RFF)$_3$R]XB-AcpP). Scrambled-sequence control PPMOs did not inhibit growth of either RR3 or XL1-Blue MRF’.

RR3 and XL1-Blue MRF’ were genetically complemented with pSbmA and used to measure the MIC of (RFF)$_3$RXB-AcpP. pSbmA fully restored susceptibility of RR3 to the PPMO, when induced with IPTG (Table 3). Interestingly, the MIC was significantly less using the complemented strains as indicators compared to the strains without pSbmA. Complementation with pSbmA also restored susceptibility to bleomycin and phleomycin.
Discussion

This is the first report we are aware of to characterize bacterial resistance to an antisense antibacterial compound. Initially, growth was occasionally and unexpectedly observed during routine MIC assays of \((RFF)_3XB\)-AcpP in cultures 4- to 8-fold above the MIC. Similar growth was never observed during MIC assays for \((RXR)_4XB-AcpP\) or \((RX)_6B-AcpP\). We speculate that the greater number of X residues (6-aminohexanoic acid) or the lack of F in the latter two PPMOs may be responsible for the apparent lack of spontaneous resistance to these PPMOs under the conditions used for the MIC assay. Alternatively, there could be more genetic loci involved in resistance to \((RFF)_3RXB\) PPMOs than in any (putative) resistance to PPMOs conjugated to other peptides such as \((RXR)_4XB\) or \((RX)_6B\). However, we have not yet rigorously pursued resistance to these later PPMOs, and it is certainly possible that spontaneous resistance may occur under appropriate conditions.

The rate of spontaneous resistance to \((RFF)_3RXB\)-AcpP was similar to the rate of spontaneous mutation for individual genes in \(E.\ coli\), which is typically between \(10^{-6}\) to \(10^{-7}\) mutations/gene/generation \((6, 24)\). This suggests that there are few genes which, when mutated, can give rise to the PPMO-resistant phenotype. However, the rate of mutation can vary widely, and any measurement of the rate of mutation is a function of many variables \((25)\), including the concentration of antibiotic used for selection, and the number of genes or loci capable of causing a resistance phenotype. Ultimately, the rate of resistance to PPMOs under in vivo conditions for specific infections will be the most meaningful measure of their usefulness in the clinic.
The spontaneous mutant PR200.1 was susceptible to all small molecule antibiotics tested. This shows that resistance to the PPMO is not caused by a change in physiology that might result in resistance to antibiotics in general. Such general changes are known to occur, and include a reduction of the net negative charge of the lipopolysaccharide of Gram-negative bacteria (7, 30), changes in capsule polysaccharide (4), changes in expression of outer membrane porins (30), alterations in outer membrane lipid composition that results in decreased membrane permeability (30), and activation or overexpression of multidrug efflux pumps (21). We tested a variety of antibiotics, some of which are hydrophilic (ampicillin, kanamycin), hydrophobic (rifampin, tetracycline), or amphiphilic (polymyxin B), and some which enter Gram-negative bacteria through the outer membrane porins (ampicillin, tetracycline) or through the outer membrane lipid bilayer (rifampin, polymyxin B). The results suggested that the mutation in PR200.1 is specific for (RFF)$_3$RXB-AcpP or PPMOs with similar peptide moieties. Later, following the identification of the mutation in $sbmA$, PR200.1 was found to be mildly resistant (4-fold) to the peptide antibiotics bleomycin and phleomycin. We hypothesized that resistance was caused by a mutation in the sequence of $acpP$ targeted by the PPMO. We have previously shown that a one-base mismatch near the 3’end of a PPMO targeted to $acpP$ in Burkholderia cepacia complex raised the MIC by a factor of at least 8-32-fold (15). However, the results in the present report found no mutation in the target region of $acpP$ in this one resistant mutant. Therefore, the hypothesis in this case was disproven. However, this is not to say that target site mutations cannot or do not occur on other as of yet uncharacterized mutants. Nevertheless, target site mutations would be statistically improbable considering that there are only 4 wobble bases in the target region of
that might possibly lead to a decrease in efficacy without changing the amino acid sequence of the targeted protein.

Another hypothesis was that resistance in PR200.1 was caused by a mutation in an oligopeptide transporter. However, oligopeptide transport mutants were just as susceptible to (RFF)$_3$RXB-AcpP as the isogenic parent strain. This showed that resistance in PR200.1 was not caused by a mutation in the known oligopeptide transporters that were tested.

In another effort to identify the mutation in PR200.1 that is responsible for resistance to (RFF)$_3$RXB-AcpP, the genome of PR200.1 was sequenced. The results showed missense mutations in only 3 genes compared to the PPMO-susceptible strain: dctA, eptA, and sbmA. In-frame, non-polar deletion mutations of each gene showed that of the three, only the sbmA deletion strain was resistant to (RFF)$_3$RXB-AcpP. Furthermore, complementation of PR200.1 with sbmA restored susceptibility to the PPMO. These results show that mutations in sbmA cause resistance to (RFF)$_3$RXB-AcpP.

The MIC of (RFF)$_3$RXB-AcpP was slightly lower using the sbmA deletion strain than PR200.1 as indicator. However, the strains originated from different parent strains, and this probably accounts for the difference in susceptibility. The parent strain of the sbmA deletion was also slightly more susceptible to the PPMO than the parent strain of PR200.1.

The transposome mutant RR3 was resistant to both (RFF)$_3$RXB-AcpP and (RXR)$_4$XB-AcpP. This differs from the spontaneous mutant PR200.1 and the sbmA deletion strain, which were resistant to (RFF)$_3$RXB-AcpP but not (RXR)$_4$XB-AcpP. This suggests that a polar affect in RR3 on the gene downstream from sbmA (yaiW) may be responsible for resistance to (RXR)$_4$XB-AcpP. yaiW is a predicted DNA-binding transcriptional regulator. Apparently a mutation in sbmA is sufficient to cause resistance to (RFF)$_3$RXB-AcpP, but not (RXR)$_4$-AcpP.
PR200.1 was susceptible to (RXR)$_4$XB-AcpP but resistant to (RFR)$_4$XB-AcpP, although these two PPMOs share the same C-N-C repeat motif. This indicates that the amino acid composition of the PPMOs may be more important than the repeating pattern of amino acids in determining resistance in PR200.1. The similarity in resistance of PR200.1 to either (RFF)$_3$RXB-AcpP or (RFR)$_4$XB-AcpP, but complete susceptibility to (RXR)$_4$XB-AcpP may suggest that X (6-aminohexanoic acid) accounts for the difference. This is supported by the result (Table 1) that resistance to (RXX)$_3$RXB-AcpP is 4-fold less than resistance to (RFF)$_3$RXB-AcpP, despite having the same repeating pattern of cationic and non-polar amino acids, but containing more X. Perhaps the unusual 6-carbon backbone of X causes a conformational change that disallows interaction with SbmA. Alternatively, sbmA mutants seem to be more resistant to peptides with F (phenylalanine). There is a positive trend between the number of F in the peptide and resistance. This is supported by the results that show higher resistance to PPMOs with more F (such as (RFF)$_3$RXB-AcpP and (RFR)$_4$XB-AcpP), lesser resistance to PPMOs with fewer F (such as RTRTRFLRRTXB-AcpP), and no resistance to PPMOs with no F (such as (RXR)$_4$XB-AcpP and (RXR)$_6$XB-AcpP), although (RXX)$_3$RXB-AcpP is an exception to this trend.

$sbmA$ encodes an active transporter for bleomycin and other peptide antibiotics (19, 26, 35, 43). Our results are consistent with SbmA acting as the active transporter for (RFF)$_3$RXB-AcpP. $sbmA$ homologs are widely conserved among bacteria (9). The homolog of $sbmA$ in *Rhizobium meliloti*, $bacA$, is required for symbiosis with alfalfa (9). The homolog in *Brucella abortus* is a virulence factor important for intracellular survival in macrophages (20). It has been proposed that the physiological substrates of SbmA are organic signaling
molecules (43). Development of an assay to measure uptake of PPMOs is currently not available, but could be used to define further the role of SbmA in resistance to PPMO.

The substrate specificity of SbmA has been investigated and found to be quite flexible. Initially, the specificity was proposed to be associated with a thiazole or oxazole structural motif (43). Later, proline-rich antimicrobial peptides were shown to be transported by SbmA (26). However, (RFF)₃RXB-AcpP has none of these structural features. If SbmA is the transporter of (RFF)₃RXB-AcpP, apparently the specificity of SbmA is not limited to thiazole-or oxazole-containing compounds or to proline-rich peptides. Our results suggest that the substrate specificity of SbmA is flexible enough to accommodate polypeptides without thiazole, oxazole, or proline. With the peptides we used in our conjugates, the specificity appears to be linked to the spacing of cationic and non-polar amino acid residues within the context of the peptide. It is also noteworthy that our all-D enantiomer conjugate (NG-05-0653) had the same MIC values as the all-L conjugate (NG-05-0200) for parental and resistant strains. This is in contrast to results shown for an all-D isomer of the proline-rich antimicrobial peptide Bac7(1-35), which was ineffective compared to the all-L form (26). It was suggested that the stereospecificity of Bac7(1-35) was attributable to its interaction with SbmA, although uptake of all-D Bac7(1-35) was not demonstrated. Perhaps the stereospecificity of Bac7(1-35) is caused by its interaction with its cytoplasmic target and not SbmA. Our results suggest that the specificity of SbmA is not necessarily limited to either the L- or D-enantiomeric form of a peptide, and is broader than previously known.

If SbmA is the plasma membrane transporter for (RFF)₃R-AcpP, we speculate that other mechanisms exist for PPMOs to cross the plasma membrane. PR200.1, RR3, and the sbmA deletion strain are still somewhat susceptible to (RFF)₃RXB-AcpP, albeit at high
concentrations. We speculate that PPMOs may be able to cross the plasma membrane by passing through the lipid bilayer in the same manner that they cross the outer membrane. There also may be additional active transporters with specificities for nucleic acid oligomers. This latter possibility is suggested by the ability of PMOs (not conjugated to a peptide) to inhibit gene expression in strains with porous outer membranes that allow passage of large oligomers (11, 12).

In summary, the results suggest that bacterial resistance to a PPMO can be determined by the peptide and not the PMO. The rate of occurrence of spontaneous resistance to (RFF)$_3$RXB-AcpP is similar to that of spontaneous changes in other bacterial phenotypes. In PR200.1 and RR3, resistance is caused by mutations in sbmA. Our results in combination with the known role of SbmA in peptide antibiotic uptake, suggest that SbmA acts as a transporter of (RFF)$_3$RXB-AcpP from the periplasm to the cytoplasm.

**Acknowledgement**

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References


Figure 1. **Viable Cell Count of 18 h Cultures.** Stationary cultures of W3110 or PR200.1 were diluted to $5 \times 10^5$ CFU/ml in Mueller-Hinton broth and divided in three. (RFF)$_3$RXB-FtsZ (FtsZ) or scrambled (Scr) PPMO (160 μM), or no PPMO was added. Cultures were grown aerobically at 37°C for 18 h, and then samples of each were diluted and plated to determine viable cells. Error bars indicate standard deviation. *indicates highly significant ($P < 0.01$) difference compared to either no PPMO or Scr-treated culture.
Figure 2. **Luciferase Activity of Cultures Treated with PPMO.** Growing cultures were treated for 7 h without PPMO (No PPMO), or with 3 concentrations of a PPMO \[(RFF)_3RXB-Luc\] targeted to a luciferase reporter gene, or a scrambled sequence control \[(RFF)_3RXB-Scr\]. After 7 h samples of each culture were measured for luciferase activity by luminometry. The experiment was repeated 3 times and the error bars indicate standard deviation. *indicates a highly significant \((P < 0.01)\) difference compared to cultures of PR200.1 with the same concentrations of \((RFF)_3RXB-Luc\), the cultures of W3110 with the same concentrations of \((RFF)_3RXB-Scr\), or the culture without PPMO.
Table 1. MIC of AcpP PPMO in Pure Cultures of *E. coli*

<table>
<thead>
<tr>
<th>PPMO No. NG- Conjugated Peptide*</th>
<th>MIC (µM [µg/ml])</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W3110</td>
<td>PR200.1</td>
</tr>
<tr>
<td>Motif 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C-N-N)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05-0200 RFFRFFRFFRXB</td>
<td>2.5 [14]</td>
<td>40 [222]</td>
</tr>
<tr>
<td>05-0653 DRdFdFdRdFdFdRdFdFRXB</td>
<td>2.5 [14]</td>
<td>40 [222]</td>
</tr>
<tr>
<td>23-248 RXXRXXRXXRXB</td>
<td>20 [102]</td>
<td>80 [204]</td>
</tr>
<tr>
<td>06-0199 KFKKFKKFKKXB</td>
<td>10 [54]</td>
<td>80 [435]</td>
</tr>
<tr>
<td>Motif 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C-N)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motif 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C-N-C)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>07-0795 RFRFFRRFRRFRXB</td>
<td>1 [6]</td>
<td>16 [94]</td>
</tr>
<tr>
<td>No Motif</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05-0246 RTRTRFLRRTXB</td>
<td>20 [111]</td>
<td>40 [111]</td>
</tr>
</tbody>
</table>

*X is 6-amino-hexanoic acid. B is β-alanine. O is ornithine. D indicates the isomeric form or the residue that follows.

**Motif 1 is (cationic-nonpolar-nonpolar, abbreviated C-N-N). Motif 2 is (cationic-nonpolar, abbreviated C-N). Motif 3 is (cationic-nonpolar-cationic, abbreviated C-N-C).
Table 2. MIC of (RFF)$_3$R-AcpP (NG-05-0200) Using Oligopeptide Transport Mutants

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Mutation/Phenotype</th>
<th>MIC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morse 2034</td>
<td>Wild-type oligopeptide transport</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>PA0183</td>
<td>opp$^{-}$/oligopeptide permease deletion</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>PA0333</td>
<td>dpp$^{-}$, opp$^{-}$/PA0183 plus dipeptide permease deletion</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>PA0643</td>
<td>tpp$^{-}$, dpp$^{-}$, opp$^{-}$/PA0333 plus tripeptide permease mutant</td>
<td>5</td>
<td>28</td>
</tr>
</tbody>
</table>
Table 3. MIC of standard antibiotics and PPMOs using transposome mutant RR3, isogenic parent strain XL1-Blue MRF’, and their \textit{sbmA} complemented strains

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>XL1-Blue MRF’</th>
<th>RR3</th>
<th>XL1-Blue MRF’ (pSbmA)</th>
<th>RR3 (pSbmA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B</td>
<td>0.8 [1]</td>
<td>0.8 [1]</td>
<td>0.8 [1]</td>
<td>0.8 [1]</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.9 [1]</td>
<td>0.9 [1]</td>
<td>0.9 [1]</td>
<td>0.9 [1]</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>0.2 [0.25]</td>
<td>0.7 [1]</td>
<td>0.01 [0.016]</td>
<td>0.01 [0.016]</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>0.3 [0.5]</td>
<td>1.3 [2]</td>
<td>0.02 [0.03]</td>
<td>0.02 [0.03]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPMO No.</th>
<th>PPMO No.</th>
<th>PPMO</th>
<th>MIC (μM [μg/ml])</th>
</tr>
</thead>
<tbody>
<tr>
<td>06-0076</td>
<td>(RXR)₄XB-AcpP</td>
<td>1 [6]</td>
<td>8 [48]</td>
</tr>
<tr>
<td>05-0655</td>
<td>(RFF)₃RXB-Scr</td>
<td>&gt;128 [&gt;714]</td>
<td>&gt;128 [&gt;714]</td>
</tr>
<tr>
<td>06-0078</td>
<td>(RXR)₄XB-Scr</td>
<td>&gt;128 [&gt;714]</td>
<td>&gt;128 [&gt;714]</td>
</tr>
</tbody>
</table>