1 Bacterial Resistance to Antisense Peptide-Phosphorodiamidate Morpholino Oligomers

2

3 Running Title: Morpholino oligomer resistance

4

5 Susan E. Puckett[1,a,#], Kaleb A. Reese[1,#], Georgi M. Mitev[1], Valerie Mullen[1], Rudd C.

6 Johnson[1], Kyle R. Pomraning[2], Brett L. Mellbye[3,b], Lucas D. Tilley[3,c], Patrick L. Iversen[3],

7 Michael Freitag[2], and Bruce L. Geller[1, 3 *]

8

9 [1] Department of Microbiology, [2]Department of Biochemistry and Biophysics, Oregon State

10 University, Corvallis, Oregon, and [3]AVI BioPharma, Inc., Corvallis, Oregon

11

12

13 #These authors contributed equally to the report

14

15 *Corresponding Author; Department of Microbiology, 220 Nash Hall, Oregon State University,

16 Corvallis, OR 97331-3804. Tel: 541-737-1845; Fax: 737-0496; e-mail: gellerb@orst.edu

17

18 [a]Currently at Department of Microbiology and Immunology, Weill Cornell Medical College,

19 New York City, NY.

20 [b]Currently at Department of Microbiology, Oregon State University, Corvallis, OR.

21 [c]Currently at Department of Microbiology and Molecular Genetics, University of Vermont,

22 Burlington, VT

23

24 Peptide phosphorodiamidate morpholino oligomers (PPMO) are synthetic DNA mimics that
25 bind complementary RNA and inhibit bacterial gene expression. (RFF)$_3$RXB- AcpP PPMO (R,
26 arginine; F, phenylalanine; X, 6-aminohexanoic acid; B, β-alanine) is complementary to 11
27 bases of the essential gene *acpP* (encodes acyl carrier protein). The MIC of (RFF)$_3$RXB-
28 AcpP was 2.5 μM (14 μg/ml) in *Escherichia coli* W3110. The rate of spontaneous resistance
29 of *E. coli* to (RFF)$_3$RXB-AcpP was 4 x 10^{-7} mutations/cell division. A spontaneous
30 (RFF)$_3$RXB-AcpP-resistant mutant (PR200.1) was isolated. The MIC of (RFF)$_3$RXB-AcpP
31 was 40 μM (224 μg/ml) in PR200.1. The MICs of standard antibiotics were identical in
32 PR200.1 and W3110. The sequence of *acpP* was identical in PR200.1 and W3110. PR200.1
33 was also resistant to other PPMOs conjugated to (RFF)$_3$RXB or peptides with a similar
34 composition or pattern of cationic and non-polar residues. Genomic sequencing of PR200.1
35 identified a mutation in *sbmA*, which encodes an active transport protein. In separate
36 experiments, a (RFF)$_3$RXB-AcpP-resistant isolate (RR3) was selected from a transposome
37 library, and the insertion was mapped to *sbmA*. Genetic complementation of PR200.1 or RR3
38 with *sbmA* restored susceptibility to (RFF)$_3$RXB-AcpP. Deletion of *sbmA* caused resistance to
39 (RFF)$_3$RXB-AcpP. We conclude that resistance to (RFF)$_3$RXB-AcpP was linked to the
40 peptide and not the PMO, dependent on the composition or repeating pattern of amino acids,
41 and caused by mutations in *sbmA*. The data further suggest that (RFF)$_3$R-XB PPMOs may be
42 transported across the plasma membrane by SbmA.
43
44

45 **Introduction**

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

56 Genomics has created an attractive potential for developing innovative strategies that
57 address the problem of antibiotic resistance. Synthetic antisense oligomers, such as peptide
58 nucleic acids (14), phosphorothioates (16), and phosphorodiamidate morpholino oligomers
59 (11, 15), silence expression of bacterial genes. Gene-silencing oligomers decrease
60 expression of reporter genes such as luciferase, activate endogenous genes such as β-
61 galactosidase, and inhibit growth and kill bacteria by targeting essential genes (10).
62 Antisense oligomers targeted to specific, essential bacterial genes reduce infections and
63 increase survival in mouse models of infection (12, 15, 40).

64 Antisense oligomers require assistance to cross the outer membrane of Gram-negative
65 bacteria because of their molecular weight and polar characteristics. Short amphipathic
66 peptides have been attached to antisense oligomers, and this has greatly improved their entry
67 into Gram-negative bacteria and increased their potency (11, 13, 27).

68 Membrane-penetrating peptides have diverse sequences, but many are cationic and
69 amphipathic. Previous investigations suggest that a repeated peptide motif with one cationic
70 residue followed by either one or two hydrophobic residues may be an important feature for
71 efficient membrane penetration (39). More recently, we have compared a variety of
72 membrane-penetrating peptides for their abilities to enhance the efficacy of peptide-
73 phosphorodiamidate morpholino oligomers (PPMO), and found differences among peptides
74 that vary in their pattern of alternating cationic and nonpolar residues and their amino acid
75 compositions (27).

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

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

90

91 **Material and Methods**

92

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

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

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

111 **PPMO.** PPMOs were synthesized at AVI BioPharma (Corvallis, Oregon) as described
112 (39). The base sequence of all PPMOs targeted to *acpP* (AcpP) is 5'-CTTCGATAGTG-3', to
113 *ftsZ* (FtsZ) is 5'-TCCATTGGTTC-3', and to *luc* (Luc) is 5'-AACGTTGAGIG. Inosine in place of

114 a guanine in the Luc PPMO was necessary to make the oligomer soluble in aqueous solutions
115 by avoiding guanine quartet structure. The scrambled base sequence control (Scr) is 5'-
116 TCTCAGATGGT-3'.

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

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

122 **Luciferase expression.** Spontaneous $(RFF)_3$RXB-AcpP-resistant mutants were made
123 chemically competent and transformed as described (29) with pSE380myc-Luc (11).
124 Overnight cultures were grown aerobically at 37°C in LB medium plus 50 μg/ml ampicillin
125 (LBA), and then diluted 2 x 10^{-2} into LBA with or without various concentrations (8, 20 and 50
126 μM) of $(RFF)_3$RXB-Luc or $(RFF)_3$RXB-Scr, and grown aerobically at 37°C for 7 h. Samples
127 were analyzed for luciferase expression by luminometry as described (11).

128 **Rate of spontaneous resistance.** The rate of spontaneous resistance to peptide-
129 PMO was measured by the method of Luria and Delbruck (23) as described (33). An
130 overnight culture was diluted to 1×10^4 cfu/ml in LB medium and divided into 20 × 1 ml
131 aliquots. Each aliquot was grown overnight at 37°C with aeration, and then 1 μl or 50 μl of
132 each was spread on 20 agar plates (60 mm × 15 mm) of LB plus 20 μM peptide-PMO. Plates
133 were grown overnight at 37°C with aeration, and colonies were enumerated.

134 **Screening transposome mutants.** EX-Tn5<R6Kγ*ori*/Kan-2>Tnp (Epicentre,
135 Madison, WI) was electroporated into *E. coli* XL1-Blue MRF', and 3 x 10^3 transductants were
136 selected on LB kanamycin (15 μg/ml) plates. The transductants were pooled and stored in

137 PBS + 15% glycerol at -75°C. The pooled transductants were thawed and 1 x 10^4 cfu was
138 spread on an LB plate that included 20 μM $(RFF)_3$RXB-AcpP. Insertion mutations from
139 PPMO-resistant mutants were sequenced by rescue cloning as described by the
140 manufacturer (Epicentre). Insertions in *sbmA* were confirmed using polymerase chain
141 reaction (PCR) as described (18). Briefly, PCR reactions contained chromosomal DNA
142 extracted from bacteria using a commercial kit (DNAeasy, Qiagen, Valenica, CA) and primers
143 (IDT Technologies, Coralville, IA) that flank the insertion site: 5'-
144 GATTGCCGTTATCTTCTGGC and 5'-GCTCAAGGTATGGGTTACTTCC. Thirty PCR reaction
145 cycles were: denature, 95°C, 0 sec; anneal 45°C, 0 sec; extend 72°C, 1 min. PCR reactions
146 were run on a 1605 air thermo-cycler (Idaho Technology, Idaho Falls, ID).
147 **Sequencing *acpP***. The *acpP* allele from each strain analyzed was amplified by
148 polymerase chain reaction (18) using as template a single bacterial colony picked from a
149 growth plate, Promega Taq polymerase (Madison, WI), and the following primers (Invitrogen,
150 Carlsbad, CA): 5'-AACGTAAAATCGTGGTAAGACC-3', and 5'-
151 TAACGCCTGGTGGCCGTTGATG-3'. The PCR products were gel-purified using Qiagen
152 MinElute PCR purification kit (Valencia, CA), and sequenced using the same primers shown
153 above at the core laboratory of the Center for Genome Research, Oregon State University.
154 **Genomic sequencing.** Genomic DNA from the W3110 wild type and PR200.1 was
155 generated by standard procedures (1). DNA was sheared by sonication and processed for
156 Illumina high-throughput sequencing as previously described (31, 32). Data analyses to find
157 individual point mutations were carried out as described (32).
158
159

160 **Results**

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

168 **Susceptibility to antibiotics and growth rate.** Colonies were isolated from a single
169 liquid culture of W3110 grown with 8 × MIC (20 μM = 112 μg/ml) of $(RFF)_3$RXB-AcpP. One
170 colony (PR200.1) was picked at random and further characterized. PR200.1 was equally
171 susceptible as the parent strain W3110 to each antibiotic tested (MIC = 4 μg/ml, 1.25 μg/ml,
172 1.25 μg/ml, 0.125 μg/ml, and 10 μg/ml for ampicillin, tetracycline, kanamycin, polymyxin B,
173 and rifampin, respectively). These results indicate that this particular PPMO-resistant isolate
174 was not resistant to antibiotics in general.

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

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

179 **MICs of AcpP PPMOs attached to various peptides.** MICs for different AcpP
180 PPMOs were measured using PR200.1 and W3110 as indicators (Table 1). All of the AcpP
181 PPMOs tested had the same base sequence, but had different peptides attached. The
182 attached peptides differed not only in their amino acid compositions, but also in the pattern of

183 repeating sequences of cationic and nonpolar residues. Repeating patterns of amino acids,
184 often including cationic and non-polar residues, are important features of membrane-
185 penetrating peptides (17, 41, 42). Four of the AcpP PPMOs, including $(RFF)_3$RXB-AcpP, had
186 peptides with a repeating amino acid motif of cationic-nonpolar-nonpolar (C-N-N), and one of
187 these was composed of D- instead of the usual L-amino acids . One AcpP PPMO was
188 conjugated to $(RX)_6$B, which has a repeating motif of cationic-nonpolar (C-N). Two other
189 AcpP PPMOs were conjugated to peptides with a repeating motif of cationic-nonpolar-cationic
190 (C-N-C): $(RXR)_4$XB or $(RFR)_4$XB. Another AcpP PMO was conjugated to RTRTRFLRRTXB,
191 which does not conform to any of the other repeat patterns. All of these PPMOs with various
192 peptides attached to the same AcpP PMO have been previously characterized and found to
193 be effective in inhibiting growth of *E. coli* (27).

194 The results show that PR200.1 was resistant to every AcpP PMO tested with the C-N-
195 N peptide motif, but was fully susceptible to $(RX)_6$B-AcpP PMO and $(RXR)_4$XB-AcpP PMO
196 (Table 1). However, PR200.1 was resistant to $(RFR)_4$XB-AcpP, which shares the C-N-C motif
197 with $(RXR)_4$XB-AcpP, but like $(RFF)_3$RXB-AcpP contains phenylalanine instead of 6-
198 aminohexaonoic acid. Compared to the susceptible parent strain W3110, resistance to
199 $(RFF)_3$RXB-AcpP in PR200.1 increased the MIC 16-fold. PR200.1 was also resistant to the
200 D-isomeric form of $(RFF)_3$RXB. The MIC of RTRTRFLRRTXB-AcpP, which lacks a repeating
201 amino acid motif but includes one phenylalanine, increased only 2-fold using PR200.1
202 compared to W3110 as indicator. The MICs of scrambled-base sequence PPMOs composed
203 with each of the same peptides used for the AcpP PPMOs were undetectable (>80 μM) in
204 every case.

205 **$(RFF)_3$RXB-PPMOs targeted to various genes.** PR200.1 was tested for susceptibility
206 to two PPMOs, each with the $(RFF)_3$ peptide motif, but different base sequences. One PPMO
207 is complementary to *ftsZ*, which is an essential gene involved in cell division. The other
208 PPMO is targeted to a luciferase reporter gene (*luc*).
209 Exponential cultures were grown for 18 h with $(RFF)_3$RXB-FtsZ, which is targeted to
210 *ftsZ*, or a scrambled (Scr) base sequence control. Samples of each culture were then plated,
211 and viable cells counted. PR200.1 grew to normal cell density, whereas the viable cell count
212 of the parent strain W3110 was reduced by over 2 orders of magnitude in the presence of
213 $(RFF)_3$RXB-FtsZ (Figure 1). The Scr had no effect on growth of either W3110 or PR200.1.
214 In other experiments, exponential cultures of W3110 and PR200.1 were grown for 7 h
215 with various concentrations of $(RFF)_3$RXB-Luc, which is targeted to a luciferase reporter gene,
216 or the scrambled base control $(RFF)_3$RXB-Scr. A plasmid that expresses luciferase had been
217 transferred into PR200.1 prior to the experiment. Samples of each culture were then
218 analyzed by luminometry for luciferase activity. The results show that $(RFF)_3$RXB-Luc did not
219 inhibit luciferase in PR200.1 at any of the 3 concentrations tested (Figure 2). In comparison,
220 W3110 showed inhibition of luciferase that was proportional to the concentration of PPMO
221 added. The scrambled sequence control did not inhibit luciferase in either strain. There were
222 no differences in the growth (optical density) of any of the cultures (data not shown).
223 **Peptide transport mutants.** The above results indicated that resistance to PPMOs is
224 linked to the peptide moiety. We hypothesized that PPMO-resistance could be caused by a
225 mutation in one of three known oligopeptide transporters. To test this, the MIC was measured
226 using various strains with mutations in one, two, or all three oligopeptide transporters (Table

227 2). The results show that $(RFF)_3$RXB-AcpP had the same MIC in PR200.1 as the parent
228 (non-mutant) strain. $(RFF)_3$RXB-Scr scrambled control showed no detectable MIC (>160 µM).

229 **Genomic sequencing.** The genomes of PR200.1 and its parent strain W3110 were
230 sequence and compared. The results indicated that a total of 3 genes had mutations in
231 PR200.1 compared to W3110: *dctA, eptA,* and *sbmA*. In *dctA*, there were 2 transition
232 mutations at bases 3958154 (T→A) and 395153 (A→G), both of which are in codon 396, that
233 caused a missense from ile to ala. In *eptA*, there was one transversion mutation at base
234 4339795 (T→A) that affected codon 259 and caused a missense from ser to thr. In *sbmA*,
235 there was one transversion mutation at base 396121 (T→G) that changed codon 87 (ser to
236 ala). No deletions or insertions were detected in any gene.

237 **Characterize deletion mutants.** In frame knock-out mutants of *dctA, eptA,* and *sbmA*
238 were tested for susceptibility to $(RFF)_3$RXB-AcpP and compared to the parent strain
239 (BW25113). The MIC of $(RFF)_3$RXB-AcpP was the same (2 µM = 11 µg/ml) using either the
240 $dctA^-$ or $eptA^-$ knock-out strains or the parent strain. The MIC using the $sbmA^-$ strain was 32
241 µM (179 µg/ml).

242 The MIC of $(RXR)_4$XB-AcpP was measured using $sbmA^-$ strain as indicator, and found
243 to be 2 µM (11 µg/ml), the same as its isogenic parent strain.

244 **Complementation with pSbmA.** PR200.1 was genetically complemented with an
245 IPTG-inducible expression plasmid that encodes *sbmA* (pSbma), or its empty control. The
246 complemented strain was grown with IPTG and used to measure the MIC of $(RFF)_3$RXB-
247 AcpP. The MIC was 1 µM and 32 µM in the induced, $sbmA^+$ complemented strain and the
248 empty vector control strain, respectively.

249 **Transposome mutants.** *E. coli* XL1-Blue MRF' was mutagenized with the
250 transposome EZ-Tn5 and a library of 1 x 10^4 mutants was spread on selection plates that
251 included 20 µM $(RFF)_3$R-AcpP. Two colonies grew on the selection plate, and the mutated
252 gene in each was sequenced. The sequences of both isolates indicated that the transposome
253 had inserted into the exact same position in *sbmA* in each isolate, suggesting that the two
254 colonies were clones. The isolates were named RR3.

255 RR3 was characterized by measuring the MICs of various standard antibiotics (Table
256 3). All standard antibiotics tested had the same MIC using either XL1-Blue MRF' or RR3 as
257 indicator, including two peptide antibiotics colistin and polymyxin B. However, RR3 was about
258 4-fold resistant to each of the peptide antibiotics bleomycin and phleomycin. PR200.1 was
259 also 4-fold resistant to bleomycin (MIC = 5.6 µM [8 ug/ml]) and phleomycin (MIC = 5.2 µM [8
260 µg/ml]) compared to W3110 (bleomycin MIC = 1.4 µM [2 µg/ml]; phleomycin MIC = 1.3 µM [2
261 µg/ml]).

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

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

272

273 **Discussion**

274 This is the first report we are aware of to characterize bacterial resistance to an
275 antisense antibacterial compound. Initially, growth was occasionally and unexpectedly
276 observed during routine MIC assays of $(RFF)_3$XB-AcpP in cultures 4- to 8-fold above the MIC.
277 Similar growth was never observed during MIC assays for $(RXR)_4$XB-AcpP or $(RX)_6$B-AcpP.
278 We speculate that the greater number of X residues (6-aminohexanoic acid) or the lack of F in
279 the latter two PPMOs may be responsible for the apparent lack of spontaneous resistance to
280 these PPMOs under the conditions used for the MIC assay. Alternatively, there could be
281 more genetic loci involved in resistance to $(RFF)_3$RXB PPMOs than in any (putative)
282 resistance to PPMOs conjugated to other peptides such as $(RXR)_4$XB or $(RX)_6$B. However,
283 we have not yet rigorously pursued resistance to these later PPMOs, and it is certainly
284 possible that spontaneous resistance may occur under appropriate conditions.

285 The rate of spontaneous resistance to $(RFF)_3$RXB-AcpP was similar to the rate of
286 spontaneous mutation for individual genes in *E. coli*, which is typically between 10^{-6} to 10^{-7}
287 mutations/gene/ generation (6, 24). This suggests that there are few genes which, when
288 mutated, can give rise to the PPMO-resistant phenotype. However, the rate of mutation can
289 vary widely, and any measurement of the rate of mutation is a function of many variables (25),
290 including the concentration of antibiotic used for selection, and the number of genes or loci
291 capable of causing a resistance phenotype. Ultimately, the rate of resistance to PPMOs
292 under in vivo conditions for specific infections will be the most meaningful measure of their
293 usefulness in the clinic.

294 The spontaneous mutant PR200.1 was susceptible to all small molecule antibiotics
295 tested. This shows that resistance to the PPMO is not caused by a change in physiology that
296 might result in resistance to antibiotics in general. Such general changes are known to occur,
297 and include a reduction of the net negative charge of the lipopolysaccharide of Gram-negative
298 bacteria (7, 30), changes in capsule polysaccharide (4), changes in expression of outer
299 membrane porins (30), alterations in outer membrane lipid composition that results in
300 decreased membrane permeability (30), and activation or overexpression of multidrug efflux
301 pumps (21). We tested a variety of antibiotics, some of which are hydrophilic (ampicillin,
302 kanamycin), hydrophobic (rifampin, tetracycline), or amphiphilic (polymyxin B), and some
303 which enter Gram-negative bacteria through the outer membrane porins (ampicillin,
304 tetracycline) or through the outer membrane lipid bilayer (rifampin, polymyxin B). The results
305 suggested that the mutation in PR200.1 is specific for $(RFF)_3$RXB-AcpP or PPMOs with
306 similar peptide moieties. Later, following the identification of the mutation in *sbmA*, PR200.1
307 was found to be mildly resistant (4-fold) to the peptide antibiotics bleomycin and phleomycin.
308 We hypothesized that resistance was caused by a mutation in the sequence of *acpP*
309 targeted by the PPMO. We have previously shown that a one-base mismatch near the 3'end
310 of a PPMO targeted to *acpP* in *Burkholderia cepacia* complex raised the MIC by a factor of at
311 least 8-32-fold (15). However, the results in the present report found no mutation in the target
312 region of *acpP* in this one resistant mutant. Therefore, the hypothesis in this case was
313 disproven. However, this is not to say that target site mutations cannot or do not occur on
314 other as of yet uncharacterized mutants. Nevertheless, target site mutations would be
315 statistically improbable considering that there are only 4 wobble bases in the target region of

316 *acpP* that might possibly lead to a decrease in efficacy without changing the amino acid
317 sequence of the targeted protein.

318 Another hypothesis was that resistance in PR200.1 was caused by a mutation in an
319 oligopeptide transporter. However, oligopeptide transport mutants were just as susceptible to
320 $(RFF)_3$RXB-AcpP as the isogenic parent strain. This showed that resistance in PR200.1 was
321 not caused by a mutation in the known oligopeptide transporters that were tested.

322 In another effort to identify the mutation in PR200.1 that is responsible for resistance to
323 $(RFF)_3$RXB-AcpP, the genome of PR200.1 was sequenced. The results showed missense
324 mutations in only 3 genes compared to the PPMO-susceptible strain: *dctA, eptA,* and *sbmA*.
325 In-frame, non-polar deletion mutations of each gene showed that of the three, only the *sbmA*
326 deletion strain was resistant to $(RFF)_3$RXB-AcpP. Furthermore, complementation of PR200.1
327 with *sbmA* restored susceptibility to the PPMO. These results show that mutations in *sbmA*
328 cause resistance to $(RFF)_3$RXB-AcpP.

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

333 The transposome mutant RR3 was resistant to both $(RFF)_3$RXB-AcpP and $(RXR)_4$XB-
334 AcpP. This differs from the spontaneous mutant PR200.1 and the *sbmA* deletion strain,
335 which were resistant to $(RFF)_3$RXB-AcpP but not $(RXR)_4$XB-AcpP. This suggests that a polar
336 affect in RR3 on the gene downstream from *sbmA* (*yaiW*) may be responsible for resistance
337 to $(RXR)_4$XB-AcpP. *yaiW* is a predicted DNA-binding transcriptional regulator. Apparently a
338 mutation in *sbmA* is sufficient to cause resistance to $(RFF)_3$RXB-AcpP, but not $(RXR)_4$-AcpP.

339 PR200.1 was susceptible to $(RXR)_4$XB-AcpP but resistant to $(RFR)_4$XB-AcpP, although
340 these two PPMOs share the same C-N-C repeat motif. This indicates that the amino acid
341 composition of the PPMOs may be more important than the repeating pattern of amino acids
342 in determining resistance in PR200.1. The similarity in resistance of PR200.1 to either
343 $(RFF)_3$RXB-AcpP or $(RFR)_4$XB-AcpP, but complete susceptibility to $(RXR)_4$XB-AcpP may
344 suggest that X (6-aminohexanoic acid) accounts for the difference. This is supported by the
345 result (Table 1) that resistance to $(RXX)_3$RXB-AcpP is 4-fold less than resistance to
346 $(RFF)_3$RXB-AcpP, despite having the same repeating pattern of cationic and non-polar amino
347 acids, but containing more X. Perhaps the unusual 6-carbon backbone of X causes a
348 conformational change that disallows interaction with SbmA. Alternatively, *sbmA* mutants
349 seem to be more resistant to peptides with F (phenylalanine). There is a positive trend
350 between the number of F in the peptide and resistance. This is supported by the results that
351 show higher resistance to PPMOs with more F (such as $(RFF)_3$RXB-AcpP and $(RFR)_4$XB-
352 AcpP), lesser resistance to PPMOs with fewer F (such as RTRTRFLRRTXB-AcpP), and no
353 resistance to PPMOs with no F (such as $(RXR)_4$XB-AcpP and $(RXR)_6$XB-AcpP), although
354 $(RXX)_3$RXB-AcpP is an exception to this trend.
355 *sbmA* encodes an active transporter for bleomycin and other peptide antibiotics (19,
356 26, 35, 43). Our results are consistent with SbmA acting as the active transporter for
357 $(RFF)_3$RXB-AcpP. *sbmA* homologs are widely conserved among bacteria (9). The homolog
358 of *sbmA* in *Rhizobium meliloti*, *bacA*, is required for symbiosis with alfalfa (9). The homolog in
359 *Brucella abortus* is a virulence factor important for intracellular survival in macrophages (20).
360 It has been proposed that the physiological substrates of SbmA are organic signaling

361 molecules (43). Development of an assay to measure uptake of PPMOs is currently not
362 available, but could be used to define further the role of SbmA in resistance to PPMO.
363 The substrate specificity of SbmA has been investigated and found to be quite flexible.
364 Initially, the specificity was proposed to be associated with a thiazole or oxazole structural
365 motif (43). Later, proline-rich antimicrobial peptides were shown to be transported by SbmA
366 (26). However, $(RFF)_3$RXB-AcpP has none of these structural features. If SbmA is the
367 transporter of $(RFF)_3$RXB-AcpP, apparently the specificity of SbmA is not limited to thiazole-
368 or oxazole-containing compounds or to proline-rich peptides. Our results suggest that the
369 substrate specificity of SbmA is flexible enough to accommodate polypeptides without
370 thiazole, oxazole, or proline. With the peptides we used in our conjugates, the specificity
371 appears to be linked to the spacing of cationic and non-polar amino acid residues within the
372 context of the peptide. It is also noteworthy that our all-D enantiomer conjugate (NG-05-0653)
373 had the same MIC values as the all-L conjugate (NG-05-0200) for parental and resistant
374 strains. This is in contrast to results shown for an all-D isomer of the proline-rich antimicrobial
375 peptide Bac7(1-35), which was ineffective compared to the all-L form (26). It was suggested
376 that the stereospecificity of Bac7(1-35) was attributable to its interaction with SbmA, although
377 uptake of all-D Bac7(1-35) was not demonstrated. Perhaps the stereospecificity of Bac7(1-35)
378 is caused by its interaction with its cytoplasmic target and not SbmA. Our results suggest that
379 the specificity of SbmA is not necessarily limited to either the L- or D-enantiomeric form of a
380 peptide, and is broader than previously known.
381 If SbmA is the plasma membrane transporter for $(RFF)_3$R-AcpP, we speculate that
382 other mechanisms exist for PPMOs to cross the plasma membrane. PR200.1, RR3, and the
383 *sbmA* deletion strain are still somewhat susceptible to $(RFF)_3$RXB-AcpP, albeit at high

384 concentrations. We speculate that PPMOs may be able to cross the plasma membrane by
385 passing through the lipid bilayer in the same manner that they cross the outer membrane.
386 There also may be additional active transporters with specificities for nucleic acid oligomers.
387 This latter possibility is suggested by the ability of PMOs (not conjugated to a peptide) to
388 inhibit gene expression in strains with porous outer membranes that allow passage of large
389 oligomers (11, 12).

390 In summary, the results suggest that bacterial resistance to a PPMO can be
391 determined by the peptide and not the PMO. The rate of occurrence of spontaneous
392 resistance to $(RFF)_3$RXB-AcpP is similar to that of spontaneous changes in other bacterial
393 phenotypes. In PR200.1 and RR3, resistance is caused by mutations in *sbmA*. Our results in
394 combination with the known role of SbmA in peptide antibiotic uptake, suggest that SbmA acts
395 as a transporter of $(RFF)_3$RXB-AcpP from the periplasm to the cytoplasm.
396

397 **Acknowledgement**
398 This work was supported by AVI BioPharma and the Howard Hughes Medical Institute
399 (through undergraduate student research fellowships to Susan E. Puckett and Valerie
400 Mullen). Preparation of Illumina sequencing libraries and data analyses were supported by
401 start-up funds from the OSU Computational and Genome Biology Initiative to Michael Freitag.
402 The authors thank Andrew Karplus for a critical discussion. Bruce Geller was employed by
403 both AVI BioPharma and Oregon State University.
404
405

406 **References**

407

408 1. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K.
409 1998. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., New York, N.
410 Y.

411

412 2. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M,
413 Wanner BL, and Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-
414 gene knockout mutants: the Keio collection. Mol. Sys. Biol. **2:**2006.0008.

415
416 3. Boucher HW, Talbot GH, Bradley JS, Edwards, Jr JE, Gilbert D, Rice LB, Scheld M,
417 Spellberg B, and Bartlett J. 2009. Bad bugs, no drugs: No ESKAPE! An update from
418 the Infectious Diseases Society of America. Clin. Infect. Dis. 48:1-12.

419

420 4. Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, and Bengoechea JA.
421 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides.
422 Infect. Immun. **72:**7107-7114.

423

424 5. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial
425 susceptibility tests for bacteria that grow aerobically: Approved standard-seventh
426 edition. Wayne, PA, USA 2006. 10.2-10.3.

427

428 6. Drake J, Charlesworth WB, Charlesworth D, and Crow JF. 1998. Rates of spontaneous
429 mutation. Genetics **148:**1667-1686.

430

431 7. Ernst RK, Guina T, and Miller SI. 2001. *Salmonella typhimurium* outer membrane
432 remodeling: role in resistance to host immunity. Microbes Infect. **3:**1327-1334.

433
434 8. Fischback MA, and Walsh CT. 2009. Antibiotics for emerging pathogens. Science
435 **325:**1089-1093.

436 9. Gazebrook J, Ichige A, and Walker GC. 1993. A *Rhizobium meliloti* homolog of the
437 *Escherichia coli* peptide antibiotic-transport protein SbmA is essential for bacteroid
438 development. Genes Dev. **7:**1485-1497.

439

440 10. Geller BL. 2005. Antisense Antibiotics. Curr. Opin. Mol. Ther. **7:**109-113.

441

442 11. Geller BL, Deere JD, Stein DA, Kroeker AD, Moulton HM, and Iversen PL. 2003.
443 Inhibition of gene expression in *Escherichia coli* by antisense phosphorodiamidate
444 morpholino oligomers. Antimicrob. Agents Chemother. **47:**3233-3239.

445

446 12. Geller BL, Deere J, Tilley L, and Iversen PL. 2005. Antisense phosphorodiamidate
447 morpholino oligomer inhibits viability of *Escherichia coli* in pure culture and in mouse
448 peritonitis. J. Antimicrob. Chemother. **55:**983–988.

449

450 13. Good L, Awasthi SK, Dryselius R, Larsson O, and Nielsen PE. 2001. Bactericidal
451 antisense effects of peptide-PNA conjugates. Nat. Biotechnol. **19:**360-364.

452

453 14. Good L, and Nielsen PE. 1998. Antisense inhibition of gene expression in bacteria by
454 PNA targeted to mRNA. Nat. Biotechnol.**16:**355-358.
455
456 15. Greenberg DE, Marshall-Batty KR, Brinster LR, Zarember KA, Shaw PA, Mellbye BL,
457 Iversen PL, Holland SM, and Geller BL. 2010. Antisense phosphorodiamidate
458 morpholino oligomers targeted to an essential gene inhibit *Burkholderia cepacia*
459 complex. J. Infect. Dis. **201:**1822-1830.
460
461 16. Harth G, Zamecnik PC, Tabatadze D, Pierson K, and Horwitz MA. 2007. Hairpin
462 extensions enhance the efficacy of mycolyl transferase-specific antisense
463 oligonucleotides targeting *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U S A.
464 **104:**7199-7204.
465
466 17. Henriques ST, Melo MN, and Castanho MARB. 2006. Cell-penetrating peptides and
467 antimicrobial peptides: how different are they? Biochem. J. **399:**1-7.
468
469 18. Kramer MF, and Coen DM. 2001. Enzymatic amplification of DNA by PCR: standard
470 procedures and optimization. Unit 15.1. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D.
471 D. Moore, J. G. Seidman, J. A. Smith, K. Struhl (ed.) Current Protocols in Molecular
472 Biology, John Wiley & Sons, Inc. New York, NY.
473

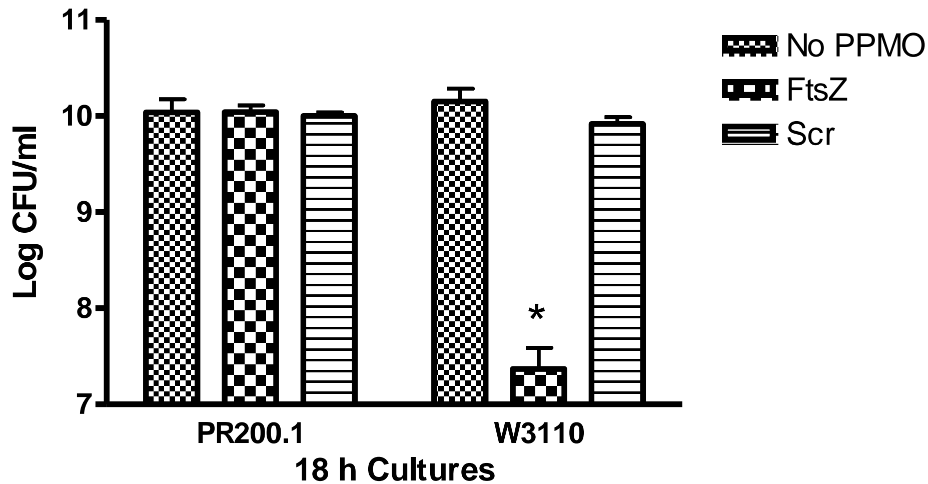
474 19. Lavina M, Pugsley AP, and Moreno F. 1986. Identification, mapping, cloning and
475 characterization of a gene (*sbmA*) required for mirocin B17 action on *E. coli* K12. J.
476 Gen. Microbiol. **132:**1685-1693.
477
478 20. LeVier K, Phillips RW, Gripper VK, Roop II RM, and Walker GC. 2000. Similar
479 requirements of a plant symbiont and a mammalian pathogen for prolonged
480 intracellular survival. Science **287:**2492-2493.
481
482 21. Li XZ, and Nikaido H. 2009. Efflux-mediated drug resistance in bacteria: an update.
483 Drugs. **69:**1555-1623.
484
485 22. Livermore DM. 2009. Has the era of untreatable infections arrived? J. Antimicrob.
486 Chemother. **64:** Suppl. 1, i29-i36.
487
488 23. Luria SE, and Delbruck M. 1943. Mutations of bacteria from virus sensitivity to virus
489 resistance. Genetics **28:**491-511.
490
491 24. Maloy S. 2011. Mutation rates.
492 http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/mutations/fluctuation.html
493
494 25. Martinez JL, and Baquero F. 2000. Mutation frequencies and antibiotic resistance.
495 Antimicrob. Agents Chemother. **44:**1771-1777.
496

497 26. Mattimuzzo M, Bandiera A, Gennaro R, Bennincasa M, Pacor S, Antcheva N, and
498 Scocchi M. 2007. Role of the *Escherichia coli* SbmA in the antimicrobial activity of
499 proline-rich peptides. Molec. Microbiol. **66:**151-163.
500
501 27. Mellbye BL, Puckett SE, Tilley LD, Iversen PL, and Geller BL. 2009. Variations in
502 amino acid composition of antisense peptide-phosphorodiamidate morpholino
503 oligomers affect potency against *Escherichia coli* in vitro and in vivo. Antimicrob.
504 Agents Chemother. **53:**525-530.
505
506 28. Neuman BW, Stein DA, Kroeker AD. Churchill MJ, Kim AM, Kuhn P, Dawson P,
507 Moulton HM, Bestwick RK, Iversen PL, and Buchmeier MJ. 2005. Inhibition, escape,
508 and attenuated growth of severe acute respiratory syndrome coronavirus treated with
509 antisense morpholino oligomers. J. Virol. **79:**9665-9676.
510
511 29. New England Biolabs, Inc. Rubidium chloride method.
512 http://www.neb.com/nebecomm/tech_reference/gene_expression/RbCl_protocol.asp
513
514 30. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited.
515 Microbiol. Mol. Biol. Rev. **67:**593-656.
516
517 31. Pomraning KR, Smith KM, and Freitag M. 2009. Genome-wide high throughput
518 analysis of DNA methylation in eukaryotes. Methods **47:**142-150.
519

520 32. Pomraning KR, Smith KM, and Freitag M. 2011. Bulk segregant analysis followed by
521 high-throughput sequencing reveals the Neurospora cell cycle gene, ndc-1, to be allelic
522 with the gene for ornithine decarboxylase, spe-1. Eukaryot. Cell **10:**724–733.
523
524 33. Rosche WA, and Foster PL. 2000. Determining mutation rates in bacterial populations.
525 Methods **20:**4-17.
526
527 34. Saka K, Tadenuma M, Nakade S, Tanaka N, Sugawara H, Nishikawa K, Ichiyoshi N,
528 Kitagawa M, Mori H, Ogasawara N, and Nishimura A. 2005. A complete set of
529 *Escherichia coli* open reading frames in mobile plasmids facilitating genetic studies.
530 DNA Research **12:**63-68.
531
532 35. Salomon RA, and Farias RN. 1995. The peptide antibiotic microcin 25 is important
533 through the TonB pathway and the SbmA protein. J. Bacteriol. **177:**3323-3325.
534
535 36. Smith MW, Tyreman DR, Payne GM, Marshall NJ, Payne JW. 1999. Substrate
536 specificity of the periplasmic dipeptide-binding protein from *Escherichia coli*:
537 Experimental basis for the design of peptide prodrugs. Microbiol. **145:**2891-2901.
538
539 37. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, and
540 Edwards, Jr. J. 2008. The epidemic of antibiotic-resistant infections: A call to action for
541 the medical community from the Infectious Diseases Society of America. Clin. Infect.
542 Dis. **46:**155-164.

543 38. Splith K, and Neundorf I. 2011. Antimicrobial peptides with cell-penetrating peptide
544 properties and vice versa. Eur. Biophys. J. **40:**387-397.
545
546 39. Tilley LD, Hine OS, Kellogg JA, Hassinger JN, Weller DD, Iversen PL, and Geller BL.
547 2006. Gene-specific effects of antisense phosphorodiamidate morpholino oligomer-
548 peptide conjugates on *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* in
549 pure culture and in tissue culture. Antimicrob. Agents Chemother. **50:**2789-2796.
550
551 40. Tilley LD, Mellbye BL, Puckett SE, Iversen PL, and Geller BL. 2007. Antisense peptide-
552 phosphorodiamidate morpholino oligomer conjugate: dose-response in mice infected
553 with Escherichia coli. J. Antimicrob. Chemother. **59:**66-73.
554
555 41. Vaara M, and Porro M. 1996. Group of peptides that act synergistically with
556 hydrophobic antibiotics against gram-negative enteric bacteria. Antimicrob. Agents
557 Chemother. **40:**1801-1805.
558
559 42. Yeaman MR, and Yount NY. 2003. Mechanisms of antimicrobial peptide action and
560 resistance. Pharmacol. Rev. **55:**27-55.
561
562 43. Yorgey P, Lee J, Kordel J, Vivas E, Warner P, Jebaratnam D, and Kolter R. 1994.
563 Posttranslational modifications in microcin B17 define an additional class of DNA
564 gyrase inhibitor. Proc. Natl. Acad. Sci. USA **91:**4519-4523.
565

566



567

568 Figure 1. **Viable Cell Count of 18 h Cultures**. Stationary cultures of W3110 or PR200.1

569 were diluted to 5 x 10^5 CFU/ml in Mueller-Hinton broth and divided in three. $(RFF)_3$RXB-FtsZ

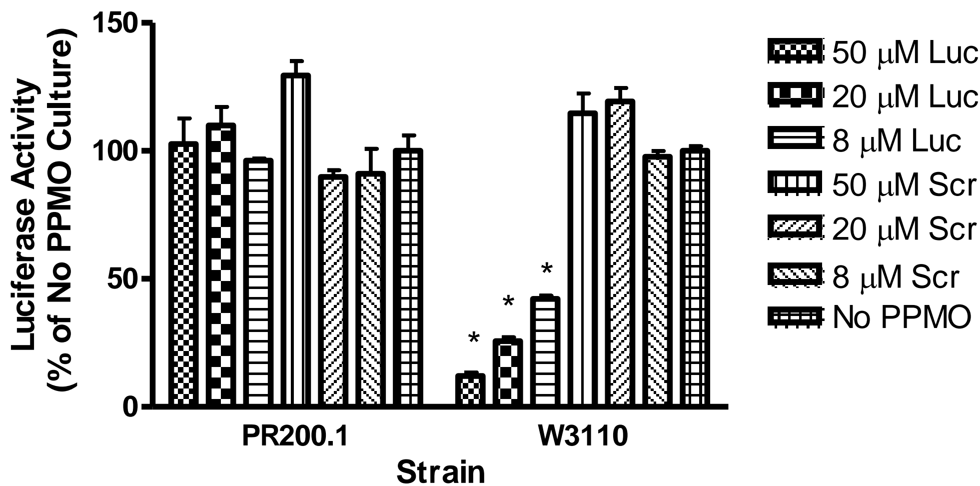
570 (FtsZ) or scrambled (Scr) PPMO (160 μM), or no PPMO was added. Cultures were grown

571 aerobically at 37°C for 18 h, and then samples of each were diluted and plated to determine

572 viable cells. Error bars indicate standard deviation. *indicates highly significant ($P < 0.01$)

573 difference compared to either no PPMO or Scr-treated culture.

574



575

576 Figure 2. **Luciferase Activity of Cultures Treated with PPMO.** Growing cultures were

577 treated for 7 h without PPMO (No PPMO), or with 3 concentrations of a PPMO [$(RFF)_3$RXB-

578 Luc] targeted to a luciferase reporter gene, or a scrambled sequence control [$(RFF)_3$RXB-

579 Scr]. After 7 h samples of each culture were measured for luciferase activity by luminometry.

580 The experiment was repeated 3 times and the error bars indicate standard deviation.

581 *indicates a highly significant ($P < 0.01$) difference compared to cultures of PR200.1 with the

582 same concentrations of $(RFF)_3$RXB-Luc, the cultures of W3110 with the same concentrations

583 of $(RFF)_3$RXB-Scr, or the culture without PPMO.

Table 1. MIC of AcpP PPMO in Pure Cultures of *E. coli*

PPMO No. NG-	Conjugated Peptide*	MIC (µM [µg/ml])	
		W3110	PR200.1
Motif 1 (C-N-N)**			
05-0200	RFFRFFRFFRXB	2.5 [14]	40 [222]
05-0653	DRDFDFDRDFDFDRDFDFDRXB	2.5 [14]	40 [222]
23-248	RXXRXXRXXRXB	20 [102]	80 [204]
06-0199	KFFKFFKFFKXB	10 [54]	80 [435]
Motif 2 (C-N)**			
06-0073	RXRXRXRXRXRXB	1.25 [7]	1.25 [7]
Motif 3 (C-N-C)**			
06-0076	RXRRXRRXRRXRXB	1.25 [7]	1.25 [7]
07-0795	RFRRFRRFRRFRXB	1 [6]	16 [94]
No Motif			
05-0246	RTRTRFLRRTXB	20 [111]	40 [111]

*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 $(RFF)_3R$-AcpP (NG-05-0200) Using Oligopeptide Transport Mutants

E. coli strain	Mutation/Phenotype	MIC	
		µM	µg/ml
Morse 2034	Wild-type oligopeptide transport	5	28
PA0183	*opp*$^-$/oligopeptide permease deletion	5	28
PA0333	*dpp*$^-$, *opp*$^-$/PA0183 plus dipeptide permease deletion	5	28
PA0643	*tpp*$^-$,*dpp*$^-$,*opp*$^-$/PA0333 plus tripeptide permease mutant	5	28

Table 3. MIC of standard antibiotics and PPMOs using transposome mutant RR3, isogenic parent strain XL1-Blue MRF', and their *sbmA* complemented strains

		MIC (µM [µg/ml])			
	Antibiotic	XL1-Blue MRF'	RR3	XL1-Blue MRF' (pSbmA)	RR3 (pSbmA)
	Polymyxin B	0.8 [1]	0.8 [1]	0.8 [1]	0.8 [1]
	Colistin	0.9 [1]	0.9 [1]	0.9 [1]	0.9 [1]
	Erythromycin	34 [25]	34 [25]	34 [25]	34 [25]
	Rifampin	6 [5]	6 [5]	6 [5]	6 [5]
	Bleomycin	0.2 [0.25]	0.7 [1]	0.01 [0.016]	0.01 [0.016]
	Phleomycin	0.3 [0.5]	1.3 [2]	0.02 [0.03]	0.02 [0.03]
PPMO No. NG-	PPMO				
05-0200	$(RFF)_3RXB$-AcpP	2 [11]	64 [355]	2 [11]	2 [11]
05-0653	[D-$(RFF)_3R$]XB-AcpP	2 [11]	64 [355]	2 [11]	2 [11]
06-0076	$(RXR)_4XB$-AcpP	1 [6]	8 [48]	0.1 [0.6]	0.1 [0.6]
05-0655	$(RFF)_3RXB$-Scr	>128 [>714]	>128 [>714]	>128 [>714]	>128 [>714]
06-0078	$(RXR)_4XB$-Scr	>128 [>714]	>128 [>714]	>128 [>714]	>128 [>714]