ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2005, p. 1184–1189 Vol. 49, No. 3
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.3.1184–1189.2005

A Novel Engineered Peptide, a Narrow-Spectrum Antibiotic, is Effective against Vancomycin-Resistant *Enterococcus faecalis*

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Received 2 September 2004/Returned for modification 23 October 2004/Accepted 5 November 2004

A novel antienterococcal peptide was prepared by fusing the enterococcal cCF10 pheromone to the channel-forming domain of colicin Ia, forming *Enterococcus faecalis* pheromonicin (PMC-EF). This peptide was bactericidal against vancomycin-resistant *Enterococcus faecalis* (VRE) organisms. Electron microscopy and vital dyes confirmed increased membrane permeability. All mice made bacteremic with VRE strains survived when they were treated with PMC-EF, while all controls died.

A major problem in the treatment of bacterial infections has been the emergence of antibiotic resistance. This is true for *Enterococcus faecium* and to a lesser extent for *Enterococcus faecalis*, which have developed resistance to many antibiotics, including vancomycin (12, 16). We have shown that a staphylococcal pheromone fused to a channel-forming peptide killed staphylococci in culture and animal infections (14). Here, we report on an application of that strategy to vancomycin-resistant *E. faecalis* (VRE) strains.

Escherichia coli cells can secrete colicins, which possess channel-forming properties bactericidal only to *E. coli*. Colicin Ia introduces a lethal 175-residue C-terminal channel into cell membranes of targeted *E. coli* (6, 8, 10, 13, 15, 17). Our plan was to fuse the C terminus of colicin Ia to cCF10, a 7-residue pheromone produced by enterococci (2, 8). This pheromone traverses recipient cell walls and interacts with a corresponding prgZ component of the cell membrane (1, 3, 4).

Mutagenesis of PMC-EF. We constructed the LVTLVFV amino acid sequence of pheromone cCF10 to follow position I626 of colicin Ia by double-stranded oligonucleotide mutagenesis (QuickChange kit; Stratagene) by using a Promega pSELECT-1 plasmid containing the colicin Ia gene (P. Gosh, University of California at San Francisco) to form *E. faecalis* pheromonicin (PMC-EF). We used the 5′-3′ oligonucleotide containing the desired LVTLVFV mutation (in bold) GCGAATAAGTT CTGGGGTATT**CTGGTTACCCTTGTGTCCGTG**TAAAT AAAATATAAGACAGGC. Controls consisted of a random peptide of the same length as cCF10 and an unrelated staphylococcal pheromone fused at the N terminus instead of the C terminus (reversed PMC-EF) (Fig. 1a). Plasmids were transfected into TG1 *E. coli* cells and purified (7, 9).

In vitro bactericidal assays. We grew vancomycin-sensitive *E. faecalis* ATCC 29212 and vancomycin-resistant *E. faecalis* ATCC 700802 (American Type Culture Collection, Manassas, Va.) as described previously (3). Shenyang Biotechnic Co. (Shanghai, People's Republic of China) synthesized cCF10.

Wild-type colicin Ia from TG1 cells transfected with colicin Ia plasmid, PMC-EF from TG1 cells transfected with reversed PMC-EF, a random protein with an SMTTVGG peptide introduced at the C terminus of colicin Ia, penicillin, and vancomycin served as controls. Turbidimetric measurements and colony counting were performed (5).

Electron microscopy and immunolabeling. We incubated VRE cells with PMC-EF, vancomycin, or growth medium alone for 2 h, then stained them with 1% phosphotungstic acid, and observed them under an electron microscope (Jeol JEM-100SX; Jeol, Akishima, Japan) at a magnification of ×15,000 to ×40,000.

We incubated VRE and BAA-42 methicillin-resistant *Staphylococcus aureus* cells with PMC-EF, PMC-EF plus free cCF10, vancomycin, or growth medium alone for 40 min, then fixed the cells in phosphate-buffered saline–paraformaldehyde, incubated specimens with rabbit anti-colicin Ia antibody (1:10 in 3% skim milk) (Babco Berkeley Antibody Co., Richmond, Calif.) followed by goat anti-rabbit antibody labeled with 5-nm-diameter gold particles (1:100 in 3% skim milk) (Wuhan Boster Biol. Tech. Co., Wuhan, People's Republic of China), and fixed the cells in phosphate-buffered saline–glutaraldehyde. Specimens were fixed in OsO_4 and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed with a Hitachi H-600 electron microscope at 80 kV (11).

The domains of the fusion peptide PMC-EF are shown in Fig. 1a and b. Figure 1c shows a polyacrylamide gel of the enterococcal chimeric protein PMC-EF (Fig. 1c, lane 2) and a control chimeric protein containing pheromone from *S. aureus* (PMC-SA) (Fig. 1c, lane 3).

Exposure of *E. faecalis* ATCC 29212 nonresistant cells to PMC-EF inhibited growth by 90% (Fig. 2a), while penicillin G

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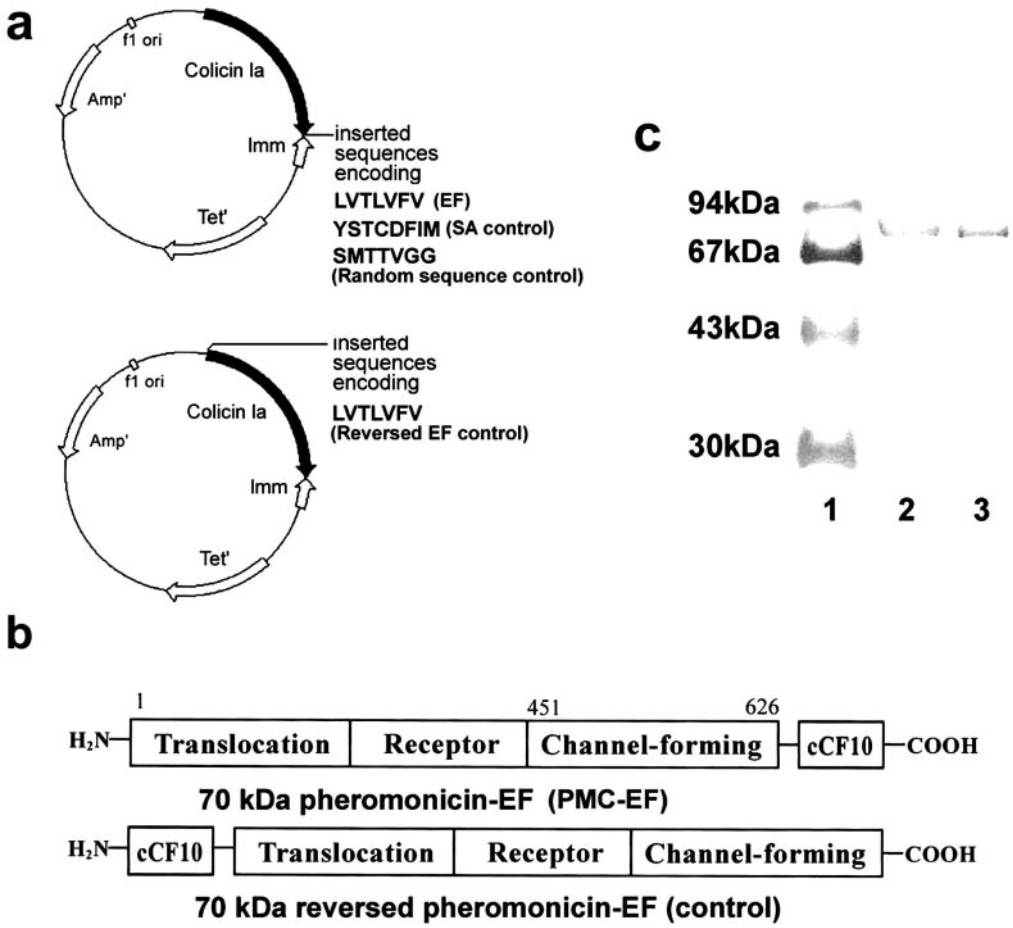

FIG. 1. Structure of PMC-EF. (a, upper panel) Shown is an 8.3-kbp colicin Ia plasmid used for site-directed mutagenesis and subsequent preparation of PMC-EF consisting of the colicin Ia plasmid with insertion of a cCF10 (EF) pheromone gene, PMC-SA with insertion of an unrelated staphylococcal pheromone (YSTCDFIM), or insertion of a random (SMTTVGG) peptide gene following amino acid position I626. (a, lower panel) Shown is a construct with insertion of cCF10 before amino acid position S1 of colicin Ia to form the reversed PMC-EF control. (b) Domain diagram of the PMC-EF construct with the cCF10 pheromone at the C terminus and the reversed PMC-EF construct with cCF10 pheromone at the N terminus. (c) Sodium dodecyl sulfate–15% polyacrylamide gel of PMC-EF purified by gradient elution from a carboxymethyl column: lane 1, molecular weight markers; lane 2, PMC-EF; and lane 3, PMC-SA.

inhibited growth by 80%. Control peptides with an unrelated 8-residue staphylococcal pheromone or a random 7-residue peptide linked at the C terminus of colicin Ia had no effects. Vancomycin (5 μg/ml) slowed initial growth of VRE cells (Fig. 2b). However, later there was no significant difference compared to untreated controls. In contrast, 5 μg of PMC-EF/ml inhibited VRE cell growth by 85%. Addition of free cCF10 pheromone (Fig. 2c) blocked PMC-EF inhibition of VRE cell growth in a concentration-dependent manner. Incubation of VRE with either free cCF10 or wild-type colicin Ia alone (Fig. 2d) did not inhibit cell growth. In contrast, PMC-EF resulted in less than 10% growth inhibition when administered to *S. aureus* or *Streptococcus pneumoniae* (Fig. 2e and f). Exposure of *S. aureus* cells to PMC-SA, a control chimeric protein containing pheromone from *S. aureus*, caused an 80% growth inhibition (Fig. 2e) (14). Withdrawal of PMC-EF failed to restore growth (data not shown).

Electron microscopy of VRE cells incubated with vancomycin (Fig. 3b) showed a morphological appearance like that of untreated cells (Fig. 3a). In contrast, after incubation with PMC-EF, 60% of VRE cells per field were distorted (Fig. 3c and d) (less than 7% per field were distorted in controls). Free cCF10 blocked adsorption of gold-labeled antibody on cytoplasmic membranes of enterococcal cells treated with PMC-EF (Fig. 3h). As shown in Fig. 3f and g, some VRE cells exposed to PMC-EF developed ruptured cell walls.

Initially after exposure to PMC-EF (Fig. 4a), the number of VRE cells stained (green) by fluorescein isothiocyanate (FITC)-labeled antibody exceeded that stained (red) with propidium iodide, a vital dye. The ratio of red to green VRE cells increased with time (Fig. 4a to d). The number of dual-labeled cells increased to a maximum at 0.5 h (Fig. 4b) and then declined, while cells with propidium iodide staining decreased, and FITC staining was almost absent by 2 h (Fig. 4d). There was no significant propidium staining and no FITC labeling of untreated cells (Fig. 4e to h).

In a mouse VRE sepsis model, all mice treated with PMC-EF survived, while all untreated mice died within 3 days. None of the *E. faecalis*-infected mice survived with vancomycin (data not shown).

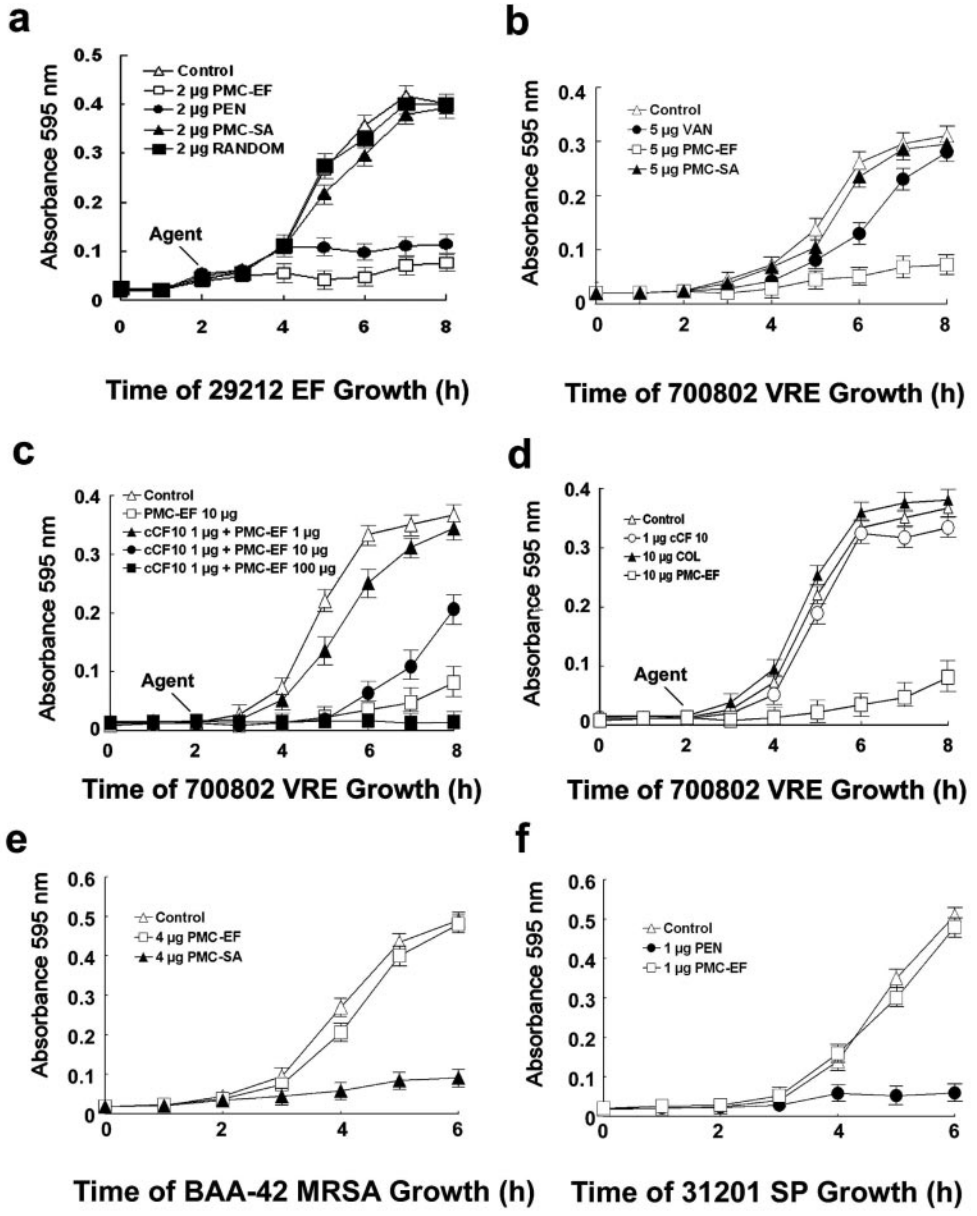

FIG. 2. Measurement of bactericidal activity of PMC-EF against *E. faecalis* by in vitro cell growth assays. The following additions were made to the media: no addition (Control), penicillin G (PEN), vancomycin (VAN), wild-type colicin Ia (COL), free cCF10 pheromone alone (cCF10), staphylococcal colicin fusion protein (PMC-SA), colicin Ia with C-terminal random 7-residue peptide (RANDOM), and enterococcal colicin fusion protein (PMC-EF). *E. faecalis* 29212, a vancomycin-sensitive *E. faecalis* strain, was incubated with all additives at 2 μg/ml (a) or all additives at 5 μg/ml (b). (c) Free cCF10 pheromone (1 μg/ml) was added to compete with increasing concentrations of PMC-EF against ATCC 700802 *E. faecalis* cells. (d) Growth of ATCC 700802 VRE cells treated with 1 μg of free cCF10/ml or 10 μg of wild-type colicin Ia or PMC-EF/ml. (e) Growth of *S. aureus* ATCC BAA-42 with 4 μg of PMC-EF or PMC-SA (an unrelated staphylococcal chimeric protein)/ml. (f) Growth of *S. pneumoniae* 31201 with 1 μg of PMC-EF or penicillin G/ml.

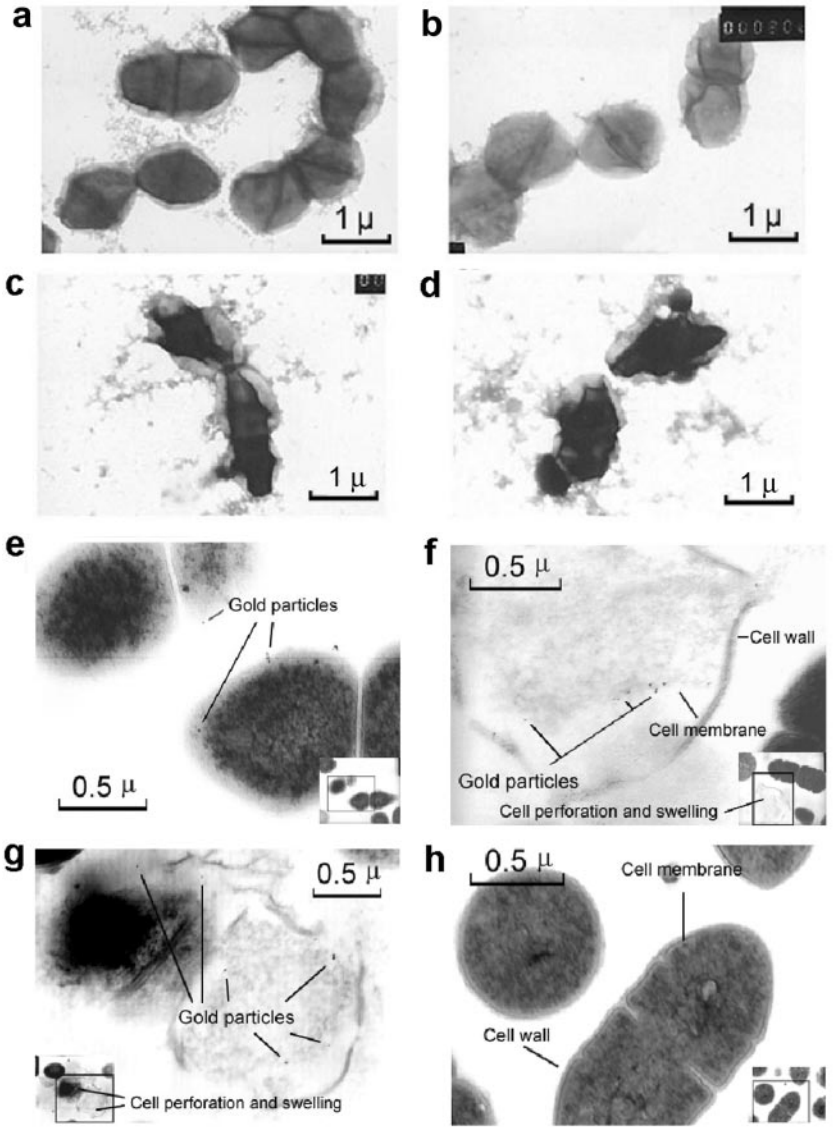

FIG. 3. Electron micrographs of ATCC 700802 VRE cells. (a) Untreated cells. (b) Vancomycin-treated cells (5 μg/ml). (c and d) PMC-EF-treated cells (5 μg/ml). (a to d) Negative staining. Magnification, ×15,000. Cells were incubated and stained with rabbit anticolicin antibody and gold-labeled goat anti-rabbit antibody. (e to g) VRE cells were treated with PMC-EF (20 μg/ml). (h) Cells treated with PMC-EF (20 μg/ml) and competed with free cCF10 (1 μg/ml). Magnification, ×30,000 to ×40,000. Insets show full-field views.

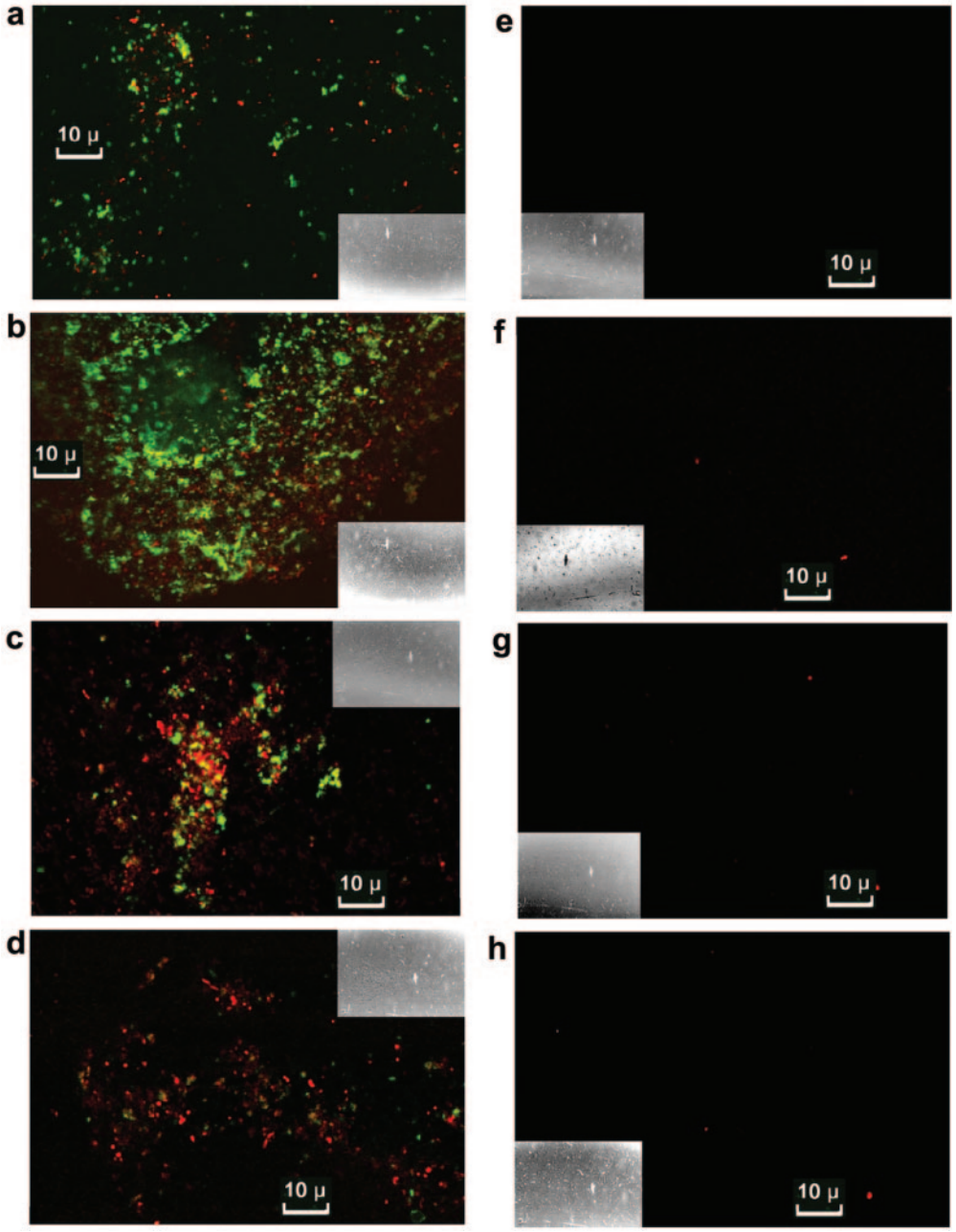

FIG. 4. Cells treated with PMC-EF (a to d) or medium alone (e to h) stained with propidium iodide and FITC-labeled antibody and examined under a confocal microscope. The following times elapsed after additions: 0.1 h, panels a and e; 0.5 h, panels b and f; 1 h, panels c and g; and 2 h, panels d and h. Insets show full-field views.

The emergence of vancomycin-resistant enterococci has been the result of increased use of vancomycin in enterococcal infections (12, 16). In addition, conjugal transfer of the *vanA* operon from enterococci has resulted in vancomycin resistance in other bacteria (12, 16). Multidrug resistance is now common among those pathogens (12, 16). Successful treatment of antibiotic-resistant bacteria will require novel agents whose mechanisms of action are different from those of conventional agents.

We conclude that a chimeric peptide containing pheromone and channel-forming domains can be targeted to antibiotic-resistant bacteria, resulting in substantial bactericidal effects. Furthermore, because a variety of pheromones are known, fusion peptides could be tailored to particular bacteria and provide novel "narrow-spectrum" antibiotics.

This work was supported by National Nature Science Foundation of China grants 39725009, 39770243, and 30276820 and National Basic Research Program of China grant G19990540001 to X.-Q.Q.; a grant from the National Institute of Diabetes and Digestive and Kidney Disorders (DK-42182) to G.Y.W.; and the Herman Lopata Chair in Hepatitis Research to G.Y.W.

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