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Abstract: Increasing antibacterial resistance presents a major challenge in antibiotic discovery. One attractive target in Gram-negative bacteria is the unique asymmetric outer membrane (OM), which acts as a permeability barrier that protects the cell from external stresses such as the presence of antibiotics. We describe a novel -hairpin macrocyclic peptide JB-95 with potent antimicrobial activity against E. coli. This peptide exhibits no cellular lytic activity, but electron microscopy and fluorescence studies reveal an ability to selectively disrupt the OM but not the inner membrane of E. coli. The selective targeting of the OM likely occurs through interactions of JB-95 with selected -barrel OM proteins including BamA and LptD as shown by photolabeling experiments. Membrane proteomic studies reveal rapid depletion of many -barrel OM proteins from JB-95-treated E. coli, consistent with induction of a membrane stress response and/or direct inhibition of the Bam folding machine. The results suggest that lethal disruption of the OM by JB-95 occurs through a novel mechanism of action at key interaction sites within clusters of -barrel proteins in the OM. These findings open new avenues for developing antibiotics that specifically target -barrel proteins and the integrity of the Gram-negative OM.

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A Peptidomimetic Antibiotic Targets Outer Membrane Proteins and Disrupts Selectively the Outer Membrane in Escherichia coli*

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Increasing antibacterial resistance presents a major challenge in antibiotic discovery. One attractive target in Gram-negative bacteria is the unique asymmetric outer membrane (OM), which acts as a permeability barrier that protects the cell from external stresses, such as the presence of antibiotics. We describe a novel β-hairpin macroyclic peptide JB-95 with potent antimicrobial activity against Escherichia coli. This peptide exhibits no cellular lytic activity, but electron microscopy and fluorescence studies reveal an ability to selectively disrupt the OM but not the inner membrane of E. coli. The selective targeting of the OM probably occurs through interactions of JB-95 with selected β-barrel OM proteins, including BamA and LptD as shown by photolabeling experiments. Membrane proteomic studies reveal rapid depletion of many β-barrel OM proteins from JB-95-treated E. coli, consistent with induction of a membrane stress response and/or direct inhibition of the Bam folding machine. The results suggest that lethal disruption of the OM by JB-95 occurs through a novel mechanism of action at key interaction sites within clusters of β-barrel proteins in the OM. These findings open new avenues for developing antibiotics that specifically target β-barrel proteins and the integrity of the Gram-negative OM.

The discovery of novel antibiotics with new mechanisms of action is an important goal in antibiotic research to combat infections caused by multidrug-resistant bacteria, in particular Gram-negative microorganisms with their unique asymmetric outer membrane (OM)3 (1). Naturally occurring cationic host defense peptides that form part of the innate immunity in many organisms have recently attracted great interest in the search for new clinically useful antibiotics (2). We explore here an approach to antibiotic discovery based upon host defense peptide-inspired macrocyclic peptidomimetics as a potential source of antibiotics displaying target selectivity and potency not seen with naturally occurring host defense peptides. In previous work (3), we described a family of macrocyclic β-hairpin peptidomimetics with potent and selective antimicrobial activity against Pseudomonas spp., which were shown to have a novel mechanism of action targeting the β-barrel outer membrane protein LptD in Pseudomonas aeruginosa and inhibiting its key lipopolysaccharide (LPS) transport function in OM biogenesis (4). We report here the discovery of a new conformationally constrained β-hairpin peptidomimetic (called JB-95) having potent antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria and, in particular, against Escherichia coli. JB-95 shows minimal inhibitory concentrations (MICs) of ~0.25 μg/ml against E. coli, including many multidrug-resistant clinical strains. We report the solution structure of JB-95 and investigations into its mechanism of action against E. coli.

Experimental Procedures

Peptide Synthesis—The methods for synthesis and characterization of all peptides have been described previously (5). JB-95 was of >95% purity by analytical reverse phase HPLC (Waters XBridge TMS (C18, 50 × 19 mm, 5 μm, 135 Å) with a gradient of 5–90% MeCN in H2O (with 0.05% trifluoroacetic acid). ESI-MS m/z 1970.1 ([M + H]+, calc. 1970.0). See the supplemental material for full 1H NMR assignments. For the synthesis of the photoprobe PAL-95, Fmoc-Glu(biotinyl-PEG)-OH (Novabiochem) and Fmoc-L-Photo-Pro-OH were used, along with other protected amino acids, following methods described earlier (3). PAL-95 purity was >95% by reverse phase HPLC (C18 Waters XBridge column using a linear gradient of 10–60% MeCN in H2O). ESI-MS m/z 593.6 [M + 4H]4+, 791.4 [M + 3H]3+. Full details for the synthesis of the fluorescent probe fJB-95 (MIC, 4 μg/ml) are given in the supplemental material.

Antibacterial Assays—MICs were determined in microtiter plates in Mueller-Hinton-I (MH-I) medium using the broth
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microdilution method (6). The kinetics of cell death was measured under the same conditions, by taking aliquots of medium and plating onto MH agar plates with growth overnight at 37 °C to determine colony-forming units.

NMR Studies—The solution structure of JB-95 was determined by 1H NMR spectroscopy. Distance restraints were obtained from NOESY and ROESY spectra with a mixing time of 250 ms. The structure calculations were performed by restrained molecular dynamics in torsion angle space by applying the simulated annealing protocol implemented in the program DYANA (7). See the supplemental material for a full description of the results.

Macromolecular Biosynthesis Assays—Potential inhibitory effects of JB-95 on macromolecular synthesis (protein, RNA, DNA, cell wall) were monitored by incorporation of radioactively labeled precursors in chemically defined medium in a microplate format as described (8), using [3H]thymidine (80 Ci/mmol), l-[3H]leucine (108 Ci/mmol), and [3H]uridine (30 Ci/mmol) (from PerkinElmer Life Sciences) and N-[3H]acetylglucosamine (30 Ci/mmol) and 2,6-[3H]diaminopimelic acid (30 Ci/mmol) (from Biotrend Chemikalien).

Membrane Permeabilization Assay with SYTOX Green—E. coli ATCC25922 was grown in MH-I at 37 °C with shaking to an A600 of 0.2–0.3. The cells were collected and resuspended in MH-I to A600 of 0.1. To this culture in a UV cuvette containing a small magnetic stirrer bar and 0.002% of polyoxyethylene 80 (Tween 80) was added SYTOX Green dye (0.5 μM), and the fluorescence signal was monitored for 400 s. The test antibiotic was added, and the fluorescence intensity was recorded during 1 h (PerkinElmer Life Sciences LS55 spectrometer; excitation at 488 nm, emission at 525 nm, and a slit width of 2.5 nm).

Fluorescence Microscopy—E. coli ATCC25922 was grown in MH-II broth at 37 °C to an A600 of ~0.5–0.7. To an aliquot (200 μl) was added JB-95 or polymyxin B (PMB), and the culture was incubated at 30 °C for 1 h with shaking. Fluorescence dye (FM4-64 (1 μg/ml), SYTOX Green (0.5 μM), DAPI (2 μg/ml)) was added, and the broth was incubated at 0 °C for 10–15 min (DAPI and SYTOX Green) or 45–60 min (FM4-64). The cells were collected by centrifugation, washed with MH-II, and then resuspended in MH-II (20 μl). The samples were imaged on a 1.1% agarose patch using a Leica CLSM SP8 gSTED 3X microscope, with laser excitation for DAPI (405 nm), SYTOX Green (514 nm), or FM4-64 (520 nm) and a photomultiplier or hybrid detector. Alternatively, to an aliquot of cell suspension in MH-II was added the fluorescence probe fJLB-95 (40 μg/ml).

After 30 min at 30 °C, the cells were washed twice with MH-II and examined as described above.

β-Lactamase and β-Galactosidase Assays—For periplasmic β-lactamase expression, E. coli ATCC25922:pET3a was grown in MH-II broth to an A600 of 0.3. The cells were collected, washed, and resuspended in PBS supplemented with CaCl2 (1 mM) and MgCl2 (0.5 mM) to an A600 of 0.2. To this suspension in a UV cuvette with 0.02% BSA was added the chromogenic cephalosporin CENTA (100 μM). After 1 min, the test antibiotic was added, and the A405 was monitored over time. The dose response with antibiotic was studied in a 24-well plate format using the cell suspension (848 μl) and CENTA (100 μM), incubated at 37 °C, and shaken at 200 rpm for 1 h, before measuring A405.

For cytoplasmic β-galactosidase expression, E. coli XL1blue::pUC19 was grown in LB medium to an A600 of 0.3. LacZa expression was induced with isopropyl 1-thio-β-D-galactopyranoside (100 μg/ml), and incubation continued at 37 °C for 1 h to an A600 of ~0.6. The cells were collected, washed twice with fresh LB medium, and then resuspended in PBS supplemented with CaCl2 (1 mM) and MgCl2 (0.5 mM) to an A600 of 0.3. The cell suspension was distributed into a 24-well plate with antibiotic, substrate (O-nitrophenyl-β-D-galactoside) was added (0.5 mM) and incubated at 37 °C with shaking for 1 h. The cells were then removed by centrifugation, and the A420 was measured.

Electron Microscopy (EM)—EM studies with E. coli ATCC25922 treated with JB-95 were performed using methods described earlier (3).

Proteomics—E. coli ATCC25922 was grown in LB to end-exponential phase with JB-95 (5 μg/ml), causing ~50% growth inhibition. Cells from treated and untreated cultures were harvested by centrifugation (three biological replicates each) and lysed by French press, and membranes were isolated by differential centrifugation (30 min at 3005 g followed by 45 min at 100,000 × g). The membrane protein pellet was resuspended in PBS, and ~10 μg of protein from treated and untreated cells was analyzed by 12% SDS-PAGE with Coomassie staining (Fig. 6A).

Proteins extracted from total membrane fractions of E. coli grown in LB medium with or without JB-95 were first separated by 12% SDS-PAGE. After reduction and carboximidemethylation, the proteins were digested with trypsin (Promega, Madison, WI), and the resulting peptides were separated by RP-HPLC and analyzed by an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) interfaced with a nanoelectrospray source. Mass spectra were further processed with an in-house processing pipeline (9) that extracts fragment ion mass spectra from Thermo RAW files using msconvert (Proteowizard, version 3.0.3831) and searches for matching peptides in an E. coli K12 protein database (substrain MG1655, NC_000913.3) containing 256 common contaminants (e.g. human keratin, trypsin) with the search engine MS-GF+ (version 9979), here using a 20-ppm mass tolerance window. Using the decoy option of MS-GF+, the list of peptide spectrum matches was filtered to an estimated overall false discovery rate of 0.1%, which amounted to a protein level false discovery rate of about 1% when requiring two unambiguous peptides (class 1a or 3a based on a PeptideClassifier analysis (10) or three spectra for protein identification in either condition). A total of 1740 proteins were identified overall. DESeq2 (11) (version 1.4.5) was used to identify the most significantly regulated proteins, here applying a q value threshold of 0.06 (Benjamini-Hochberg adjusted p value indicating that roughly three false positives are expected among the top 56 differentially regulated proteins). Proteomics data associated with this paper can be downloaded from the ProteomeXchange under accession number PXD002588.

Functional Analysis—For prediction of β-barrel OM proteins and subcellular localization, we relied on the publicly available BOMP (12) and PSORTb version 3.0 (13) Web servers (see Table 2), as described (14).
Quantitative PCR Analysis—E. coli ATCC25922 cells were grown to end-exponential phase in the presence or absence of JB-95. Cell harvest, RNA extraction, and cDNA synthesis were as described previously (15). The expression of E. coli ATCC25922 genes wcaI (EG11790), wzc (EG13568), murC (EG10619), arnA (EG14091), ompF (EG10671), lptD (EG11569), lamB (EG10528), and bamA (EG12676) was analyzed by quantitative RT-PCR (qRT-PCR) using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Basel, Switzerland) and an Mx3000P instrument (Agilent). Each PCR was run in triplicate with three dilutions of cDNA (15, 7.5, and 3.75 ng) using 15 μl Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix and 5 μl individual primers in a total volume of 24 μl. Melting curves were generated for verifying the specificity of the amplification. The primers used are listed in the supplemental material.

Photolabeling Experiments—Photolabeling experiments with E. coli K12 MG1655 and E. coli ATCC25922 and the probe PAL-95 (Fig. 1A) were performed using methods described in detail elsewhere (3).

Results

Antimicrobial Activity—A screening effort uncovered the backbone macrocyclic tetradecapeptide, called JB-95 (cyclo-(Trp1-Arg2-Ile3-Arg4-Ile5-DArg6-Trp7-Lys8-Arg9-Leu10-Arg11-Arg12-D-Pro13-Pro14)); see Fig. 1A). JB-95 has good antimicrobial activity against a panel of Gram-negative and Gram-positive bacteria (Table 1). However, the antimicrobial activity was considerably higher against E. coli (MIC = 0.25 μg/ml) and was maintained across a panel of E. coli clinical strains showing resistance to a variety of known antibiotics. The enantiomer of JB-95 (ent-JB-95) shows 2–4-fold reduced antimicrobial activity against all tested organisms. The kinetics of E. coli ATCC25922 cell killing in a microplate format revealed a bactericidal action over several hours at 2–8 × MIC, but not the rapid killing caused by the cell lytic peptide protegrin I (PG-I) (Fig. 1B).

Structural NMR Studies—The average solution structure of JB-95 was determined by 1H NMR spectroscopy in aqueous
A detailed analysis of two-dimensional $^1$H NOESY and ROESY spectra revealed a dense network of cross-strand nuclear Overhauser effects (NOEs), which, along with other data, including amide chemical shift dispersions, $J_{\text{HNH}}$ coupling constants, amide hydrogen/deuterium exchange rates, and temperature coefficients (see the supplemental material), clearly indicate a stable conformationally arrested $\beta$-hairpin structure within the JB-95 macrocycle. Structure calculations were performed by restrained molecular dynamics in torsion angle space with the program DYANA (7). A bundle of 20 conformations incurring the lowest DYANA target function were selected, and a single representative structure is shown in Fig. 2. All structures contain type II $\beta$-turns within the template (d-Pro$^{13}$-L-Pro$^{14}$) and at the tip of the loop (d-Arg$^{6}$-L-Lys$^7$), with regular $\beta$-strands connecting the two turn structures (16).

The NMR studies show that JB-95 adopts a stable $\beta$-hairpin conformation in aqueous solution. Permeabilization of the OM—JB-95 showed no significant lytic activity on human red blood cells at a concentration of 100 $\mu$g/ml, which is well above the MIC. The permeabilizing effect of JB-95 on E. coli ATCC25922 was investigated using the fluorescent dye SYTOX Green. E. coli cells grown in MH-I broth and then treated with SYTOX Green and JB-95 showed no rapid increase in fluorescence over 1 h (Fig. 1C), whereas treatment of cells with PMB leads within 20 min to a rapid characteristic increase in green fluorescence, as both IM and OM are permeabilized and the dye gains access to and binds nucleic acids in the cytoplasm.

Macromolecular Synthesis Assays—We investigated the influence of JB-95 on macromolecule biosynthesis in E. coli ATCC25922 by monitoring the incorporation of radioactively labeled precursors in chemically defined medium in a microplate format (8). Labeling starts with exponentially growing cultures at an $A_{600}$ of 0.3, and the incorporation of label is per-
formed over a fixed period of only 20 min to reveal direct effects of the antibiotic, before secondary effects and cell death become prominent. No significant inhibition by JB-95 was observed of protein, RNA, or DNA biosynthesis, whereas control experiments with known antibiotics gave rise to the anticipated inhibitory effects (not shown). Interestingly, JB-95 reproducibly caused a significant stimulation of radioactivity incorporated from \([^{3}H]\)acetylglucosamine into macromolecules (Fig. 1D). In contrast, only a minor stimulation of tritium incorporation was observed in labeling experiments using \([^{3}H]\)diaminopimelic acid. The results are not consistent with a direct inhibition of cell wall/membrane biosynthesis.

**Microscopy Studies**—The effects of JB-95 on *E. coli* cells grown in MH with concentrations of JB-95 causing 50% growth inhibition for 1 h at 30 °C were analyzed using a high-resolution stimulated emission depletion fluorescence microscope, with staining of membranes by the membrane dye FM4-64 and of nucleoids by DAPI and using SYTOX Green to detect permeabilized cells (Fig. 3). The stained nucleoids were not influenced significantly by JB-95, and apart from a small number of dead cells, no significant staining (<10% of cells) was observed by SYTOX Green. Relative to untreated cells, however, a large proportion (>50%) of cells treated with JB-95 were elongated and contained unusual accumulations of membrane-like material stained by FM4-64 (Fig. 3).

A derivative of JB-95 labeled with Alexafluor-488 (called flJB-95; see Fig. 1A) was prepared to study the uptake of the antibiotic by fluorescence microscopy. This derivative shows an MIC of ~4 \( \mu \)g/ml and so retains a good antimicrobial activity against *E. coli*. Cells were shaken with flJB-95 for 30 min in MH broth and then examined using stimulated emission depletion microscopy. The staining pattern (Fig. 3A) revealed that the probe was concentrated into clusters or islands, consistent with localized binding sites on the cell surface.

Evidence for a perturbation in membrane morphology was revealed by transmission electron microscopy (TEM) of cells grown in LB with JB-95 at concentrations causing ~50% inhibition of growth. In comparison with untreated controls, sections of treated *E. coli* cells appeared with a minimal periplasmic space and showed distinct ruptures in the OM, without any apparent disturbance to the IM (Fig. 4, B and C). Many cells also showed accumulations of unusual membrane-like material between the IM and the OM (Fig. 4D). These microscopy studies indicate a dramatic effect of JB-95 on the outer cell membrane. When JB-95-treated cells were examined by scanning EM, the most notable feature compared with controls was the appearance of knoblike structures over the surface of most cells, which was not seen in the control (Fig. 4, E and F).

**Susceptibility to Detergents and Antibiotics**—*E. coli* cells grown with JB-95 at concentrations causing ~50% growth inhibition were plated onto agar containing 0.5% SDS and 0.5 mM EDTA or a selection of other antibiotics. The results revealed a dramatic increase (~10^4-fold) in the sensitivity of JB-95-treated cells to growth on agar containing SDS/EDTA compared with untreated cells, consistent with a compromised OM permeability barrier allowing exposure of the IM to the lethal actions of detergent (Fig. 5A). In agar disk diffusion assays, the

**FIGURE 3. Fluorescence microscopy of *E. coli* cells.** *E. coli* ATCC25922 cells grown in MH-II without drug and stained with SYTOX Green, DAPI, and FM-4-64 (A) and cells treated with either JB-95 (5 \( \mu \)g/ml; B–E) or PMB (0.03 \( \mu \)g/ml; F–H) for 1 h at 30 °C and labeled with fluorescent dye were analyzed using a Leica CLSM SP8 gSTED microscope (scale bar, 4 \( \mu m \)). A, control cells, no drug; with JB-95; B, SYTOX Green staining (no signal visible); C, DAPI staining; D, FM-4-64 staining; E, superimposition of B–D; with PMB; F, FM-4-64 staining; G, SYTOX Green staining; H, superimposition of F and G; I, cells stained with flJB-95 (see Fig. 1A) for 30 min in MH broth.
Effects on the OM Proteome—The influence on the OM proteome of *E. coli* ATCC25922 grown with JB-95 concentrations causing ~50% growth inhibition was investigated. Whole membrane fractions isolated by differential centrifugation from both treated and nontreated control cells were analyzed by SDS-PAGE. The gels reveal a dramatic decrease in the relative amounts of some membrane proteins compared with the untreated control (Fig. 6A). To identify membrane proteins differentially expressed in the presence of JB-95, a semiquantitative proteomic analysis was performed using in-gel digestion and ESI-LC-MS/MS analyses. Data analysis was performed using DESeq2 to identify the most significantly regulated proteins between treated and untreated cells (each measured in triplicate) (11, 14). The protein expression profiling experiment identified a total of 1740 proteins. Applying a stringent selection of differentially expressed proteins (q ≤ 0.06), we identified 19 significantly up-regulated and 37 down-regulated proteins (Fig. 6B) in JB-95-treated cells, as recorded in Table 2. We observed that β-barrel OM proteins and the larger group of PSORTb OM proteins are significantly enriched among differentially regulated proteins (p < 10⁻^15, respectively). Most notable among the up-regulated are proteins involved in capsule biosynthesis and export (wzc, Wza, and Gmd), lipid A modifications (ArnA, ArnC, and EptA), OM stress responses (ClpX, DegP, PhoQ, and RstB), and drug efflux (AcrD). Equally notable among the significantly down-regulated proteins is the large number of OM β-barrel proteins, porins, and transporters (Table 2). The level of the β-barrel folding protein BamA was not significantly changed by JB-95. However, suppressive effects on BamA might be compensated by up-regulation as part of an envelope stress response (σ²). To independently assess the proteomics results, qRT-PCR analysis was performed on independent biological replicate cultures, selectively targeting genes involved in capsule and murein biosynthesis (*wcaI*, wzc, and *murC*); a UDP-L-Ara4n-formyl transferase encoded by *arnA*; and *ompF*, *lptD*, *lamB*, and *bamA*. These results revealed genes that are strongly up-regulated (*wcaI* (+10-fold), *wzc* (+6-fold), *arnA* (+3-fold)), others that are down-regulated (*ompF* (−23-fold) and *lamB* (−5-fold)), and some whose levels are not significantly changed by JB-95 (*lptD* (−1.3-fold), *bamA* (1.3-fold), and *murC* (1.6-fold)). A good correlation is seen between the results at the mRNA and protein levels for many of these genes (Table 2), including *bamA*, whose level is not significantly affected by JB-95.

Photolabeling Experiments—Photolabeling experiments were carried out with the photoprobe PAL-95, which is identical to JB-95 except for the replacement of L-Pro by the photoreactive diazirine-containing L-photoproline (3), and of L-Arg by a Glu(PEG2-biotin) (residue Z in Fig. 1A). PAL-95 retained a high antimicrobial activity against *E. coli* (Table 1). Experiments were performed with both the clinical isolate (ATCC25922) and a genetically well characterized K12 strain. Results of photolabeling experiments in *E. coli* K12 MG1655 are shown in Fig. 7. After photolabeling cells with PAL-95, the OM proteome was extracted and analyzed by two-dimensional gel electrophoresis. Photolabeled, biotinylated proteins were detected after blotting to a membrane by chemiluminescence. Comparison of the blots with Coomassie-stained two-dimensional gels from unla-
beled OM proteome allowed identification of photolabeled BamA and LptD on the basis of their unique and characteristic positions in the two-dimensional gel, including characteristic changes in the mobility of LptD in the gel before and after reduction by DTT, as described earlier (18). Other photolabeled proteins were detected around 67 and 40 kDa at positions expected for BtuB and for LamB and FadL, respectively. The most abundant OM proteins (OmpA/F) are not labeled by the photoprobe. Photolabeling experiments with PAL-95 and *E. coli* ATCC25922 gave similar results and also showed labeling of BamA, LptD, LamB, and FadL, but not BtuB (not shown). These results show that JB-95 interacts with several β-barrel proteins in the OM of *E. coli*, including in both strains the essential proteins LptD and BamA required for the biogenesis of the OM.

**Discussion**

The macrocyclic β-hairpin-shaped peptide JB-95 displays antimicrobial activity against Gram-negative bacteria and *Staphylococcus aureus* (Table 1). However, the activity against *E. coli* is an order of magnitude higher, suggesting a selective mechanism of action that merited further investigation. JB-95 shows no cellular membranolytic activity, and the kinetics of bacterial cell death reveals a clear difference between the actions of PG-I and JB-95 (Fig. 1B). PG-I kills a wide spectrum of Gram-positive and Gram-negative bacte-
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TABLE 2

Differential membrane protein analysis

| Class                                | Locus ID   | Localization | BOMP prediction | Description | Gene name | Log2 -fold change 1 versus 2 |
|--------------------------------------|------------|--------------|-----------------|-------------|-----------|-------------------------------|
| Energy production and conversion     | EG10995    | Cytoplasmic  | t-Ase-AII        | aspA        | -2.3      |
|                                      | EG10232    | CM           | dmsA             | dmsA        | -3.2      |
|                                      | EG10233    | Unknown      | DMSO reductase   | dmsA        | -2.9      |
|                                      | EG10330    | CM           | Fumarate reductase | fbdA       | -2.2      |
|                                      | EG10331    | CM           | Fumarate reductase | fbdB       | -1.9      |
|                                      | EG10393    | Cytoplasmic  | Anaerobic glyceraldehyde dehydrogenase | glpC | -3.0      |
|                                      | EG10701    | Cytoplasmic  | Formate C-acyltransferase | pffB | -1.4      |
|                                      | EG11227    | Periplasmic  | Formate dehydrogenase-N | fdegB | -3.5      |
|                                      | EG11801    | CM           | Hydrogenase-2 large subunit | hybC | -2.6      |
|                                      | EG13006    | CM           | Hydrogenase-2 small subunit | hybE | -2.4      |
|                                      | EG13844    | CM           | S- and N-oxide reductase, A subunit | ynfB | -3.9      |
|                                      | EG10673    | Periplasmic  | Oligopeptide-binding protein | goppA | -2.7      |
|                                      | EG11005    | Cytoplasmic  | Tryptophanase    | trpA        | -1.6      |
|                                      | EG10386    | Periplasmic  | Glutamine-binding protein | glnA | -2.5      |
|                                      | EG12130    | Cytoplasmic  | DnaK-like chaperone Hsc66 | hscA | 3.4      |
|                                      | EG10283    | Cytoplasmic  | Fructose-1,6-bisphosphatase | frb | 2.9      |
|                                      | EG10528    | OM           | 4                | Maltooligosyl maltodextrinase | maltD | -3.0      |
|                                      | EG10560    | Cytoplasmic  | Maltooligosyl phosphorylase | maltP | -2.5      |
|                                      | EG10432    | Cytoplasmic  | Glutamate 1-semialdehyde aminomutase | hsdM | 2.3      |
| Lipid metabolism                     | EG10280    | OM           | 5                | Fatty acid transport protein | fadA | -2.3      |
| Cell wall, membrane, envelope biogenesis | EG10669    | OM           | 1                | Outer membrane protein A | ompA | -2.0      |
|                                      | EG10670    | OM           | 5                | Outer membrane protein C | ompC | -2.7      |
|                                      | EG10671    | OM           | 5                | Outer membrane protein F | ompF | -2.7      |
| Coenzyme metabolism                  | EG10528    | OM           | 4                | Outer membrane phospholipase A | oppA | -3.2      |
|                                      | EG10560    | Cytoplasmic  | 3                | Outer membrane channel for nucleosides | iux | -2.5      |
|                                      | EG11124    | OM           | 4                | Outer membrane protein W | oppW | -3.0      |
|                                      | EG11569    | OM           | 1                | LPS assembly complex LptD/E | lptD | -3.2      |
|                                      | EG11787    | Cytoplasmic  | Fucose biosynthesis, GDP-α-mannose 4,6-dehydratase | gmd | 3.5      |
|                                      | EG11790    | Cytoplasmic  | Putative colic acid biosynthesis glycosyl transferase | wcaA | 3.7      |
|                                      | EG12135    | OM           | 5                | Outer membrane protein X | oppX | -1.8      |
|                                      | EG12513    | OM           | 1                | Assembly module for autotransporter export | tanA | -1.1      |
|                                      | EG13492    | OM           | 5                | MBL interacting protein | mgbA | -3.1      |
|                                      | EG13566    | OM           | 2                | Colanic acid export channel lipoprotein | wca | 3.5      |
| Cell motility                        | EG13568    | CM           | 3                | Tyrosine protein kinase; colanic acid production | wzc | 4.3      |
|                                      | EG14090    | CM           | 3                | Undecaprenyl phosphate-aminoribosyl synthase | arrC | 1.9      |
| Post translational modifications     | EG13491    | CM           | 4                | UDP-glucuronate dehydrogenase | arrA | 3.1      |
|                                      | EG10321    | Extracellular | Flagellin, structural gene, H-antigen | fliC | -3.0      |
|                                      | EG10139    | Cytoplasmic  | ATPase subunit of ClpX protease | clpX | 2.0      |
|                                      | EG10463    | Periplasmic  | Periplasmic, membrane-associated senso endoprotease | degp | 2.1      |
| Inorganic ion transport and metabolism | EG10014    | CM           | Aminoglycoside efflux pump; RND-type transporter | acrD | 3.7      |
|                                      | EG10126    | OM           | 3                | Vitamin B12 outer membrane receptor | btuB | -4.1      |
|                                      | EG10286    | OM           | 1                | OMF ferric citrate receptor | fecE | -4.9      |
|                                      | EG10302    | OM           | 4                | OMF transporter for ferrichrome-iron | fnuA | -3.3      |
|                                      | EG10514    | CM           | Potassium-translocating ATPase, B subunit | kdpD | 3.8      |
|                                      | EG12525    | CM           | Magnesium transporter, ATP-dependent | magT | 4.5      |
|                                      | EG13774    | OM           | 1                | Putative TonB-dependent OM receptor | yscD | -3.7      |
|                                      | EG10260    | Cytoplasmic  | Isocitratimutase, aryl carrier protein (ArCP) | trntB | 3.6      |
| Signal transduction                  | EG10702    | CM           | Histidine kinase sensor protein | phoQ | 2.4      |
| Intracellular trafficking and secretion | EG11233    | CM           | Sensory histidine kinase of RdAAB two-component system | rsbB | 3.1      |
| Others                               | EG12866    | OM           | 0                | OM factor (OMF) of tricarboxylic efflux pumps | ionC | -2.3      |
|                                      | EG10988    | CM           | Aspartate, malate chemoreceptor, methyl-accepting | tar | -3.2      |
|                                      | EG1613     | CM           | Lipid A phosphoethanolamine transferase | cpxA | 1.8      |
|                                      | EG12257    | Unknown      | Oxidase involved in cellulose production | casC | -3.9      |
|                                      | EG12693    | Unknown      | DUF535 family protein, function unknown | ybhX | 4.5      |
|                                      | EG1329     | Unknown      | DUF4147 family periplasmic tridomain protein | ydeH | -4.0      |

* Gene number and nomenclature according to Ecogene version 3.0.
* Localization identified as described under "Experimental Procedures." CM, cytoplasmic membrane.
* BOMP identified as described under "Experimental Procedures." (0 = did not find the protein to be an integral OM protein, but there were integral OM homologs; 1 = least reliable prediction; 5 = most reliable prediction).
* Log2 -fold change of protein expression, comparing induced cells (1, with JB-95) and uninduced (U, no drug).

ria within 20–30 min of cell contact at micromolar concentrations through a lytic mechanism of action (19). Furthermore, exposing cells to JB-95 does not lead to rapid cytoplasmic uptake of SYTOX Green, in contrast to cytoplastic staining seen after treatment with PG-I or PMB (Figs. 1C and 3).

TEM studies provide evidence that JB-95-treated E. coli suffer multiple ~50-nm breaches of the OM (Fig. 4) without visible effects on the IM. This differential effect on IM and OM merits further attention and discussion. First, amphiphilic cationic antimicrobial peptides (CAMPs) with a membranolytic mode of action, such as PG-I and PGLa, rapidly destabilize both inner and outer membranes of E. coli at concentrations close to the MIC (19–21). Permeabilization of both the OM and IM of E. coli by the human antimicrobial peptide LL-37, with uptake into the cytoplasm of SYTOX Green, has been documented by real-time fluorescence microscopy (22). Indeed, many CAMPs, including examples isolated from the innate immune systems of various organisms as well as others of purely synthetic origin, physically impair membrane bilayers (23–25). Several models have been described to explain such activity, including formation of porelike structures and micellization of the membrane.
Mechanism of Action of a Peptidomimetic Antibiotic

The antibiotic PMB permeabilizes the OM before targeting and disrupting the cytoplasmic membrane in *E. coli* (33, 34). As a result, PMB causes release into the medium of not only periplasmic β-lactamase but also cytoplasmic β-galactosidase (35, 36), as confirmed in our studies (Fig. 5). Earlier electrical measurements with reconstituted planar bilayers suggested an initial electrostatic interaction of PMB with the LPS outer leaflet, followed by formation of transient membrane lesions of about 3 nm in diameter (37, 38). The lesions allow PMB and other small molecules and ions to permeate the OM in both directions (self-promoted uptake) (39) but appear to be quite different in size and nature from the lesions induced by JB-95 (Fig. 4). Although we observe complete release into the medium of periplasmic β-lactamase after treatment of cells with JB-95, almost no release occurs of β-galactosidase from the cytoplasm, in contrast to observations made with PMB (Fig. 5). Moreover, the damage to the OM caused by JB-95 accounts for the greatly enhanced sensitivity of JB-95-treated cells to SDS/EDTA on agar (Fig. 5A).

The integrity of the OM in Gram-negative bacteria is essential for survival and is monitored by several transcriptional signaling systems, which can initiate damage repair responses. One of these is the σE stress response, which is activated by a dual signal comprising incorrectly folded OM proteins and by off-pathway intermediates in LPS transport and assembly in the periplasm (40, 41). In response to these signals, σE stimulates the transcription of many genes, including all of the machinery required for the transport and assembly of LPS and OM proteins into the OM (42–44). However, σE also induces expression of small regulatory RNAs, MicA and RydB, which act to inhibit synthesis of several β-barrel OM proteins (45, 46). The Rcs phosphorelay is another signaling system in Gram-negative bacteria, which is induced by OM and cell wall damage and controls the expression of genes involved in motility, extracellular polysaccharide and biofilm formation, virulence, and periplasmic quality control (47). Rcs regulates production of capsular polysaccharide (colanic acid) and extracellular polysaccharide, which help stabilize the OM and combat envelope stress (48). Last, the PhoPQ two-component regulatory system in Gram-negative bacteria is important for bacterial survival in host tissues and senses CAMPs and induces responses that regulate the protein and lipid content of the IM and OM (49, 50). In particular, PhoPQ-induced modifications occur to the lipid A segment of LPS before transport to the cell surface, such as the addition of cationic moieties, including 4-amino-4-deoxyarabinose, that reduce the net negative charge of the LPS and contribute to bacterial resistance to CAMPs and PMB (51).

This background is helpful in understanding the response of *E. coli* to JB-95. Thus, the stimulated incorporation of radiolabeled N-acetylg glucosamine into macromolecules (Fig. 1D) is probably due to a stimulation of capsular polysaccharide production. The appearance in scanning EM pictures of knoblike structures over the surface of cells treated with JB-95 (Fig. 4F) is reminiscent of previously reported surface-exposed poly-GlcNAc (52). The dimensions of the knobs appear quite different from the surface blebs that have been observed upon inhibition of σE in *E. coli* (53) or the membrane protrusions typical of PMB-treated cells (54, 55). Moreover, the results of proteomic and qRT-PCR studies show up-regulation triggered by JB-95 of key proteins involved in capsular polysaccharide biosynthesis and excretion (Wza, Wzc, and Wcal) (Table 2). The unusual accumulations of membrane-like material observed by fluorescence and TEM (Figs. 3D and 4D) might be explained by a build-up of intermediates in capsular polysaccharide or LPS biosynthesis, linked to a C55-undecaprenoid lipid carrier (44, 56). The lipid-bilayer accumulations appear approximately 3 times thicker than a membrane phospholipid bilayer (compare...
Mechanism of Action of a Peptidomimetic Antibiotic

OM; Fig. 4D, arrows). Moreover, because down-regulation of LptD was observed here in the proteomic studies, impaired LPS transport to the cell surface should be expected, which typically leads to dramatic membrane ultrastructural changes (57–59). Mutations in the inner membrane LPS and phospholipid flip-fapse MsBA have also been shown to cause inner membrane reduplications and invaginations due to inhibition of lipid export (60).

The proteomic studies reveal that many β-barrel OM proteins are down-regulated in JB-95-treated cells (Fig. 6 and Table 2). These results can be at least partly explained by a σK-dependent response acting through the small regulatory RNA MicA and/or RydB, which have been shown to inhibit the synthesis of many β-barrel OM proteins in E. coli (45, 46) (Table 2). Interestingly, the essential OM LPS translocase LptD was significantly depleted in the proteomics study (Table 2), although by qRT-PCR only a minor change was seen. In contrast, the regulation of the essential β-barrel folding machine BamA was largely unaffected by JB-95 in both the proteomic and qRT-PCR assays. Because BamA is known to be essential for the correct folding and insertion of most β-barrel proteins into the OM, including LptD (42), an alternative explanation for the depletion of many β-barrel OM proteins may be that JB-95 inhibits the function of BamA. The OM protein Wza, which is up-regulated by JB-95 (Table 2), has a transmembrane domain of eight α-helices (61) that is not dependent on BamA for insertion into the OM (62). Other significant JB-95-induced proteomic changes are increases in levels of proteins active in maintenance of the periplasm (DegP and ClpX) (63) and modifications to lipid A through the PhoPQ response (PhoQ, ArnA, ArnC, and EptA) (51).

High-resolution fluorescence microscopy of E. coli cells exposed to the fluorescently labeled form of JB-95 (flJB-95) shows the peptide concentrated in a punctated pattern (Fig. 3H) in the OM rather than binding uniformly over the cell surface. Early EM and recent fluorescence labeling studies have shown that many OM proteins in E. coli associate into islands or patches in the OM (64–67). The labeling patterns seen with flJB-95 suggest that the peptide is associating with OM protein patches. This conclusion is supported by the photolabeling studies, which show that JB-95 interacts with several β-barrel OM proteins (Fig. 7), interactions that might mediate the disruptive effects on the OM. For example, membrane thinning caused by contact of lipid groups with membrane-bound β-barrel proteins might provide sensitive sites where OM disruption mediated by JB-95 could occur (68). Alternatively, some of the photolabeled β-barrel OM proteins, including BamA and LptD in two different E. coli strains, expose β-strands by transient opening of the β-barrel to form lateral gates during transport, folding, or translocation of substrate molecules across the membrane (69–75) and might be vulnerable to interactions with peptidomimetics having folded β-hairpin structures. These proposals might be testable in future work using reconstituted β-barrel proteins in vitro. Finally, optimization of both the antimicrobial and drug-like properties of molecules like JB-95 could have important implications for the development of a new class of antibiotics specifically targeting the Gram-negative OM.

Author Contributions—J. A. R. and L. E. conceived the study and overview all experimental work. J. B. and M. U. synthesized peptides. J. B., M. U., F. L. M., and K. Z. performed microbiological, microcopy, photolabeling, and fluorescence studies. U. O., C. H. A., and G. P. assisted with proteomics studies; K. M. performed structural studies by NMR. All authors reviewed the results and approved the final version of the manuscript.

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Supporting Information
A Peptidomimetic Antibiotic Targets Outer Membrane Proteins and Disrupts Selectively the Outer Membrane in *Escherichia coli*

1. Peptide synthesis.

2. NMR studies of the solution conformation of JB-95

3. qPCR primers

1. Peptide synthesis.

**Synthesis of the fluorescence probe flJB-95.**

The following synthetic route to the fluorescently labelled flJB-95 was followed:

![Chemical structure of flJB-95](image)

The cyclic peptide precursor was prepared by solid-phase assembly of a linear peptide precursor followed by macrocyclization in solution, using methods described earlier (1). Standard Fmoc-protected amino acids and Fmoc-Glu(PEG3-N$_3$)-OH were used for solid-phase peptide synthesis.

To the cyclic peptide (1.37 mg) in PBS (700 µL) was added BTTAA (2-4-{(bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl]-1H-1,2,3-triazol-1-yl}acetic acid(2)) (0.82 mg), sodium ascorbate (0.1 M, 30 µL), AlexaFluor-488 alkyne (0.25 mg, *Molecular Probes, Inc.*) in DMSO (100 µL) and CuSO$_4$ (0.1 M, 30 µL). After 1 h another aliquot of sodium ascorbate (0.1 M, 30 µL) and CuSO$_4$ (0.1 M, 30 µL) was added and stirred for another 1 h. The reaction was quenched by addition of water (1 mL) containing 0.1% TFA. The product was purified by reversed phase HPLC on an Agilent Eclipse XDB-C18 semi-preparative column employing a MeCN/H$_2$O gradient with 0.1% TFA. Yield 0.6 mg of an orange powder, 95% purity by reverse phase HPLC. Retention time = 9.55 min (*Grace VYDAC® 218TP54, C18 column, 5 µm, 4.6x250 mm, flow 1 ml/min, gradient 20-60% MeCN/H$_2$O +0.1% TFA over 6 column volumes. High resolution ES-MS: m/z 679.58826 (M+4H$^+$), calc. mass 679.58786.)
2. NMR Studies of the Solution Conformation of JB-95

$^1$H NMR measurements were performed either in H$_2$O/D$_2$O (9:1) or pure D$_2$O at pH 5.6 and pH 3. Spectra were acquired on a Bruker AV-600 spectrometer at 300 K. 1D and 2D TOCSY $^1$H NMR spectra were also recorded at various temperatures (280 K, 290 K, 310 K and 320 K) for determination of the amide temperature coefficients. Water suppression was performed by presaturation. $^1$H-NMR assignments (given below) were made using 2D DQF-COSY, TOCSY, NOESY and ROESY spectra. All spectra were referenced on internal trimethylsilyl propanoic acid (TSP).

$^1$H Chemical shifts of JB-95 in 90% H$_2$O/10%D$_2$O, pH 5, 300 K.

|       | NH   | H-C(α) | H-C(β) | Others                  |
|-------|------|--------|--------|-------------------------|
| Trp$^1$ | 7.87 | 4.88   | 3.31, 3.31 | H($\delta^1$) 7.36; H($\epsilon^3$) 7.74; H($\zeta^2$) 7.52; H($\zeta^3$) 7.13; H($\eta^1$) 7.27; NH($\epsilon^1$) 10.24 |
| Arg$^2$ | 8.75 | 4.96   | 1.72, 1.80 | CH$_2$(γ) 1.51, 1.64; CH$_2$(δ) 3.13, 3.16; NH(ε) 7.22; NH$_2$(η) -,- |
| Ile$^3$ | 8.65 | 4.50   | 1.77   | CH$_2$(γ') 1.03, 1.31; CH$_3$(γ') 0.83; CH$_3$(δ) 0.80 |
| Arg$^4$ | 8.56 | 5.04   | 1.60, 1.77 | CH$_2$(γ) 1.39, 1.46; CH$_2$(δ) 3.15, 3.15; NH(ε) 7.16; NH$_2$(η) -,- |
| Ile$^5$ | 8.37 | 4.17   | 1.68   | CH$_2$(γ') 1.03, 1.38; CH$_3$(γ') 0.83; CH$_3$(δ) 0.80 |
| DArg$^6$ | 8.81 | 3.99   | 1.38, 1.52 | CH$_2$(γ) 0.40, 1.07; CH$_2$(δ) 2.85, 2.85; NH(ε) 6.78; NH$_2$(η) -,- |
| Trp$^7$ | 8.93 | 4.90   | 3.10, 3.61 | H($\delta^1$) 7.27; H($\epsilon^3$) 7.68; H($\zeta^2$) 7.51; H($\zeta^3$) 7.13; H($\eta^1$) 7.26; NH($\epsilon^1$) 10.15 |
| Lys$^8$ | 8.14 | 4.52   | 1.85, 1.85 | CH$_2$(γ) 1.38, 1.46; CH$_2$(δ) 1.71, 1.71; CH$_2$(ε) 3.01, 3.01; CH$_3$(ζ) -,- |
| Arg$^9$ | 8.59 | 4.94   | 1.75, 1.75 | CH$_2$(γ) 1.47, 1.65; CH$_2$(δ) 3.16, 3.16; NH(ε) 7.29; NH$_2$(η) -,- |
| Leu$^{10}$ | 8.87 | 4.72   | 1.55, 1.61 | CH(γ) 1.54; CH$_3$(δ) 0.88, 0.88 |
| Arg$^{11}$ | 8.63 | 4.93   | 1.76, 1.87 | CH$_2$(γ) 1.52, 1.66; CH$_2$(δ) 3.18, 3.18; NH(ε) 7.23; NH$_2$(η) -,- |
| Arg$^{12}$ | 8.74 | 4.90   | 1.73, 1.92 | CH$_2$(γ) 1.62, 1.64; CH$_2$(δ) 3.17, 3.25; NH(ε) 7.23; NH$_2$(η) -,- |
| dpro$^{13}$ | -    | 4.71   | 1.90, 2.28 | CH$_2$(γ) 2.02, 2.14; CH$_2$(δ) 3.50, 3.87 |
| Pro$^{14}$ | 4.35 | 1.42, 1.95 | CH$_2$(γ) 0.85, 1.65; CH$_2$(δ) 3.55, 3.64 |

$^3$J$_{HN}$ coupling constants were determined from 1D spectra or from 2D NOESY spectra by inverse Fourier transformation of in-phase multiplets. $^{13}$C,$^1$H]-HSQC spectra at natural abundance were recorded in pure D$_2$O. Chemical shift deviations (CSDs) from statistical random coil values ($\Delta\delta$=δ$_{obs}$-δ$_{coil}$) for Hα, Cα and Cβ resonances were measured. In peptide segments of three or more consecutive residues with predominantly β-sheet conformations, positive Hα and Cβ CSDs (downfield shifts) in the range of >0.1 ppm and >0.5 ppm are expected, whereas negative Cα CSDs <0.5 ppm are expected. The opposite trends apply to α-helical regions. $^3$J$_{HN}$ values are correlated with the backbone torsion angle φ via the Karplus relation and should be <6 Hz in α-helices and >8 Hz in β-sheets. Amide proton temperature coefficients (-$\Delta\delta_{NH}$/T, ppb/K) are typically smaller than -4 to -5 ppb/K for amide protons that are shielded from solvent and/or are
involved in intramolecular hydrogen bond formation. Relative H/D exchange rates of amide protons are slowed when the NH is involved in intramolecular hydrogen bonding or otherwise shielded from solvent accessibility.

Cα and Cβ 13C-chemical shifts for JB-95, 3JHN, (Hz) values, amide temperature coefficients (−Δδ/T (ppb/K)) and amide NH relative H/D exchange rates (+ slow, ++ medium, +++ fast) in 90% H2O/10% D2O, pH 5, 300 K.

| Residue | C(α)  | C(β)  | 3JHN (Hz) | −Δδ/T (ppb/K) | rel. NH kexch |
|---------|-------|-------|-----------|---------------|---------------|
| Trp5    | 56.62 | 31.05 | 8.6       | 1.7           | +             |
| Arg2    | 55.48 | 31.46 | 8.6       | 7.7           | +++           |
| Ile³    | 59.81 | 41.71 | 9.4       | 3             | +             |
| Arg⁴    | 55.22 | 32.0  | 9.1       | 6.7           | ++            |
| Ile⁵    | 60.4  | 39.30 | 9.5       | 2.1           | +             |
| d-Arg⁶  | 58.02 | 28.96 | 4.4       | 7.6           | +++           |
| Trp⁷    | 56.58 | 29.8  | 9.3       | 9             | +++           |
| Lys⁸    | 56.03 | 34.63 | 8.7       | 3             | ++            |
| Arg⁹    | 55.45 | 31.57 | 9.1       | 7.1           | +++           |
| Leu¹⁰   | 54.28 | 45.1  | 9.0       | 2.9           | +             |
| Arg¹¹   | 55.52 | 31.91 | 8.6       | 6.9           | +++           |
| Arg¹²   | 52.98 | 32.08 | nd        | 2.4           | +             |
| d-Pro¹³  | 61.42 | 30.73 |           |               |               |
| Pro¹⁴   | 63.68 | 32.17 |           |               |               |

JB-95 appeared as a single species in solution, with trans peptide bonds. For example, a strong Hα-Hδ NOE was observed between Arg12-dPro13. The pattern of CSD values indicates a regular β-hairpin conformation with two extended β-strands between residues 1-5 and 8-12 characterized by positive ΔδHα, positive ΔδCβ and negative ΔδCα values. The strands are separated by a turn characterized by negative ΔδHα, negative ΔδCβ and positive ΔδCα values at dArg⁶ and opposite CSD values at Trp⁷. These data are consistent with the formation of a type II′ β-turn. 3JHN,Cα coupling constants show large values >8 Hz except for residue 6 in the turn region, which is clear evidence for predominantly extended backbone conformations. Furthermore, H/D exchange experiments show slowly exchanging amide protons at Trp1, Ile3, Ile5, Lys8, Leu10 and Arg12. These residues are expected to occupy hydrogen-bonding positions in a regular β-hairpin register, due to intra-molecular hydrogen-bonding. The H/D exchange results correlate very well with the low NH temperature coefficients seen for these residues with values of < 4 ppb/K. The hydrogen bonds identified in these measurements were used as distance restraints in the calculation of the NMR-based structures.

Distance restraints were obtained from NOESY and ROESY spectra with a mixing time of 250 ms. The structure calculations were performed by restrained molecular dynamics in torsion angle space by applying the simulated annealing protocol implemented in the program DYANA (3). Starting from 100 randomized conformations a bundle of 20 conformations is selected, which incur the lowest DYANA target energy function. Well-ordered average solution structures could be determined based on numerous inter-strand NOEs in aqueous solution, which reveal a high population of β-hairpin conformations.
Characteristic backbone NOE connectivities of the β-hairpin fold could be observed in the NOESY spectrum between HN protons of Trp1-Arg12, Ile3-Leu10 and Ile5-Lys8. The calculated solution NMR structures converge to a narrow range of backbone conformations with an average rmsd to the mean structure of 0.9 Å. As assumed based on the CSD pattern in the tip region, a type II’ β-turn was found with characteristic Hα-HN and HN-HN NOEs between DArg⁶-Lys⁸ and Trp⁷-Lys⁸, respectively.

Statistics for the final 20 NMR structures calculated for JB-95 calculated using DYANA are given below.

| statistics for JB-95 | 
|---------------------|
| Number of NOE upper-distance limits | 150 |
| Intraresidue | 80 |
| Sequential | 37 |
| Medium- and long-range | 33 |
| Residual target function value (Å²) | 0.93 ± 0.18 |
| Mean rmsd values (Å) | 
| All backbone atoms | 1.32 ± 0.80 |
| All heavy atoms | 2.83 ± 1.01 |
| Residual NOE violations | 
| Number > 0.2 Å | 10 |
| Maximum (Å) | 0.34 |

3. qPCR primers

A list of primers used for qPCR analyses is given below.

Oligonucleotides used for qPCR:

| Primer name | Sequence (5' - 3') |
|-------------|--------------------|
| wcaI_F      | TTCGCGTTCGATGATGAATA |
| wcaI_R      | TTCACCAATATTGCCGGAGT |
| wzc_F       | GATGTCGAGTCTGGTCAGCA |
| wzc_R       | CAGCACACCCACGATAGAAA |
| murC_F      | ATGGTTTCCAGCATCTACGC |
| murC_R      | GTATCCATGTGGTCGCTTC |
| arnA_F      | CCGCCATTAAACACCGGTAAT |
| arnA_R      | CGACCAGACGGAATTTCT |
| ompF_F      | ACGCTTCTACGATGTTGGT |
| ompF_R      | AGCCGCTGGTGTTTTGTAAT |
| lptD_F      | TGAAGTCCTACGATGTTGGT |
| lptD_R      | GAAACGGTGGCATTGAAGTT |
| lamB_F      | TGATTGTCTCTCTCTCATCG |
| lamB_R      | GAATACCCAGCGTCGTTTCG |
| bamA_F      | CCCGTTCACCACTCGACTCT |
| bamA_R      | GGTCACTTTGTTGCGTTAT |
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