Supporting Information

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SI Experimental Procedures

Bacterial Strains and Culture. *Escherichia coli* strains were grown in Luria–Bertani broth or agar at 37 °C and were used to propagate plasmids. *Enterococcus faecalis* strains OG1RF (1) and 0852 (2) were inoculated from single colonies grown on Brain Heart Infusion (BHI) agar at 37 °C into BHI broth and were grown statically overnight for 15–18 h before all assays unless otherwise noted. Antibiotics were added at the following concentrations: for *E. coli*, kanamycin 50 mg/L and erythromycin (Erm) 750 mg/L; for *E. faecalis* strains, Erm, 25 mg/L; fusidic acid, 25 mg/L; kanamycin, 500 mg/L; and rifampin, 25 mg/L.

Genetic Manipulations. *E. faecalis* OG1RFΔ*srtA* (3) or *mprF2* was transformed with plasmid pAK1::*srtA*-HA [previously called pAL1::*srtA*-HA, native (4)] in which epitope-tagged sortase A (SrtA) is expressed on a plasmid and transcribed from the native *srtA* promoter.

E. faecalis OG1RF genes targeted for mutation were identified based on the annotated complete genome of *E. faecalis* V583 (5); all references to genomic loci are based on this annotation (GenBank accession number AE016830). Multiple peptide resistance factor (MprF) homologs in *E. faecalis* were identified by BLASTP searches of the sequenced enterococcal genomes using MprF protein sequences from *Staphylococcus aureus* and *Listeria monocytogenes* (6, 7).

To create in-frame deletions of *mprF1* and *mprF2* in strain OG1RF, regions ∼1 kb upstream and downstream of the genes were amplified from OG1RF using primer pairs dEF0031F/EF0031_sewR or dEF1027F_IF/newEF1027_sewR for upstream regions and EF0031_sewF/dEF0031R or newEF1027_sewF/dEF1027R_IF for downstream regions. These products were sewn together and amplified using the primer pairs dEF0031F/dEF0031R or dEF1027F_IF/dEF1027R_IF. These PCR products were cloned into pCR2.1 (Invitrogen) according to the manufacturer's protocol and were sequenced. Correct clones subsequently were subcloned into the temperature-sensitive shuttle vectors pJRS233 or pGCP213 (8, 9), and the flanking PstI or EcoRI sites were used to generate deletion constructs pJRS213-Δ*mprF1* and pGCP213-Δ*mprF2*, respectively. Deletion constructs then were transformed into OG1RF by electroporation, and the transformants were selected at 30 °C on Erm. Chromosomal integrants were selected by growth at 42 °C in the presence of Erm. Selection for excision of the integrated plasmid by homologous recombination was accomplished by growing the bacteria at 30 °C in the absence of Erm. Loss of the *mprF1* or *mprF2* loci in Erm-sensitive bacteria was demonstrated by PCR using the primer pairs dEF0031F/dEF0031R or dEF1027F_IF/dEF1027R_IF.

The complementation construct of *mprF2* was made in pGCP123 (9). The coding sequence for *mprF2*, including its promoter, was amplified from OG1RF with EF1027compF_IF/EF1027compR_IF, digested with EcoRI and BamHI, and ligated into pGCP123. Resulting plasmids were confirmed by sequencing, transformed into *E. faecalis*, and were selected for on BHI Kan.

Human β-Defensin 3-Cy3 Synthesis, Purification, and Activity Testing. Synthetic human β-defensin 3 (hBD3) (100 μg) (10) was dissolved in 100 μL 10 mM sodium-phosphate buffer, pH 7.0 [instead of sodium bicarbonate buffer (pH 8), to favor amino-terminal labeling and to minimize labeling at lysins]. To these solutions 3 μL Alexa Fluor 594 (Component A microscale protein-labeling kit; Alexa Fluor 594 succinimidyl ester; Invitrogen) were added, and the mixtures were incubated for 1 h at room temperature in the dark. Thereafter samples were acidified with 1% aqueous (vol/vol) trifluoroacetic acid and were separated by reversed-phase HPLC (Jupiter C2C18, Phenomenex) with recording at 215 nm, 280 nm, and 350 nm. Aliquots of HPLC fractions were assayed for antimicrobial activity [radial diffusion assay; *E. coli* was used as the read-out (11)]. Fractions containing strong antimicrobial activity and strong absorbance at >300 nm (which indicates the presence of a Cy3 chromophor) were analyzed by ESI-MS. Antimicrobially active fractions showing positive ion mode signals, which corresponded to the addition of one or two Cy3 molecules to each β-defensin molecule, were pooled, lyophilized, and stored in the dark until further use.

Fluorescent Microscopy. Overnight cultures of wild-type *E. faecalis* and *mprF* mutant cells were subcultured 1:10 into BHI and were grown statically for ∼2 h, harvested at mid-log phase (OD_{600} 0.5), washed once by resuspension in 0.01 M low-salt phosphate buffer (PB), and then diluted an additional 1:4 with PB.

For immunofluorescent microscopy, 100 μL of cells diluted in PB or treated with human β-defensin 2 conjugated to a fluorophore (hBD2-Cy3) were resuspended in 4% (vol/vol) paraformaldehyde and incubated for 20 min. Then 20 μL of fixed cells were spread onto poly-L-lysine slides and were dried in the dark for 15 min at room temperature. The cell wall then was lysed on the slide by adding 20 μL of lysozyme (20 mg/mL) to the cells and incubating for 2 h at 37 °C. Removal of the cell wall was validated by staining with 20 μL of the polysaccharide-binding Calcofluor White Stain (catalog no. 18909; Sigma Aldrich) for 1 min immediately after lysozyme treatment. Absence of cell wall was confirmed by absence of Calcofluor red fluorescence on the cell surface. After incubation the cells were washed with PB, blocked with 2% (wt/vol) BSA in PB (P-BSA) for 20 min, incubated with 20 μL of primary antibody rabbit α-HA (1:500 dilution in P-BSA) (H6908; Sigma Aldrich) or rabbit α-SecA (1:500 dilution in P-BSA) (12) at 4 °C overnight. Slides then were washed extensively with PB, incubated with Alexa Fluor 488 goat anti-rabbit secondary antibodies for visualizing SecA (catalog no. A-11034; Invitrogen Inc.) or Alexa Fluor 568 goat anti-rabbit secondary antibodies for visualizing SrtA-HA (catalog no. A-11011; Invitrogen, Inc.), both diluted 1:1,000 with P-BSA, incubated for 1 h at room temperature, washed extensively with PBS, mounted with Vectashield mounting media (Vecalotor Laboratories, Inc), covered with 0.16–0.19 mm thick coverslips, dried, and imaged. Immunofluorescent imaging was performed using an inverted epifluorescence microscope (Zeiss Axio observer Z1; Carl Zeiss GmbH) fitted with a 100× oil immersion objective with numerical aperture 1.4 optovar magnification changer 1.5×. For unbiased image analysis, exposure times on the wide-field microscope were fixed to 1,500 ms for all experiments for unbiased image analysis. The costaining images were acquired using AF488/FITC filter cube sets fitted with a 460–490 nm bandpass excitation filter and a 515–550 nm bandpass barrier filter and with AF568/cy3 filter cube sets fitted with a 530–550 nm bandpass excitation filter and a 590-nm-long pass barrier filter. Control labeling experiments were performed in parallel with controls omitting the primary antibody and were consistently negative at the concentration of secondary antibody used in these studies. Images were processed using Adobe Photoshop CS5.1.

EM. *E. faecalis* OG1X was grown to midlog phase (∼OD_{600} 0.5), and the cells were adjusted to ∼10^2 cfu/mL in 0.01 M sodium phosphate buffer, pH 7.0. Cells were incubated with a sub-

inhibitory concentration of 200 μg/mL hBD2 (Peptides International) for 10 min at 37 °C, washed in PB, and resuspended in 3% (vol/vol) paraformaldehyde/0.1% glutaraldehyde. Cells then were infiltrated in 2.3 M sucrose and frozen in liquid nitrogen. Cryosectioned samples were labeled with the primary antibody goat α-hBD2 (AbD Serotec) at 1:100 and were detected by secondary rabbit α-goat conjugated with 10 nm gold 1:100 (Biocell, BBInternational). Sections were analyzed in a Tecnai 10 at 80 kV (FEI Company), and digital images were taken in a Megaview III (Soft Imaging System GmbH).

Cationic Antimicrobial Peptide Killing Assays. Overnight cultures of *E. faecalis* were diluted 1:20 into BHI and were grown for ∼2 h to OD_{600} 0.4–0.6. The cultures then were normalized to OD_{600} 0.5 and were further diluted 500-fold in PB. Then 50 μL of bacterial culture was incubated with concentrations of hBD2 ranging from 6.25–50 μg/mL (Peptide Institute Inc.) for 2 h at 37 °C. Bacteria then were serially diluted, and surviving bacteria were determined by cfu enumeration. Bacteria were resuspended in, and the assays were performed in, 0.01 M sodium phosphate buffer, pH 7.0.

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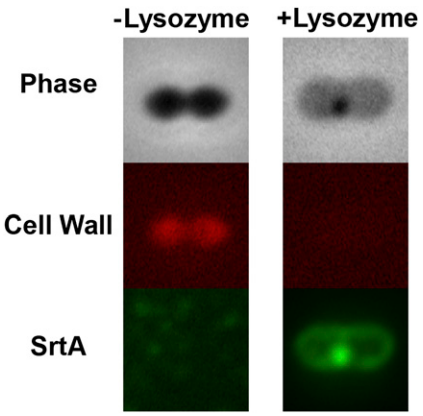

Fig. S1. Cell wall degradation by lysozyme is required for SrtA immunofluorescence. *E. faecalis* OG1RF Δ*srtA* pAK1::*srtA*-HA was subjected to immunolabeling with α-HA for the localization of SrtA in the absence of presence of lysozyme. Cell wall removal by lysozyme was confirmed by the loss of staining by Calcofluor White.

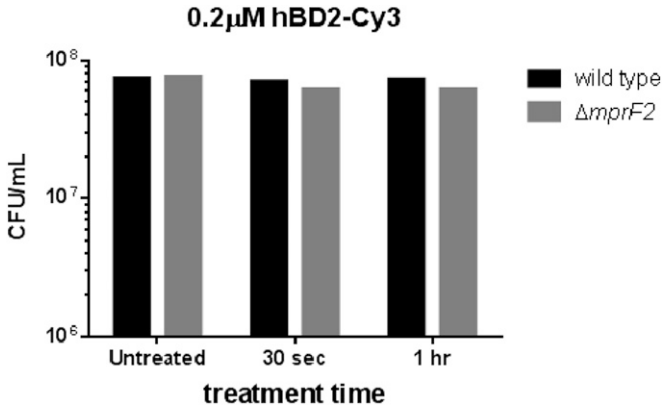

Fig. S2. hBD2-Cy3 at 0.2 μM does not inhibit *E. faecalis* growth. Wild-type OG1RF or Δ*mprF2* cells were incubated with 0.2 μM hBD2-Cy3 for 30 s to mimic the exposure time for fluorescent microscopy assays or for 1 h to ensure that prolonged exposure to hBD2-Cy3 was not inhibitory.

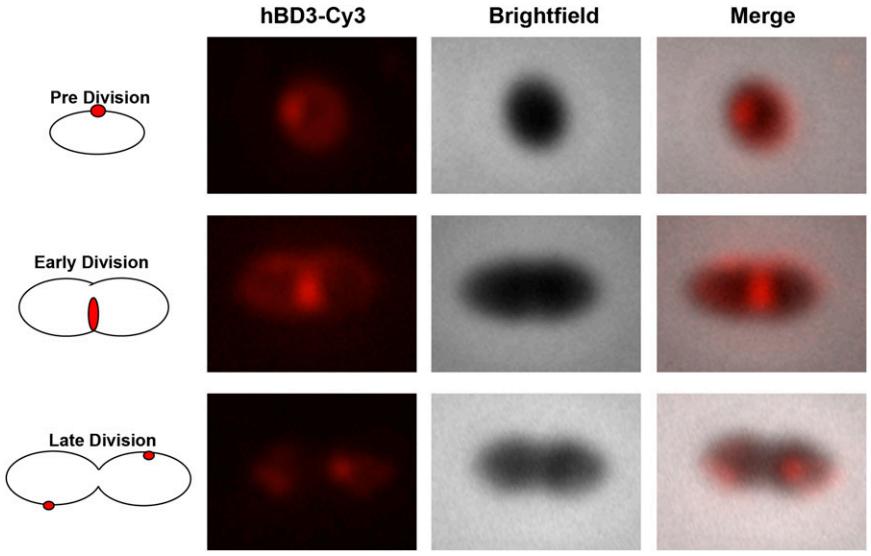

Fig. S3. hBD3 targets live *E. faecalis* OG1RF wild-type cells in a focal manner. Representative images of live *E. faecalis* OG1RF incubated with hBD3-Cy3 and imaged without fixation or processing. (*Left*) Cy3 fluorescence images. (*Center*) Phase-contrast images. (*Right*) Merged fluorescence and phase-contrast images.

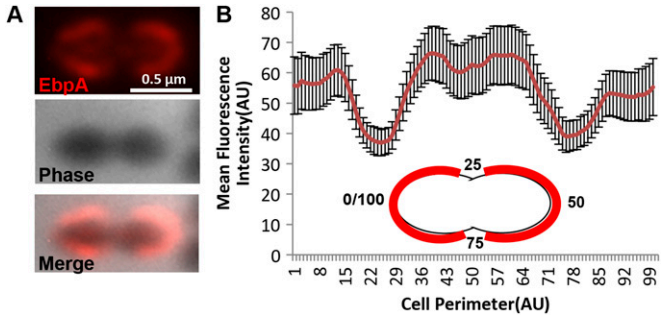

Fig. S4. Endocarditis and biofilm-associated (Ebp) pili are distributed in a nonfocal, hemispherical pattern around the mature cell wall. (*A*) Representative images of fixed *E. faecalis* OG1RF labeled with α-Epb subunit A (EbpA) (9), as described previously (4). (*B*) The mean fluorescent intensity around the cell perimeter was determined by Projected System of Internal Coordinates from Interpolated Contours software (MATLAB). Cells in which no fluorescence was detected were excluded from the analyses.

Table S1. Primers used in this study

Primer name	Sequence (5′-3′)
dEF0031F_IF	GCTCTAGAAGGATGCGCCAAGGTTTTGA
EF0031_sewR	GCAAAGAGTACGCTGGTCCGAATATCCGCCCAGCTCATGC
EF0031_sewF	GCATGAGCTGGGCGGATATTCGGACCAGCGTACTCTTTGC
dEF0031R_IF	GCTCTAGACGCCTTAGGTTTCCGCTCAC
dEF1027F_IF	GATATCTGCAGAATTCTTGGAAAAATTACGAAAACGAAA
EF1027_sewR	AACAGGGTCATTTATTTGCCACGACAGATTTTGCGGTTACAT
EF1027_sewF	ATGTAACCGCAAAATCTGTCGTGGCAAATAAATGACCCTGTT
dEF1027R_IF	TACCGAGCTCGGATCCTGGGCACCAATTAAAACGAT
EF1027compF_IF	GCTTGATATCGAATTCTATGGCATCCCAGTGATTGA
EF1027compR_IF	TAGAACTAGTGGATCCTTAATTGCGCTTTCCCATTC