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Focal targeting by human β-defensin 2 disrupts localized virulence factor assembly sites in Enterococcus faecalis


S. pyogenes and Enterococcus faecalis, SecA often localizes at Corynebacterium diphtheriae, S. mutans, S. agalactiae, and S. pneumoniae. In S. pyogenes, focal localization of the ExPortal-factor assembly sites. More recently, the specificity of focal targeting by defensins is important for efficient bacterial killing, and that pathogens have evolved mechanisms to balance focal localization of secretion and sortase enzymes with modification of these localization sites to combat defenses.

Significance

In Gram-positive bacteria, many virulence factors are assembled and attached to the growing cell wall by sortase enzymes, which are localized to one or two sites in the cell membrane. Mislocalization of sortase enzymes negatively impacts their function. We demonstrate that β-defensins target Enterococcus faecalis at discrete sites, resulting in the mislocalization of sortase and associated secretion enzymes. The multiple peptide resistance factor protein, which adds cationic residues to anionic phospholipid domains in the membrane, limits focal defensin targeting in E. faecalis. This work suggests that focal targeting by defensins is important for efficient bacterial killing, and that pathogens have evolved mechanisms to balance focal localization of secretion and sortase enzymes with modification of these localization sites to combat defenses.


The authors declare no conflict of interest.

See Commentary on page 19880.

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on the presence of anionic phosphatidylglycerol lipid domains in the membrane (19).

Cationic antimicrobial peptides (CAMPs), part of the innate immune repertoire, have a variety of bacterial targets, including anionic membrane constituents such as LPS and teichoic acid, cell-wall precursors, and the phospholipid-rich bacterial cell membrane (20–22). Accordingly, bacteria have evolved a number of mechanisms to resist CAMP killing (23). One well-studied example is multiple peptide resistance factor (MprF), a membrane protein that aminoacylates anionic phospholipids, usually adding cationic amino acids to the phospholipid head groups, in a variety of Gram-positive organisms to protect these organisms from killing by cationic peptides (24–27). Despite microbial resistance mechanisms, defensins present an enticing class of anionic ribosomal agents for targeting anionic lipid-associated domains of localized virulence factor assembly on the bacterial surface.

In this study, we examine whether secretion- and sorting-associated microdomains in E. faecalis, a leading cause of nosocomial and opportunistic infections, are specifically targeted by CAMPs. We show that human β-defensins interact with E. faecalis at discrete foci, interrupting localized sites of secretion and sorting in the membrane. Further, we show that modification of anionic membrane lipids confers resistance to and limits focal targeting by cationic antimicrobial peptides, providing a link between localized interaction of CAMPs and efficiency of killing.

Results

SecA and Sortase A Are Focally Localized on Whole E. faecalis Cells.

We have shown previously that SecA colocalizes with both sortase A (SrtA) and sortase C (SrtC) at discrete foci, often near the septum, in E. faecalis (7). These findings were achieved by immunoelectron microscopy (IEM) labeling of E. faecalis thin sections to image membrane and cytoplasmic proteins while bypassing barriers to antibody penetration presented by the cell wall and cell membrane. To extend our earlier analyses, we have compared hBD-2-mediated cell-wall deacetylation and detergent-mediated membrane permeabilization on whole bacterial cells and have performed immunofluorescent microscopy (IFM) analyses to probe wild-type cells and wild-type cells expressing a fully functional SrtA that was epitope tagged with human influenza HA (7).

The localization pattern of SecA was characterized at three size stages (Experimental Procedures) as cells progressed through division using an antibody raised against E. faecalis SecA (α-SecA). Consistent with our previous IEM observations, SecA predominately localized to the equatorial mid-cell at each stage of cell division, appearing as single foci at the septum at early stages of division (Fig. 1 A and B) and appearing in a single- or multifocal pattern at nascent sites of cell division during late stages of cell division (Fig. 1 C). As expected for the detection of cytoplasmic antigens, lysozyme treatment and membrane permeabilization were required for SecA immunolabeling.

SrtA localization was very similar to that of SecA, with prominent single, equatorial foci at early stages of division (Fig. 1 D and E) and a multifocal pattern at late stages of division (Fig. 1 F). Lysozyme treatment was required for labeling the membrane protein SrtA, confirming that the enterococcal cell wall serves as a barrier to antibody penetration (Fig. S1). Our previous IEM studies demonstrating SrtA and SecA colocalization did not stratify the cells by the stage of cell division and therefore likely reflected colocalization in the most abundant cells in the population, i.e., those in the early stages of division (Experimental Methods). Here we show that IFM patterns of SrtA and SecA colocalization are similar in pre- and early-division cells but differ in late-division cells (Fig. 1 C and F), possibly reflecting different rates or modes of movement from the active to the nascent division site.

Human β-Defensin 2 Targets E. faecalis in a Focal Manner That Coincides with the Midcell.

To test whether CAMPs target the E. faecalis bacterial membrane in a localized manner, we examined the localization pattern of fluoresecently labeled human β-defensin 2 (hBD2) (28) upon interaction with the bacterial cell. To visualize the initial interaction of the peptide with live bacteria and to prevent cellular lysis, subinhibitory concentrations of hBD2 were used. We incubated the live E. faecalis strain OG1RF [a laboratory strain, originally isolated from the oral cavity (29)] or 0852 [a low-passage urine isolate (30)] grown to midlog phase with 0.2 μM hBD2 directly conjugated to a fluorophore (hBD2-Cy3) for 1 or 5 min, respectively. Exposure to 0.2 μM hBD-Cy3 had no effect on cell viability (Fig. S2). We observed by fluorescent microscopy a cell cycle-associated pattern of focal hBD2-Cy3 localization that coincided with the sites of the current or nascent division plane (Fig. 2 A and B). Early in the division cycle, a ring-like pattern of hBD2-Cy3 was visible around the nascent septum. In bacteria undergoing cell division, hBD2-Cy3 localized to puncta at the current or next division plane (Fig. 2 A and B). Treatment of E. faecalis OG1RF with hBD3-Cy3, which retains the same biological activity as native hBD3 and which bears a higher net positive charge than hBD2, revealed similar punctate targeting of the bacterial mid-cell (Fig. S3). Together, these observations suggest that the antimicrobial peptides hBD2 and hBD3 interact with the E. faecalis surface at distinct foci at or near the septum.

To validate hBD2-Cy3 interaction patterns observed on live E. faecalis strain OG1RF and 0852 cells and to ensure that localization was not a consequence of nonspecific fluorophore interactions with the cell, we incubated live E. faecalis cells with a subinhibitory concentration of biologically active hBD2 lacking a fluorophore. Bacteria treated with hBD2 then were fixed, cryosectioned, labeled with α-hBD2 and gold-labeled secondary antibodies, and subjected to immunogold transmission electron microscopy. Single puncta of hBD2 in association with the membrane of single cells were observed in hBD2-treated cells (Fig. 2 C) but not in untreated control cells (Fig. 2 D).

Focal Localization of SrtA and SecA Is Disrupted upon Treatment by Antimicrobial Peptides. If hBD2 targets wild-type E. faecalis at sites of secretion and sorting, we postulated that hBD2 should either colocalize with SecA and SrtA or perturb their localization. We therefore exposed live E. faecalis OG1RF pkA1:srtA-HA cells in exponential phase to hBD2-Cy3, followed by fixation, lysozyme treatment with or without membrane permeabilization, and immunolabeling for each protein. We observed typical focal SrtA-HA and SecA localization in the absence of defensin (Fig. 3 A and C). However, focal targeting by hBD2-Cy3 resulted in dispersal of SrtA-HA and SecA foci (Fig. 3 E and G).
To quantify the altered patterns of SrtA and SecA localization in response to defensin exposure, we focused on cells in the early stages of division (Fig. 1) because they were most abundant within the log-phase population under study (Experimental Procedures). For quantitative image analysis, we used Projected System of Internal Coordinates from Interpolated Contours (PSICIC), a program originally developed for elongated cells such as *E. coli* and *Caulobacter crescentus* (31, 32). We extended the program to record the fluorescence intensity at the perimeter of ovococcal and diplococcal *E. faecalis* cells, enabling the rapid quantification of localized fluorescence measurements at the membrane of the bacteria. To control for cell-cycle differences in localization, we analyzed cells with perimeters between 4.8–8 μm for lysozyme-treated cells and between 3.6–6 μm for untreated cells, corresponding to early division. At this stage, position 25 (in arbitrary units, AU) of the cell perimeter corresponds to one side of the cell.

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**Fig. 2.** hBD2 interacts focally with *E. faecalis*. (A and B) Representative images (Top, Cy3 fluorescence; Middle, phase contrast; Bottom, merge of fluorescence and phase contrast) of live *E. faecalis* OG1RF (A) and 0852 (B) cells incubated with hBD2-Cy3 and imaged without fixation or processing. (Scale bar in A, 0.5 μm.) (C and D) Immunoelectron microscopy of *E. faecalis* OG1X incubated with a subinhibitory concentration of hBD2 (C) or untreated (D), followed by fixation and immune-labeling with α-hBD2 and a gold-labeled secondary antibody. (Scale bars in C and D, 200 nm.)

**Fig. 3.** Antimicrobial peptides disrupt SrtA and SecA focal localization. *E. faecalis* OG1RF ΔsrtA pAK1::srtA-HA cells were incubated with buffer (A–D) or subinhibitory concentrations of hBD2-Cy3 (E and F) before fixation and processing for fluorescent microscopy. (A and E) SrtA-HA was visualized via antibodies against the HA tag. (C and G) SecA was visualized via α-SecA antibodies. Representative images are shown. (Scale bar, 0.5 μm.) The mean fluorescent intensity around the cell perimeter from at least 200 early-division cells from two independent experiments was determined by PSICIC software. Cells in which no fluorescence was detected were excluded from the analyses. Units are displayed in arbitrary units (AU). Cartoons (B and D) depict the predominant site of SrtA-HA and SecA localization in hBD2 untreated cells; the coordinates of the perimeter that correspond with the x-axes (B, D, F, and H) are shown also. In B, D, F, and H, the bold line indicates the mean and error bars indicate the SEM fluorescence at each point on the perimeter. All images were deconvolved using ZEN (blue edition) by Carl Zeiss Microimaging GmbH.
Septum, and 75 AU corresponds to the other side. Consistent with earlier observations (Fig. 1), the mean fluorescent intensity corresponding to sites of SrtA and SecA foci peaked at the septa in the absence of defensin (Fig. 3 B and D). In contrast, after hBD2-Cy3 treatment, the defensin localized most prominently at the septum, whereas SrtA and SecA displayed a diffuse localization pattern significantly different from that in untreated cells (P < 0.0001; Kolmogorov-Smirnov test) (Fig. 3 F and H). In comparison with focal membrane proteins, cell-wall-anchored proteins in wild-type cells were not focally localized but instead were distributed in a relatively diffuse and homogenous pattern throughout the cell wall (Fig. S4). From these experiments we conclude that SrtA and SecA localization is altered after treatment with hBD2-Cy3.

Anionic Lipid Microdomains Are Present in E. faecalis. Membrane targeting and permeabilization is critical for CAMP activity in which the peptides intercalate and form pores that ultimately kill the bacteria (20). We therefore postulated that the focal interaction of hBD2 with E. faecalis might be governed by anionic lipid domains, because CAMPs preferentially interact with anionic lipids. In support of this hypothesis, the fluorescent anionic lipid probe nonyl acridine orange (NAO) specifically stained the E. faecalis membrane at discrete domains (Fig. 4).

MprF Reduces Focal Targeting by hBD2. Many bacteria encode MprF to mediate resistance to cationic antimicrobials (27, 33, 34). E. faecalis contains two paralogs of this gene, mprF1 and mprF2. Recently, MprF2 was shown to modify anionic phospholipids in E. faecalis strain 12030, resulting in Lys-phosphatidylglycerol (Lys-PG), Ala-PG, and Arg-PG, whereas mprF1 did not have a role in PG aminoacylation (27). To examine the contribution of E. faecalis OG1RF MprF1 and MprF2 in focal interaction with antimicrobial peptides, we created in-frame deletions in the gene encoding each protein. Consistent with findings in E. faecalis strain 12030, strain OG1RFΔmprF2 was more sensitive to CAMP killing after treatment with increasing concentrations of hBD2, but OG1RFΔmprF1 was not (Fig. 4B). In complementation analyses, a plasmid expressing wild-type mprF2 (ΔmprF2/mprF2) restored resistance to hBD2 killing to levels identical to wild type (Fig. 4B).

To examine whether MprF2 plays a role in the focal interaction between hBD2-Cy3 and E. faecalis, we incubated live OG1RFΔmprF2 with subinhibitory concentrations of hBD2-Cy3 (Fig. S2). The fluorescent defensin interacted with wild-type and OG1RFΔmprF2 cells in a similar focal pattern, but increased fluorescence intensity was observed at the foci in the mutant strain (Fig. 4C). The increased hBD2-Cy3 binding to mprF2 mutant cells was quantified in early-division cells. E. faecalis ΔmprF2 cells were associated with a quantitative increase in fluorescence compared with wild-type cells, and the increased fluorescence occurred at the same septal region of the cell as in wild-type cells (Fig. 4D). Complementing the ΔmprF2 deletion with mprF2 on a plasmid restored hBD2 focal targeting to wild-type levels (Fig. 4E). Complementation experiments were performed in the presence of antibody for plasmid maintenance, resulting in an overall decrease in fluorescence intensity in all strains for unknown reasons. Taken together, these data show that E. faecalis MprF2 confers resistance to killing by hBD2 with concomitant decreased hBD2 targeting. These findings support the hypothesis that specific and focal targeting of the bacterial membrane by hBD2 not only disrupts SrtA and SecA foci but also plays a critical role in the killing potential of the peptide.

Discussion

We have shown that CAMP defensins interact with E. faecalis at discrete foci at the cell membrane and that this targeted interaction disrupts the focal localization of secretion and virulence factor assembly proteins. Further, we provide evidence that the MprF2 protein implicated in neutralizing negatively charged head groups of anionic lipids plays a protective role in focal cationic defensin targeting of enterococci. From this work we propose a working model in which anionic lipid-enriched microdomains in the membrane coordinate localized secretion and virulence factor assembly and that these microdomains are specifically targeted by cationic defensins (Fig. 5A).

Our model presupposes that these lipid domains would contain anionic lipids in both membrane leaflets, so that anionic head groups face both the cytoplasmic and extracellular space to coordinate endogenous protein localization and exogenous peptide targeting, respectively. A subset of anionic lipids within the domain would be modified by MprF2 mediating electrostatic repulsion of CAMPs (24), giving rise to the observed enhanced hBD2 binding in the absence of mprF2 (Fig. 5B). These assumptions are plausible in light of a recent report showing that both leafllets of the E. faecalis cell membrane contain equivalent amounts of lysylphosphatidylglycerol and other forms of

![Figure 4](image-url)

**Fig. 4.** Antimicrobial peptide foci are more intense in mprF mutants. (A) Live E. faecalis OG1X were incubated with NAO, which specifically interacts with anionic lipids. (B) Relative survival after exposure to increasing concentrations of hBD2 was measured for OG1RF wild-type, ΔmprF2, and ΔmprF2/mprF2 cells. (C) E. faecalis OG1RF wild-type, ΔmprF1, or ΔmprF2 cells incubated with 0.2 μM Cy3-hBD2 throughout the cell cycle. (D) Mean fluorescent intensity around the cell perimeter of at least 340 early-division cells per strain, grown in the presence of kanamycin to ensure plasmid maintenance. (D and E) Intensity analysis performed in PSICIC (MATLAB); error bars reflect the SEM.
modified PG as well as approximately equal amounts of overall PG and modified PG in the membrane (35).

We have shown previously that the septal focal localization of SrtC enzymes involved in pilus biogenesis in enterococci requires a highly cationic region on the cytoplasmic tail of the protein (see SortaseC in Fig. 5). Neutralizing or rendering the cytoplasmic tail negatively charged resulted in diffused SrtC localization and a concomitant reduction in piliation, arguing that focal SrtC localization is linked to its efficient function in pilus biogenesis. In that study, we proposed that sortase focal localization could be mediated via electrostatic interactions between anionic lipid domains and the cationic cytoplasmic tail of the localized protein (7). Consistent with this model, we also showed SecA to colocalize with sortase enzymes. Furthermore, it is established that secretion proceeds more efficiently in the presence of anionic lipids (17). Numerous bacteria, including E. faecalis, possess anionic lipid microdomains in their membranes, as visualized by incorporation of the fluorescent dye NAO (18, 36–38). Anionic lipids are specifically enriched at focal sites of secretion in S. pyogenes (18), and depletion of anionic lipids results in the mislocalization of translocon foci in B. subtilis (19). Secretion of Listeria monocytogenes listeriolysin O is diminished in an mprF mutant, suggesting a link between secretion and anionic lipid modifications in that organism as well (39). Together, these observations support a model of localized anionic lipid domains that are protected by MprF2 and that coordinate sites of focal secretion and sorting.

At each stage of the cell division cycle, hBD2 and hBD3 interact with E. faecalis predominantly at the septum or nascent septum, where SecA and SrtA foci also are observed, and hBD2 exposure results in dispersal of these focal domains of virulence factor assembly. What is the target of hBD2 that leads to dispersed localization of SrtA and SecA? Many CAMPs kill bacteria via direct interactions with the cell membrane leading to pore formation and cell death. However, some CAMPs have additional targets that facilitate their toxic effects (40). Particularly well studied are peptides that interact specifically with the peptidoglycan biosynthesis intermediate lipid II at high affinity, including the lantibiotic nisin, fungal defensin plectasin, human neutrophil peptides, and hBD3, allowing them to “dock” at sites of cell-wall synthesis resulting in the sequestration of lipid II away from its functional location and/or resulting in localized membrane pore formation (22, 41–45). Because nascent PG synthesis, and hence lipid II appearance, occurs in rings emanating from the septal area in ovococci such as E. faecalis, it would not be surprising for lipid II targeting CAMPs to localize to the septal area as well, as we observe. However, a high-affinity interaction between hBD2 and lipid II has not been reported. Therefore, we postulate that, regardless of the initial point of interaction of the defensin with the cell, lipid II in the case of hBD2, and a possible yet-to-be described target for hBD3, both molecules ultimately interact with membrane microdomains, leading to mislocalization of secretion and sorting of proteins. Akin to this notion, treatment of S. pyogenes with subinhibitory concentrations of the cyclic cationic peptide antibiotic polymyxin B results in localized sites of interaction of the peptide with the bacterium as well as reorganization of SecA from single foci to multiple foci or diffuse localization and a reduction in secretion of some streptococcal toxins (46). Conversely, subinhibitory concentrations of some antimicrobial peptides, such as the cathelicidin LL-37, also can induce the expression of virulence factors in S. pyogenes, suggesting possible opposing responses to CAMPs at sublethal but physiologically plausible levels (47).

Antimicrobial peptides have been proposed as attractive candidate therapies against increasingly multidrug-resistant bacteria because they have retained antibacterial efficacy despite millennia of coassociation between microbes and the host. Although bacteria have evolved CAMP-resistance mechanisms, these mechanisms are not highly effective compared with mechanisms of resistance to modern antibiotics (48). However, bacteriostatic antibiotics can antagonize killing of both E. coli and S. aureus by cathelicidin antimicrobial peptides; this phenomenon is linked to the inability of the peptide to interact specifically at the septum of cells when cell division has been inhibited (49). Similarly, it is possible that bacteriostatic antibiotics would inhibit hBD focal targeting to the septum of E. faecalis. As such, understanding the mechanisms of CAMP–microbe interaction, killing, and resistance mechanisms is increasingly important for leveraging and improving their antimicrobial capacity. To this end, we demonstrate a link between focal CAMP targeting and killing efficacy: MprF mutants that simultaneously bind peptide more strongly at discrete foci are more sensitive to killing by the peptide. Moreover, our studies show that, in addition to bactericidal activities at high concentrations, subinhibitory CAMP treatment can target and disrupt sites of virulence factor assembly, providing a possible mechanism for CAMPs as antivirulence therapies. Finally, the accessibility of this region to the extracellular environment and its critical role in processing and secreting virulence factors make it a possible site both for localized interaction between the bacterium and the host and for targeting of optimized antimicrobial peptide therapeutics.

**Experimental Procedures**

Strains, growth conditions, and cloning methods are detailed in SI Experimental Procedures. Primers are listed in Table S1. Detailed protocols for hBD3-Cy3 synthesis, purification, and activity testing; fluorescent microscopy and quantitative analysis; electron microscopy; and CAMP-killing assays are described in SI Experimental Procedures.

**Fluorescent Defensin Labeling.** hBD2-Cy3 (0.2 μM final concentration) (FC3-072-48, Phoenix Pharmaceuticals, Inc.) or hBD2-Cy3 (0.1 μg/mL final concentration) was added to midlog-phase bacteria, diluted in phosphate buffer, and incubated for 30 s (for OG1RF experiments) or 5 min (for 0852 experiments). Anionic phospholipid location was assessed by adding NAO.
(catalog no. A-1372; Molecular Probes) to bacterial growth cultures at a final concentration of 1 µM, which did not inhibit the growth of E. faecalis, as described previously (18). After labeling, cells were immediately washed, spread onto poly-L-lysine precoated slides (catalog no. 22247-1; Polysciences, Inc.), and imaged or processed for immunolabeling.

Quantitative Analysis of Fluorescent Foci. Cells were divided into three cell-cycle stages based on the perimeter of the cells at each stage of the cell cycle, as defined by inequalities: 3.6 µm ≥ P ≤ 4.8 µm; 4.8 µm > P ≤ 8 µm; and P > 8 µm, where P, P₀, and P₁ represent the perimeter of cells at pre-, early-, and late-progression stages of the cell cycle. Early-division cells were the most abundant size within the log-phase population (72%) and therefore were chosen for quantitative analysis. Cell perimeters were detected on phase-contrast images using the PSICIC software (32). The perimeter fluorescence intensity profiles of detected cells were calculated by sampling intensity values of the pixels identified by PSICIC at the cell border. These intensity values then were plotted against the total distance along the cell border at which they were found. To calculate the average perimeter profiles for many cells, individual cell profiles were normalized along the x-axis (distance) by sampling the profiles at evenly spaced points along the x-axis using MATLAB’s interp1 function with the original profile as the reference curve. This used the default “linear” method. Quantitative analysis was performed on at least 100 cells per condition, from at least two independent experiments.

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