Human Host Defense Peptide LL-37 Prevents Bacterial Biofilm Formation

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The ability to form biofilms is a critical factor in chronic infections by Pseudomonas aeruginosa and has made this bacterium a model organism with respect to biofilm formation. This study describes a new, previously unrecognized role for the human cationic host defense peptide LL-37. In addition to its key role in modulating the innate immune response and weak antimicrobial activity, LL-37 potently inhibited the formation of bacterial biofilms in vitro. This occurred at the very low and physiologically meaningful concentration of 0.5 μg/ml, far below that required to kill or inhibit growth (MIC = 64 μg/ml). LL-37 also affected existing, pregrown P. aeruginosa biofilms. Similar results were obtained using the bovine neutrophil peptide indolicidin, but no inhibitory effect on biofilm formation was detected using subinhibitory concentrations of the mouse peptide CRAMP, which shares 67% identity with LL-37, polymyxin B, or the bovine bactenecin homolog Bac2A. Using microarrays and follow-up studies, we were able to demonstrate that LL-37 affected biofilm formation by decreasing the attachment of bacterial cells, stimulating twitching motility, and influencing two major quorum sensing systems (Las and Rhl), leading to the downregulation of genes essential for biofilm development.

Pseudomonas aeruginosa, a gram-negative bacterium, is one of the three major causes of infections in hospitalized patients, causing around 180,000 infections per year in North America alone (16). This important opportunistic human pathogen can cause nosocomial pneumonia, catheter and urinary tract infections, and sepsis in burn wound and immunocompromised patients (17, 27, 39, 52). Moreover, P. aeruginosa is the most prevalent and significant pulmonary pathogen in patients with cystic fibrosis (CF), causing chronic infections, and is the most common cause of eventually fatal lung disease (21, 40).

The ability to form biofilms is the crucial factor in chronic infections by P. aeruginosa (19, 48) and has made this bacterium a model organism with respect to biofilm formation. Once established, these sessile communities constitute a protected mode of growth that promotes survival in a hostile environment and are difficult to treat due to their high inherent resistance to antimicrobial agents (12, 14, 50). In addition to P. aeruginosa lung infections in CF patients (19, 48), examples of biofilm infections are chronic wound and sinus infections, ear infections, endocarditis, and medical device infections, including the colonization of catheters and implants such as joints or stents (11, 13, 23, 38).

The exploitation of stresses already imposed on organisms by the in vivo environment or host defense systems represents an intriguing new approach to combating infections (23). With this in mind, we analyzed the interaction between cationic host defense (antimicrobial) peptides and P. aeruginosa. These peptides represent a promising class of antimicrobials and are ubiquitous in nature as components of innate immune defense systems (4, 5, 8). They are found at mucosal surfaces or in phagocytic granules and are characterized as having 12 to 50 amino acids, including 2 to 9 basic (Arg or Lys) residues and around 50% hydrophobic amino acids (8). Certain peptides possess direct antimicrobial activity against gram-positive and gram-negative bacteria, fungi, and protozoa, and synthetic peptides in particular can demonstrate MICs as low as 0.25 to 4 μg/ml (37). These peptides often have a broad spectrum of abilities to modulate immunity as part of the innate immune response, and they demonstrate promise as a new approach to antimicrobial therapy (4, 5, 8). The major human cationic host defense peptide, LL-37, is found at mucosal surfaces, in the granules of phagocytes, and in most bodily fluids at concentrations of around 2 to 5 μg/ml, and it is found at much larger concentrations at sites of chronic inflammation, e.g., 30 μg/ml in the CF lung. Although this peptide often is designated a cationic antimicrobial peptide, we have argued previously (4, 5) that its antimicrobial activity is strongly antagonized under physiological salt concentrations (e.g., its MIC for many common pathogens is 32 to 96 μg/ml in the growth medium that usually is utilized for the assessment of antibiotic MICs), and thus its most important antimicrobial property in vivo relates to its potent antiinflammatory (antiendotoxic) activity and selective ability to modulate favorable immune responses.

In this study we demonstrate that, at very low concentrations, far below those that kill or inhibit growth, LL-37 strongly prevents bacterial biofilm formation in vitro. Moreover, we were able to show that LL-37 also affects existing, pregrown P. aeruginosa biofilms. Similar results were obtained using the bovine neutrophil peptide indolicidin. However, no inhibitory effect on biofilm formation was detectable using subinhibitory
concentrations of polymyxin B, bovine bactericin homolog Bac2a, or mouse peptide CRAMP. Microarray studies were performed to obtain an insight into the mechanism.

MATERIALS AND METHODS

Bacteria, media, peptides, and MICs. P. aeruginosa PAO1 (51) was grown at 37°C in either complex Luria-Bertani (LB) broth, BM2 minimal medium [62 mM potassium phosphate buffer, pH 7.0, 7 mM (NH4)2SO4, 2 mM MgSO4, 10 μM FeSO4, 0.4% (wt/vol) glucose], or BM2-swarm medium, comprising BM2 minimal medium with 0.5% (wt/vol) Casamino Acids substituted for 7 mM (NH4)2SO4 (32). For the cultivation of biofilms, P. aeruginosa PAO1 was grown either in complex LB broth or in minimal medium [9 g Na2HPO4, 1.5 g KH2PO4, 1.0 g NH4Cl, 0.2 g MgSO4·7 H2O, 0.02 g CaCl2·2 H2O, 1.2 mg Fe(III)NH2-citrate, 0.1 mg ZnSO4, 0.03 mg MnCl2, 0.3 mg H3BO3, 0.2 mg CoCl2·6 H2O, 0.01 mg CuCl2, 0.02 mg NiCl2, 0.03 mg Na2MoO4 in 1,000 ml distilled water (43)]. Solid medium contained either 0.3% (wt/vol) agar for assessing swimming motility, 0.5% agar for swarming, and 1% agar for twitching motility assessments. Peptides LL-37, indolcinnin, CRAMP, and Bac2A were synthesized by Fmoc chemistry as previously described (18) and were 96% pure, while polymyxin B was purchased from Sigma. These peptides were used at the following MICs: LL-37, 64 μg/ml; indolcinnin, 64 μg/ml; CRAMP, 4 μg/ml; Bac2A, 50 μg/ml; and polymyxin B, 2 μg/ml (7, 18, 26, 55, 56).

Biofilm experiments. Abiotic solid-surface assay (SSA) biofilm formation was analyzed in polypropylene 96-well microtiter plates after 20 h of incubation at 37°C as described previously (15, 50). After crystal violet staining, the absorbance was measured at 595 nm using a microtiter plate reader (Bio-Tek Instruments Inc.).

For more sophisticated biofilm analyses, P. aeruginosa was grown in minimal medium (43) in continuous-culture flow cells (channel dimension, 1 by 1 by 120 mm) in the absence or presence (4 μg/ml) of LL-37 at 23°C as previously described (31). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 2 × 106 cells ml−1 and incubated without flow for 4 h at 23°C. The flow was then started with a mean flow of 0.3 ml min−1, corresponding to a laminar flow with a Reynolds number of 5. Biofilms were stained and visualized using the live/dead BacLight bacterial viability kit (Molecular Probes Inc.). Live SYTO9-stained cells and dead propidium iodide-stained cells were visualized with a Leica TCS microscope using appropriate optical filters.

Motility assays. Swimming motility was evaluated on BM2 plates containing 0.3% (wt/vol) agar (Difco). Swimming was examined on BM2-swarm plates containing 0.5% (wt/vol) agar (Difco) as described previously (32). All swarm experiments were repeated five times independently. In any one experiment, swimming plates were poured from the same batch of agar and then dried in the open and inverted at 37°C for 2 to 4 h. Bacteria were inoculated on swarm agar plates at 1- to 2-μl aliquots of a BM2 overnight culture, and plates were incubated for 20 h at 37°C. Swimming was similarly evaluated using BM2-agar plates with 0.3% agar. Twitching was measured after 24 to 48 h of incubation at 37°C on plates containing 1% (wt/vol) agar and LB broth (24).

Microtector attachment assay. The attachment assay was carried out in wells of polypropylene microtiter plates. Overnight cultures grown in BM2 medium were washed twice and resuspended in BM2 medium containing 0.5% (wt/vol) Casamino Acids, and the optical density at 600 nm (OD600) was adjusted to 1.0. After the inoculation of the microtector wells, cells were allowed to adhere for 1 h before being washed three times using a multichannel pipette to remove unattached cells. Attached cells were stained with crystal violet as performed in the SSA biofilm experiments. The number of attached cells was analyzed by measuring absorbance at 595 nm. Mean adhesion values for each condition were determined for 16 wells, and similar results were observed in three repeated attachment experiments.

DNA microarray experiment. Microarray experiments were performed on five independent cultures. P. aeruginosa PA01 was grown in flow-cell chambers as described above in the absence or presence (4 μg/ml) of LL-37. After 4 days of incubation, cells were harvested from the biofilm. RNA isolation, cDNA synthesis, hybridization to microarray slides (The Institute for Genomic Research [TIGR], Pathogenic Functional Genomics Resource Center), and the analysis of DNA microarray slides using ArrayPipe version 1.7 were performed as previously described (28). Only genes that exhibited a change, compared to the results for the untreated control, of twofold or more with a P value of ≤0.05 were considered in this study.

Microarray accession number. The microarray data have been deposited in MIAMEExpress under accession number E-FPMI-12.

RESULTS

Subinhibitory concentrations of LL-37 inhibit biofilm formation. In examining the effects of the cationic human host defense peptide LL-37 on Pseudomonas aeruginosa, we observed a MIC of 64 μg/ml in microtector plates but an apparent inhibition of static cultures at lower concentrations. This encouraged us to investigate the effect of LL-37 on P. aeruginosa biofilm formation. Initially, abiotic SSAs were performed using cells of strain PAO1 and different concentrations of LL-37. As shown in Fig. 1, subinhibitory concentrations as low as 0.5 μg/ml peptide (1/128 of the MIC) led to an approximately 40% decrease in biofilm mass. This inhibitory effect increased with increasing concentrations of LL-37, with a maximum of 80% inhibition at a concentration equal to a quarter of the MIC.

To test whether the inhibitory effect on biofilm formation was related to the growth inhibition of the bacteria, growth was measured in liquid LB medium supplemented with different concentrations of peptide under shaking conditions at 37°C. These studies demonstrated that subinhibitory concentrations of up to 16 μg/ml LL-37 did not affect the planktonic growth of P. aeruginosa (data not shown).

Since LL-37 revealed strong antibiofilm properties even at very low concentrations, we analyzed the effect of other commonly studied antimicrobial peptides on P. aeruginosa biofilm formation. Cells of P. aeruginosa were grown for 20 h at 37°C in 96-well microtector plates containing LB medium and subinhibitory (one-fourth the MIC) concentrations of the different peptides. Results similar to those of the LL-37 analysis were obtained using the bovine peptide indolcinnin at 16 μg/ml, whereas no inhibitory effect on biofilm formation was detectable using the mouse peptide CRAMP (67% identical to the LL-37 sequence) at 1 μg/ml, bovine bactericin homolog Bac2A at 12.5 μg/ml, or polymyxin B at 0.5 μg/ml, the last of which was already known to have no inhibitory effect on P. aeruginosa biofilm formation and functioned as a negative control in this experiment (Fig. 2).
Flow-cell analyses. Since LL-37 is a component of the human innate immune system and is found in various mucosal secretions as well as at epithelial surfaces, we were particularly interested in the antibiofilm properties of this peptide. To investigate the effect of LL-37 on *P. aeruginosa* biofilm formation in more detail, continuous-culture flow-cell analyses were performed in the absence and presence of low concentrations of LL-37, imitating the natural concentrations of peptide found at the human mucosal surface. A stationary-phase culture of *P. aeruginosa* PA01 grown overnight was inoculated into sterile square glass capillaries containing minimal medium with and without subinhibitory concentrations of LL-37 (4 μg/ml). After 4 days of incubation at room temperature, biofilms were stained and observed using confocal laser-scanning microscopy. LL-37-treated and untreated control biofilms exhibited significant differences. The untreated biofilm showed a fully mature, structured biofilm with an average thickness of about 50 ± 5 μm and numerous microcolonies with diameters ranging from 25 to 100 μm (Fig. 3A). However, the biofilm that was cultured in the presence of subinhibitory concentrations of LL-37 (4 μg/ml, or 1/16 the MIC) throughout the 4 days of incubation was 50% thinner than the biofilm formed in the untreated control, showing a thickness of 25 ± 5 μm, and was less structured (Fig. 3B). In this biofilm, dead cells were not localized inside the microcolonies, indicating that LL-37 was not cytotoxic.

**LL-37 affects established *P. aeruginosa* biofilms.** To examine whether LL-37 can affect established *P. aeruginosa* biofilms in vitro, we initially grew biofilms in minimal medium without peptide addition in flow-cell chambers as described above. After incubation for 2 days, untreated biofilms were differentiated, and the mature biofilm architecture, including microcolonies, could be observed (data not shown). We then changed the medium and switched to minimal medium containing 4 μg/ml LL-37 and incubated it for another 48 h prior to staining and analysis. Compared to the 50-μm untreated biofilm, this 2-day LL-37-treated biofilm revealed a thickness of only 20 ± 5 μm and lacked the characteristic architecture of the mature untreated biofilms (Fig. 3C). Microcolonies that could be detected after the first 2 days of incubation in the absence of LL-37 were no longer present. Dead cells were found to be evenly distributed in the biofilm layer.

**Microarray analysis.** To investigate the mechanism of biofilm inhibition, we examined the effect of LL-37 on the gene expression profile of *P. aeruginosa*. Microarray analysis was performed to analyze the global gene expression of bacteria grown under biofilm conditions in a flow cell in the presence and absence of LL-37. An analysis of five independent experiments demonstrated the statistically significant (P < 0.05 by Student’s t test), greater-than-1.5-fold change in expression levels of 786 genes (selected examples are shown in Table 1; also see Table S1 in the supplemental material). Of these, 311 were upregulated and 475 downregulated.

Type IV pili are involved in twitching motility. Interestingly, several genes related to biogenesis and function were upregulated by two- to threefold in the presence of LL-37 (Table 1). In contrast, genes associated with the assembly of flagella, which are involved in initial adherence during biofilm formation, were found to be downregulated by two- to threefold (Table 1). However, most interestingly, we observed the downregulation of a large number of quorum-sensing-controlled genes in the presence of subinhibitory concentrations of LL-37. In addition to the lasl gene, coding for the quorum-sensing autoinducer synthesis protein Lasl, and the rhlR gene, coding for the quorum sensing regulator RhlR, which represent key components of the two major quorum-sensing systems in *P. aeruginosa* and which were found to be downregulated by two- to threefold (Table 1), respectively, we identified more than 50 genes known to be part of the Las or Rhl regulon (45, 54) to be downregulated up to 15-fold (see Table S1 in the supplemental material).

**LL-37 stimulates bacterial surface motility and reduces attachment.** To test the importance of these gene expression changes, the twitching motility and attachment of *P. aeruginosa* were analyzed in the presence of different concentrations of LL-37. LL-37 stimulated twitching motility in a dose-dependent manner (Fig. 4). Even in the presence of low concentrations of peptide (4 μg/ml), twitching was significantly (P < 0.01 by Student’s t test) increased. In contrast to twitching, there was no observed influence of LL-37 on either swimming or swarming motility, even though these processes are required for biofilm formation (32, 47).

Since the adherence of bacterial cells to surfaces has an important influence on biofilm development as the initial step in forming a microbial community on a solid surface, and since we observed a downregulation of genes associated with flagella biosynthesis, which are involved in initial attachment of the bacteria to a solid surface, we investigated the attachment behavior of *P. aeruginosa* in the presence of LL-37. Attachment assays were carried out in microtiter plates using BM2 medium containing 0, 4, 16, and 32 μg/ml peptide. Low concentrations of LL-37 statistically significantly (P < 0.01 by Student’s t test) decreased the attachment of *P. aeruginosa* to the plastic surface (Fig. 5). How-
DISCUSSION

In this study, we describe a new, previously unrecognized role of the human cationic (antimicrobial) peptide LL-37 in host defense. In addition to its immunomodulatory effects on epithelial cells, monocytes, and dendritic cells in the innate immune response and rather weak antimicrobial activity (5, 53), it prevents the formation of bacterial biofilms in vitro. This occurs even at very low concentrations that are far below those that kill or inhibit growth. LL-37 is a 37-residue, α-helical peptide that is produced at mucosal surfaces by epithelial cells (2), upregulated in response to infection and inflammation, and can be released by the degranulation of neutrophils (for a review, see reference 5). It can be found on unstimulated surfaces at concentrations of 2 to 5 μg/ml and at concentrations exceeding 30 μg/ml in inflamed epithelium.

The interaction of LL-37 with bacterial cells is not yet fully understood, although other antimicrobial peptides are known ever, this effect was even more substantial at increasing concentrations of LL-37.
to have various targets within the bacterial cell, including cell membranes, DNA, RNA, and cellular proteins, among others (37). However, we were able to demonstrate that LL-37 affects the development of biofilms in at least three ways. First, the initial attachment of \textit{P. aeruginosa} cells to the surface was significantly reduced in the presence of LL-37. This would lead to a smaller number of bacteria actually involved in the initial steps of biofilm development. Second, LL-37 promotes twitching, a specialized form of surface motility mediated by the type IV pili, by stimulating the expression of genes related to type IV pilus biosynthesis and function. Increased surface motility would cause bacteria to wander across the surface instead of forming biofilms and is known to result in thin and flat biofilms without the mushroom-like structure (35, 49). Third, using microarray technology, we also were able to demonstrate that LL-37 affects the two major quorum-sensing systems of \textit{P. aeruginosa}, namely the Las and the Rhl systems, by downregulating key components. This resulted in a downregulation of more than 50 genes (Table 1) that are part of the respective regulons (45, 54). Among other genes that are known to be essential for biofilm development and maintenance are \textit{rhlA} and \textit{rhlB}, the rhamnosyltransferase genes, and the \textit{lasB} gene, encoding the elastase LasB (33, 34).

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FIG. 4. Twitching motility of \textit{P. aeruginosa} in the presence of different concentrations of LL-37. \textit{P. aeruginosa} cells were spot inoculated on twitching plates containing LB medium, 1% (wt/vol) agar, and different concentrations of the antimicrobial peptide LL-37. Twitching zones were determined after 24 h of incubation at 37°C. All experiments were done in triplicate with four technical repeats, and statistical significance was determined using Student’s \textit{t} test (**, \textit{P} < 0.01).

FIG. 5. Attachment of \textit{P. aeruginosa} cells in the presence of different concentrations of LL-37. The attachment assay was carried out in wells of polypropylene microtiter plates as described in Material and Methods. After the inoculation of the microtiter wells, cells were allowed to adhere for 1 h before being washed three times using a multichannel pipette to remove unattached cells. Attached cells were stained with crystal violet, and the number of attached cells was analyzed by measuring the absorbance at 595 nm. Mean adhesion values for each condition were determined for 16 wells, and similar results were observed in three repeated attachment experiments. Statistical significance was determined using Student’s \textit{t} test (**, \textit{P} < 0.01).
Results similar to our findings were reported recently for the inhibition of P. aeruginosa biofilms by lactoferrin (49). This cationic human glycoprotein, which is present in external secretions, especially milk (1), was found to inhibit bacterial biofilm formation due to its iron-chelating properties, which also resulted in increased twitching motility. To test whether LL-37 exhibits similar iron binding properties even though it is a positively charged molecule, we performed biofilm and motility assays in the presence of different peptide and iron concentrations ranging from 0 to 16 μg/ml and 0 to 100 μM, respectively. However, under all analyzed conditions, the addition of extra iron to the liquid medium did not restore the biofilm defect or the increased twitching motility (data not shown). Moreover, no evidence was provided by the microarray analysis that the P. aeruginosa cells were iron limited during LL-37 treatment, ruling out the possibility of iron-chelating effects of LL-37 in our experiments.

Since the formation of biofilms, especially in lung infections of CF patients, protects bacteria during infections and allows survival in a hostile environment (14, 50), the inhibition of biofilm formation by this major peptide of the human mucosal surface may serve as an additional mechanism to prevent bacterial survival in the human host, in concert with other initial defense mechanisms. With the reduction of biofilm formation, secondary immune responses may then be better able to combat the infecting organism (49). Interestingly, it has been shown for several bacteria, including P. aeruginosa and Staphylococcus aureus, which both are involved in CF lung infections, that these bacteria possess proteinases that are able to degrade and inactivate LL-37 and lactoferrin even during lung infections (6, 44, 46). In this context, it is also worth mentioning that evidence was provided recently for LL-37 being a key factor in the mucosal immunity of the urinary tract. It was demonstrated by Chromek and coworkers (9) that LL-37 production was highly increased in epithelial cells after contact with bacteria and that LL-37 protected the urinary tract against invasive Escherichia coli using mice as model organism; however, no mechanism for this has been demonstrated to date. Since biofilm formation is involved in urinary tract infections, LL-37, with its antibiofilm properties, could help protect the urinary tract from infection.

Since it has been demonstrated that medically important antibiotics, including aminoglycosides, fluoroquinolones, and tetracycline, among others, work poorly in chronic infections and, in contrast, even act as intermicrobial signaling agents that stimulate bacterial biofilm formation at subinhibitory concentrations (20, 25), new antimicrobial agents are needed to combat chronic infections. Recently, screening methods for antibiofilm agents have been developed (10, 36), and several high-throughput screenings of natural compounds were performed to identify substances with antibiofilm properties that could be used as potential agents in biofilm-associated infections (3, 22, 29, 41, 42); however, only limited success has been achieved to date. The antibiofilm properties of certain cationic peptides, combined with their antimicrobial and immunomodulatory activities, could provide the basis for the development of novel drugs that could help fight chronic infections.

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