Lipopolysaccharide Phosphorylation by the WaaY Kinase Affects the Susceptibility of *Escherichia coli* to the Human Antimicrobial Peptide LL-37*

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Background: LL-37 is a human antimicrobial peptide that acts by damaging bacterial membranes.

Results: The absence of WaaY kinase decreases *E. coli* susceptibility to LL-37.

Conclusion: Lipopolysaccharide phosphorylation by WaaY is relevant to LL-37 activity.

Significance: This work provides insight into the first interaction of LL-37 and the bacterial outer membrane.

The human cathelicidin LL-37 is a multifunctional host defense peptide with immunomodulatory and antimicrobial roles. It kills bacteria primarily by altering membrane barrier properties, although the exact sequence of events leading to cell lysis has not yet been completely elucidated. Random insertion mutagenesis allowed isolation of *Escherichia coli* mutants with altered susceptibility to LL-37, pointing to factors potentially relevant to its activity. Among these, inactivation of the *waaY* gene, encoding a kinase responsible for heptose II phosphorylation in the LPS inner core, leads to a phenotype with decreased susceptibility to LL-37, stemming from a reduced amount of peptide binding to the surface of the cells, and a diminished capacity to lyse membranes. This points to a specific role of the LPS inner core in guiding LL-37 to the surface of Gram-negative bacteria. Although electrostatic interactions are clearly relevant, the susceptibility of the *waaY* mutant to other cationic helical cathelicidins was unaffected, indicating that particular structural features or LL-37 play a role in this interaction.

Antimicrobial peptides (AMPs) are evolutionarily ancient components of innate host defense, present in both animals and plants. They often have broad spectrum activity against many pathogenic microorganisms as well as the capacity to modulate the activity of cellular components of the immune system (1, 2). AMPs can be divided into several groups or families according to phylogenetic or structural criteria (3). In mammals, two prevalent families have been recognized: defensins and cathelicidins (4). The former are characterized by compact β-sheet structures stabilized by conserved S–S bridges (5, 6). Cathelicidins instead include a quite heterogeneous group of AMPs having common a conserved, anionic, cathelin-like N-terminal proregion, whereas the cationic C-terminal antimicrobial domain is highly variable. Furthermore, the number of different cathelicidins present in a single species also varies from only one, as in humans, to over a dozen, as in pigs (7–9).

The only human cathelicidin, hCAP18 (10, 11), is produced by phagocytes, mucosal epithelial cells, and keratinocytes (12). The C-terminal bactericidal portion, LL-37, is a linear, cationic peptide with 37 residues and a net charge of +6 at physiological pH and is released from the precursor by the action of protease 3 (13). It is active against both Gram-positive and Gram-negative bacteria and some fungi (14). In addition, it binds lipopolysaccharide (LPS) (11) and protects the host from endotoxic shock by preventing LPS binding to CD14(+) cells, thereby suppressing the production of cytokines by those cells (15). A number of other biological functions have also been attributed to LL-37, ranging from immunomodulatory roles to the stimulation of angiogenesis and epithelial wound healing (16).

The antimicrobial activity of LL-37 is quite medium-dependent, being influenced by the presence of ions, especially anions, which can profoundly modify the structure of the peptide and promote a transition from a disordered form to an α-helical and oligomeric form (17, 18). It also binds, especially in the latter form, to organic components of its biological medium. As with many other helical AMPs, the positive charge of LL-37 promotes the initial interaction with anionic components of the microbial surface, whereas its amphipathic structure favors the subsequent insertion into and permeabilization of the cytoplasmic membrane. The exact mechanism of the cell lysis, however, has not been completely elucidated (19, 20), although it seems that the oligomeric nature of the peptide is relevant (18). In particular, the process by which the peptide migrates across the outer membrane to reach the cytoplasmic membrane and the role of different bacterial components affecting this migration remain largely unknown.

In this study, while searching for bacterial determinants affecting the sensitivity to LL-37, we have identified LPS heptose phosphorylation as a relevant factor. Our findings indicate...
a specific role of the LPS inner core in the binding of LL-37 to the surface of Gram-negative bacteria.

Experimental Procedures

**Bacterial Strains, Media, and Growth Conditions**—Bacterial strains used in this study are listed in Table 1. The wild-type strains and their mutants were grown at 37 °C under aerobic conditions in Luria-Bertani (LB) or in Mueller-Hinton (MH) broth. The antimicrobial and flow cytometry assays were performed on mid-log phase cultures. When required, antibiotics were added at the following concentrations: ampicillin (75 µg/ml), kanamycin (25–50 µg/ml), and tetracycline (15 µg/ml).

**Antimicrobial Peptides**—Human LL-37; the cathelicidins BMAP-27, CRAMP, and Bac7(1–35); and ovine SMAP-29 were synthesized, purified, and stored as described previously (21).

**Transposon Mutagenesis and Recombinant DNA Techniques**—A transposon mutant library of the *Escherichia coli* strain HB101 strain was created using the EZ-Tn5™ <KAN-2>Tnp Transposome kit (Epicenter Biotechnologies), following the instructions of the supplier. Briefly, *E. coli* HB101 cells were made competent as described previously (22) and transformed with the EZ-Tn5™ <KAN-2>Tnp construct by electroporation. Mutagenized cells were selected on a medium supplemented with 25 µg ml⁻¹ kanamycin. The mutant clones were collected from the plates, pooled, resuspended in 20% glycerol, and stored at -80 °C. Approximately 60,000 clones were obtained.

Southern blot analyses were performed using genomic DNA that was purified from 20 random kanamycin-resistant colonies using the Sarkosyl-Proprase lysis method (23), digested with EcoRV, and transferred on an Amersham Biosciences Hybond-N+ nylon membrane (GE Healthcare). Hybridization was carried out under high stringency conditions, using a Tn5 fragment as a probe. The randomness of the insertion in the genome of mutants was confirmed. The Tn5 fragment was amplified by PCR directly from the transposon-transposase complex, supplied with the EZ-Tn5™ <KAN-2>Tnp kit, using TN5-F and TN5-R primers (Table 1), and labeled with the digoxigenin-based DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). The membranes were washed, and signal was detected according to the manufacturer’s instructions.

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, ligation using T4 ligase, and transformation of *E. coli* cells, were performed using stan-
Deletion of waaY Decreases Sensitivity of E. coli to LL-37

Results

Isolation of E. coli Mutants with Decreased Susceptibility to LL-37 after Tn5 Mutagenesis—Transposon mutagenesis of the E. coli strain HB101 was performed and resulted in a library of ~60,000 clones. Mutants showing a decreased susceptibility to the human host defense peptide LL-37 were isolated by plating aliquots from this library on solid MH agar medium containing 30 μM LL-37, the concentration required to completely inhibit the growth of the wild-type strain (as opposed to just 2 μM inhibiting growth in liquid medium). Twenty colonies, named HCR1–HCR20, were isolated, and their decreased susceptibility to LL-37 was confirmed by subculturing clones in liquid medium containing 2 μM peptide (data not shown). Southern blot analysis carried out with a Tn5 fragment as a probe on all HCR clones showed that 15 of 20 displayed the same hybridization profile, whereas the other five mutants carried the Tn5 transposon in genomic fragments of different length (Fig. 1).

Identification of Genes Conferring Decreased Susceptibility to LL-37—To identify the interrupted genes in six mutants displaying different hybridization profiles (HCR1, HCR3, HCR12, HCR18, HCR19, and HCR20), the genomic DNA was subcloned in a plasmid vector and selected for kanamycin resistance, and the resulting clones were sequenced. The identified genes and the Tn5 insertion sites are reported in Table 2. The relation of five of these genes to LL-37 susceptibility is not immediately apparent from the known functions of their products (see Table 2), but the subcellular location of most of them in the outer membrane (ydbA, mdtQ, and mdtE) or periplasm (ynfF) suggests that they might have a role in modulating the interactions of the cathelicidin with the bacterial membranes.
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We focused on the sixth interrupted gene (waaY) identified in HCR1 because it was also found in all of the other 14 clones that displayed a similar hybridization profile, although with the insertion occurring in at least five different sites within the same gene, as revealed by PCR analysis (data not shown). This result strongly suggests a correlation between the function of the waaY gene product and bacterial susceptibility to LL-37, so that it is likely to be associated with the peptide’s mode of action.

In E. coli, the waaY gene is located in the waa operon (formerly rfa) (Fig. 2A), a long transcription unit involved in LPS synthesis (29). In particular, waaY encodes a kinase that specifically adds a phosphate group to the second heptose residue in the inner core of LPS during the synthesis process (Fig. 2B). The waaZ and waaU genes are located just downstream of waaY in the operon and are likely to encode for glycosyltransferases (30). Therefore, we checked by RT-PCR analysis whether the expression of those genes was affected in HCR1, but unlike waaY, they were properly transcribed, excluding polar effects (Fig. 3).

**Inactivation of the waaY Gene Reduces the Susceptibility of E. coli to LL-37 in a Specific Manner**—MIC assays indicated that the susceptibility to LL-37 was reduced 2-fold in HCR1 compared with the wild-type strain (Table 3). Growth kinetics assays showed that the mutant strain was as vital as the wild type (Fig. 4A), but whereas 1 μM LL-37 completely inhibited the growth of the wild-type strain over a 4-h period, it only slowed that of the mutant strain (Fig. 4B). The difference in the susceptibility was most evident in bactericidal activity assays in the presence of 2 μM LL-37, which showed a 4-log drop in the wild-type strain but only a 1-log drop in the mutant one. Furthermore, when the HCR1 strain was complemented by a plasmid carrying a functional copy of the waaY gene (HCR1C strain), the susceptibility to LL-37 was restored to the wild-type level or even increased (Fig. 4, B and C).

To confirm the role of the WaaY kinase in mediating the susceptibility to LL-37, a waaY deletion mutant strain from the Keio library (JW3600) (31) and its parental strain BW25113 were also tested, giving similar results, with a 2-fold increase in MIC (2 μM for BW25113 versus 4 μM for JW3600) and a similar effect on growth kinetics (data not shown). On the other hand, the susceptibility of the waaZ- and waaU-null mutants to LL-37 was unaffected (data not shown). Their gene products are reported to affect LPS core structure in other regions (KdoII and HeplV, respectively) (32).

On the whole, these results indicate that inactivation of the waaY gene confers a phenotype that is less susceptible to LL-37 due to a specific modification of the LPS core oligosaccharide. To test how general this effect is, the HCR1 strain was treated with different cathelicidins (Table 4). No differences in terms of MIC values (Table 3) or effect on bacterial growth rate (not shown) was observed between HCR1 and the wild-type HB101 in the presence of the bovid α-helical cathelicidins BMAP-27, CRAMP, and SMAP-29 or of the linear, proline-rich peptide Bac7(1–35). These results indicate that the inactivation of waaY confers a phenotype that specifically reduces the susceptibility to LL-37.

**LL-37 Has a Reduced Capacity to Bind to and to Permeabilize E. coli HCR1**—The WaaY kinase phosphorylates HepII, and this could favor the binding of LL-37, which is cationic. To evaluate whether the decreased susceptibility of the HCR1 strain to LL-37 derived from an altered capacity of the peptide to bind to the bacterial surface, HB101 and HCR1 cells were incubated with subinhibitory concentrations of a BODIPY-labeled, fluorescent derivative of LL-37 (LL-37-BY), and surface fluorescence was then analyzed by flow cytometry. Note that labeling with BODIPY does not alter the charge of the peptide, and only a slight decrease in its potency was observed, even if the difference in the susceptibility to the peptides between the wild type and the waaY mutant remained identical (Table 3). The tagged LL-37 was found to bind efficiently to the surface of HB101 cells in a dose-dependent manner, whereas surface binding to the HCR1 mutant was significantly lower (Fig. 5, A and B), suggesting that the reduced LPS anionicity influences the initial interaction of the peptide with the surface of target cells. Surface binding comparable with that of the wild-type HB101 was instead observed for HCR1C cells, so that the complementation of waaY restores the interaction to a normal level (Fig. 5, A and B).

After the initial interaction with the outer membrane, part of the LL-37 molecules translocate to the bacterial cytoplasmic membrane, probably a primary target. To determine whether the effect of knocking out the waaY gene and reducing surface binding affected the efficiency of bacterial permeabilization by the peptide, we compared the percentage of cells that became positive for the fluorescent probe PI after exposure of wild-type or mutant cells to the peptide. As shown in Fig. 5C, LL-37 caused ~50% permeabilization of HB101 cells within 30 min, whereas the percentage was markedly reduced in the mutant HCR1 cells (~25%) under the same conditions at both 0.5 μM (Fig. 5C) and 0.25 μM concentrations (data not shown). The HCR1C strain, harboring multiple copies of the waaY gene, showed an even higher level of membrane binding and permeabilization than the wild type (Fig. 5). These results strongly support the correlation between the initial peptide binding to the phosphate groups in the inner core of LPS and its subsequent capacity to damage the cytoplasmic membrane barrier.
A large body of experimental evidence indicates that membrane permeabilization is a key step in the killing of microorganisms by many antimicrobial host defense peptides, including the human cathelicidin LL-37. This can occur either via pore formation or a more generalized membrane compromising (17, 19), but the details of these possible modes of action are not completely understood. In particular, the factors affecting the approach of the peptide to the inner membrane from the extracellular milieu and the structural features that favor its transit through the outer layers of the cell wall are still unclear.

In the present study, we have identified an \textit{E. coli} mutant, HCR1, with decreased susceptibility to LL-37 due to the interruption of the \textit{waaY} gene by a transposon insertion. This gene encodes an LPS kinase, suggesting that decreased susceptibility derives from less phosphorylated LPS, thereby affecting the peptide’s initial accumulation at the outer membrane; translocation to the periplasm; and ultimately its capacity to accumulate on, interact with, and then permeabilize the cytoplasmic membrane.

### Table 2

| Mutant | Mutated gene | Size (bp) | Tn5 position | Function of the gene product | Reference |
|--------|--------------|----------|--------------|-------------------------------|-----------|
| HCR1   | \textit{waaY}(\textit{rfaY}) | 699      | 393–401      | HepII-kinase to form LPS-inner core | 29        |
| HCR3   | \textit{ydBA} \_2   | 3324     | 3128–3136    | Fragment 1 of a split CDS predicted outer membrane protein | 56        |
| HCR12  | \textit{ynfR}  | 2424     | 222–230      | Oxidoreductase subunit of a putative selenate reductase | 56        |
| HCR18  | \textit{yohG} (\textit{mdtQ}) | 1089     | 818–826      | Outer membrane component of a multidrug efflux system | 56        |
| HCR19  | \textit{gudB} | 1401     | 185–193      | Glutamate decarboxylase of the glutamate-dependent acid resistance system | 56        |
| HCR20  | \textit{yhiLI} (\textit{mdtE}) | 1158     | 342–350      | Membrane component of the MdtEF-TolC multidrug efflux transport system | 56        |

\textit{a} Starting from start codon

### Table 3

| Peptide           | MIC (\textmu g/mL) |
|-------------------|--------------------|
| LL-37             | 1                  |
| LL-37-BY          | 2                  |
| CRAMP             | 0.50               |
| SMAP-29           | 0.25               |
| BMAP-27           | 0.5                |
| Bac7(1–35)        | 0.125              |
| Polymyxin B       | 0.125              |
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### Figure 2

Map of the \textit{waaQ} operon and representation of the structure of the LPS of \textit{E. coli} K12. A, map of the \textit{waaQ} operon of \textit{E. coli} K12. Arrows, experimentally verified promoters. Dashed arrow, predicted promoter (32, 53, 54). The position of the Tn5 transposon insertion into \textit{waaY} is indicated. B, schematic representation of the structure of LPS of \textit{E. coli} K12, based on two published schemes (29, 55). The site of action of \textit{WaaY} is highlighted in the box. \textit{KDO}, 3-deoxy-\textalpha-D-manno-2-octulosonic acid; \textit{Hep}, L-glycero-\textalpha-D-manno-heptose; \textit{P}, phosphate; \textit{PPEtn}, 2-aminoethyl diphosphate.

### Figure 3

RT-PCR analysis of \textit{waaY}, \textit{waaZ}, and \textit{waaU} transcripts in HB101 and HCR1 strains. RNA was isolated as described under “Experimental Procedures,” and the resulting cDNA was analyzed by gel electrophoresis for the presence of \textit{waaY}, \textit{waaZ}, and \textit{waaU} transcripts of the \textit{waa} operon, with expected sizes of 699, 852, and 1074 bp, respectively.

**Discussion**

A large body of experimental evidence indicates that membrane permeabilization is a key step in the killing of microorganisms by many antimicrobial host defense peptides, including the human cathelicidin LL-37. This can occur either via pore formation or a more generalized membrane compromising (17, 19), but the details of these possible modes of action are not completely understood. In particular, the factors affecting the approach of the peptide to the inner membrane from the extracellular milieu and the structural features that favor its transit through the outer layers of the cell wall are still unclear.

In the present study, we have identified an \textit{E. coli} mutant, HCR1, with decreased susceptibility to LL-37 due to the interruption of the \textit{waaY} gene by a transposon insertion. This gene encodes an LPS kinase, suggesting that decreased susceptibility derives from less phosphorylated LPS, thereby affecting the peptide’s initial accumulation at the outer membrane; translocation to the periplasm; and ultimately its capacity to accumulate on, interact with, and then permeabilize the cytoplasmic membrane.
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The LPS layer of the Gram-negative bacterial outer membrane functions as an effective barrier against some external solutes (33) but has a rather variable structure. Lipid A, the membrane anchor, is linked to core oligosaccharide (OS), a short chain of sugar residues that have multiple phosphorylation sites, and in some strains can then extend into O-antigen, which consists of a variable number of oligosaccharide repeats (32). The genes responsible for the biosynthesis, phosphorylation, and modification of core OS in E. coli and Salmonella enterica Typhimurium are clustered in the waa locus (32), which includes a long transcription unit (32) (see Fig. 2A). The product of waaY is a kinase responsible for the phosphorylation of HepII, the second of the three L-glycero-D-manno-heptose (Hep) residues in the LPS inner core region in E. coli and S. enterica Typhimurium (29). Its absence reduces the negative charge of the core OS without altering the stability of the LPS layer (32).

We found that the HCR1 strain, lacking this kinase, was able to grow both on solid and in liquid media containing LL-37 at concentrations that inhibit the parental strain. Its advantage over the wild-type strain was most evident for short exposure times in liquid medium. This suggested a modification of the initial interaction between LL-37 and the bacterial surface. Note that interruption of the waaY gene by Tn5 insertion does not cause polar effects on the expression of downstream genes, and knocking these genes out in any case does not affect the susceptibility, so the effect seems specific to the activity of the WaaY kinase. Furthermore, complementation of the waaY mutation completely restored the sensitivity to the parental strain level.

LPS is among the first structures that AMPs encounter in their trajectory toward the inner membrane of Gram-negative bacteria, and it has been shown to play a relevant role in the mode of action of antimicrobial peptides, such as defensins (34). LPS, with its negatively charged groups, participates in electrostatic interactions with these cationic molecules (35). It is well known that some bacteria can, in fact, acquire some protection against AMPs by reducing the net negative charge of lipid A (36–39), and this has been very recently extended to commensal gut bacteria (40). Further strong support for the role of waaY in decreasing sensitivity to AMPs comes from a recent work showing that the S. enterica Typhimurium can mutate its homologue of waaY, resulting in a resistant phenotype, under selective pressure from LL-37 (41).

We cannot exclude the possibility that lack of the phosphate is only indirectly involved in reducing sensitivity to LL-37 (e.g. by affecting the composition and/or conformation of the LPS). However, because waaY inactivation results in a significant reduction of the amount of LL-37 bound to the cell surface (Fig. 5A), the simplest explanation is that the phosphate group on HepII is an important initial docking site for this peptide. In this respect, it is also interesting to note that S. enterica Typhimurium produces PmrG, a specific phosphatase capable of dephosphorylating HepII, leading to an increased resistance to Fe(III). This ion seems to share the same binding sites with polymyxin B (42). A similar mechanism might therefore be more generally used to modulate the susceptibility toward other types of cationic AMPs. Significantly, PmrG is regulated by the two-component regulatory system PmrA/PmrB, involved in diverse other mechanisms conferring resistance to cationic AMPs, including a transferase that modulates the negative charge on lipid A by introducing aminoarabinose (43, 44). A PmrG homologue is present in E. coli (45), although it has not yet been experimentally established whether it has a similar role.

The key step in the inactivation mechanism of many cationic AMPs is the permeabilization of the bacterial cytoplasmic membrane, which occurs when peptide molecules accumulate on the bacterial surface over a threshold level. A reduced binding to and migration across the outer membrane would significantly affect this process. Flow cytometric assays confirm that the lack of HepII phosphorylation results in both reduced binding (Fig. 4A) and permeabilization of HCR1 cells by LL-37 when compared with the wild-type cells (Fig. 5).

An important aspect of waaY inactivation is that the general stability of the LPS layer seems unaffected when HepII is not phosphorylated. Conversely, it is affected upon inactivation of the waaP gene, which removes the kinase required for Hep
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phosphorylation. In E. coli and Salmonella spp., HepII can be phosphorylated by the WaaY kinase only if HepI has previously been phosphorylated. Conceptually, the complete loss of the phosphates from the OS, if \textit{waaP} is deleted, should lead to an even lower susceptibility to AMPs. However, it also leads to significant structural changes of the LPS and a pleiotropic phenotype known as deep-rough (46, 47), characterized by hypersensitivity to detergents and hydrophobic antibiotics (29) and by a loss of virulence \textit{in vivo} (48). The \textit{waaP} knockout mutant was, in fact, found to be hypersensitive to LL-37 as well as to the detergent SDS (data not shown). Thus, phosphorylated HepI is important for outer membrane stability because it is likely to be needed for cross-linking of adjacent LPS molecules by divalent cations and polyamines (49), so it cannot be dispensed with. On the other hand, HepII phosphorylation is less significant for LPS stability; therefore, it can be dispensed with as a bacterial stratagem to decrease the susceptibility toward some cationic AMPs.

It may be relevant that this holds true for LL-37 but not other \(\alpha\)-helical cathelicidins or structurally different proline-rich ones, suggesting a specific type of interaction that may depend on particular structural characteristics of LL-37. This peptide is unlike most cationic, helical AMPs, including closely related primate orthologues, which only adopt a helical conformation upon contact with bacterial membranes. Due to a particular pattern of side-chain electrostatic attractions, it shows a relatively stable helical structure also in aqueous, physiological conditions, which drives it to aggregate and to approach the cytoplasmic membrane and interact with it in an oligomeric form (50). Binding to LPS appears to further aid the helical structuring and aggregation (35, 51), which may impede the transit through the outer membrane and the accumulation at the cytoplasmic membrane, and phosphorylated HepII could play a role in this interaction.

In conclusion, with this study, we have obtained new insight into the mode of action of the human host defense peptide LL-37, establishing a role for a specific phosphate group of the inner core of LPS that adds to that of other lipid A phosphates, known to be important for the binding of cationic AMPs in general (52). Moreover, modulating HepII phosphorylation alters sensitivity of bacterial cells to an important effector of the innate immunity, LL-37. This could represent one of the several strategies that contribute to pathogen survival in the host. The resistance conferred by switching off the waaY gene is not outstanding \textit{per se}, and we report that it is limited to LL-37. However, it could become relevant in combination with other alterations that increase other aspects of resistance.

### TABLE 4

| Peptide*    | Sequence                        | aa* | q’  |
|-------------|---------------------------------|-----|-----|
| LL-37       | LLGDFFRKSKEKIGKEFKRIVQRKDFLRNLPRTES-OH | 37  | 6+  |
| CRAMP       | GLLRKGGEKIGEKLEKQEKQKNNFTQKLVPQQE-OH   | 34  | 6+  |
| SMAP-29     | RGLRRLGRKIAHGVRKKYQTPVTLRIIRA-NH2   | 28  | 10+ |
| BMAP-27     | GRKFRFKKFKKLFKKLSPVPLLLHE-NH2     | 26  | 11+ |
| BacC(1–35)  | RRPRPRLPRPRPLPPRPGPRPPLPFPP-OH     | 35  | 11+ |

* LL-37 is from humans, CRAMP is from mice, SMAP-29 is from sheep, and BMAP-27 and BacC(1–35) are from cattle.

* Amino acid.

* Peptide charge, taking into account C-terminal amidation (−NH₂) and considering His as neutral.

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**FIGURE 5.** Binding of LL-37 to and permeabilizing effect on wild-type HB101 and mutant HCR1 \textit{E. coli} cells. Fluorescence of \textit{E. coli} HB101, HCR1, and HCR1C cells incubated with 0.1 \(\mu\)M (A) and 0.25 \(\mu\)M (B) LL-37-BODIPY for 10 min at 37 °C and then analyzed by flow cytometry. The averaged mean fluorescence intensity (MFI) ± S.D. (error bars) of three independent experiments is shown. *, \(p < 0.01\) peptide-treated HB101 cells versus peptide-treated HB101 and HCR1C cells. C, percentage of PI-positive cells after incubation of \textit{E. coli} HB101 (solid line), HCR1 (dotted line), and HCR1C (dashed line) with 0.5 \(\mu\)M LL-37 at different times. The background level of permeabilized, fluorescent cells obtained by using peptide-untreated samples was always lower than 3% and was removed from the corresponding peptide-treated sample. The averaged percentages of PI-positive cells ± S.D. from three independent experiments are shown. *, \(p < 0.05\) peptide-treated HCR1 cells versus peptide-treated HB101 and HCR1C cells at the 30 and 60 min time points.
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Author Contributions—K. B. and S. F. designed and performed most of the experiments, M. M. performed assays to characterize the mutant phenotype, M. B. carried out flow cytometric analyses, A. T. and R. G. provided conceptual advice, and A. T. contributed to the writing of the manuscript. M. S. designed the study, supervised the experiments, and edited the manuscript with contributions from all of the other authors.

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