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A Bacterial Mutant Library as a Tool to Study the Attack of a Defensin Peptide

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Human defensins are small, cysteine-rich host-defense peptides that contribute to innate immunity.[1] Human Defensin 5 (HD5ox) is a 32-residue α-defensin that is highly expressed in the small intestine and exhibits antimicrobial activity against a variety of bacterial and fungal species. Despite its importance in human health and disease, the mechanisms of action of HD5ox and many other peptides of the defensin family are not completely understood. Defensins are often generalized as membrane-permeabilizing peptides, but antibacterial mechanisms other than membrane disruption have been identified. We established an unbiased genetic screen based on a mutant library of the model organism Escherichia coli K-12 to identify bacterial pathways that modulate HD5ox susceptibility. The screen yielded thirty-one genes that confer hypersensitivity to HD5ox when knocked-out, and included genes responsible for membrane biosynthesis and integrity. These genes were independently confirmed as being important for HD5ox-mediated killing of E. coli. Moreover, the unbiased screen uncovered an interaction between HD5ox and lipopolysaccharide.

HD5ox is an α-defensin and features a triple-stranded β-sheet secondary structure that is stabilized by three conserved intramolecular disulfide bridges (CysI—CysVI, CysII—CysIV, CysIII—CysV) in the oxidized form (Figure 1A,B).[2] The peptide is produced by small intestinal Paneth cells[3] and released into the lumen upon bacterial challenge.[4] HD5ox displays antimicrobial activity against an impressively broad spectrum of species that include Gram-negative Escherichia coli and Gram-positive Staphylococcus aureus.[5] Such broad-spectrum and seemingly unspecific activity typifies many defensins, and these peptides are often described to act by membrane permeabilization. Indeed, several studies demonstrated that membrane permeabilization occurs upon incubation of bacteria with various defensins,[6] and HD5ox treatment results in E. coli inner membrane damage.[7] Nevertheless, recent investigations delineate that the mechanism of action of defensins should not be generalized and that membrane disruption is only one factor governing antibacterial activity. Alternative antimicrobial or host-defense mechanisms are now appreciated for fungal plectasin,[8] human defensin 6,[9] and human β-defensins 2[10] and 3.[11] Defensins differ significantly in amino acid sequence, overall charge, and

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.
hydropobicity; these factors confer different modes of action. Thus, a case-by-case evaluation is needed for each defensin. For HD5\textsubscript{ox}, it remains unclear how this peptide kills bacteria and thereby contributes to maintaining a healthy microbial flora in the gut and other organ systems\textsuperscript{[12]} although differences in antibacterial activity have been observed for Gram-negative and –positive strains\textsuperscript{[5,7]} A robust tool that provides information about bacterial protection against and response to the attack by defensins would facilitate understanding of the mechanism of action of HD5\textsubscript{ox} as well as other defensin family members, and unveil similarities and differences between these important contributors to innate immunity in humans and other organisms. To address this need, we report an unbiased genetic screen optimized for identifying genes that affect HD5\textsubscript{ox} activity against \textit{Escherichia coli}. This screening approach described herein will be adaptable to other defensin peptides and bacterial mutant libraries.

We established a genetic screen for defensins by using the Keio Collection, a genome-wide collection of single gene deletion mutants in the non-pathogenic model organism \textit{E. coli} K-12. The Keio Collection contains 3,985 strains created by replacing single genes with a kanamycin resistance cassette\textsuperscript{[14]} The main advantage of using genetic mutant libraries is that they provide a direct link between a phenotypic screen (easy read-out) and the identity of the gene knock-out. Screens using bacterial mutant libraries have led to the identification of genes or pathways affected by certain antibiotics and other bioactive compounds and have afforded valuable contributions to mechanism-of-action studies\textsuperscript{[15]} The main disadvantage of using the Keio Collection and other libraries is that essential genes are not included and therefore cannot be screened. Nevertheless, there are relatively few essential genes in \textit{E. coli}, and the Keio Collection covers 93\% of all annotated genes.

We screened the Keio Collection against HD5\textsubscript{ox} to identify gene knock-outs that increased the susceptibility of \textit{E. coli} to HD5\textsubscript{ox}. These genes account for bacterial protection against HD5\textsubscript{ox}. An antimicrobial activity assay (AMA) in liquid culture was employed because the antimicrobial activity of HD5\textsubscript{ox}, like many defensins, is attenuated by agar\textsuperscript{[16]} The AMA was optimized for the most suitable concentration of HD5\textsubscript{ox}, media composition, growth time, and temperature (Supporting Information). Most Keio mutants exhibit growth rates, hereafter described as “fitness,” that differ from that of the wild type (WT), resulting in variable cell densities as measured by OD\textsubscript{600} in the AMA. We also observed that the cell density affected the sensitivity of \textit{E. coli} to HD5\textsubscript{ox}. Therefore, we performed the AMA in the presence and absence of HD5\textsubscript{ox} and used a normalized fitness ratio (termed, see Supporting Information) in order to obtain robust data for the screen. Strains showing WT sensitivity to HD5\textsubscript{ox} are characterized by $\phi = 1$ whereas strains which are hypersensitive to HD5\textsubscript{ox} are characterized by $\phi < 1$. Strains exhibiting a $\phi > 1$ may be linked to a resistant phenotype and are not considered in this study.

In an initial screening round, the entire Collection was subjected to the AMA. Selected strains with initial $\phi$ values indicating hypersensitivity (see Supporting Information, n=367) were subjected to a second screening round. Only strains with a mean $\phi < 0.7$ from the two screening rounds were selected and passed on to the third screening round (n=166). After three screening rounds, ninety-seven mutants displayed a hypersensitive phenotype (overall mean $\phi$ of three screening rounds < 0.7, Supporting Table S2). The ninety-seven mutants
identified as hypersensitive to HD5\textsubscript{ox} were further evaluated in a more robust AMA where the cell densities of the strains were normalized (rounds 4 and 5). For thirty-one mutants, the mean $\psi$ over five rounds of testing was $< 0.7$. These hypersensitive mutants were ranked according to the mean $\psi$ value obtained from two rounds of AMA with normalized cell densities to produce a final list of hits (Figure 1B).

To confirm the results of the Keio screen, AMAs were carried out for select membrane-related mutants complemented with ASKA plasmids. The ASKA clone collection is a plasmid library where each plasmid contains one annotated \textit{E. coli} gene expressed under an inducible promoter.\cite{17} WT sensitivity to HD5\textsubscript{ox}, ascertained by $\psi$ value, to HD5\textsubscript{ox} was restored for about 50\% of the complemented strains (Supporting Figure S4). Furthermore, successful complementation was observed for four of the five most sensitive mutants (\textit{rfaF}, \textit{lpcA}, \textit{rfaD}, \textit{surA}), validating the experimental screening approach.

Next, the thirty-one hits were analyzed with two publicly available bioinformatics resources. The EcoCyc web resource\cite{18} was employed to identify enrichments for pathways or biosynthetic processes, cellular compartments, and gene ontology terms, and the STRING database\cite{13} was used to establish networks based on gene relations. The STRING analysis afforded a wide network of connectivity and possible interactions for the hypersensitive hits (Figure 1C). These mutations are associated with the biosynthesis of membrane components, outer membrane protein folding and localization, and protein secretion systems. The “big picture,” obtained from both enrichments and network analyses, is that sensitive mutants are linked to components of the \textit{E. coli} outer membrane (OM).

One pathway, the biosynthesis of $\alpha$-glycero-$\beta$-D-manno-heptose, a sugar moiety found in the core of lipopolysaccharide (LPS), was highly enriched (p-value $1.8 \times 10^{-9}$, when the enrichment was performed for pathways and all gene ontology terms using EcoCyc). $\alpha$-glycero-$\beta$-D-manno-heptose is biosynthesized in the cytosol as an ADP-activated derivative, and four enzymes are involved in its biosynthesis from sedoheptulose-7-phosphate: LpcA, GmhB, RfaE, and RfaD.\cite{19} The four corresponding knock-out mutants are among the highest-ranked hypersensitive hits obtained from the Keio screening of HD5\textsubscript{ox} (mutant ($\psi$): \textit{rfaE} (0.28), \textit{lpcA} (0.30), \textit{rfaD} (0.30), \textit{gmhB} (0.42), Figure 1B).

LPS is an essential component of the OM of most Gram-negative bacteria.\cite{20} In \textit{E. coli} K-12, LPS consists of a Lipid-A anchor, a core made up of ten sugar units, and the enterobacterial common antigen (ECA, Figure 2).\cite{21} In addition to the genes related to the biosynthesis of the LPS core, one mutant related to an enzyme that attaches an $\alpha$-glycero-$\beta$-D-manno-heptose unit to the Lipid-A anchor (RfaF) and mutants related to ECA biosynthesis were also identified as sensitive in the screen (Figure 2).

LPS provides a permeability barrier for the bacterial cell, and bacteria with LPS deficient in $\alpha$-glycero-$\beta$-D-manno-heptose show increased sensitivity to certain antibiotics.\cite{22} The four mutants of the $\alpha$-glycero-$\beta$-D-manno-heptose biosynthesis pathway have been identified as sensitive to other antimicrobial compounds in other Keio screens. For example, the Collection was screened against a variety of clinically used antibiotics to create an
“antibiotic barcode,” and the rfaE mutant was observed to be hypersensitive to eleven of twenty-two antibiotics.[23]

On the basis of the lectin-like properties of HD5ox[24] and the screening results, we reasoned that the peptide interacts with LPS, and performed additional AMAs where HD5ox was pre-incubated with LPS prior to addition of a log-phase culture of E. coli K-12 (Figure 3A). Colistin (polymyxin E), which binds the Lipid-A portion of LPS,[25] and vancomycin, an antibiotic that binds to Lipid II,[26] were used as controls. When the compounds were pre-incubated with LPS, attenuated antibacterial activity was observed for colistin whereas exogenous LPS had negligible effect on the activity of vancomycin. Decreased antibacterial activity was also observed for HD5ox in the presence of LPS, suggesting that LPS interacts with this peptide as previously observed for other defensins,[27] and provides protection of E. coli against the peptide.

To ascertain the interaction of HD5ox with LPS in an in vitro assay, the Limulus Amebocyte Lysate (LAL) assay was performed, which allows for the quantification of the bound fraction of LPS, using colistin and vancomycin as controls (Figure 3B).[27–28] In agreement with the AMA, the LAL assay revealed LPS binding for both colistin and HD5ox. LPS binding was not observed with increasing concentrations of vancomycin. These experiments further confirm that HD5ox binds to LPS. Taken together, these LPS-binding assays suggest that LPS is important for bacterial defense against HD5ox, and confirm the results of the screen as relevant for HD5ox activity.

One possible explanation for the observed hypersensitivity of LPS-deficient mutants to HD5ox is the loss of barrier function provided by LPS, leading to facilitated OM permeabilization/damage or cellular entry. To probe whether a compromised OM affords enhanced HD5ox antibacterial activity, the E. coli imp4213 mutant was evaluated in the AMA. This mutant exhibits increased OM permeability to a variety of molecules including maltodextrins and antibiotics.[29] It was also more susceptible to HD5ox than the parent strain (Figure 3C), confirming that a compromised OM enhances E. coli susceptibility to HD5ox. Along these lines, enterobacterial E. coli OM polysaccharides were recently found to have a protective function against HD5ox.[30]

Because mutants in LPS biosynthesis and mutants related to membrane integrity exhibited increased sensitivity to HD5ox in the AMA, we evaluated whether the observed hypersensitivity to HD5ox would manifest as a different morphological phenotype. The WT and the five most sensitive E. coli mutants were studied by phase-contrast microscopy (Figure 4 and Supporting Figure S5). The morphologies observed when treating the WT cells with HD5ox included clumping, cell elongation, and formation of blebs. When treating the five most sensitive mutants with HD5ox, similar phenotypes were obtained. These results indicate that the hypersensitivity to HD5ox observed for the mutants does not correlate with altered cellular morphologies, at least at the level observable by phase-contrast microscopy, but rather to the lack of LPS.

In summary, screening of the Keio Collection robustly identified genes that confer HD5ox hypersensitivity when knocked out. One result from this unbiased approach and subsequent
bioinformatic analyses is that many of the hypersensitive mutants are related to *E. coli* OM integrity, and that the most sensitive hits are all related to LPS biosynthesis. Using independent techniques, we confirmed the interaction of HD5$_{ox}$ with LPS that we identified by means of the unbiased screen.

A compromised OM results in enhanced susceptibility to HD5$_{ox}$, indicating that the *E. coli* OM provides protection against attack by HD5$_{ox}$. This observation is significant because *E. coli* contributes to human health and disease in organ systems where HD5$_{ox}$ is expressed; *E. coli* strains include both commensals of the human gut as well as pathogens that cause intestinal and urinary tract infections.\(^{[31]}\) Moreover, knowledge about which genes, when knocked-out, render the bacteria hypersensitive to HD5$_{ox}$, may facilitate the identification of adjuvant targets and therefore be useful for designing molecules that enhance the antibacterial activity of HD5$_{ox}$.\(^{[32]}\)

Several possibilities for the origin(s) of the hypersensitive phenotype exist and are relevant for mechanism-of-action studies, and warrant further exploration. Because the OM of Gram-negative bacteria prevents various small molecules and antibiotics from entering the cells, one working model is that the OM serves a barrier function and inhibits HD5$_{ox}$ entry and access to one or more targets. In this model, the association of HD5$_{ox}$ with LPS inhibits HD5$_{ox}$ entry. Another possibility is that HD5$_{ox}$ more readily damages the compromised OMs of the mutant strains, which affords the hypersensitive phenotype; however, the phase-contrast microscopy images presented in this work provide no clear evidence for enhanced OM damage for the hypersensitive mutants.

To the best of our knowledge, this work constitutes the first genetic screen reported for a defensin peptide. We expect that the optimized screening conditions developed for HD5$_{ox}$ in liquid culture will be applicable to other defensins and will be useful for elucidating similarities and differences in bacterial susceptibility as well as mechanism of action for various defensin family members. We envision that the screen will serve as resource for defensin studies and ultimately afford a “defensin bar-code” like what currently exists for small-molecule antibiotics.\(^{[23]}\)

**Experimental Section**

**Antimicrobial Activity Assay for Keio Screen**

Strains were inoculated from agar plates or glycerol stocks in 150 µL of LB-Lennox (kanamycin 25 µg/mL) in a flat-bottom 96-well plate using a 96-metal-prong replicator and incubated at 37 °C overnight. The next morning, an aliquot (1–2 µL) of overnight culture was transferred to 150 µL of LB (NaCl 50 mg/mL, kanamycin 25 µg/mL) using the replicator and incubated for 2 h at 37 °C. After that time, an aliquot (1–2 µL) of the culture was first transferred with the replicator to 20 µL of sterile water, and then further transferred from the water (1–2 µL) to 50 µL of assay mixture using the replicator. The assay mixture consisted of 10 mM potassium phosphate pH 7.4 and 1% trypticase soy broth (TSB) with or without 8 µM HD5$_{ox}$. The plates were incubated at 37 °C for 1 h in a shaking incubator (150 rpm). After that time, a 50-µL aliquot of 2x AMA media (adapted from Orchard *et al.*,\(^{[33]}\)) Supporting Information) was added and the plates were sealed with parafilm. The plates
were incubated first at 37 °C for 2 to 3 h, then at 30 °C overnight in a shaking incubator (150 rpm). The OD$_{600}$ was measured 20 h after addition of the AMA media on a plate reader after stirring the cultures with the replicator.

**LAL Assay**

The Thermo Pierce LAL Endotoxin Quantitation kit was used. LPS from *E. coli* was provided with the kit. The assay was performed following the manufacturer's instructions with modifications (Supporting Information).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**

Membrane-related mutants are sensitive to HD5. A) NMR solution structure of HD5. (PDB: 2LXZ) and primary amino acid sequence. Grey lines indicate native disulfide bonds. B) Ranking of sensitive mutants obtained from the Keio screen. The first column lists the gene knock-outs. The second column shows the results of three screening rounds ($\phi_{(1-3)}$). In the third column, the $\phi$ values obtained from two confirmation rounds using an AMA with normalized cell count are outlined ($\phi_{(4-5)}$). Hits are ranked according to $\phi_{(4-5)}$. C) Adaptation of the confidence view of an interaction network analysis of hypersensitive hits.
from the Keio screen using the STRING database. Spheres represent proteins and the corresponding gene names are indicated, and the lines represent connectivity. The relative thickness of each line indicates the relative confidence of interaction between the genes. The spheres of the five most sensitive mutants are depicted in blue.
Figure 2.
Sensitivity of mutants deficient in LPS biosynthesis. A) Structure of ADP-\(\text{L-glycero-}\beta-\text{D-manno-heptose}\). B) \(\phi\) values indicative of the sensitivity of LPS-related mutant to HD5\(_{\text{ox}}\). C) Scheme of LPS from \textit{E. coli}, which consists of a Lipid-A anchor, an inner core of ten sugar moieties, and the enterobacterial common antigen (ECA).
Figure 3.
LPS binds to HD5\textsubscript{ox} and decreases its antibacterial activity. (a) AMA against \textit{E. coli} K-12 with exogenous LPS. Compounds and LPS were pre-incubated for 10 min before cells were added. (b) LAL assay to quantify bound LPS with increasing concentrations of colistin, vancomycin, or HD5\textsubscript{ox}. Compound and LPS mixtures were incubated for 30 min before addition of LAL. The fraction of free LPS was calculated using an LPS standard curve and converted to bound LPS (bound LPS (in Endotoxin Units/mL) = 1 – free LPS).\textsuperscript{[28]} (c) AMA using the \textit{E. coli} leaky membrane mutant \textit{imp}4213. The mutant is more sensitive to HD5\textsubscript{ox} compared to the WT.
Figure 4.
Phase-contrast microscopy reveals that WT (*E. coli* BW25113) and the *rfaF* mutant exhibit similar morphological changes upon treatment with 40 µM HD5ox (1 h, 37 °C, ~10⁸ CFU/mL). Scale bar represents 5 µm.