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Peptides Selected for Binding to a Virulent Strain of *Haemophilus influenzae* by Phage Display Are Bactericidal

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Nontypeable *Haemophilus influenzae* (NTHi) is an obligate parasite of the oropharynx of humans, in whom it commonly causes mucosal infections such as otitis media, sinusitis, and bronchitis. We used a subtractive phage display approach to affinity select for peptides binding to the cell surface of a novel invasive NTHi strain R2866 (also called Int1). Over half of the selected phage peptides tested were bactericidal toward R2866 in a dose-dependent manner. Five of the clones encoded the same peptide sequence (QKRTSIATEGCLPS; clone hi3/17), while the remaining four clones encoded unique peptides. All of the bactericidal phage peptides but one were cationic and had similar physical-chemical properties. Clone hi3/17 possessed a similar level of activity toward a panel of clinical NTHi isolates and *H. influenzae* type b strains but lacked bactericidal activity toward gram-positive (*Enterococcus faecalis, Staphylococcus aureus*) and gram-negative (*Proteus mirabilis, Pseudomonas aeruginosa, and Salmonella enterica*) bacteria. These data indicate that peptides binding to bacterial surface structures isolated by phage display may prove of value in developing new antibiotics.

*Haemophilus influenzae* is a small gram-negative human-restricted bacterium found almost exclusively in the nasopharynx and is the causative agent of mucosal infections such as otitis media, sinusitis, and bronchitis. Non-typeable *H. influenzae* (NTHi) strains lack the antigenic capsular polysaccharides (serologic types a to f) of the encapsulated *H. influenzae* strains. NTHi has also been responsible for sporadic outbreaks of invasive disease such as meningitis and bacteremia in immunocompetent hosts, warning of its potential virulence (5, 6, 16, 24, 25). Since 1985, the nearly universal administration of the *H. influenzae* type b conjugate vaccine (Hib) has virtually eliminated all invasive diseases due to type b encapsulated strains in the developed world; however, these vaccines do not confer protection against NTHi strains. Moreover, inappropriate antibiotic treatment is contributing to the emergence of antibiotic-resistant strains of NTHi (34).

There has been significant progress in identifying vaccine candidates to prevent NTHi disease utilizing outer membrane proteins (OMPs); however, this continues to be a challenge (26). The genotypic and phenotypic diversity of OMPs is greater among NTHi strains than among encapsulated strains (11). NTHi strains are able to evade host immune defenses through mutation of OMPs and phase variation of their lipopolysaccharides, thereby increasing the diversity of antigens presented to the immune system (3).

Since it was first reported (8, 28, 30), phage display has become routinely used for the isolation of peptide ligands to a wide variety of targets, including small molecules (28), receptors (2), and whole-cell epitopes (8, 35). In particular, phage display offers a powerful tool for the selection of peptides binding to specific motifs on whole cells since it is a nontargeted strategy that enables the identification of surface structures that may not have been considered as targets or have not yet been identified. This is of particular relevance to *H. influenzae* since ca. 57% of its genes have no known function (1), and yet some may encode for cell surface motifs that could be a vaccinogen for vaccines or could be a target for antibacterial drug discovery. In addition, whole-cell phage display offers the possibility of identifying phage-displayed peptides that inhibit the function of previously unidentified targets and thereby inhibit bacterial growth.

We used a subtracted whole-cell phage display approach to isolate peptides binding to the cell surface of NTHi strain R2866. Selected phage peptides were specifically bactericidal toward *H. influenzae* strains in a dose-responsive manner. These phage peptides were cationic and bound to a 21- or a 49-kDa OMP of NTHi strain R2866, as demonstrated by immunoblotting. The data suggest that these defensin-like peptides bind to a specific motif on the bacterial cell surface.

**MATERIALS AND METHODS**

**Bacterial strains.** Table 1 lists the strains used in these studies. *H. influenzae* was grown on chocolate agar (36 g of Difco GC medium/liter, 10 g of hemoglobin/liter, 10 ml of Difco Supplement B [Becton Dickinson]/liter, 5,000 U of bacitracin/liter, and 15 g of Bacto-Agar/liter or supplemented brain heart infusion (sBHI) broth (37 g of brain heart infusion medium [Becton Dickinson]/liter containing 10 mg each of β-NAD and hemin-chloride/liter and 5,000 U of bacitracin/liter) (27). *Staphylococcus aureus, Proteus mirabilis, and Enterococcus faecalis* were grown as described above, except that bacitracin was omitted. *Salmonella enterica* was grown in Luria-Bertani (LB) broth. For all solid media, 15 g of Bacto agar/liter was added. Multilocus sequence typing was performed as described by Meats et al. (21), whereas biotyping was performed as described by Killian (17) and Holmes et al. (14).

**Antimicrobial peptides.** Polymyxin B sulfate (catalogue no. P0972) was purchased from Sigma Chemical Company, St. Louis, Mo. Magainin II was generously supplied by Mark Anderson, Magainin Pharmaceuticals, Plymouth Meeting, Pa. LL-37 was synthesized on a Perkin-Elmer/Applied Biosystems 433 synthesizer and purified by reversed-phase C18 high-pressure liquid chromatography by Alan Weber. The purity of the peptide was confirmed by analytical high-pressure liquid chromatography detecting the peptide by determining the...
absorbance at 215 nm. Human neutrophil peptide-1 (α-defensin-1) and human β-defensin-2 were obtained from Peptides International, Louisville, Ky.

Assay of antimicrobial activity. Representative affinity-selected phage-displayed peptides were tested for their ability to inhibit the growth of selected bacterial stains in vitro. Each phage clone was amplified by *Escherichia coli* infection, purified twice by polyethylene glycol precipitation, and resuspended in Tris-buffered saline (TBS; pH 7.5) as described above. Phage concentrations were calculated as virions per microliter by using UV absorbance (31). A version of the microdilution susceptibility test was used to determine the antimicrobial activity of each of the phage-displayed peptides. In brief, each of the bacterial strains was grown to an optical density at 600 nm (OD600) of 0.6 in the appropriate media, as described above, and then diluted to 1.0×10⁶ CFU/ml with the same media to grow the bacterial strains. Each well in a 96-well microtiter plate (Corning catalogue no. 3595) was inoculated with 150 μl of 1×10⁶ to 5×10⁹ CFU of bacteria/ml in the appropriate media, and 50 μl of twofold dilutions of phage-displayed peptides in TBS (pH 7.5). Controls included TBS without phage peptides and TBS containing dilutions of the unselected f88-4/15-mer phage library. After 16 to 18 h at 37°C without shaking, the ODs of the bacterial cultures were measured at OD600 in a plate reader (Dynatech MR5000). The number of viable bacteria in the 96-well plates was determined by performing colony counts and plating onto appropriate agar plates. All assays were performed in triplicate with two separate phage preparations. Colony counts on appropriate agar plates were also used to determine the initial inoculum bacteria used in the assay. The MIC was defined as the lowest concentration of phage-displayed peptides that completely inhibited detectable growth (OD600 of <0.005) of the test bacteria. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of phage-displayed peptides resulting in a 99.9% kill of the bacteria after 16 to 18 h of incubation.

The MICs of antimicrobial peptides were determined in a similar manner. Peptides were dissolved in a solution containing 0.01% acetic acid and 0.1% bovine serum albumin (Sigma) sterilized with a 0.2-μm microfilter. The solution was then diluted to 10⁵ CFU/ml with the same media, as described above, and the MIC determination, with 5×10⁴ KID (K91BlueKan cells) used to affinity select for peptides binding to R2866. In the affinity selection procedure, the depleted library was added to 1.0×10⁹ CFU of R2866 (resuspended in PBS-G) in a final volume of 4 ml of PBS-G. This bacterium-phage mixture was gently agitated on an orbital shaker as described above. After 20 min, phage binding to R2866 was separated from nonbinding phage in solution by a series of 10 washing and centrifugation steps (1,000 × g for 1 min) with 1 ml of PBS-G (pH 7.0) each time. The phage bound to R2866 was eluted from the pellet R2866 by the addition of 200 μl of elution buffer (0.1 M HCl [pH 2.2] with glycine and 1 g of bovine serum albumin/lotter) for 10 min at room temperature. The phage eluted from R2866 was immediately neutralized with 1 M Tris-Cl (pH 9.0), and the titer of an aliquot of the eluate was determined as TU so that the yield of phage binding to R2866 [i.e., (output phage/input phage) × 100] could be determined (31). The remaining phage was amplified by infecting starved *E. coli K91BlueKan* cells (35). The amplified phage were twice purified by polyethylene glycol precipitation and resuspended in TBS buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl [pH 7.5]) (30). The titer of the amplified phage was determined in *E. coli K91BlueKan* cells prior to being reapplied to freshly prepared R2866 as described above. Phage isolation against R2866 was continued for a total of three affinity selection and two amplification steps. Phage eluted from the third and final round of affinity selection against R2866 was used to infect *E. coli K91BlueKan* cells. These cells were plated on LB plates containing 40 mg of tetracycline/liter to select for bacteria containing phage. Bacterial colonies, each containing a single phage population, were randomly selected and propagated for subsequent analyses.

**Table 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s) and/or source</th>
<th>MLSTa</th>
<th>Biotypeb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R652</td>
<td><em>H. influenzae</em> strain Rd KW20 sequenced by the TIGR</td>
<td>47</td>
<td>IV</td>
<td>10</td>
</tr>
<tr>
<td>R3001</td>
<td><em>H. influenzae</em> bronchoalveolar lavage of a patient with cystic fibrosis</td>
<td>ND</td>
<td>II</td>
<td>21a</td>
</tr>
<tr>
<td>R2866</td>
<td>Invasive nontypeable <em>H. influenzae</em>; complete genome sequence available at NCBI</td>
<td>99</td>
<td>V</td>
<td>24</td>
</tr>
<tr>
<td>R2140</td>
<td><em>H. influenzae</em> biogroup aegyptius; CDC F3031</td>
<td>ND</td>
<td>III</td>
<td>6</td>
</tr>
<tr>
<td>E1a</td>
<td>Hib Eagan</td>
<td>44</td>
<td>I</td>
<td>37</td>
</tr>
<tr>
<td>R538</td>
<td>Reference Hib ATCC 7975</td>
<td>ND</td>
<td>I</td>
<td>NA</td>
</tr>
<tr>
<td>R1513</td>
<td>P. aeruginosa; ATCC 27853; quality control strain for antibiotic susceptibility testing</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S. aureus</td>
<td>UMC clinical lab</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S. enterica</td>
<td>UMC clinical lab</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>UMC clinical lab</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E. feca1</td>
<td>UMC clinical lab</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Meat et al. (21). ND, not done; NA, not applicable.
b Holmes et al. (14) and Kilian (17).
c UMC, University of Missouri—Columbia.

DNA sequencing and peptide analysis. Single-stranded DNA isolated from the phage clones (30) was sequenced from the 3′ end of the pVIII gene by using an ABI Prism 377 automated sequencer (Applied Biosystems) at the DNA Core (University of Missouri–Columbia). The DNA sequences were translated into amino acids by using the “translate” program on the proteomics server of the Swiss Institute of Bioinformatics Expert Protein Analysis System (ExPaSy [http://www.expasy.ch]). The isoelectric points of the predicted peptide sequences were calculated by using “compute MW/pI,” also present on the ExPaSy proteomics server. Sequence alignments were performed by using the CLUSTAL W sequence alignment program (33; available at http://www.ebi.ac.uk/clustalw/). GeneDoc (http://www.psc.edu/biomed/genedoc/) was used as a tool for visualizing, editing, and analyzing multiple sequence alignments of the peptides.

Immunoblotting. OMPs were purified from NTHi strain R2866, as described previously (27). In brief, R2866 cells were resuspended in PBS (pH 7.0) and disrupted by passage through a French pressure cell (SLM Instruments) at 15,000 lb/in². Soluble and membrane fractions of R2866 were separated by ultracentrifugation at 140,000 × g for 4 h at 4°C in a Ti80 rotor. Protein concentrations were determined by using a Bio-Rad protein assay kit. For Western immunoblot analyses, OMPs (5 μg/sample) from R2866 were boiled for 5 min in sodium dodecyl sulfate (SDS) reducing buffer prior to being separated at 150 V on Bio-Rad SDS–12% polyacrylamide gel electrophoresis (PAGE) minigels (18). Proteins were transferred onto Immobilon polyvinylidene difluoro...
ride (PVDF) transfer membranes (Millipore) by using the Mini-Trans-Blot transfer cell (Bio-Rad). The PVDF membranes were blocked at room temperature for 2 h in blocking solution (10% bovine serum albumin in PBS). The membranes were then incubated with 4 × 10^12 virions of phage-displayed peptides in PBS for 1 h at room temperature. After being washed in PBS for 20 min, the membranes were incubated with a 1:2,500 dilution of mouse anti-M13 antibody (Amersham Pharmacia Biotech) for 1 h in blocking buffer. The membranes were washed as described above and then incubated with a 1:2,500 dilution of rabbit anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (Sigma) for 1 h. After the membranes were washed as described above, the bands on the immunoblots were detected by using the TMB membrane peroxidase substrate system (Kirkegaard & Perry Laboratories). SDS-PAGE broad-range prestained markers (Bio-Rad) were used to estimate the size of the bands binding the recombinant phage.

RESULTS

Isolation of phage-displayed peptides binding to R2866. We used a 15-mer random peptide phage display library (f88-4/15) to affinity select for peptides that bound to cell surface epitopes of an invasive NTHi, strain R2866. Since we wanted to isolate peptides binding to strain-specific epitopes, we chose to preadsorb the starting library against a nonvirulent NTHi (Rd KW20) prior to affinity selecting for peptides binding to R2866. We reasoned that, without this preadsorption step, phage clones binding to more abundant and/or accessible epitopes shared by Rd KW20 and R2866 would have been the predominant outcome during the affinity selection steps (15). The depleted library was then used to affinity select for peptides binding to R2866. After each round of selection, the yield of phage peptides eluted from the cell surface of R2866 was determined prior to reamplification by E. coli infection. A >2,000-fold enrichment for NTHi-binding phage had occurred during the three rounds of affinity selection against R2866. Most of this enrichment occurred between the first and second rounds of selection (700-fold), with only a minor increase in the final round (2.9-fold).

After the third round of selection against R2866, 16 individual clones were randomly selected from the eluted phage population and independently propagated (Table 2). An in vitro growth assay was used to identify potential clones inhibiting the growth of R2866 in liquid culture. NTHi strain R2866 was suspended in sBHI medium and individually incubated with each of the 16 phage-displayed peptides (see Materials and Methods). After an overnight incubation at 37°C, nine of the clones were found to inhibit the growth of R2866, as determined by direct measurement of the ODs of the bacterial cultures. The remaining seven clones showed no activity against R2866 and were not significantly different from the unselected library control in this regard (data not shown).

Single-stranded DNA was isolated from the nine clones inhibiting the growth of R2866 and the peptide-encoding region on gene 8 sequenced. Five of the clones encoded the same predicted peptide sequence, as represented by hi3/17 (KQRTSIRATEGCLPS [Table 2]). They also contained the same genomic sequence, indicating that they were likely to be siblings. The remaining four clones encoded unique peptide sequences, apart from hi3/11, which had the same “KOR” motif as the group represented by hi3/17 at its amino terminus (Table 2). With the exception of hi3/2 (pI 6.75), all of the phage-displayed peptides were cationic with calculated isoelectric point values (pI) between 8.60 and 9.99 (Table 2).

The phage peptides were aligned and shaded according to their physical-chemical properties (Fig. 1). Selection of specific groups of amino acid residues occurred during the affinity selection procedure. For example, all of the peptides contained a positively charged amino acid residue (K, R) at position 1 of the aligned peptides (position 2 for hi3/2 in Fig. 1). A strong preference for polar amino acid residues occurred at positions 2, 3, 5, 9, and 10 of the aligned peptides (Fig. 1). A small amino acid residue occupied positions 8 and 14 of the aligned peptides (position 2 for hi3/2 in Fig. 1). A positively charged amino acid residue (K, R) at position 1 of the aligned peptides (position 2 for hi3/2 in Fig. 1). A strong preference for polar amino acid residues occurred at positions 2, 3, 5, 9, and 10 of the aligned peptides (Fig. 1). A small amino acid residue occupied positions 8 and 14 of the aligned peptides, and an aliphatic residue occurred at position 10 (Fig. 1). A small amino acid residue occupied positions 8 and 14 of the aligned peptides (Fig. 1). A small amino acid residue occupied positions 8 and 14 of the aligned peptides, and an aliphatic residue occurred at position 10 (Fig. 1). These conserved properties were not present in the phage peptides that were not identified as being antimicrobial (data not shown).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Frequency</th>
<th>Amino acid sequence</th>
<th>pI</th>
<th>MIC</th>
<th>MIC&lt;sub&gt;c&lt;/sub&gt;</th>
<th>MBC&lt;sub&gt;99.5&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>hi3/17</td>
<td>5/15</td>
<td>KQRTSIRATEGCLPS</td>
<td>9.50</td>
<td>2 × 10&lt;sup&gt;9&lt;/sup&gt; (0.8)</td>
<td>3 × 10&lt;sup&gt;9&lt;/sup&gt; (1.2)</td>
<td></td>
</tr>
<tr>
<td>hi3/2</td>
<td>1/15</td>
<td>GRHRTSVPTDEVFTT</td>
<td>6.75</td>
<td>1 × 10&lt;sup&gt;10&lt;/sup&gt; (4.1)</td>
<td>1.5 × 10&lt;sup&gt;10&lt;/sup&gt; (6.2)</td>
<td></td>
</tr>
<tr>
<td>hi3/12</td>
<td>1/15</td>
<td>KKSHPSPSEWGNLT</td>
<td>8.61</td>
<td>1 × 10&lt;sup&gt;10&lt;/sup&gt; (4.1)</td>
<td>2 × 10&lt;sup&gt;10&lt;/sup&gt; (8.25)</td>
<td></td>
</tr>
<tr>
<td>hi3/11</td>
<td>1/15</td>
<td>KQDRSRSGYTAPTTLV</td>
<td>9.99</td>
<td>1 × 10&lt;sup&gt;10&lt;/sup&gt; (4.1)</td>
<td>2 × 10&lt;sup&gt;10&lt;/sup&gt; (8.25)</td>
<td></td>
</tr>
<tr>
<td>hi3/13</td>
<td>1/15</td>
<td>RNHGTDRAINTIPPLS</td>
<td>9.61</td>
<td>2.3 × 10&lt;sup&gt;10&lt;/sup&gt; (9.4)</td>
<td>2.3 × 10&lt;sup&gt;10&lt;/sup&gt; (9.4)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> That is, the frequency of the phage clone isolated from the f88-4/15-mer library.

<sup>b</sup> pI, calculated isoelectric point.

<sup>c</sup> MIC is defined as the minimum concentration of phage-displayed peptides resulting in no detectable growth.

<sup>d</sup> MBC<sub>99.5</sub> is defined as the lowest concentration of phage-displayed peptides necessary to kill 99.5% of the bacteria.

FIG. 1. Alignment of phage-displayed peptides. The deduced amino acid sequences from hi3/17, hi3/2, hi3/12, hi3/11, and hi3/13 were aligned by using CLUSTAL W (33). Dashes indicate gaps used to maximize the alignment. The physical-chemical mode of GeneDoc was used to assign each column of the alignment to one of the 12 predefined groups of physiochemical properties. Red writing on a blue background indicates positively charged amino acids; black writing on a green background indicates polar amino acids; green writing on a yellow background indicates small amino acid residues; white writing on a black background indicates hydrophobic amino acids; and red writing on a gray background indicates aliphatic amino acids.
The phage-displayed peptides were bactericidal toward R2866. A microdilution susceptibility test was used to determine the in vitro antimicrobial activity of the phage-displayed peptides. Twofold dilutions of the phage peptides from Table 1 were incubated with R2866 in sBHI broth in the 96-well plate format. After an overnight incubation at 37°C, the number of CFU remaining in the microtiter plates was determined by plating onto solid sBHI media (see Materials and Methods). All of the phage peptides exhibited a dose-response effect against R2866 over a range of virion concentrations (Fig. 2). At the highest concentrations, all of the phage peptides in Table 2 were strongly bactericidal toward R2866, reducing the viable counts by almost 100%, as determined by plating onto sBHI agar. At lower virion concentrations, the growth of R2866 was inhibited compared to unselected library control (Fig. 2).

The phage clones also varied in their potency against R2866 (Table 2 and Fig. 2). hi3/17 possessed the greatest activity against R2866 with an average MIC of $2 \times 10^9$ virions/μL, assuming that all 150 peptides displayed on pVIII are simultaneously accessible (Table 2). Phage-displayed peptides hi3/2, hi3/11, hi3/12, and hi3/13 showed MICs from $1.0 \times 10^9$ to $2.3 \times 10^10$ virions/μL corresponding to a peptide concentration of between 4.1 and 9.4 μM (Table 2). Overall, hi3/17 showed at least fivefold higher activity toward R2866 (Table 2). The cognate peptides, corresponding to hi3/17, hi3/2, hi3/12, and hi3/13, were synthesized and tested for their antimicrobial activity. At a concentration of 200 μM, these linear peptides did not have detectable antimicrobial activity toward H. influenzae R2866. This could be because of protease resistance or an essential structural conformation of the peptide that is specifically induced by expression on the phage coat (31).

Specificity of hi3/17 activity. The in vitro antimicrobial activity of hi3/17 was tested against H. influenzae and other bacterial strains. hi3/17 at a concentration of $3 \times 10^9$ virions/μL showed no bactericidal activity against gram-positive (E. faecalis and S. aureus) and gram-negative (P. mirabilis, P. aeruginosa, and S. enterica) bacteria. Surprisingly, hi3/17 inhibited the growth of S. aureus even though we could not demonstrate bactericidal activity against this strain. hi3/17 was bactericidal against all of the NTHi strains tested (Rd KW20, R3001, and R2140) and Hib strains E1a and R538 at a concentration of $3 \times 10^9$ virions/μL, indicating that hi3/17 recognized an epitope that was not isolate specific.

Characterization of the binding specificity of phage-displayed peptides. We tested the individual phage clones for their ability to bind to OMPs from NTHi strain R2866. An immunoblot with the phage clones revealed that the phage clones hi3/2, hi3/12, and hi3/13 bound to a band of ca. 21 kDa under reducing conditions, whereas phage clone hi3/17 bound to a band with a mobility of 49 kDa (Fig. 3).

DISCUSSION

Cell surface molecules on bacteria play an essential role during pathogenesis because they act at the interface between the host and its immune system. Although sequencing of microbial genomes has yielded potential targets for antibacterial drug discovery, the lack of functional genomic information has largely precluded these genes from the drug discovery process (7). Whole-cell phage display can be used to isolate specific peptide ligands capable of disrupting bacterial physiology, without prior information on the receptors involved. The phage peptides were initially isolated on the basis of their ability to bind
Phytophthora capsici successfully used to isolate peptide ligands that disrupted plant bacterial cell function. In the past, this approach was individually tested in function-based assays for their ability to to whole NTHi cell surface epitopes, and then these were individually tested in function-based assays for their ability to disrupt bacterial cell function. In the past, this approach was successfully used to isolate peptide ligands that disrupted plant (Phytophthora capsici and Eimeria acervuline) pathogens (4, 9).

Given that the cell surface epitopes on NTHi have not been characterized in detail, we chose to isolate phage clones binding to a wider range of epitopes rather than to the most abundant or highly accessible epitopes on the cell surface, as is usually the case in a direct selection strategy (15). In our experiments, the initial phage library was preadsorbed against an avirulent strain of NTHi (Rd KW20) prior to affinity selecting for peptide binding to R2866. This strategy largely eliminated the predominant clones from the depleted phage population while preserving peptides binding to R2866 and Rd KW20. This is in line with the expectation that preadsorption removes peptides binding to the high-density antigens, while preserving those binding to the less common antigens (15).

The success of this strategy was reflected in the high proportion of bactericidal phage-displayed peptides isolated from the preadsorbed library (9 of 16 clones isolated; Table 2). Five of these clones encoded the same peptide sequence (KQRTSIRATEGCLPS), as represented by hi3/17, whereas the remaining four encoded unique peptide sequences (Table 2). A conventional amino acid alignment revealed that there was no clear consensus pattern among the five groups of peptides; however, it was evident that strong selection pressure had occurred for amino acid residues with similar physical-chemical properties (Fig. 1). These conserved physical-chemical properties were not present in the phage peptides that were not antimicrobial (data not shown).

NTHi strains R2866 and R652 have been sequenced (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), while the complete genomic sequence of a type b strain (strain 10801, MLST 6) is available from the Sanger Institute (http://www.sanger.ac.uk/Projects/H_influenzae/). Themultilocus sequence type (MLST) has not been determined for R2140, but the 17 H. influenzae biogroup aegyptius strains that have been MLST typed fall into two groups that are distinct from NTHi and from encapsulated H. influenzae (http://www.mlst.net). Overall, it is likely that the H. influenzae strains used here express diverse surface molecules.

NTHi is commonly found as a commensal of the human upper respiratory tract in humans, where it can also act as an opportunistic pathogen (23). The epithelial airways of mammals are protected from bacterial infection by an elaborate host defense system that includes the secretion of antimicrobial peptides, chemokines, and cytokines (13). The bactericidal phage-displayed peptides in the present study were isolated on the basis of their ability to bind to NTHi, and yet they shared similarity to a family of natural antimicrobial peptides called defensins. Defensins are a large group of cationic antimicrobial peptides that play an important role in the lung innate defense system (13, 29). Certain defensins are secreted in response to microbial infection and exhibit broad-spectrum activity against express much less capsular polysaccharide (unpublished observations). The genome of strain R2866 contains approximately 200 genes in 40 genetic islands that are not present in the R652 genome. Strain R3001 has not been characterized genetically, but its different biotype suggests that it is likely to be distantly related to all of the other strains studied. The MLST has not been determined for R2140, but the 17 H. influenzae biogroup aegyptius strains that have been MLST typed fall into two groups that are distinct from NTHi and from encapsulated H. influenzae (http://www.mlst.net). Overall, it is likely that the H. influenzae strains used here express diverse surface molecules.

<table>
<thead>
<tr>
<th>Antimicrobial peptide</th>
<th>Activity (MIC [µg/ml]) against H. influenzae strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R652</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.5</td>
</tr>
<tr>
<td>LL-37</td>
<td>10.0</td>
</tr>
<tr>
<td>Magainin II</td>
<td>2.5</td>
</tr>
<tr>
<td>Human β-defensin-2</td>
<td>4.0</td>
</tr>
<tr>
<td>Defensin HNP-1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

a The mean MIC of three triplicate assays is given.
a range of bacteria, fungi, and viruses (12). Like defensins, the bactericidal phage-displayed peptides isolated in the present study were also cationic (apart from hi3/2) with isoelectric points between 8.60 and 9.99 (Table 2). The activity of the phage-displayed peptides against NTHi was dose dependent with MIC in the low micromolar range (Table 2 and Fig. 2). This concentration is a conservative estimate, however, since it assumes that all of the peptides on the phage surface are simultaneously accessible to the bacterium. An unusual feature of these peptides is that they lacked activity as linear molecules (data not shown) but were highly active (MICs in micromolar range) when expressed on the phage. This activity was in the same range of the activity of cationic peptides: polymyxin B (0.36 to 1.44 μM), LL-37 (0.5 to 1.03 μM), Magainin II (1.01 to 2.02 μM), human β-defensin-2 (0.92 to 1.85 μM), and α-defensin-1 (1.45 μM) (Table 3).

Little is known about how the innate immune system responds to respiratory pathogens such as H. influenzae; however, the importance of defensins in preventing infections by NTHi was demonstrated in mice, where the knockout of a single defensin, β-defensin-1, resulted in delayed clearance of H. influenzae from the lung (22). It has also been shown that β-defensin-2 can inhibit the growth of NTHi in vitro (19, 32). Given that the bactericidal phage-displayed peptides were isolated on the basis of their ability to bind to NTHi and had similarities to defensins, it is plausible that there are surface molecules on NTHi that bind to defensins produced in the human lung. Western blot analyses indicated that hi3/17 (group 1 peptides; Table 2) bound to denatured cell surface epitope of ~49 kDa, whereas group 2 peptides (hi3/2, hi3/12, and hi3/13) all bound to an epitope of ~21 kDa (Fig. 3).

Natural antimicrobial peptides have attracted attention as a novel source of therapeutic agents; however, given problems with in vivo toxicity and low biological activity, research has focused on the understanding the structure-activity of these peptides so that new antimicrobial peptides can be designed (36). Most of the peptides synthesized to date have either been variants of the natural antimicrobial peptides or isolated from synthetic combinatorial libraries (20). In both of these processes, peptides tend to retain broad-spectrum activity against a range of microorganisms. In contrast, the bactericidal phage-displayed peptides isolated in the present study were highly specific toward all of the H. influenzae strains tested, including NTHi and type b strains. It is likely that any peptides having broad-spectrum activity are likely to have been lost from the primary phage library because of the requirement to propagate the phage in an E. coli host.

In conclusion, we describe the utility of using live whole bacterial cells as a target for the isolation of antimicrobial phage-displayed peptides. The peptides were highly active when expressed as part of the phage coat but lacked activity in their linear cognate form, precluding their direct use in drug development. The target receptor(s) on H. influenzae may be good candidates for vaccine or drug development with the phage peptides as the “bait” for the ligand.

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REFERENCES


