Identification by the Phage-display Technique of Peptides That Bind to H7 Flagellin of *Escherichia coli*

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The four peptides interacting with H7 flagellin of *Escherichia coli* were selected from a phage display library. The library was selected four times, and the interacting phage peptides were competitively eluted with H7 flagellin. An enzyme-linked immunosorbent assay (ELISA) showed that these peptides were reactive with the H7 flagellin in a dose-dependent manner. Among them, a D1 phage clone showed the highest binding affinity to the H7 flagellin. We synthesized the D1 peptide (LHIHRPTLSIQG) corresponding to the peptide-encoding region of the D1 phage clone. The synthetic peptide showed micro-molar affinity (EC₅₀ value = 1.9 μM) for the H7 flagellin. Furthermore, this D1 phage interacted more specifically with the H7 flagellin than with the other flagellins (H1, H5, H12, or H23) of *E. coli*. *In situ* hybridization clearly showed that the peptide only detected those cells harboring the H7 flagellin gene (*flc*). The peptide may specifically bind to the H7 flagellin on the cell surface. These results suggest that the phage-display technique could be used as a tool for identifying peptides as an alternative to using a ligand as a diagnostic reagent in food products or in clinical testing.

Key words: H7 flagellin; flagella; antigen; phage display

A vast number of bioassay systems and biosensors depend on antibodies as recognition reagents. Although antibodies have the desired sensitivity and selectivity for this purpose, it may not be feasible in some cases to use antibodies for routine applications due to their weak stability against heat and mechanical shock. An additional drawback is the time- and labor-intensive process of obtaining antisera from animals by the conventional polyclonal antibody technique which can yield a variable product. It is even difficult to develop and produce monoclonal antibodies that provide a more consistent product. Several studies have addressed these limitations, including the use of nucleic acid aptamers⁴⁻⁵ and phage-display peptides as sensor reagents.³⁻⁵

We have previously explored the feasibility of using short peptides obtained by a phage-display peptide library.³ Phage display has been well established as a powerful selection technique by which a peptide or protein is expressed as a fused coating protein of a bacteriophage, resulting in displaying the fused protein on the surface of the virion. Phage display has been used to create a physical linkage between a vast library of random peptide sequences to the DNA encoding each sequence, enabling rapid identification with a high throughput of peptide ligands against a variety of target molecules by an *in vitro* selection process called biopanning.³ This display technique has the advantage of rapid and easy detection, allowing a displayed random peptide library to be conveniently screened.

We isolated and identified the phage-display peptides that bind to the H7 flagellin of *Escherichia coli*. H7 flagellin is the most abundant component of *E. coli* O157:H7 flagella. This *E. coli* strain is a known human pathogen⁹ that has commonly been found in contaminated food products and identified as the infection source resulting in severe bloody diarrhea and abdominal cramps.⁷,⁸ The flagellins comprise the antigens that allow serological distinction among different H serotypes.⁹ Considerable efforts have therefore been made to establish a rapid detection method for unique epitopes of the H7 antigen.¹⁰⁻¹² This article describes the characterization of phage clones selected with the H7 flagellin by using the phage-display technique described. The novel peptides selected with the target could have the potential to be used alone or in combination to detect *E. coli* O157:H7.

**Materials and Methods**

**Materials.** The phage-display peptide library (Ph. D. -12) was purchased from New England Biolabs (US). Bovine serum albumin, IPTG (isopropyl-β-D-thiogalactopyranoside), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), ampicillin, DAPI

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coli ER2738 (NEB, US) and amplified. These amplified ml linker peptide (GGGSK) was procured from Sawady Technology Co. (Tokyo, Japan).

Purification of E. coli flagellin. The flagellin proteins were purified from each Escherichia coli YK4130 strain that was harboring the flagellin gene, fliC (i.e., H1, H5, H7, H12, or H23). Each strain expressed flagella on the cell surface. The recombinant strains were grown in 100 ml of a Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride), 100 μg ml⁻¹ of ampicillin and 50 μg ml⁻¹ of kanamycin at 30°C for 18 h. The cells were collected by centrifugation (10,000 × g for 10 min) and washed twice with 10 ml of phosphate-buffered saline (PBS). After re-suspending the cell pellet in 2 ml of PBS, the flagellin was obtained from the cell-pellet solution by vigorously shaking in a Vortex mixer for 30 min. The flagellin in the supernatant in the soluble form was further purified by ultrafiltration (Ultrafree, MWC 100,000; Millipore, Japan). The purity of the flagellin was confirmed by SDS-PAGE (12.5% acrylamide slab gel, 1 mm in thickness), the protein band being stained with Coomassie brilliant blue R-250. The protein concentration was measured by an assay kit (Bio-Rad), using immunoglobulin as the standard protein.

Electron Microscopy. The E. coli YK4130 strains were grown under the above-mentioned cell-growth conditions. Cells were centrifuged and fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) osmium tetroxide. Samples were stained with 4% uranyl acetate and lead citrate. After treatment, the stained cells of each strain were viewed with a Hitachi H-7000 electron microscope.

Biopanning of the H7 flagellin-binding phage. Phage binding to H7 flagellin was screened essentially as described. The phage library was panned in an immunotube (Nunc, Denmark) coated with 1 ml of H7 flagellin (10 μg ml⁻¹) in PBS containing 0.01% Tween 20 (TPBS) overnight at 4°C. The immunotube was blocked with PBS containing 5% BSA for 2.0 h at 37°C. Phages (4×10⁹ pfu) in PBS were added to the H7 flagellin-coated tube. After incubating for 1 h at 25°C, the unbound or weakly bound phages were washed out by rinsing five times with TPBS. The phages bound to the immunotube were eluted after incubating for 30 min with 1 ml of H7 flagellin (50 μg ml⁻¹). The recovered phages were used to infect E. coli ER2738 (NEB, US) and amplified. These amplified phages were purified by precipitation with 0.2 vol. of PEG/NaCl (20% (w/v) polyethylene glycol and 2.5 M NaCl) and used for the next panning round. After four rounds of the panning cycle, 48 single clones infected with E. coli ER2738 were isolated from the LB/IPTG/X-gal plate. These isolated clones were each grown in 500 μl of the LB medium with ER2738 in a 24 multi-well plate (Sumitomo, Japan). The supernatant of each culture was analyzed by ELISA as described next. The phage titer was calculated from the number of plaques on the LB/IPTG/X-gal plate, according to the manufacturer’s manual.

Analysis of phages bound to H7 flagellin. The phage-binding assay was done by ELISA, a volume of 100 μl/well being used for each incubation. The wells of 96-well microtiter plates (Costar, US) were directly coated overnight with H7 flagellin (10 μg/ml) at 4°C, before being blocked with 5% BSA under the conditions described for biopanning. Phage diluents were added to each well in duplicate and incubated for 1.5 h at 37°C. After the plates were washed three times with TPBS, an HRP/anti-M13 monoclonal conjugate in PBS (1:2000) was added to each well. The plates were incubated for 1.5 h at 37°C, before being washed with TPBS. The absorbance at 405 nm was measured with an MTP-300 microplate reader (Corona, Japan) after development with ABTS (2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate), KPL).

DNA sequence analysis. Single-stranded DNA from individual phage clones was purified with a QIA prep M13 system (QIagen). The nucleotide sequences of the isolated DNA were analyzed by a dye terminator cycle-sequencing reaction (ABI Prism dye terminator cycle sequencing kit, Perkin-Elmer) according to the manufacturer’s instructions, and analyzed with a 3700 DNA sequencer (Applied Biosystems). The primer used for the sequencing had the nucleotide sequence of 5'-CCCTCATAGTTAGCGTAACG-3'.

ELISA of the purified phages. Each phage purified by PEG/NaCl precipitation was assayed to examine whether the clone specifically recognized the H7 flagellin. The ELISA conditions used were similar to those used for the binding analysis, with the exception of the concentration of the H7 flagellin. The purified H7 antigen was serially diluted with PBS and added to the wells of 96-well microplates. Each phage was prepared as a solution of 1.0×10⁻⁷ pfu/ml in PBS, and 100 μl was added to each well.

Characterization of the synthetic peptides. The synthetic peptide was assayed to examine whether it specifically recognized the H7 flagellin. The ELISA conditions used were similar to those described for the binding analysis, with the exception that a streptavidin-horseradish peroxidase conjugate (Amer sham Biosciences, UK) was used instead of the HRP/anti-M13 monoclonal conjugate. The synthetic
The H7 flagellin was used to coat the plates for inhibition ELISA. The synthetic peptide was serially diluted with PBS and added to each well of 96-well microplates.

The H7 flagellin (10 μg/ml) was used to coat the plates for inhibition ELISA. The synthetic peptide (20 μg/ml) was respectively incubated with 40 μg/ml of the H1, H5, H7, H12, or H23 flagellin. After each incubation, the solution was added to the wells. The synthetic peptides that bound to the H7 flagellin immobilized wells were detected by the streptavidin-horseradish peroxidase conjugate. The synthetic peptide incubated without any flagellin was used in a control experiment.

\textit{In situ hybridization. E. coli} YK4130:H7 cells were grown under the already described conditions. After harvesting by centrifugation, the cell pellet was washed twice with TPBS and then suspended in the same buffer. The washed cells were incubated with 10 μg ml\(^{-1}\) of the synthetic peptide at 4–8°C for 18 h, before the unreacted peptides were removed by centrifugation (5,000 \(\times\) g for 5 min). The resulting cell pellet was suspended in PBS and added to 20 μg/ml of Cy3-labeled streptavidin (Amersham Biosciences, UK). After incubating for 1.0 h at room temperature, the unreacted Cy3-labeled streptavidin was removed by centrifugation (5,000 \(\times\) g for 5 min). The resulting cell pellet was suspended in PBS, and DAPI was added to the suspension for staining nucleic acid. The cells were fixed on a PTFE membrane filter (Millipore, Japan) with a slide glass, \textit{E. coli} YK4130 cells (\(A\alpha\)C) being used as a negative control. Microscopic observation was done with a BX-60 fluorescence microscope (Olympus, Japan) equipped with a PXL1400 cooled charge-coupled device (CCD) camera system (Photometrics).

\textbf{Results and Discussion}

\textit{Purification of the E. coli H7 antigen}

As shown in Fig. 1a, the \textit{E. coli} YK4130:H7 cells expressed flagella on the cell surface much more abundantly than \textit{E. coli} YK4130 cells (\(A\alpha\)C) which did not form flagella on the cell surface (Fig. 1b). The other strains harboring each flagellin gene also expressed abundant flagella on the cell surface (data not shown). The \textit{E. coli} flagellins could be simply purified from each strain by vigorously shaking the cells (30 min at room temperature; see the Materials and Methods section) and by the subsequent ultrafiltration procedure. Each purified flagellin by SDS-PAGE under reducing conditions corresponded to 50–66 kDa, consistent with the reported masses\(^{13}\) (Fig. 2).

\textit{Biopanning of the phage clones specific for the H7 antigen}

In order to identify the peptides specific for the H7 flagellin, a phage library expressing the degenerate peptide was serially diluted with PBS and added to each well of 96-well microplates.

\textbf{Fig. 1.} Transmission Electron Micrograph of a Negatively Stained Cell of \textit{E. coli} Strain YK4130:H7 (a) and YK4130 (b). Bars, 2 μm.

\textbf{Fig. 2.} SDS-PAGE Patterns of the Purified \textit{E. coli} Flagellins. Lanes: 1, standard protein markers (NEB): (MBP-\(\beta\)-galactosidase [175 kDa], MBP-paramyosin [83 kDa], glutamic dehydrogenase [62 kDa], aldolase [47.5 kDa], triosephosphate isomerase [32.5 kDa], \(\beta\)-lactoglobulin A [25 kDa], lysozyme [16.5 kDa]); 2, H1 flagellin; 3, H5 flagellin; 4, H7 flagellin; 5, H12 flagellin; 6, H23 flagellin.
12-mer peptide was screened by biopanning, using the purified H7 flagellin as already described. Elution by the low-pH method\[^{16}\] was unsuccessful for isolating specific phage clones against the H7 flagellin, even after the fourth round of biopanning. Although several clones were isolated from the fourth round of selection by low-pH elution, most of these were observed to bind not only to the target H7 flagellin, but also non-specifically to the polystyrene surface of the immunotube. These non-specific-binding peptides contained many aromatic amino acids such as tryptophan and tyrosine (data not shown here). Considering that peptides containing abundant aromatic amino acids have shown relatively strong binding affinity in previous works,\[^{17}\] it seems that these aromatic amino acids might have high affinity for such plastic materials as polystyrene and polyvinyl chloride. In order to isolate specific phages to the target, the elution procedure used in biopanning was changed to the competitive method\[^{16}\] with the target. Four rounds of panning with competitive elution were done to concentrate the specific interactive phage clone with the target. An increasing proportion of interacting phages was observed with each selection, this being confirmed by measuring the yield of eluted phage particles (Table 1).

Forty-eight individual phage clones from the fourth eluate were selected and analyzed by ELISA. Seventeen clones were found to be reactive, and four phage clones (i.e., C1, C3, D1 and F6) showed apparently higher affinity by ELISA (Fig. 3). DNA sequencing of the positive phage clones found the amino acid sequences shown in Table 2. The sequences of these clones were almost all prolinerich, which may confer an important secondary structure. No unique consensus sequences were found in these clones.

**Characterization of the positive phage clones and the synthesized peptides**

The most strongly binding clones were chosen and further investigated by ELISA. Clones C1, C3, D1, and F6 showed the strongest interaction with the H7 flagellin (Fig. 3). These clones were amplified on a large scale and purified by the PEG/NaCl precipitation method. Each purified phage was then analyzed by ELISA. Since anti-M13 antibody was used as a detection tool in ELISA, non-specific phages in high concentrations caused an ELISA signal due to non-specific binding to polystyrene or blocking reagents.\[^{17}\] Thus, we assayed the purified phages at various concentrations of the H7 flagellin to confirm their binding specificity only to the immobilized targets. As shown in Fig. 4, all of the phage clones showed the signal in a concentration-dependent manner, indicating a specific binding avidity of the selected phage clones for the H7 flagellin.

| Table 1. Biopanning against H7 Flagellin of *Escherichia coli* |
|-----------------|-----------------|-----------------|
| Round           | Input Phage (pfu) | Elute Phage (pfu) | Yield |
| First           | $1.5 \times 10^{12}$ | $1.1 \times 10^5$ | $7.3 \times 10^{-8}$ |
| Second          | $6.5 \times 10^{12}$ | $4.6 \times 10^6$ | $7.1 \times 10^{-7}$ |
| Third           | $4.1 \times 10^{12}$ | $6.2 \times 10^7$ | $1.5 \times 10^{-5}$ |
| Fourth          | $4.7 \times 10^{12}$ | $7.5 \times 10^7$ | $1.6 \times 10^{-5}$ |

**Fig. 3.** Screening Results for Clones Selected with the H7 Flagellin.

The absorbance at 405 nm is shown for each clone when examined against an H7 flagellin-coated well. Measurements were taken in duplicate.
Table 2. Deduced Amino Acid Sequences of Peptides Binding H7 Flagellin Selected from a 12-Mers Phage Display Library

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>YALGSNPLRLPW</td>
</tr>
<tr>
<td>D1</td>
<td>LHIHRPTLSIQG</td>
</tr>
<tr>
<td>F1</td>
<td>QDVHLPQSRYT</td>
</tr>
<tr>
<td>D2</td>
<td>HEATQINARLDP</td>
</tr>
<tr>
<td>C3</td>
<td>MLPSPGALRNPD</td>
</tr>
<tr>
<td>D3</td>
<td>SFWTTSSWALR</td>
</tr>
<tr>
<td>E3</td>
<td>LLADTHHRPWT</td>
</tr>
<tr>
<td>A4</td>
<td>SSIRPPFPAPV</td>
</tr>
<tr>
<td>D4</td>
<td>HLTYSFRPHTL</td>
</tr>
<tr>
<td>F4</td>
<td>TPQVFPHSTRL</td>
</tr>
<tr>
<td>A5</td>
<td>WHQTYTSSWES</td>
</tr>
<tr>
<td>B5</td>
<td>VPTLSTVSLQT</td>
</tr>
<tr>
<td>C5</td>
<td>MGFTAPRFPHY</td>
</tr>
<tr>
<td>D5</td>
<td>EGQYKSNLFT</td>
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<td>VMTYPYSRAYH</td>
</tr>
<tr>
<td>C6</td>
<td>MPDILLRNLS</td>
</tr>
<tr>
<td>F6</td>
<td>LISSPRPVYTP</td>
</tr>
</tbody>
</table>

Fig. 4. ELISA Results for the Purified Phage Clones.

After assessing the selected peptide sequences from the ELISA results, we synthesized a peptide which was displayed on the D1 phage clone (Fig. 3). Since we had detected the molecules with ELISA by using the streptavidin conjugate, the peptide was labeled with biotin via a flexible linker (GGGSK). As expected, the peptide showed binding affinity to the purified H7 flagellin in a dose-dependent manner as indicated in Fig. 5a. The effective concentration (EC50) value of the synthetic peptide was calculated to be 1.9 μM. The other peptides probably demonstrated micro-molar affinity.

The inhibition assay (Fig. 5b) showed significantly decreased peptide binding to the target after incubation with the H7 or H23 flagellin. Significant cross-reactivity of the peptide with the H23 flagellin was apparent. Dispensing the synthetic peptide into a solution of the H23 flagellin during incubation decreased the binding to the immobilized H7 flagellin. This is not surprising, considering that the amino acid sequence of the H7 flagellin is very similar to that of H23. The peptide could recognize epitopes of the H7 flagellin in similar regions to those of the H23 flagellin. In comparison, the H1, H5, or H12 flagellin was not confirmed to affect peptide binding to the target. The peptide was found to be more strongly reactive with the H7 flagellin than with the others (H1, H5, H12, and H23) in this assay.

In situ hybridization

We further investigated the binding specificity of the synthetic peptide to the H7 flagellin by in situ hybridization, using E. coli YK4130:H7 cells. As shown in Fig. 6a, a strong Cy3 red signal from the target cells was observed by in situ hybridization of the probe peptide, and a DAPI blue signal was also observed at the same position on E. coli YK4130:H7 (Fig. 6a) by fluorescence microscopy. In the case of E. coli YK4130, however, only the DAPI signal was apparent as shown in Fig. 6b, with no Cy3 red signal. These results clearly indicate that the peptide could recognize the target antigen specifically on the cell surface.

In conclusion, we produced and characterized four peptides directed against the H7 flagellin of E. coli. These unique peptides, whether alone or in combination, could be used for detecting pathogenic E. coli O157:H7 instead of using antibodies. With some assay systems, the sensitivity and avidity of peptides are not sufficient to generate a signal derived from the recognition of a target. However, peptides make it easier to improve the affinity for a target than antibodies. Multivalent forms of a peptide have been successfully used to improve the affinity for a target. Furthermore, most linear peptides, in comparison with antibodies, do not require special conditions for storage such as refrigeration. These peptides can maintain their activity when exposed for a long period to an elevated temperature and have the advantage over conventional antibodies that they can be stored, shipped, and used under extreme field conditions. The results of this study demonstrate that the phage-display technique can be used to identify peptides as the lead to a bacterial detection tool.
Fig. 5. H7 Flagellin-binding Characteristics of the Synthetic Peptide.

ELISA was used for all of the assays. (a) Serial dilutions of the synthetic peptide were put on to the immobilized H7 flagellin. (b) Inhibition of the H7 flagellin binding to the synthetic peptide was examined by incubating the peptide first with one of the flagellins (H1, H5, H7, H12, or H23), and then dispensing the solution into an ELISA plate coated with the H7 flagellin. Each value is the mean of triplicate wells.

Fig. 6. Micro-photographs of E. coli Strains YK4130:H7 (a) and YK4130 (b) with the Cy3-Labeled D1 Peptide (red signal) and DAPI-Stained (blue signal).

Bars, 5 μm.

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References


