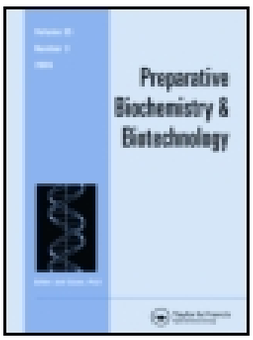

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Rapid label-free detection of *E. coli* using a novel SPR biosensor containing a fragment of tail protein from phage lambda

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ABSTRACT

In efforts to speed up the assessment of microorganisms, researchers have sought to use bacteriophages as a biosensing tool, due to their host-specificity, wide abundance, and safety. However, the lytic cycle of the phage has limited its efficacy as a biosensor. Here, we cloned a fragment of tail protein J from phage lambda and characterized its binding with the host, *E. coli* K-12, and other microorganism. The N-terminus of J was fused with a His-tag (6HN-J), overexpressed, purified, and characterized using anti-His monoclonal antibodies. The purified protein demonstrated a size of ~38 kDa upon SDS-PAGE and bound with the anti-His monoclonal antibodies. ELISA, dot blot, and TEM data revealed that it specifically bound to *E. coli* K-12, but not to *Pseudomonas aeruginosa*. The observed protein binding occurred over a concentration range of 0.01–5 μg/ml and was found to inhibit the *in vivo* adsorption of phage to host cells. This specific binding was exploited by surface plasmon resonance (SPR) to generate a novel 6HN-J-functionalized SPR biosensor. This biosensor showed rapid label-free detection of *E. coli* K-12 in the range of 2×10^4 -2×10^9 CFU/ml, and exhibited a lower detection limit of 2×10^4 CFU/ml.

KEYWORDS

Bacteriophage lambda; *E. coli* K-12; SPR; tail protein

Introduction

The sensitive, selective, and rapid monitoring of pathogenic microorganisms are vital for food and water safety, bioterrorism prevention, and public health. The conventional methods for detecting microorganisms (e.g., those based on culture and colony counting, immunology, and polymerase chain reaction) are reliable but tend to be time-consuming (24–52 hr), and labor intensive, and cost ineffective.[1,2] Biosensors have shown tremendous potential to overcome these limitations, as they offer numerous benefits, including high degrees of sensitivity and specificity, rapidity, the potential for miniaturization, and portability for on-site detection.[3,4] Numerous biosensors have been reported for the detection of various environmental pollutants,[3,5,6] including microorganisms.[4,7] A biosensor typically consists of a biological sensing element that is capable of recognizing a target molecule (analyte) in the environment, and a transducer that transmits a signal when the sensing element binds its specific target molecule; such signals can be optical,[8] electrochemical,[9,10] flow cytometric,[11] mass perturbance-based,[12] mechanical resonator-based,[13] or SPR.[14,15] Different biosensing elements have been exploited to improve the specificity, sensitivity, and rapidity of biosensors for pathogen detection, including antibodies,[12] DNA,[16] RNA,[17] aptamers,[18] peptides,[19,20] and carbohydrates.[21] However, these biosensing elements are relatively unstable under environmental conditions, limiting their long-term storage and field use. Moreover, they cannot differentiate between pathogenic and non-pathogenic organisms with sufficiently high sensitivity and selectivity, and their production remains time-consuming and costly.[15]

The bacteriophage has gained interest as a unique biosensing element for the development of pathogen-detecting platforms, due to its abundance, reliability, fast binding, and stability.[4] Phages infect their specific host bacteria through the binding of their capsid or tail proteins to specific receptors on the surface of a host bacterium. The recognition and binding of a phage is highly specific, and thus may be used for bacterial typing.[22] This specificity and selectivity makes phages excellent candidates for use as biosensing elements in biosensors. Phages also have the benefit of resisting adverse conditions, including those related to temperature, pH, and ionic strength. The incorrect orientation of an immobilized phage on the surface of a biosensor can lower the efficiency of cell capture,[23] meaning that the proper orientation of the phage on the sensor platform is important for biosensor development. Several microorganisms, including *E. coli*,[15,24–27] *S. aureus*,[14,15] and *B. anthracis* spores,[28] have been detected by exploiting intact phages as recognition elements with different sensing platforms. However, intact phage-based detection has some disadvantages, such as the lysis of captured bacteria by intact phages, which decreases

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the biosensor signal, and the drying effect of long-term exposure, which decreases the binding capacity.[29] Some intact phages show enzymatic activity toward their host bacterial surface receptor, and thus yield inconsistent signals on a biosensor platform. Moreover, intact phages are relatively large for use in distance-dependent sensing platforms, such as those based on SPR.[4] The use of phage proteins involved in phage-bacterium binding has been proposed as a means to resolve these drawbacks. A few attempts have been made to utilize the receptor-binding proteins of phages for bacterial detection.[22,29] For example, cysteine-tagged P22 phage receptor binding proteins were used to detect *S. typhimurium*,[29] and a glutathione-S-transferase fusion protein from the phage, NCTC 12673, was used to detect *C. jejuni*.[30,31]

Genetic analyses of tail protein J from phage lambda[32] have suggested that the C-terminal portion of the 1132-amino-acid protein might be sufficient for binding to LamB, the surface receptor of its target, *E. coli* K-12.[33] LamB is a nonspecific channel in the outer membrane, allowing the diffusion of small molecules, such as maltose and maltodextrins. Wang et al. constructed a fusion protein in which the C-terminal portion of the J protein was fused to maltose-binding protein (MBP), and confirmed its ability to bind LamB. However, it was shown that the binding of the fusion protein to LamB only partially reduced the uptake of maltose, and that one-third of LamB molecules did not bind to the fusion protein,[33] suggesting that the presence of the large MPB (~42 kDa) in the fusion protein might cause steric hindrance.

In an effort to overcome this issue and also to develop an SPR biosensor, a His-tag was used here to produce a fusion protein of C-terminal fragment of J. This strategy was based on the assumption that a small ligand might have less steric hindrance effects and increase the SPR response to bacterial attachment, as described in a previous report.[15] The C-terminal portion of J protein was cloned and a $(His)_6$-tag at its N-terminus (6HN-J) was constructed. The purified 6HN-J protein was examined for its binding specificity to *E. coli* by various approaches, such as ELISA, dot blot, TEM, and an *in vivo* adsorption assay. Following its validation, the fusion protein was exploited in an SPR biosensor.

Experimental

Chemicals

The media components were purchased from Difco (Detroit, MI) . The viral DNA isolation kit was purchased from Macherey-Nagel (Düren, Germany). The gel extraction and plasmid isolation kits were purchased from Qiagen (Hilden, Germany). *Sal*I, *Hind*III, Taq polymerase, and T4-DNA ligase were purchased from TaKaRa (Shiga, Japan). The pET Express & Purify kits (In-Fusion Ready) were purchased from Clontech (Mountain View, CA). The horseradish peroxidase (HRP)-conjugated anti-His monoclonal antibody was purchased from Clontech (Mountain View, CA). The IgG (H + L) anti-rabbit immunoglobulin-gold (12 nm) conjugate was purchased from Jackson Immuno Research Lab (West Grove, PA). The ultrasensitive HRP Substrate for Western Blot was purchased from TaKaRa (Shiga, Japan). The TMB peroxidase EIA substrate kit for ELISA was purchased from Bio-Rad (Hercules, CA). The Series S Sensor Chip nitrilotriacetic acid (NTA), His-Capture Kit, and HBS-P + running buffer were purchased from BIAcore (GE Healthcare, Uppsala, Sweden). All other reagents were of analytical grade and were used as received without further purification.

Bacteria and culture conditions

Escherichia coli DH5α (*hsdR*, *recA*, *thi-1*, *relA1*, and *gyrA*96) was used to maintain plasmids. *E. coli* K-12 *and Pseudomonas aeruginosa* ATCC 27853 were obtained from the KCTC (Korean Collection for Type Cultures) and grown in tryptic soy broth at 37 °C. *E. coli* BL21 (DE3) (*hsdS*, *gal*, *ëclts*857, *ind*1, *sam*7, *nin*5, and *lac*UV5-T7gene1) was used for plasmid maintenance and expression of the fusion protein. *E. coli* DH5α and BL21 (DE3) cells harboring plasmids (p6HN-J) were cultured at 37 °C in TYS media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 30 μg/ml ampicillin. The numbers of plaque forming units (PFUs) were determined by serially diluting phage samples and using the soft LB overlay method to plate them with *E. coli* K-12 host cells on LB agar.

Construction of plasmid p6HN-J

The phage lambda stock was prepared by CsCl gradient centrifugation using standard methods.[34] Phage lambda DNA was isolated with a commercially available kit, and PCR amplified with primers designed to introduce *Sac*I and *Hind*III sites at each end (forward 5'-AAG GCC TCT GTC GAC GAT TAT TAC TTT TAT ATC CGC-3', reverse 5'-AGA ATT CGC AAG CTT TCA GAC CAC GCT GAT GCC CAG-3'). The resulting PCR products were gel isolated, cloned into the N In-Fusion Ready vector (pET6XHN-N) using an In-Fusion Ready cloning kit, and transformed into *E. coli* DH5α. The desired plasmid (p6HN-J) was identified by colony PCR and restriction analysis of miniprepped DNA, and transformed into *E. coli* BL21 (DE3) by the $CaCl_2$ method. Cloning, ligation, and transformation were carried out using standard methods.[34]

Protein purification

E. coli BL21 (DE3) cells harboring p6HN-J were incubated with isopropyl-β-D-thiogalactopyranoside (IPTG) to trigger overexpression of the fusion protein, which was purified using a HisTALON purification kit according to the manufacturer's recommendations. Briefly, an overnight cell culture was sub-cultured in 500 ml LB-ampicillin, grown to $OD_{600\,nm} = 0.8$, and induced with 1 mM IPTG for 4 hr. The induced cells were harvested by centrifugation at 10,000 × *g* for 20 min, and the cell pellets were frozen at −20 °C. The frozen cells were suspended in a 1/10 volume (relative to the initial culture volume) of the provided 1X equilibration buffer (50 mM Na_3PO_4, 300 mM NaCl, 20 mM imidazole;

pH 7.4) and sonicated on ice, three times for 10 s each with a 30-s pause between each burst. The cell extract was centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was carefully transferred to a clean tube for SDS-PAGE analysis. The pellet was suspended with 10 ml equilibration buffer, sonicated on ice (for 15 min with 30 s interval at 60% strength; Branson digital sonifier 450; Danbury, CT), and centrifuged at 10,000 × *g* for 20 min at 4 °C. The pellet was suspended with 5 ml Tractor buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris–HCl; pH 7.4) containing 6 M urea, incubated in ice for 1 hr, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was diluted with 10 ml Tractor buffer to yield a final concentration of 2 M urea, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was applied to a HisTALON cartridge (cat. No. 635650, Clontech; Mountain View, CA) that had been previously equilibrated with 10 ml equilibration buffer. The column was then washed with 10 ml wash buffer (50 mM Na_3PO_4, 300 mM NaCl, 40 mM imidazole; pH 7.4) and eluted with 5 ml elution buffer (50 mM Na_3PO_4, 300 mM NaCl, 300 mM imidazole; pH 7.4). One ml of the eluted fraction was collected, and the protein amount was quantified.

Western and dot blot analyses

For Western blot analysis, pellet and purified protein fractions were resolved by SDS-PAGE and transferred to nitrocellulose membranes using standard techniques.[34] Each membrane was incubated with 20 ml blocking buffer (5% nonfat dry milk in 0.2% Tween-20/PBS) for 1 hr at room temperature and then with a HRP-conjugated anti-His monoclonal antibody (diluted 1:1000–20,000 in blocking buffer) for 1 hr at room temperature with shaking. The membrane was washed three times with washing buffer (0.2% Tween-20 in PBS) for 10 min each wash, incubated with a chemiluminescent HRP substrate for 5 min with shaking, and exposed to an X-ray film. For dot blot analysis, overnight-cultured (approximately ~10^9 cells/ml) *E. coli* K-12 and *P. aeruginosa* cells were heat killed in a 100 °C water bath for 15 min, serially diluted (10°, 10^{-1}, and 10^{-2}), spotted on a nitrocellulose membrane (2 µl per spot), blocked with blocking buffer, and incubated with 6HN-J or 1% BSA in PBS (control) for 1 hr at 37 °C with shaking. The membrane was then washed, incubated with HRP-conjugated anti-His monoclonal antibody (diluted 1:1000–20,000 in blocking buffer), and developed as described above for the Western blot analysis.

ELISA

A microtitration plate was coated with 30 µl/well of *E. coli* K-12, *P. aeruginosa* cells (approximately 5 × 10^9 cells/ml), or BSA (1 mg/ml) with 100 µl/well coating buffer (100 mM carbonate buffer, pH 9.6) for 2 hr. The immobilized cells were then incubated with 6HN-J proteins (0.01–300 µg/ml) for 1 hr at 37 °C in an orbital shaker, blocked with 200 µl/well blocking buffer (1% BSA in PBS) for 2 hr at 37 °C, washed three times for 3 min with 200 µl/well washing buffer (0.05% Tween-20 in PBS) per wash, and incubated with 100 µl/well HRP-conjugated anti-His monoclonal antibody (typically diluted 1:1000–5000 in blocking buffer) for 1 hr at room temperature with shaking. The cells were washed five times for 3 min each and exposed to 100 µl/well TMB peroxidase substrate solution for 10 min (until color development), and the results were read at $OD_{655\,nm}$.

In vivo *inhibition of phage lambda*

Overnight-culture cells (approximately 5 × 10^9 cells/ml) were centrifuged, suspended in 700–800 µl of 10 mM $MgSO_4$, and aliquoted (100 µl) to e-tubes. The cells were incubated with purified 6HN-J (0.01, 0.1, 1, 10, or 25 µg ml^{-1}) for 1 hr at 37 °C, and then with phage lambda (diluted to ~500 PFU with SM + gelatin solution) for 20 min. Cells were centrifuged at 10,000 × *g* for 5 min. Supernatants containing unbound phages were transferred to clean Falcon tubes, incubated with 100 µl cells for 20 min at 37 °C, and plated on LB agar using the soft LB overlay method. Pellets containing host cells with bound phages were suspended with 100 µl of 10 mM $MgSO_4$ and plated on LB agar using the soft LB overlay method. The plates were incubated at 12–16 hr at 37 °C, and the numbers of plaques were counted.

TEM

Overnight-culture cells were centrifuged, suspended with 1 ml PBS (approximately 5 × 10^9 cells/ml), and one drop (20–50 µl) of the suspended cells were placed on Parafilm. A Formvar-coated copper grid was placed on the drop. The assemblage was incubated for 2 min, and then sequentially exposed to PBS-4% paraformaldehyde solution for 5 min, PBS-50 mM NH_4Cl solution for 5 min, PBS-1% BSA-1% normal goat serum solution for 5 min, purified 6HN-J solution for 20–30 min, and anti-His antibody (1:100) for 30 min. The grid was rinsed 5 times with 1X PBS and incubated with IgG (H + L) anti-rabbit immunoglobulin-gold (12 nm) conjugate for 10 min. The grid was washed several times with PBS, dried, and observed by TEM (Hitachi H-7600).

Surface plasmon resonance (SPR)

The binding kinetics of 6HN-J and microorganisms were studied using a BIAcore T200 instrument (GE Healthcare, Little Chalfont, UK). 6HN-J was diluted in running buffer (HBS-P+; 0.01 M HEPES, 0.15 M NaCl, 0.05% surfactant P20, pH 7.4) and immobilized on a Series S Sensor Chip NTA using a standard ligand-capture method (Ni/His-tag immobilization) with a His Capture Kit. The four cells of each chip were treated separately during the immobilization procedure. Flow cell 1 was spared the attachment of Ni^{2+} and 6HN-J, and was used as blank reference surface; meanwhile, flow cells 2–4 were coupled with Ni^{2+} and His-tagged 6HN-J. The NTA chip was primed with washing buffer (HBS-P + with 3 mM EDTA) and running buffer. The NTA chip was then implemented by a continuous flow of

regeneration buffer (350 mM EDTA) for 1 min, running buffer for 1 min, 0.5 mM $NiCl_2$ for 1 min, washing buffer for 1 min, ligand sample (6HN-J; 1:200 diluted with HBS-P + buffer) for 1 min, and different numbers of microorganisms (live cells or cells that had been heat-killed in boiling water for 15 min) for 20 min (Supplementary data). Serial dilutions of the microorganism were prepared in running buffer and flowed through cells 1–4. Binding analysis was conducted at a flow rate of 5 µl/min at 25 °C throughout the experiment, with the exception of the washing step (50 µl/min). The sensor surface was regenerated for 1 min using 350 mM EDTA. In each run, the association phase and subsequent dissociation phase were monitored for 20 and 10 min, respectively (Supplementary data). The binding sensorgrams of microorganisms were estimated from the obtained reference-subtracted sensorgrams (e.g., the sensorgrams of flow cells 2–4 minus that of flow cell 1) *via* a steady-state affinity model using the Biacore T200 evaluation software version 3.0 (GE Healthcare Companies, Little Chalfont, UK).

Results and discussion

Overexpression, purification, and Western blot of 6HN-J

Phage lambda has advantages and disadvantages over other sensing elements.[4] However, although its host specificity could be an interesting element for biosensor construction, its lytic cycle could be a limitation in this context. To resolve this, we considered using recombinant tail protein J instead of intact phage as a sensing element for SPR biosensor construction. A construct encoding the distal portion (residues 784–1132) of the J, which was inferred to interact with surface membrane protein LamB of *E. coli* K-12,[33] was PCR amplified to yield a product of more than ~1000 base pairs (data not shown). The purified PCR product was then fused with a $(His)_6$-tag by insertion into a linearized vector (pET6XHN-N) to generate p6HN-J, in which the coding sequence was under the control of the IPTG-inducible T7*lac* promoter. The vector (p6HC-J) encoding the C-terminally tagged protein fragment was not overexpressed under IPTG (data not shown). This indicated that the N-terminal His-tag had a better orientation for the 6HN-J compared to the C-terminal His-tag. This is in agreement with a previous report that N-terminally Cys-tagged proteins captured bacteria more efficiently than C-terminally Cys-tagged proteins due to preferential orientations.[29] The 6HN-J protein was found to be overproduced as an insoluble pellet fraction, as assessed by SDS-PAGE (lane 5 of Fig. 1A). The insoluble pellet was solubilized in 6 M urea, refolded by dilution with equilibration buffer, and purified on an affinity column. The purified 6HN-J had an apparent size of ~38 kDa on SDS-PAGE (Fig. 1A), which was consistent with the predicted size. Thus, the relatively small 6HN-J could be used in an SPR-based biosensor, where detection is distance-dependent.

The identity of purified 6HN-J was confirmed by Western blotting with an anti-His monoclonal antibody (Fig. 1B). The anti-His monoclonal antibody clearly bound with the overexpressed protein band in the IPTG-induced

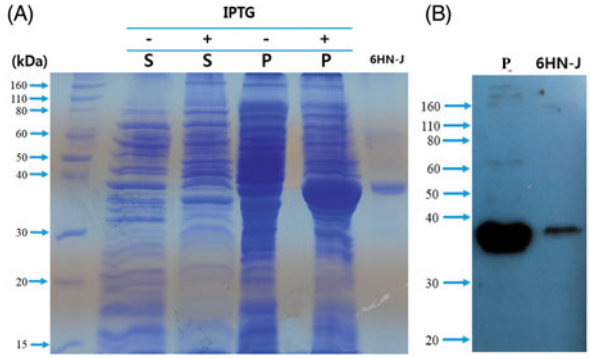

Figure 1. SDS-PAGE (A) and Western blot (B) of the 6HN-J. (A) *E. coli* cells harboring p6HN-J were induced in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 1 mM IPTG. The cells were lysed and centrifuged into pellet (P) and soluble (S) fractions. The purified 6HN-J protein (lane 6) had an apparent molecular weight of ~38 kDa on 12% SDS-PAGE. Lane 1, molecular size markers. (B) The resolved proteins were electro-transferred to a nitrocellulose membrane and incubated with an HRP-conjugated anti-His monoclonal antibody (1:4000). Lane 1: pellet fraction with IPTG; lane 2: the purified 6HN-J.

pellet fraction (Fig. 1B, lane 1) and the purified 6HN-J protein fraction (Fig. 1B, lane 2). The results indicate that the overexpressed and purified protein bands corresponded to the expected His-tagged fusion proteins.

ELISA and dot blot analyses

The ability of the purified 6HN-J to bind *E. coli* K-12 and other microbe was quantified by ELISA (Fig. 2A). As shown in Figure 2, the purified 6HN-J was found to bind tightly to *E. coli* K-12 cells; this binding was observed at concentrations of 0.01–0.5 µg/ml and plateaued or decreased thereafter. In contrast, there was no detectable specific binding of the purified 6HN-J with *P. aeruginosa* or BSA (Fig. 2A).

Next, we used dot blotting to further assess whether the purified 6HN-J protein could bind to the surfaces of *E. coli* K-12 and/or *P. aeruginosa* (Fig. 2B). The anti-His monoclonal antibody reacted strongly with the spots of *E. coli* K-12 pretreated with the purified 6HN-J protein, whereas negligible binding was observed with the BSA-pretreated spots (Fig. 2B). There was no specific binding of the anti-His monoclonal antibodies with spots corresponding to *P. aeruginosa*, regardless of whether the membrane had been pretreated with 6HN-J or BSA. Thus, the BSA-protected spots did not show any significant binding to host or non-host cells, whereas the purified 6HN-J protein bound to *E. coli* K-12 but not to *P. aeruginosa* (Fig. 2B). These results indicate that the 6HN-J protein exhibits excellent specificity for *E. coli* K-12 cells.

TEM

The binding of the 6HN-J to the surface of cells was visualized with TEM. Consistent with the results of ELISA and dot blot, binding of anti-rabbit immunoglobulin-gold was observed in *E. coli* K-12 cells pretreated with the purified 6HN-J protein (Fig. 3A), but not non-pretreated cells (Fig. 3C). This result is in agreement with a previous report[32] showing that the anti-MBP serum reacted only with

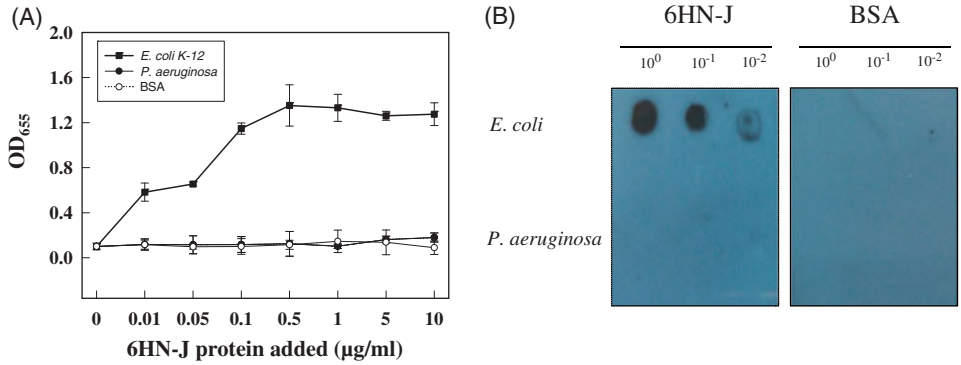

Figure 2. ELISA (A) and dot blot (B) analyses. (A) Overnight cultures of *E. coli* K-12, *P. aeruginosa,* and BSA were used to coat the wells of microtitration plate (30 μl; ~5×10^9 cells/ml) for 2 hr at 37 °C, and incubated with the indicated amounts of purified 6HN-J for 1 hr at 37 °C. The cells were incubated with the HRP-conjugated anti-His monoclonal antibody (1:1000), exposed to HRP substrate, and examined at OD_{655}. (B) A nitrocellulose membrane was dotted with heat-killed *E. coli* K-12 and *P. aeruginosa* (2, 2×10^{-1}, and 2×10^{-2} μl), incubated with purified 6HN-J protein (final concentration, 5 μg/ml) or 1% BSA in PBS (control), and treated with HRP-conjugated anti-His monoclonal antibody (1:2500).

spots corresponding to LamB and MBPwt on a sheet preincubated with MBP-J fusion protein. In this work, we did not observe any specific binding with *P. aeruginosa* (Fig. 3B).

In vivo *inhibition of phage lambda adsorption*

We next tested how the binding of the 6HN-J to intact *E. coli* K-12 cells affected the *in vivo* adsorption of phage lambda onto these cells (Fig. 4). As shown in Figure 4, the *in vivo* adsorption of phage lambda was dose-dependently inhibited by the 6HN-J (open circles), with ~50% inhibition seen in the presence of 1 μg ml^{-1} of 6HN-J and almost complete inhibition seen in the presence of 25 μg ml^{-1} of the protein. Conversely, the numbers of unbound phage (those in the supernatant) increased with the amount of 6HN-J (closed circles). This inhibitory effect may reflect that the 6HN-J competed with phage lambda for the same binding site at the surface receptor of *E. coli* K-12. Our findings could be supported by the previous report that phage lambda competed with the purified MBP-J fusion protein for the same binding site on LamB.[33] However, we found that the dose-dependent inhibition of phage adsorption was concentration-dependently reversed with a higher concentration (over ~50 μg ml^{-1}) of 6HN-J (data not shown). This may reflect that protein–protein interactions at a higher concentration cause steric crowding and block the bacterial adhesion sites on 6HN-J, as described in a previous report.[14]

SPR

The binding kinetics of 6HN-J with microbes were assessed using an SPR instrument (BIAcore T200) with a Series S Sensor Chip NTA. The N-terminal histidine of 6HN-J bound to the Ni^{2+} on flow cells 2–4 of the NTA chip surface such that the C-terminal portion of 6HN-J was appropriately oriented to capture bacteria. Flow cell 1, which was not coupled with Ni^{2+} or 6HN-J, was used as blank reference; this enabled us to subtract the nonspecific bindings of microbes from the obtained sensorgrams using the Biacore T200 evaluation software version 3.0 (GE Healthcare Companies, Little Chalfont, UK). The responses were higher

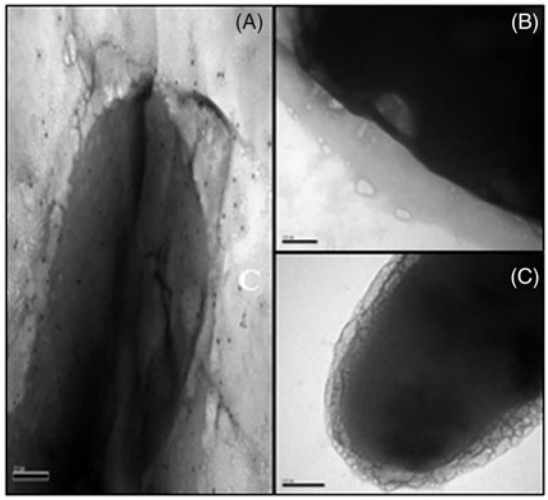

Figure 3. Electron microscopy study of 6HN-J binding. A Formvar-coated copper grid was placed on a drop of suspended culture cells (~ 5×10^9 cells/ml). The assemblage was then sequentially treated as described in the Experimental section. The grid was incubated with IgG (H + L) anti-rabbit immunoglobulin-gold (12 nm) conjugate. *E. coli* K-12 (A), *P. aeruginosa* (B), and blank *E. coli* K-12 cells (C) are shown.

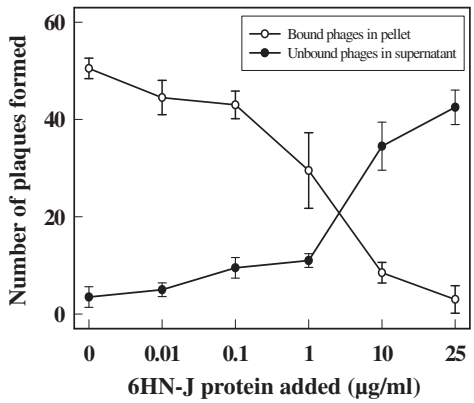

Figure 4. *In vivo* inhibition of phage lambda binding. *E. coli* K-12 cells were incubated with the indicated amounts of purified 6HN-J (0.01, 0.1, 1, 10, and 25 μg/ml) for 1 hr at 37 °C, exposed to wild-type phage (~500 PFU) for 20 min at 37 °C, and then centrifuged. Pellets containing cells with bound phages were suspended and plated using the soft LB overlay method (open circles). Supernatants containing unbound phages were incubated with *E. coli* K-12 and plated using the soft LB overlay method (closed circles).

when ~5 μg/ml 6HN-J was coupled on the sensor surface (1:200) compared to ~10 μg/ml of 6HN-J (1:100) (Fig. 5). Therefore, we used 1:200 diluted 6HN-J (60 s flow), which yielded a better binding sensorgram, for all subsequent binding assays. Heat-killed microbes were used for the binding assays because although the sensorgrams of live and heat-killed microbes were very similar, the former showed slight oscillations (data not shown). The binding sensorgrams of 6HN-J with the microorganisms were estimated from the obtained reference-subtracted sensorgrams. Typical binding kinetics (association and dissociation) were obtained with *E. coli* cells in the range of $2 \times 10^4 - 2 \times 10^9$ CFU/ml (Fig. 6A). The binding of *E. coli* cells increased up to ~300 s and plateaued until dissociation occurred after 1200 s when the regeneration buffer was applied. Using S/N > 3, we found that the lower limit of detection was $\sim 2 \times 10^4$ CFU/ml, which is similar to that of a previous report in which the detection limit of *S. aureus* was found to be 10^4 CFU/ml with the lytic phage SPR-based SPREETA™ sensor (Texas Instruments company, Dallas, TX).[14] The detection limit of *Campylobacter jejuni* using the phage NCTC 12673 receptor binding protein-derivatized SPR was 10^2 CFU/ml,[31] while that of *Salmonella typhimurium* using the P22 phage tail spike protein-immobilized SPR showed 10^3 CFU/ml.[29] *E. coli* was also detected by other methods: RT-PCR coupled to fluorescence (detection limit of 10^2 CFU/ml), fiber optic immunosensor (2.9×10^3 CFU/ml), QCM immunosensor (10^3 CFU/ml), and impedimetric immunosensor (10^4 CFU/ml).[2] Thus, the sensitivity of the biosensor developed here for *E. coli* detection is in a range similar to those of various detection approaches using SPR or other methods. The SPR response representing *E. coli* binding was found to be dose-dependent: the calibration plot (regression curve; $y = 6x + 3.3$; $R^2 = 0.96$) showed good linearity for *E. coli* cell numbers from 2×10^6 to 2×10^8 CFU/ml (Fig. 6B). However, short physical attachment with *P. aeruginosa* was observed. Sensorgrams for *P. aeruginosa* demonstrated that these cells appeared to attach up for to 300 s and then spontaneously detach without reaching a steady binding plateau (Fig. 6C), indicating that the bindings were nonspecific. Based on the SPR data, we speculate that the phage tail protein might randomly and transiently (perhaps for 300 s) attach to a cell surface in search of a specific receptor, and then either bind steadily to the receptor to initiate phage translocation (e.g., *E. coli*) or, in the absence of the receptor, detach from the cell surface (e.g., *P. aeruginosa*). Our results are consistent with a previous report suggesting that phage will first rapidly recognize and adhere to the cell surface in a reversible fashion, followed by the slower irreversible binding of a secondary minor tail protein to another receptor molecule on the surface of the bacterium.[35] Silva group also reported that the first step of bacteriophage adsorption involves random collisions between phage and bacteria by Brownian motion, dispersion, diffusion, or flow as the phage searches for a specific receptor. In this reversible step, binding to bacterial surface components is not definitive and the phage can detach from the bacterial surface. The specific connection between bacterial receptor and phage-binding

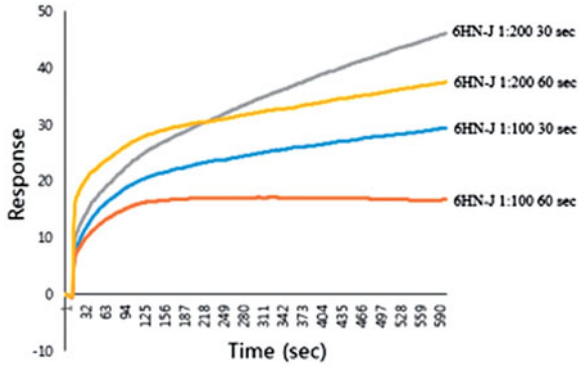

Figure 5. SPR responses of 6HN-J attachment. Purified 6HN-J (1 mg/ml) was diluted (1:100 or 1:200 with HBS-P + buffer) and flowed for 30 or 60 s over the sensor surface of the NTA-Ni^{2+} chip at a flow rate of 5 μl/min. Then, equal numbers (10^8 CFU/ml) of *E. coli* cells were flowed for 10 min.

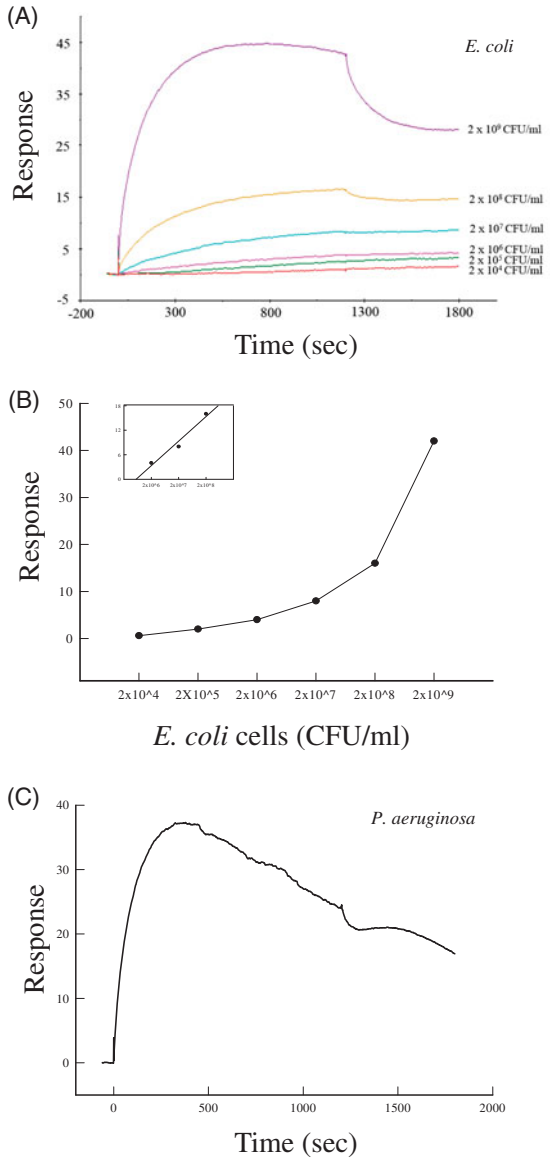

Figure 6. Detection of microorganisms using the SPR biosensor. (A) 6HN-J (1:200) was attached to the NTA-Ni^{2+} chip for 1 min, and different amounts ($2 \times 10^4 - 2 \times 10^9$ CFU/ml) of *E. coli* cells were flowed over the sensor surface for 20 min at a flow rate of 5 μl/min. (B) A dose-dependent response was observed. A calibration plot (regression curve; $y = 6x + 3.3$; $R^2 = 0.96$) for *E. coli* binding shows good linearity from 2×10^6 to 2×10^8 CFU/ml. (C) *P. aeruginosa* (3×10^9 CFU/ml) cells were flowed for 20 min under the same conditions.

domains triggers conformational rearrangements in other phage molecules resulting in irreversible bindings.[36]

Further experiments are underway to enhance the sensitivity of our SPR strategy by using nanoparticles as a signal enhancer in the sandwich format for the direct detection of bacteria in real-world samples.

Conclusions

The use of intact phages as biosensors has been limited by problems with host cell lysis, which decreases the signal. Here, we overcame these limitations by using a fragment of tail protein J from phage lambda as a sensing element for the SPR-based detection of *E. coli* K-12. The N-terminus of tail protein J fragment was fused with a $(His)_6$-tag. This fusion protein (~38 kDa) showed specific binding to *E. coli* K-12 but not to *P. aeruginosa* by ELISA, dot blot, TEM, and *in vivo* inhibition assay. The binding of N-terminal $(His)_6$ to Ni^{2+} enabled the fusion protein to be bound to the NTA chip in an orientation that allowed the C-terminal portion of 6HN-J to capture bacteria. We observed rapid detection of (within 20 min) and typical binding kinetics with *E. coli* K-12 in the range of $2 \times 10^4 - 2 \times 10^9$ CFU/ml, with a lower detection limit of 2×10^4 CFU/ml. In contrast, we observed only nonspecific bindings with *P. aeruginosa*. We thus herein report a novel SPR biosensor harboring a fragment of phage tail protein, show that it yields rapid and label-free detection of bacteria, and propose that it could be expanded for real-time monitoring of target microorganisms in the environment.

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