Investigation of *Listeria* Phage Endolysin Cell-wall Binding Domain (CBD) Surface Display in *Escherichia coli*

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Abstract. Cell surface display of target proteins has been widely used in biotechnology and industry. In this study, cell-wall binding domain (CBD) from *Listeria* phage endolysin A500 was fused with anchoring domains of YadA and OmpA, respectively, in *Escherichia coli*, aiming at binding of *E. coli* cells to *Listeria* cells. The fusion proteins were expressed after induction and their surface localizations were verified by Western blot. CBD-YadA fusion was displayed on the cell surface, however, was toxic to *E. coli*. OmpA-CBD fusion was translocated to the outer layer of the cell membrane but with compromised availability on the cell surface. Therefore, functional surface display of CBD in *E. coli* requires another anchor for fusion strategy.

Introduction

Listeria monocytogenes is a widely spread foodborne pathogen causing listeriosis with mortality rate of 30%. Contaminated foods contain dairy products, minimally processed vegetables, and ready-to-eat meat products. *Listeria* phages have been demonstrated as promising candidates in detection, differentiation and biocontrol of *Listeria* during food processing and storage. Constructed phages with reporter genes such as *luxAB* and *celB* have been tested with high sensitivity [1, 2]. Especially the approval of several *Listeria* phage commercial agents for food safety purpose promotes research in phage application [3]. Endolysins, the potential candidates for antimicrobials, are bacteriophage-encoded murein hydrolases which can degrade host cell and release phage progeny. The modular structure of *Listeria* phage endolysins consists of two distinct parts: N-terminal enzymatically active domain (EAD) and C-terminal cell-wall binding domain (CBD) [4]. The EAD part determines the catalytic activity and the CBD part has high binding specificity to *Listeria* cell wall.

Cell surface display is a rapidly expanded technique in molecular biology and used in whole-cell bioremediation, high-throughput screening and vaccine development. In Gram-negative bacteria, different strategies for displaying proteins have been explored by using carrier proteins such as OmpA, LamB and OprF [5]. Lpp-OmpA is composed of the signal sequence, first nine N-terminal amino acids of *E. coli* lipoprotein (Lpp)

and 46-159 amino acids of the OmpA. Proteins for instance, hemagglutinin and cyclodextrin glucanotransferase with size ranging from 27 to 74 amino acids have been expressed in this way for biocatalysis and antibody library screening [6]. YadA, originated from *Yersinia*, is a non-fimbrial adhesin mediating adherence to hosts. Constructed chimeric YadA proteins can translocate the YadA passenger domain across the outer membrane in *Yersinia enterocolitica*, which provides a clue for potential utilization of YadA anchor domain in cell-surface display [7].

In this study, we aimed at constructing an *E. coli* strain which would have the ability to bind to pathogenic *L. monocytogenes* and kill pathogens by introducing anti-listerial peptide (such as bacteriocin) gene. To achieve it, we constructed *L. monocytogenes* phage endolysins CBD domain surface display systems using anchoring domain YadA and Lpp-OmpA, respectively. The fusion proteins were tested for their expression, location and display on the cell surface.

Materials and Methods

Bacteria, Plasmids and Culture Conditions

The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* host strains were cultured on Luria-Bertani (LB) medium at 37 ℃. Kanamycin (50 μg/ml) and ampicillin (100 μg/ml) were added when appropriate. The expression of CBD-YadA was induced with anhydrotetracycline (1-200 ng/ml), and the expression of OmpA-CBD was induced with 0.02 mM isopropyl-β-D-thiogalactopyranoside (IPTG). *L. monocytogenes* WSLC 1018 (gift from Prof. Martin Loessner, ETH Zürich, Switzerland) was cultivated at 30 ℃ in BHI medium (Oxoid).

Table 1. *E. coli* strains and plasmids

Strains and plasmids	Description
E. coli strains	
DH5α	Transformation host [8]
BL21(DE3)	Transformation host (Invitrogen)
TG1	Transformation host [9]
ECO615	TG1/pLEB588 [10]
ECO759	TG1/pLEB733
ECO760	TG1/pASG-IBA4
ECO762	TG1/pLEB735
ECO766	BL21(DE3)/pET-LOAvi
ECO767	BL21(DE3)/pLEB737
Plasmids	
pHPL500	Vector containing *CBD500* gene [11]
pET-LOAvi	Cell surface display vector containing lpp-ompA sequence [12]
pASG-IBA4	Vector for CBD-YadA surface display (IBA GmbH)
pLEB588	pHELIX2 + *pepR* promoter of *Lactobacillus rhamnosus* [10]
pLEB733	SS_{usp45}-*papA* in pLEB588
pLEB735	*CBD-yadA* in pASG-IBA4
pLEB736	P_{pepR}-SS_{usp45}-*papA* in pLEB735
pLEB737	Vector for OmpA-CBD surface display, derivative of pET-LOAvi

Construction of CBD500 Plasmids

CBD500-YadA fusion was constructed by overlap-PCR with primers CBD500F, CBD500R, yadAF and yadAR (all primer sequences are listed in Table 2). Pediocin gene *papA* from *Lactobacillus plantarum* WHE 92 was amplified by PCR with primers PedF and PedR. The signal sequence of *usp45* was amplified by PCR with primers uspF and uspR. The SS_{usp45} and *papA* fragments were cut with *Nae*I and *Dra*I, ligated, and the ligation mixture was used as a template to amplify SS_{usp45}-*papA* fusion with primers uspF and PedR. The P_{pepR}-SS_{usp45}-*papA* cassette was amplified from pLEB733 with primers pepRF and PedR2. Primers CBD500F2 and CBD500R2 were used to construct OmpA-CBD500 fusion. Plasmids, verified by restriction enzyme digestion and DNA sequencing, was introduced into *E. coli* BL21(DE3).

Table 2. Primers used in this study

Name	Sequence (5' to 3')
CBD500F	ACGCCATGGCAAAACACTAATACAAAT
CBD500R	ACGCCATGGCAAAACACT AATACAAAT
yadAF	ACGCCATGGCAAAACACTAATACAAAT
yadAR	ACGCCATGGCAAAACACTAATACAAAT
PedF	GAGCTCGAGTTTAAATACTACGGTAATGGGGTTAC
PedR	GACGTCGACTAGCATTTATGATTACCT
PedR2	AGTCCACTACGTGCTAGCATTTATGATTACCT
uspF	GACGTCGACATGAAAAAAAAGATTATCTCAGC
uspR	GAGCTCGAGGCCGGCGTAAACACCTGACAACGG
pepRF	ACTGCACATTGTGTGCTTTGATACTCACCAATG
CBD500F2	ACGCCATGGCAAAACACTAATACAAAT
CBD500R2	AACGGATCCTTAGTGATGGTGATGGTGATGTTTTAAGAAGTATTC

Cell Fractionation

Bacterial cell fractionation was carried out as previously described [13]. Cell suspension was then sonicated on ice. The supernatant was centrifuged at 25,000×g for 1 h to obtain total membrane fraction in pellet. The supernatant was regarded as the soluble cytoplasmic/periplasmic fraction. The pellet was resuspended with 20 mM Tris-HCl buffer (containing 1% sodium lauroyl sarcosine, pH 7.4) for solubilizing inner membrane. The outer membrane fraction was re-pelleted by centrifugation at 25,000×g at 4 ℃ for 1 h. The supernatant was regarded as the inner membrane fraction.

SDS-PAGE and Western Blot Analysis

The protein samples were analysed on 12% SDS-PAGE. Separated proteins were electroblotted onto polyvinylidene-fluoride (PVDF) membranes (0.45 μm) (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The anti-His/AP antibody was used for Western blot analysis (Invitrogen WesternBreeze chromogenic kit) according to manufacturer's instructions.

Whole Cell ELISA

Induced cells of pASG-IBA4 or pET-LOAvi constructs were harvested, washed with PBS (pH 7.0), and resuspended in the same buffer to an OD600=1. Cells were coated

onto the wells of 96 wells Nunc microtiter plate overnight at 4 ℃ and blocked with 3% BSA in PBS. The wells were washed with PBST (PBS and 0.1% Tween 20). The pET-LOAvi constructs were incubated with anti-His/AP antibody at a final dilution of 1:3000 for 1 h. Wells were washed with PBST before adding alkaline phosphatase yellow (pNPP) as a substrate. The absorbance of each well was measured at 405 nm. For pASG-IBA4 constructs, anti-Strep-tag antibody (1:500), anti-rabbit antibody with horseradish peroxidase conjugate (1:5000), and TMB as a substrate were used. After stopping the color reaction with 0.5 M H_2SO_4, the absorbance was measured at 450 nm.

Results

Surface Expression of CBD500 with the Outer Membrane Anchor of YadA

CBD500 (GenBank: X85009.1), originated from *L. monocytogenes* phage endolysin Ply500 has specificity to bind to the surface of *Listeria* cells [4]. By whole cell ELISA the CBD-YadA fusion was shown to be displayed on the cell surface in strain ECO762 in Fig. 1A. Addition of anti-listerial peptide pediocin secretion to ECO762 cells decreased the production of the CBD500-YadA fusion protein (Fig. 1). Furthermore, tetracycline induction of CBD500-YadA expression inhibited cell growth and induced cell lysis (data not shown). Since the CBD500-YadA fusion was toxic for *E. coli*, and the co-production of pediocin further weakened its expression, the next aim was to test another membrane anchor for the surface display of CBD500.

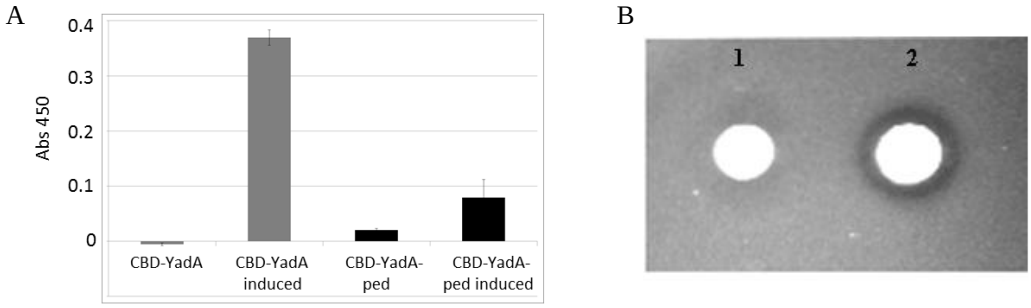

Figure 1. Analysis of *E. coli* YadA constructs. A: Whole cell ELISA of CBD500-YadA and CBD500-YadA-pediocin (ped) fusions expressed on the surface of tetracycline-induced ECO762 (TG1/CBD500-YadA) and ECO764 (TG1/CBD500-YadA + P_{pepR}-SS_{usp45}-*papA*) cells respectively. Co-production of pediocin reduces the CBD500-YadA expression. B: Inhibition of *L. monocytogenes* WSLC 1018 by pediocin producing *E. coli*. 1, ECO762 cells; 2, ECO764 cells. Two µl of the culture suspensions were spotted on *Listeria* lawn on BHI agar plate, and incubated overnight.

Availability of the OmpA-CBD500 Fusion Protein on the Cell Envelope

OmpA-CBD500 was expressed at reasonable level after IPTG induction. Fig. 2A shows a visible new protein band estimated to be around 30 kDa could be detected as expected by SDS-PAGE. The localization of the CBD500 on the external surface of *E. coli* was determined by Western blot. Strong bands could be observed in the outer membrane fraction after IPTG induction, indicating the CBD500 fusion was mainly localized at the outer layer of the cell membrane (Fig. 2A). The availability of the CBD500 domain on the surface of intact cells was analysed by whole cell ELISA. The signal of ELISA was weak, though higher than the negative control (Fig. 2B). Displaying CBD500 via OmpA anchor did not show evident binding effect when mixing induced *E. coli* cells with listerial cells (data not shown).

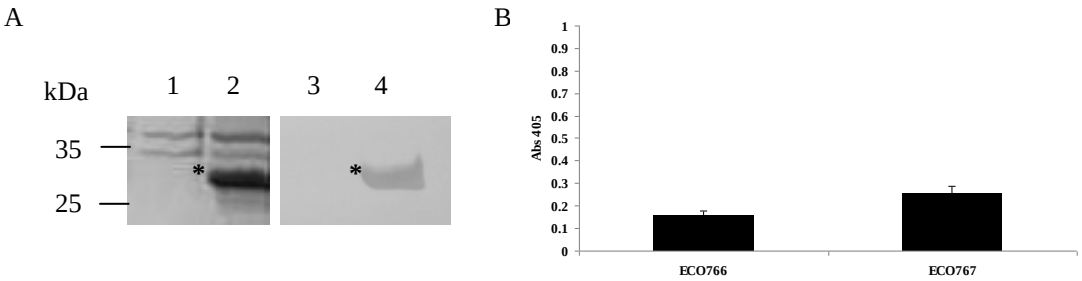

Figure 2. Analysis of *E. coli* OmpA constructs. A: Localization of OmpA-CBD500. 1, SDS-PAGE of outer membrane proteins without IPTG induction; 2, SDS-PAGE of outer membrane proteins with IPTG induction; 3, Western blot of outer membrane proteins without IPTG induction; 4, Western blot of outer membrane proteins with IPTG induction. The OmpA-CBD500 fusion protein is marked with an asterisk. B: Whole cell ELISA of ECO766 (BL21(DE3)/pET-LOAvi) and ECO767(BL21(DE3)/OmpA-CBD500) after IPTG induction.

Discussion

Previously, *Listeria* phage endolysins CBDs have been fused with green fluorescence protein (gfp) to study their binding mechanisms with listerial cells [4]. However, there is no report about presenting CBDs on the microbial surface via outer membrane protein fusions and investigating the interaction by in vivo model. An evident outcome is surface display of anchor YadA is not favorable but lethality in *E. coli* cells. Expression of CBD500 fused to OmpA was successful, but the protein on the surface was poorly available for antibodies. It is speculated the presented CBD500 is not far enough away from the lipopolysaccharide layer, which may prevent the interaction between CBD500 and *Listeria* cell wall ligands. Or, the CBD500 has penetrated into outer membrane after translocation, leading to few accessible CBD500 for binding. Another concern is the putative incorrect folding of CBD500 due to high level expression under T7 promoter. Stathopoulos et al [14] have pointed out earlier this display system is probably not compatible to extensive secondary and tertiary structures of the passenger protein. As the three dimensional structure of CBD500 and the specific region responsible for binding have not been revealed, one can speculate conformation change may have happened in the fusion potentially explaining the inefficient interactions with ligands.

Conclusions

CBD500 displaying *E. coli* strains were constructed using YadA and OmpA as carrier proteins. Fusion proteins were expressed on the cell surface with compromised availability on the cell envelope. Utilization of outer membrane protein anchors for surface display may require introduction of long proteins linkers or protein domains to ensure functional presentation of the domain to be displayed.

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