

# Development of Sporobeads Coated With Hecad1/2 for Rapid Detection and Capturing Of Pathogenic *Listeria Monocytogenes*

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**Abstract** - Internalin proteins localized at the surface of pathogenic *Listeria monocytogenes* interfere with E-cadherin to adhere and internalize into mammalian cells. E-cadherin has five extracellular, immunoglobulin-like domains (EC1 to EC5), of which the first domain is sufficient to mediate *L. monocytogenes* invasion. Immunomagnetic beads employ antibodies that react either with pathogenic or non-pathogenic *Listeria*. To obtain cost-effective and easy-to-produce beads for detecting pathogenic *L. monocytogenes*, we cloned and efficiently expressed human E-cadherin domains 1 and 2 (hEcad1/2) in the *Bacillus subtilis* spore coat. The cDNA sequence encoding hEC1/2 protein 23.7 kDa (MH511517.1) was inserted in pET22b and expressed and purified from *Escherichia coli* BL21. We used the CotY as a significant structural component of the *B. subtilis* spore coat to express hEcad1/2. We constructed a recombinant plasmid p1CSV-CotY-N-hEcad1/2 incorporating the cotY-hEcad1/2 gene under the control of the *cotY* promoter. The constructed plasmid was transformed into *B. subtilis* KO7 by double cross-over method and an amylase-inactivated mutant was generated. After spore induction, the developed sporobeads showed a high binding affinity for *L. monocytogenes* 4b. This result demonstrated the potential of human E-cadherin ectodomain 1 and 2 in a practical application involving the detection and capture of pathogenic *L. monocytogenes*.

**Keywords:** *Listeria monocytogenes*, detection, E-cadherin, expression, *Bacillus subtilis*, sporobead

## 1. Introduction

*Listeria monocytogenes* is one of the most important food-borne pathogens which causes a major opportunistic illness by crossing the intestinal epithelial barrier to access deeper tissues. In physiologically stressed and immunocompromised individuals, *Listeria* can cross the blood-brain barrier to spread to distant organs [1]. Adhesion and internalization of *L. monocytogenes* to the host intestinal epithelium are crucial for establishing infection and spreading to extraintestinal sites. In pathogenic *Listeria* species, internalin proteins (InlA, InlB, InlC, InlF, and InlP) control the stages of infection. The internalins InlA, InlB, and InlF are located at the bacterial cell surface and have a modular structure including an N-terminal cap domain, varying C-terminal repeats, an LRR-domain of 22 amino acid repeats, and an interrepeat region (IR) domain. These proteins target the adhesion molecule E-cadherin on the cell surface to bind and internalize into mammalian cells [2, 3].

E-cadherin has five extracellular, immunoglobulin-like domains (EC1 to EC5), of which the first domain is sufficient to mediate *L. monocytogenes* invasion. The leucine-rich repeat (LRR) domain of InlA surrounds and specifically recognizes hEC1. In other words, hEC1 occupies and fills the central cavity created by the curved LRR-domain of InlA (fig. 1) [2]. The pathogenicity of *L. monocytogenes* relies on virulence factors such as internalins [4]. In previous studies, bacteria expressing either InlA or InlB and beads coated with these proteins were successfully internalized in non-phagocytic cells [5]. In another related study, *L. monocytogenes* was shown to infect the E-cadherin-expressing cardiomyocytes in vitro [6].

Immunomagnetic separation (IMS) methods have been extensively used to detect *L. monocytogenes*. These methods employ antibodies that react with pathogenic or non-pathogenic *Listeria* [7]. The pathogenicity of *L. monocytogenes* relies on virulence factors such as internalins [8], and the hEcad ectodomain is adequate to start interaction with internalin, which connotes the cytoplasmic domain plays no role in adhesion [9].

Compared to the immobilization of proteins on synthetic surfaces, which is a costly and laborious procedure, the self-immobilization on *B. subtilis* spore coat has been introduced as a cost- and time-efficient alternative. Therefore, this study aimed to express human E-cadherin domains 1 and 2 (hEcad1/2) on the spore coat of *B. subtilis* and validate its potential in capturing pathogenic *L. monocytogenes*.

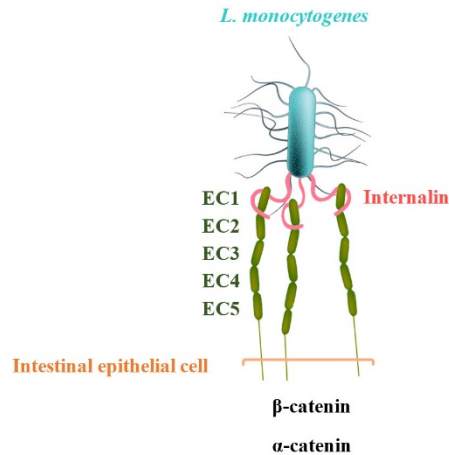


Fig 1: Schematic of internalin interaction with E-cadherin

## 2. Materials and Methods

### 2.1. Construction of h-Ecad1/2 expression plasmid

The sequence coding for the extracellular domain of Human E-cadherin (657-bp hEcad1/2) (MH511517.1) was codon optimized and synthesized by Genescript and cloned into the pET22b with a 5' overhang containing a *XhoI* site, a 3' overhang containing *NcoI* site, and a 6× His-tag fused at the N-terminus of the expressed protein.

### 2.2. Expression of h-Ecad1/2

The expression plasmid pET22b-hEcad1/2 was cloned into competent *E. coli* BL21(DE3) cells by electroporation and positive transformed cells were selected on LB agar (merck) containing 100 µg/ml ampicillin. *E. coli* BL21 transformed with pET22b-hEcad1/2 was grown in 500 ml LB broth (merck) at 37 °C with vigorous shaking until OD 600 nm of 0.4-0.5 was reached, and an aliquot (*t*<sub>0</sub>) of supernatant was taken. Expression of the His-Tag- hEcad1/2 was induced by the addition of 1 mM isopropyl-1-thio-β-d-galactoside (IPTG) to a final concentration of 1 mmol/L, and one-milliliter sample was taken every hour, up to 5 h after induction. The cell pellet was harvested by centrifugation at 12,000 ×g for 5 min at 4 °C, resuspended in sonication buffer (50mM Tris-Hcl, 500mM NaCl) (pH 7.0), and sonicated at 4°C using a Sonorex Super 10P (Bandelin) ultrasonic bath. Cell debris was centrifuged at 12,000 ×g for 20 min at 4°C. The supernatant was prepared for the following purification.

### 2.3. Purification of hEcad1/2

The recombinant protein was purified using a HisTrap™ FF Crude column. The column was filled with Ni sepharose medium and equilibrated with binding buffer (20 mmol Na<sub>3</sub>PO<sub>4</sub>, 500 mmol NaCl, 5 mmol imidazole, pH 7.4). The sample was loaded at a flow rate of 0.5 ml/min, and the column was then washed with 10-15 column volumes of binding buffer. The column-bound proteins were eluted using elution buffer (20 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 1 mmol/L EDTA, and 1 mol/L NaCl, pH 8.0) at a flow rate of 1 ml/min. Protein fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue.

### 2.4. SDS/PAGE and Western Blotting

The expression of target hEcad1/2 protein was analyzed using Bio-Rad mini-protean TGX precast gel on the PowerPac Basic (Bio-Rad, USA), electrophoresed at 100 V for 1 h, and then stained with Coomassie brilliant blue R250. Following SDS-<sup>-</sup>, proteins were transferred from SDS-PAGE to polyvinylidene fluoride (PVDF) membranes on tank-blotting devices (Bio-Rad) at 400 mA for 60 min.

Western blot analysis was carried out using mouse anti-Histidine-Tag monoclonal primary antibody (Sigma) and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma) and imaged by Bio-Rad ChemiDoc Touch Imaging system.

## 2.5. Construction of recombinant integration plasmid p1CSV-CotY-N-hEcad1/2 and pre-cloning in *E. coli* ECE371

The hEcad-specific primers, hec-F: 5'-TCTAGATAAGGAGGAAGCCGGCATGGACTGGGTTATTCCTCCCATCAG-3' (*Xba*I site is underlined) and hec-R: 5'-ACTAGTATTAACCGGTCGGAGGATTATCGTTGGTGTCTCAG-3' (*Ngo*MIV site is underlined), were designed to amplify a 657-bp hEcad1/2 gene from the vector pET22b-hEcad1/2. The PCR product was purified using GeneJET PCR purification kit (Thermo Scientific) and then 5'-phosphorylated with T4 Polynucleotide Kinase (New England Biolabs) and linearized by T4 DNA ligase (Fermentas, USA). The purified PCR product (hEcad1/2 gene) was double digested with *Xba*I and *Age*I while the p1CSV-CotY-N vector (rfp-cassette) was excised with *Xba*I and *Ngo*MIV restriction enzymes and then fused to the end of the *cotY* gene to yield the p1CSV-CotY-N-hEcad1/2 recombinant expression plasmid (Fig. 2).

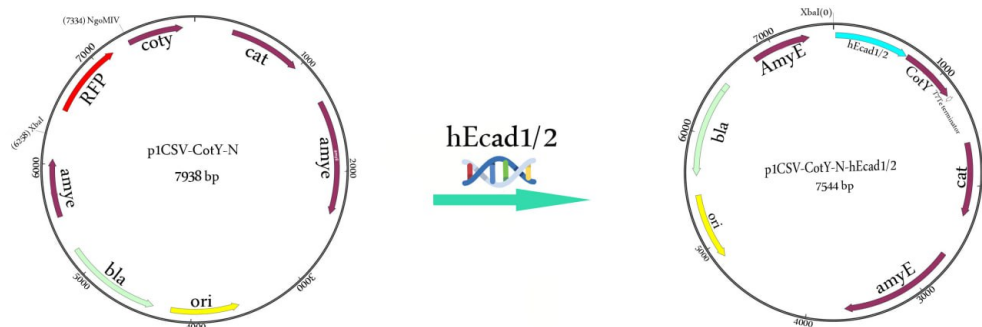


Fig. 2: Steps in construction of recombinant p1CSV-CotY-N-hEcad1/2 plasmid

The vector contains a *bla* gene that mediates ampicillin resistance (100 µg/ml) in *E. coli* and chloramphenicol resistance (5 µg/ml) gene for selection in *B. subtilis* and integrates into the *amyE* locus by double crossing-over. For sequencing, *E. coli* ECE371 was transformed with p1CSV-CotY-N, and sequencing of the cloned hEcad gene was performed by Sanger sequencing.

## 2.6. Construction of recombinant *Bacillus subtilis* KO7

*B. subtilis* KO7 (Bacillus Genetic Stock Center ID 1A1133) was used to express the hEcad1/2 protein on the spore coat. The gene of hEcad1/2 was fused to the end of the *cotY* gene to yield the p1CSV-CotY-N-hec1/2 recombinant expression plasmid (Fig. 2). The integrated recombinant plasmid was linearized by *Sca*I and transformed into *B. subtilis* competent cells by double cross-over recombination with chloramphenicol selection (5 µg/ml). *B. subtilis* KO7 cells harboring p1CSV-CotY-N-hec1/2 were grown at 37 °C in LB medium containing chloramphenicol (5 µg/ml) to induce expression of the hEcad1/2 protein. The expected integration into the *amyE* locus was confirmed by the starch test, as described previously [10].

## 2.7. Preparation of spores

The exhaustion method was used to induce sporulation of *B. subtilis* in Difco Sporulation Medium (DSM), as previously described [11]. Cultures were harvested 48 h after incubation in DSM at 37 °C. Spores were collected, washed with 1 M NaCl, and purified using lysozyme treatment to break any residual sporulating cells.

## 2.8. Capture of *L. monocytogenes*

*L. monocytogenes* serotype 4b was activated and enriched in BHI broth at 37 °C for 24 h. After washing by 10 mM phosphate buffer solution (PBS) and centrifugation at 8000 rpm for 5 min, one hundred microliters of *L. monocytogenes* was added onto the ten microliters of spore, and the capturing efficiency was visualized under the microscope after a 10-min reaction.

## 3. Results

### 3.1. Verification of pET-22b containing hEcad1/2

The vector was digested with *NcoI* and *XhoI* enzymes. As expected, a 671-bp band was observed after the digestion of pET22-hEcad1/2 (Fig. 3).

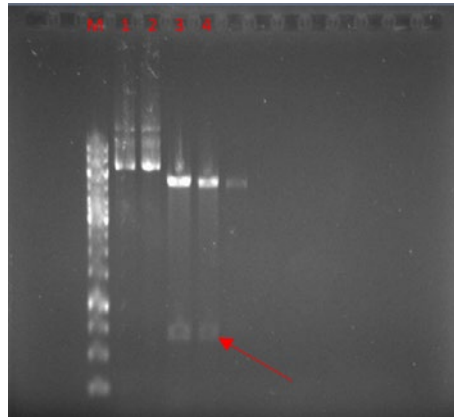


Fig. 3: Double digestion of pET-22b-hEcad1/2 with *NcoI* and *XhoI* enzymes. M: DNA ladder (1kb); Lane 1&2: undigested DNA sample; lane 3&4: digested DNA sample. As expected, a 671-bp band is seen in lane 3&4 (shown by the arrow).

### 3.2. Protein expression and purification from *E. coli*

The BL21 samples induced with IPTG were run on Bio-Rad mini-protean TGX precast gel to evaluate the intracellular expression of the hEcad1/2 protein. As shown in Fig. 4A, a band of 23.7 kDa corresponding to the molecular weight of the hEcad1/2 was observed. For the periplasmic expression of hEcad1/2, we used *pelB* signal peptide, and as shown in Fig. 4B, Western blotting confirmed the expression of His-tagged protein.

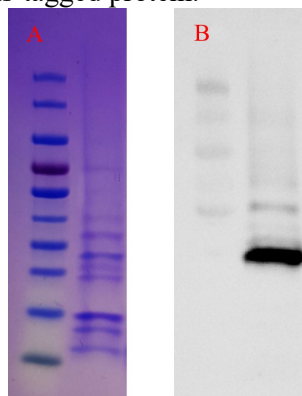


Figure 4: (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of hEcad 1/2 expression in *E. coli* BL21 containing pET22-hEcad1/2 plasmid (cytoplasmic construct), (B) Western blot analysis with anti-His antibody.

### 3.3. Construction and cloning of recombinant p1CSV-CotY-N-hEcad1/2

The recombinant plasmid p1CSV-CotY-N-hEcad1/2 was constructed by inserting the hEcad1/2 DNA fragment into the p1CSV-CotY-N backbone vector (Fig. 2). Obtained colonies had an antibiotic selection, and the gene amplification from the

construct showed the expected size. We confirmed no DNA mutation in the constructed plasmid by DNA sequencing (data not shown) and then transformed p1CSV-CotY-N-hEcad1/2 into competent *B. subtilis* KO7 cells.

### 3.4. Identification of *cotY*-hEcad1/2 integrated into the chromosome of *B. subtilis*

Amylase activity assay was employed to identify *cotY*-hEcad1/2 integration mutants. The integration of *cotY*-hEcad1/2 at *amyE* locus disrupted the secretion of amylase; as a result, no colorless halo was observed around the colonies on the Starch Agar plates stained by iodine, but in control strain, a white halo appeared around the colonies due to the secretion of amylase (Fig. 5).

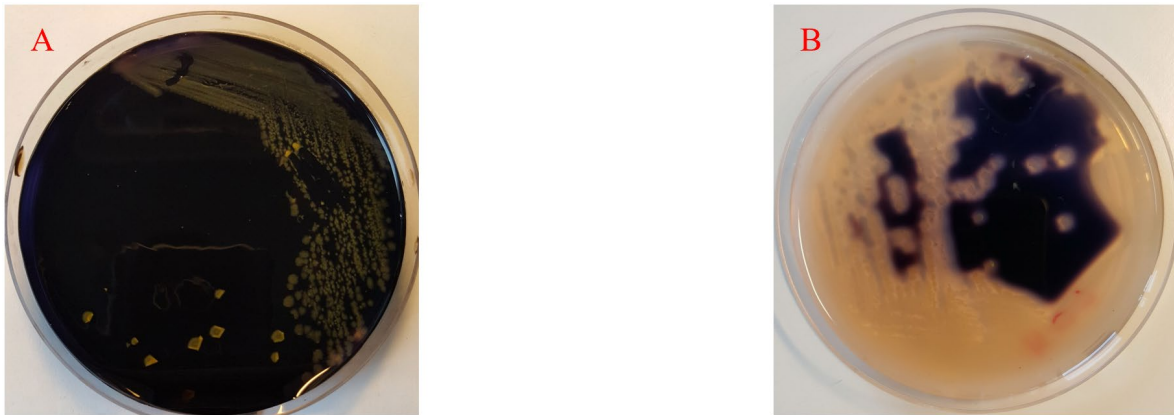


Fig. 5: Verification of transgenic *B. subtilis* by amylase activity test. (A) The insertion of *cotY*-hEcad1/2 destroyed *amyE* and produced an amylase-deficient strain, (B) while the wild-type strain presented a white halo around the colonies due to the secretion of amylase.

### 3.5. Detection of *L. monocytogenes*

The reliability of the developed sporobeads for the detection of pathogenic *L. monocytogenes* was evaluated under the Zeiss Axioskope 2 plus microscope. As illustrated in Fig. 6, the sporobeads showed high-affinity binding to the *L. monocytogenes* 4b.

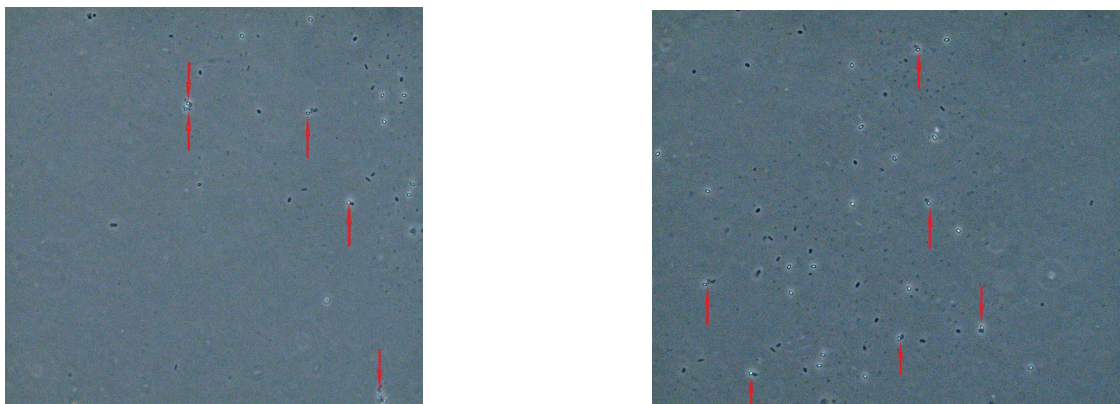


Fig. 6. Sporobead capture of the *L. monocytogenes* 4b under Zeiss Axioskope 2 plus microscope. Arrows show positive interaction between spherical sporobead (white) and *L. monocytogenes* (short rods).

#### 4. Conclusion

This is the first report of recombinant human E-cadherin ectodomain 1/2 expressed on the *B. subtilis* spore coat the biological activity. Surface-displayed hEcad1/2 showed a higher affinity to *L. monocytogenes* 4b. This system could real value in detection and capturing of pathogenic *L. monocytogenes*.

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