

# Expression, Purification and Characterization of a Recombinant *Staphylococcus epidermidis* Fibronectin-Binding Protein

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**Abstract:** *Staphylococcus epidermidis* is the leading cause of implant-related infections and is responsible for serious complications including sepsis, bacterial emboli, and even death. Bacterial adhesion is mediated by specific interactions between cell surface receptors and blood plasma or extracellular matrix proteins, such as fibronectin (Fn), that initially adsorb onto an implanted medical device. Here we isolate and partially characterize a recombinant *S. epidermidis* fibronectin-binding protein, Embp32, previously cloned in *Escherichia coli* M607. The His-tagged Embp32 was extracted from *E. coli* and purified via nickel chelation affinity chromatography. Molecular weight of the isolated Embp32 was determined to be 32.5 kDa via SDS-PAGE, which agreed with the known amino acid sequence. Bacterial adhesion inhibition assays, intended to verify the biological activity of the recombinant Embp32, were not successful.

## 1. INTRODUCTION

Of the two million cases of nosocomial infections reported annually in the United States, 50% are associated with indwelling medical devices [3]. An overwhelming number of these infections are due to *S. epidermidis*, a symbiotic inhabitant of the patients' epidermal layer [3]. Until recently, the role of *S. epidermidis* and other coagulase-negative staphylococci as pathogens had not been recognized [6]. Infections caused by *S. epidermidis* are frequently related to indwelling cardiovascular devices [6,10,11] such as cardiac pacemakers, prosthetic heart valves, cerebrospinal fluid shunts, and intravascular catheters. Rates of infection for cardiovascular implants average approximately 4%, but can be as high as 40% for cardiovascular ventricular assist devices [3]. Estimated combined costs of medical and surgical treatment for infections of cardiovascular implants can range from \$35,000 to \$50,000 per case [3]. In addition, mortality rates associated with device-related infections are highest for cardiovascular implants [3].

*S. epidermidis* is regarded as an opportunistic pathogen that readily adheres to medical devices at the site of surgery [2,9,10,11]. Adhesion of *S. epidermidis* and other bacteria is a two-step mechanism. The first step of adhesion is a non-specific and reversible physical adherence of the bacteria onto the surface [1]. The second step of attachment entails specific intermolecular reactions between bacterial surface receptors and extracellular

matrix proteins adsorbed on the surface of the device [2,9,10,11]. Many studies have shown that *S. epidermidis* adhesion to implant materials is mediated by specific binding to Fn adsorbed on the surfaces [7,9,11]. However, little is known about the structure of the *S. epidermidis* fibronectin-binding receptor (Embp32) or its binding mechanism [10,11].

Previous investigations have described the isolation and detailed characterization of Fn receptors for the related bacterial species, *S. aureus* [4]. Identical work for *S. epidermidis* has not been completed, although Williams *et al.*, [11] have recently identified a fibronectin-binding domain in the *S. epidermidis* proteome using recombinant phage technology. The group subsequently cloned this binding segment, denoted Embp32, into *E. coli*. In the current study, we isolated the recombinant Embp32 from the *E. coli* clone and characterized the protein via SDS-PAGE and an adhesion inhibition assay.

## 2. MATERIALS AND METHODS

### Chemicals

Tris-HCl 12% polyacrylamide precast gels and protein standards were purchased from Bio-Rad (Hercules, CA); human plasma Fn from Chemicon (Temecula, CA); Luria-Bertani (LB) broth and trypticase soy broth (TSB) from Fisher Scientific (Tustin, CA); B-PER and Slide-A-Lyzer Dialysis Cassettes from Pierce Biotechnology (Rockford, IL); Ni-NTA (nickel

nitrilotriacetic) agarose resin from Qiagen (Valencia, CA); and Live/Dead *BacLight*<sup>TM</sup> Bacterial Viability kit from Molecular Probes (Carlsbad, CA). Antibiotics, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), ammonium chloride, imidazole, protease inhibitor cocktail, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO).

### *Bacteria*

*E. coli* M607 containing a plasmid encoding for the Fn-binding region (protein denoted Embp32) of the *S. epidermidis* cell wall associated Fn-binding protein was a generous gift from Dr. Sean Nair at University College London, London, U.K. The *embp32* gene was placed downstream of a *LacZ* promoter and was linked to a 6-histidine tag to aid in purification by nickel chelation chromatography. *S. epidermidis* RP62A was purchased from The American Type Culture Collection (ATCC# 35984).

### 2.1 Cell culture

*E. coli* M607 were grown on LB media plates containing 1.5% agar, 100  $\mu$ g/ml ampicillin, 25  $\mu$ g/ml kanamycin, 20  $\mu$ g/ml streptomycin, and 20  $\mu$ g/ml spectomycin. Plates were incubated overnight at 30°C. One colony was selected from a plate and inoculated into 10 g/L LB containing the antibiotics above. Cells were incubated overnight at 30°C in rotary waterbath shaker (New Brunswick Scientific G-76) at 150 rpm.

*S. epidermidis* RP62A were grown on TSB plates containing 1.5% agar and incubated overnight at 37°C. One colony was selected from a plate and inoculated into 5 g/L TSB and incubated overnight at 37°C.

### 2.2 Protein expression

Cell culture solution was diluted 1:10 into fresh LB media containing antibiotics. Cells were incubated for 2 h at 30°C in a rotary shaker (Innova 4400). Protein expression was induced through the *LacZ* promoter with 0.1 mM IPTG and cells incubated for an additional 4 h. Cells were harvested in 110 ml aliquots by centrifugation at 13,000 x g and 4°C. Cell pellets were stored at -20°C for later use.

### 2.3 Protein purification

Cell pellets were resuspended in B-PER reagent containing 750 mM ammonium chloride, 20 mM imidazole and appropriate amount of protease inhibitor and gently mixed at 4°C for 30 min. Solution was centrifuged at 23,000 x g at 4°C; supernatants were pooled and added to 1 ml Ni-NTA agarose resin. The slurry was mixed for 1 h at 4°C and then loaded onto 1 ml disposable columns. The column was washed sequentially with (1) B-PER containing 20 mM imidazole and 500 mM ammonium chloride, (2) B-PER containing 20 mM imidazole and 250 mM ammonium chloride, (3) phosphate buffered solution (PBS) containing 20 mM imidazole and 0.15 M NaCl, and (4) PBS containing 20 mM imidazole and 0.15 M NaCl. His-tagged Embp32 was eluted from the Ni-NTA resin with PBS containing 250 mM imidazole and 0.15 M NaCl. All washes, elutions, and a sample of resin were saved at 4°C for subsequent SDS-PAGE analysis. Purified protein samples were dialyzed against PBS containing 0.15 M NaCl using Slide-a-Lyzer dialysis membrane cassettes (MW cutoff = 10,000) to remove imidazole from solution.

### 2.4 Protein Characterization

All samples collected from protein purification steps were subjected to SDS-PAGE analysis. Molecular weight of Embp32 was determined by comparison to protein standards. The empirical molecular weight was compared to the theoretical value calculated using the known amino acid sequence (as determined by R.J. Williams, *et al.* [11]) and by means of the LabVelocity internet program [8].

### 2.5 Adhesion inhibition assay

Lab-Tek Chamber Slide wells were exposed to Fn solutions of either 2.5  $\mu$ g/ml Fn or 25  $\mu$ g/ml Fn. Plates were incubated for 1 h at 37°C. Each well was rinsed twice with PBS to remove any loosely adherent Fn. The surfaces of the wells were passivated by addition of 1% bovine serum albumin (BSA) followed by incubation at 37°C for 30 min. Wells were rinsed twice with PBS and 97.5  $\mu$ g/ml Embp32 was added to appropriate wells. All other wells were filled

with PBS alone. Plates were then incubated for an additional hour at 37°C and rinsed twice with PBS. *S. epidermidis* culture was adjusted to an optical density of 1.0 (approximately  $1 \times 10^8$  cells/ml) and the cell suspension was placed in all wells and incubated for 2.5 h at 37°C. Loosely adherent bacteria were removed by gently rinsing the wells three times with PBS.

### 2.6 Fluorescence microscopy

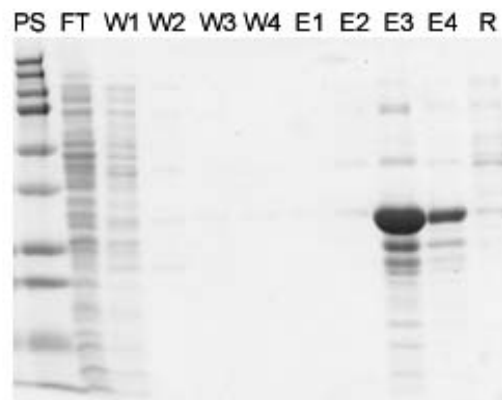
Adherent bacteria were stained using Live/Dead BacLight™ Bacterial Viability stain kit according to the manufacturer's protocol. Excess liquid was aspirated from surfaces and cells viewed with an epifluorescent microscope (Zeiss Axioskop 2, Jena, Germany) equipped with an Optronics MagnaFire Digital Camera System (Optronics, Goleta, CA), a 100x oil immersion objective, and appropriate filters. Five random fields were selected per well and imaged using the MagnaFire software program. Cell numbers were quantified with the aid of ImageJ software.

## 3. RESULTS

Characterization of Embp32 via SDS-PAGE. Embp32 was successfully expressed in and isolated from *E. coli* M607 and was purified using nickel chelation affinity chromatography. The presence of the Embp32 protein in the eluted samples was verified via SDS-PAGE (darkest bands seen in lanes E3 and E4 of Figure 1). However, contaminating proteins were also present in the elution fractions containing the target protein. This indicates that optimization of the purification procedure is needed.

Molecular weight of the Embp32 isolate was determined to be approximately 32.5 kDa. The SDS-PAGE results from Williams *et al.* [11] indicated that Embp32 was approximately 35 kDa. Using the program LabVelocity [8] and the known amino acid sequence of the Embp32, we determined that the theoretical molecular weight of Embp32 is 32.5 kDa. This calculation corroborated our own SDS-PAGE results.

The LabVelocity program was also used to determine the extinction coefficient of Embp32, which was calculated as  $8940 \text{ cm}^{-1} \text{ M}^{-1}$ . The extinction coefficient enabled us to determine the concentration of purified Embp32 solutions. The concentration of isolated stock Embp32



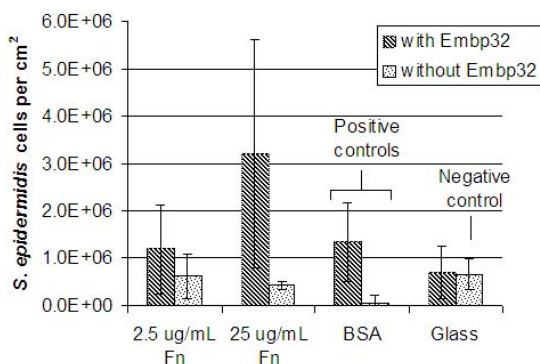
**Figure 1** SDS-PAGE results. Lanes are labeled as follows: PS, protein standards; FT, column flow-through; W1-W4, washes 1-4; E1-E4, elutions 1-4; R, leftover Ni-NTA agarose resin. The target protein (Embp32) was eluted in the 3rd and 4th elution fractions (darkest bands in lanes E3 and E4).

solution used in the following inhibition assay was found to be 0.43 mg/ml.

Characterization of Embp32 via adhesion inhibition assay. Bacterial adhesion inhibition assays were carried out to verify the ability of Embp32 to inhibit *S. epidermidis* specific binding to Fn. Adhesion inhibition would show that the isolated Embp32 is biologically active and can block Fn-binding proteins present on the *S. epidermidis* cells, negating adhesion to adsorbed Fn. However, results in Figure 2 indicate that Embp32 did not block binding of *S. epidermidis* to Fn. In fact, more cells adhered to Fn in the presence of Embp32 than to Fn alone. Interestingly, addition of Embp32 to the BSA negative controls also increased bacterial adhesion compared to BSA alone. As expected, BSA prevented bacterial adhesion; however, the positive controls (glass) exhibited much lower adhesion than anticipated. It should also be noted that Embp32 adsorbed on glass does not appear to increase cell adhesion in the absence of other proteins. The effect of Embp32 to increase cell adhesion was only seen when Fn or BSA was preadsorbed on the surface.

## 4. DISCUSSION

Nickel chelation affinity chromatography proves to be a promising method for the purification of the recombinant protein, Embp32. The affinity



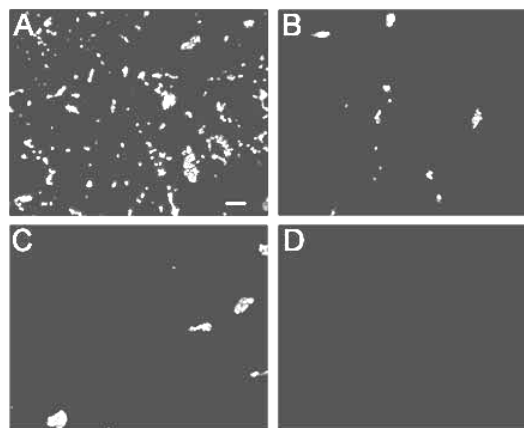
**Figure 2** Binding of *S. epidermidis* to Fn-coated surfaces with and without Embp32. Fn coated surfaces with Embp32 are indicated by hashed bars. Surfaces without Embp32 are indicated by spotted bars. Standard deviation of samples is marked by error bars.

chromatography procedure allowed the target Embp32 protein to be selectively bound to the matrix of the column while various extraneous proteins were mostly excluded. The presence of some contaminating proteins in the elution fractions containing Embp32 indicates that the purification protocol needs to be refined in order to assure more consistent results for future investigations. Embp32 could be further purified using multiple passes through the Ni-NTA column. Another possible improvement may be to wash the column with an imidazole gradient, rather than a single large step increase between the wash and elution solutions. A gradient wash would remove more weakly bound proteins from the matrix before the strongly-bound Embp32 is eluted.

The isolated Embp32 protein was determined via SDS-PAGE to have an approximate molecular weight of 32.5 kDa. Embp32 molecular weight was also verified using the program LabVelocity [8] and the protein's known amino acid sequence. Our observed molecular weight of 32.5 kDa is slightly lower in comparison to the results of Williams *et al.* [11], however, this may be insignificant.

Following the isolation of Embp32, we attempted to characterize the protein's functionality via a bacterial adhesion inhibition assay. Contrary to expectations, the addition of Embp32 increased *S. epidermidis* adhesion to

both adsorbed Fn and BSA as seen in Figure 3. This indicates that Embp32 was not in a biologically active form. It is possible that Embp32 may have been denatured either during the purification procedures or during storage subsequent to purification. No increase in cell adhesion was observed on glass adsorbed with Embp32 compared to glass alone. For this reason, nonspecific adhesion of *S. epidermidis* to Embp32 is not likely the cause of increased adhesion to Fn and BSA. However, the amount of adsorbed Embp32 was not quantified so it is possible that it was not consistent between surfaces, resulting in conflicting observations. It is also possible that contaminants in the Embp32 elution could have contributed to bacterial binding to surfaces despite Embp32 inhibition of specific Fn adhesion.



**Figure 3** Sample fluorescence images of adhered live *S. epidermidis* stained with Live/Dead stain. (A) 25  $\mu\text{g/ml}$  Fn with Embp32; (B) 25  $\mu\text{g/ml}$  Fn without Embp32; (C) BSA with Embp32; (D) BSA without Embp32.

Future work will be needed to further purify and characterize Embp32 and verify its biological activity *in vitro*. Enzyme-linked immunosorbent assays and radiolabeling will also be used to quantify Embp32 binding to Fn. The purified Embp32 can then be used to develop antibodies and small molecule inhibitors of *S. epidermidis* adhesion to Fn-coated biomaterials.

## ACKNOWLEDGMENTS

We thank Kyung Park for her help throughout this project. The National Science Foundation

(Grant EEC-9529161), the National Institutes of Health (Grant R21EB000987-3), and the University of Washington Engineered Biomaterials (UWEB) faculty/staff members are also gratefully acknowledged for their support.

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