Heterologous Expression and Purification of a 238 kDa Large Biofilm Associated Surface Protein (Bap) in Escherichia coli

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Abstract

Biofilm-associated protein (Bap) is a large surface protein (~238 kDa) that plays a significant role in the development of Staphylococcus biofilms. Surface proteins in S. aureus are functionally redundant, which implies that a null mutant that affects one surface protein might only be partially defective in the studied function. Therefore, the objective of this study was to clone, overexpress and purify the full Bap protein in E. coli to enable us to characterize the protein in detail for future experiments. The challenging part of this study was to resolve the problem of plasmid instability of recombinant constructs, which is speculated to be due to the large size of the gene and the presence of 13 direct tandem repeats, when conventional E. coli strains such as DH5α and XL1-Blue were used as a cloning host. The full bap gene (~6.8 kb) was amplified by long-range Taq polymerase and cloned in an expression vector pET21b in E. coli stbl2 and in BL21(DE3)-pLysS for over expression. DNA sequencing of the cloned gene confirms 100% identity with bap gene in situ (S. aureus V329). Successful expression of the full length of Bap protein in E. coli BL21(DE3)-pLysS was confirmed by the SDS-PAGE and Western blotting using Anti-His tag antibody. To the best of our knowledge, it is first attempt to clone and overexpress full-length Bap protein in E. coli. The use of recombinant Bap gene will allow us to study and aid in its biophysical characterization.

Keywords: Biofilm associated surface protein (Bap); Heterologous expression; Molecular cloning; Gene expression; PCR

Introduction

Staphylococcus aureus is one of the most common root causes of nosocomial infections because of its dominant biofilm forming property. Staphylococci infection is common in both humans and animals. Biofilms have been implicated in almost 60% of all bacterial infections [1]. The bacterial biofilm formation is governed by several factors that are under the control of diverse genetic elements [2]. One of the factors is the expression of Biofilm-associated protein which confers the capacity to form biofilm. It also plays a crucial role in bacterial infection process even in the absence of ica operon, which is responsible for polysaccharide intercellular adhesion (PIA)/poly-β-1,6-N-acetylglicosamine (PNG) synthesis [3]. Bap was the first protein among the family of large surface proteins that is reported to be involved in initial attachment to surfaces and assist in cell–cell interactions [4]. The gene bap has been reported to be widespread among natural isolates of coagulase-negative Staphylococcus species, like S. epidermidis, S. chromogenes, S. xylosus, S. simulans and S. hyicus [5]. Of late, a number of surface proteins are reported to have structural features of member proteins of this family are an extracellular signal and proteinase K can be used to modulate the S. aureus biofilms [9-11]. Functional as well as the evolutionary significance of bap gene has to be discerned. Surface proteins in S. aureus are functionally redundant, which implies that a null mutant that affects one surface protein might only be partially defective in the studied function. Therefore, cloning in a surrogate host is the best way to study them. Therefore, in this study, cloning, overexpression and purification from a surrogate host, Escherichia coli were attempted.

The aim of this study was to clone full-length bap (~6.8 Kb) gene in E. coli and heterologous expression of the Bap protein under extremely controlled condition to facilitate in vitro study of the Bap protein and its functional characterization. Apart from large size of the gene, the presence of the 13 tandem repeats in C-region of the gene, which confers instability due to homologous recombination, were the main challenges in molecular cloning of bap gene. To overcome these challenges, combinations of different hosts and incubation temperatures were tried. In this study, an expression recombinant plasmid, named pET21b-bap, was constructed to express recombinant protein and the high-purity protein was obtained with Ni–NTA affinity chromatography.

Materials and Methods

Bacterial strains, expression vector and culture conditions

Staphylococcus aureus V329 containing full length bap gene was obtained from Dr. I Iasa, Spain and was used in the study. Cloning host E. coli stbl2 (Invitrogen) and expression host E. coli BL21(DE3)-pLysS

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A primer pair, sabF and sabR (Table 1) were used to detect presence of gene from the published sequence of bap of S. aureus V329 [13]. Amplification was carried out with a reaction mixture of 50 µl which contained 1 µl of template DNA, 25 µl of 2x Dynazyme PCR master mix (Finnzymes) and 50 pmol of each of forward and reverse primer. The final volume was made up with nuclease-free water. The PCR programme included initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (52°C for 30 sec) and extension (72°C for 1 min 30 sec). Final extension was carried out at 72°C for 5 min. The PCR product was characterized by gel electrophoresis on 1% agarose gel with pre-stained ethidium bromide (5 mg/ml) in 0.5 M TAE electrophoresis buffer. A GeneRuler<sup>TM</sup> DNA ladder mix (Fermentas, Germany) was used as molecular weight marker.

### Confirmation of bap gene release from vector by double digestion assay

The gene was released from the cloned plasmid vector by double digestion using restriction enzymes (XhoI and NdeI). The digested samples were analysed by 1% agarose gel electrophoresis.

### Nucleotide sequencing of the gene and double digestion with restriction enzymes

The entire open reading frame of cloned bap gene in pET21b(+) vector was fully sequenced to exclude polymerase errors and inadvertent mutations. A total of 11 primers were designed by using an online programme by Genscript, each targeting 600 bp of the bap gene (Supplementary Table S1).

### Over-expression and purification the 6X-Histidine-Bap fusion protein in E. coli BL21(DE3)

*Escherichia coli* BL21(DE3) harbouring pET21b-bap was cultured in LB broth containing 1% glucose and carbenicillin (50 µg ml<sup>-1</sup>) at 30°C. Overnight grown cultures were diluted 1:100 into fresh 500 mL LB broth supplemented with carbenicillin (50 µg ml<sup>-1</sup>) and incubated at 30°C with shaking at 150 rpm to optical density (OD<sub>600</sub>) 0.8. Culture of this stage was optimized for the soluble expression of Bap. After attaining 0.8 OD, 1 mM IPTG was added and the culture was incubated at 30°C for 5 h at 200 rpm.

Induced cells were harvested and lysed in lysis buffer (50 mM Tris (pH 7.9), 500 mM NaCl, 1 mM protease inhibitor phenylmethanesulfonyl fluoride, and 1 mg/ml lysozyme) followed by sonication for 5 min at 4°C. The lysate was cleared by centrifugation at 12000 rpm for 20 min at 4°C, and the supernatant was loaded onto an Ni-NTA column pre-equilibrated with equilibration buffer (50 mM Tris-HCl (pH 7.9) and 500 mM NaCl). The column was washed with 50 column volumes of wash buffer (50 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 10 mM imidazole), and the protein was eluted out with elution buffer (50 mM Tris (pH 7.9), 500 mM NaCl, and 500 mM imidazole).

### SDS PAGE and Western blotting

Purification and molecular weight of expressed His-tagged Bap protein was confirmed by carrying out by SDS-PAGE with broad range

| Primers | Sequence (5'-3') |
|---------|-----------------|
| sab F   | CCTATATCGAAGGTTAGAATTTCACGAC |
| sab R   | GCTTGTGAAGTTAATCTGTACCTGC |
| BFNd1   | GAGGTGAGTAACATATGGAAATACAAAGGTGTTCCTAC |
| BRXh1   | CAATATTTAAGCTCGAGTTTTTTATCATTTTCTAAGACG |

**Table 1:** List of primers used in the study.
pre-stained protein ladder having 10 – 230 kDa markers (New England Biolabs, India). The acrylamide concentration was 10% in the resolving gel and 5% in the stacking gel. Expression and purification of Bap was monitored by SDS – PAGE followed by coomassie blue staining.

For Western blotting, semi-dry electro-blotting transfer method was used. Proteins bands were transferred to polyvinylidene fluoride (PVDF) membranes using iBLOT gel transfer stacks (Life Technologies) by electroblotting. The Non-fat-dried milk bovine blocking solution (Sigma) was used at working concentration of 3%. Membranes were washed with PBS-Tween and then incubated for 1 h at room temperature with a 1:10,000 dilution of Anti-His_6-Tag monoclonal mouse antibody (Life Technologies). The bound antibodies were detected by using BCIP and NBT.

**Results**

**Construction of recombinant plasmid pET21b-bap**

Long range DNA polymerase was able to amplify full 6.8 kb bap gene with restriction enzymes sites (Nde I and XhoI) at the ends. After double digestion with restriction enzymes, Nde I and XhoI, it was cloned into the expression vector pTE21b upstream to 6X-His tag sequence (Figure 1). Recombinant plasmid (pET21b-bap) was transformed in to cloning host E. coli stbl2.

**Plasmid instability of recombinant pET21b-bap plasmid in E. coli (DH5-α) and expression of truncated Bap protein**

Recombinant pET21b-bap plasmid had instability issue when E. coli (DH5-α) was used as a host strain to clone full bap gene fragment. Figure 2A shows the presence of two bands in plasmid extract from transformed E. coli (DH5-α) culture that was grown at 37°C. Presence of two different sized plasmids suggested that occurrence of homologous recombination among the direct tandem repeats. Western blotting of E. coli BL21(DE3)-pET21b-bap lysates post-IPTG induction (1 mM) with anti-His_6-Tag antibodies showed the presence of Bap protein, however with truncated size of ~90 kDa (Figure 2B).
Confirmation of the presence of full-length bap gene in recombinant plasmid

Transformed E. coli Stbl2 colonies with pET21b-bap plasmid were checked for the presence of bap gene using internal primers sabF and sabR (Table 1) in colony PCR (supplementary Figure S1). Positive colonies were used to isolate recombinant plasmid and PCR was performed with internal primers (Figure 3b). Insert release assay confirmed the size of inserted bap gene (Figure 3c). Figure 3c shows that released DNA fragment was ~6.8 kb equivalent to full bap gene, whereas undigested plasmid had a size of 12.2 kb (5.4 kb plasmid + 6.8 kb bap gene). Whole gene sequencing using battery of primers (supplementary Table S2) confirmed the DNA sequence of the cloned gene was 100% identical to bap in situ.

Overexpression and purification of Bap-His\textsubscript{6} fusion protein

Successful expression of full-length of Bap protein in E. coli BL21(DE3)-pLysS was accomplished at 30°C with 1 mM IPTG induction for 4 h. Subsequently, the Bap-His\textsubscript{6} fusion protein was purified from E. coli BL21(DE3)-pLysS via its His-tag using Ni-NTA affinity chromatography under native conditions. SDS-PAGE of the affinity-purified fusion proteins revealed an approximately 238-kDa protein, which was again confirmed by western blotting experiments using Anti-His\textsubscript{6} tag antibody (Figures 4A and 4B).

Discussion

Staphylococcus aureus and other coagulase-negative staphylococci
are the most dominant bacteria among human implant-associated infections. In general, pathogenicity and persistence of these infections are associated with biofilm forming capability. Many surface proteins are reported to be involved in biofilm formation such as SasG [14], FnBPA and FnBPB [15], and the biofilm-associated protein Bap [3]. The Bap protein is encoded by a 6831 bp-long bap gene. It is speculated that Bap family proteins could be a novel antigen for protection studies. However, very little is understood about its secondary and tertiary structural features. Overexpression and purification of Bap is a prerequisite to carry out its biophysical characterisation. Therefore in this study, we have attempted to do the same.

This study appears to be the first study where full Bap protein was expressed with His-tag to aid in purification procedure. Earlier, we tried commonly used cloning hosts such as E. coli DH5α and XL1-blue, however, when transformed colonies were investigated for the presence of bap gene, recombinant pET21b-bap plasmid had instability issue. It was found that the recombinant plasmid size was less than that of expected size i.e. 12.2 kb (Figure 2A). It is speculated that apart from the large size of recombinant plasmid, such instability could also be due to the occurrence of homologous recombination events among 13-delta tandem present in the C-region of the bap gene. Presence of two different sized plasmids suggested that occurrence of homologous recombination among the direct tandem repeats. The presence of truncated Bap protein with truncated size in Western blotting of E. coli BL21(DE3)-pET21b-bap lysates post-IPTG induction (1 mM) with anti-His6-Tag antibodies substantiated the above speculation (Figure 2B). Therefore, E. coli sbl2 was used as a cloning host as these cells are suitable for the cloning of unstable inserts such as retroviral sequences or direct repeats [16]. Following transformation with pET21b-bap construct and culturing the transformed Sbl2 cell culture at lower temperature (at 30°C) the presence of full bap gene confirmed in various ways to avoid any anomaly, as shown in Figure 3.

Since PCR can introduce some errors during DNA polymerisation [17], it was also necessary to make sure that 6.8 kb long amplified DNA fragment was free of any unintended mutations. To confirm the identity of amplified bap gene with the bap gene in situ (S. aureus V329), DNA sequencing was performed with a battery of primers, each targeting 600 bp of gene (supplementary Table S2). Whole gene sequencing confirmed that the cloned DNA fragment was 100% identical to bap in situ.

For the expression of the Bap in E. coli BL21(DE3)-pLysS, the recombinant plasmid pET21b-bap was transformed into the expression host E. coli BL21(DE3)-pLysS. In pET expression system, cloned gene is transcribed by T7 promoter that binds specifically to T7 RNA polymerase and is not recognized by the E. coli RNA polymerase thereby suppresses the leaky expression. On the other hand T7 RNA polymerase gene remains under the control of the IPTG inducible lacUV5 promoter. Background expression from pET expression plasmids is further minimised by the co-expression of T7 lysozyme (by either plasmid pLysS or pLysE), which is a natural inhibitor of T7 RNA polymerase. T7 RNA polymerase transcribes five times faster than E. coli RNA polymerase hence is suitable for expressing long genes without falling off. While doing induction and expression of Bap, using lower temperature (30°C) enhanced the stability of bap gene, which was unstable due the presence of direct repeats via homologous recombination at higher temperature. Using C-terminal His-tag has its own advantages as detection of C-terminal His-tag by anti-His-tag antibody in western blotting confirmed that the bap gene was fully expressed i.e. 238-kDa as shown in Figures 4A and 4B.

Earlier, bap gene was reported to be present only in some isolates of S. aureus from bovine mastitis [3,18,19]. Of late some bap-harbouring S. aureus isolates, recovered from human and animals species, have been reported which suggests that a slow transfer of bap gene among S. aureus strains is very much possible [13,19]. Horizontal gene transfer might spread the bap gene among other pathogenic S. aureus strains because the bap gene is present in Pathogenicity Island SaPblov2, which is a mobile genetic element in S. aureus V329 [4,20]. Spreading of Bap among staphylococci and the presence of homologous proteins among other pathogens suggest the urgent need of complete characterisation of such proteins. Apart from this, studying functioning and biophysical characterisation of Bap is warranted as various other surface proteins exhibit similar structural similarity, such as SasG [14], SasC [21] and accumulation-associated protein (Aap) [22,23] Bap is homologous to the accumulation-associated protein Aap, which mediates biofilm accumulation in S. epidermidis [23].

Conclusion

In this study, we have successfully cloned, overexpressed 6.83 kb long bap gene E. coli and purified the overexpressing soluble fractions of Bap using Ni-NTA column. The procedures of expression and purification of Bap in this study could be used to produce large amounts of protein for its further biophysical characterisation. In near future, biophysical characterisation studies of Bap will be carried out, which will further pave a way towards understanding and development of suitable anti-biofilm strategies against S. aureus biofilm related nuisance.

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