Shukla and Rao. Clon Transgen 2016, 5:2
DOI: 10.4172/2168-9849.1000153

Cloning & Transgenesis
ISSN: 2168-9849

Research Article Open Access

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 construct, 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 DH5-α 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-acetylglucosamine (PNAG) 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. hycus* [5]. Of late, a number of surface proteins are reported to have structural homology with *bap* and constitute Biofilm-associated proteins family (*Bap* family). Such proteins have been identified in different organisms' viz., *Bap* in *Staphylococcus aureus*, *Esp* in *Enterococcus faecalis* [6], *LapA* in *Pseudomonas putida* [7] and *BapA* in *Salmonella* [8]. Common features of member proteins of this family are an extracellular signal sequence, very high molecular weight, repetitive structure and a typical cell wall anchoring domain [4]. In the recent past, there is an increase in the list of biofilm associated surface proteins in the NCBI database despite not knowing the physical basis of function. Therefore, it is necessary to decode how actually these proteins perform in their adhesive functions.

The work on *Bap* protein is still in infancy and till date, besides the pioneering work by Cucarella et al. [3], there is not much information available. In one of our previous studies, we showed that how *Bap* is important in *S. aureus* biofilm stability and how non-antibiotic factors such as Calcium ion concentration 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 lasa, 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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Received June 30, 2016; **Accepted** July 25, 2016; **Published** August 02, 2016

Citation: Shukla SK, Rao TS (2016) Heterologous Expression and Purification of a 238 kDa Large Biofilm Associated Surface Protein *(Bap)* in *Escherichia coli*. Clon Transgen 5: 153. doi: 10.4172/2168-9849.1000153

(Promega) were used as a cloning host and expression host respectively (Table S1). Expression vector pET21b (+) was procured from Novagen (Madison, WI) and was used for protein expression in *E. coli*. *S. aureus* was cultured in tryptic soy broth (TSB) supplemented with 0.25% glucose. *E. coli* strains stbl2 and BL21 (DE3) were grown in Luria-Bertani medium [12].

Genomic DNA isolation and plasmid extraction

The genomic DNA was isolated from log phase culture of *S. aureus* V329 and *E. coli* strains using a QiaAmp DNA mini kit according to the manufacturer's protocol, except that the staphylococcal cells were lysed by lysostaphin (5 mg ml^{-1}; Sigma) at 37°C for 2 h before DNA extraction. Plasmid DNA was isolated from *E. coli* strain using a Qiagen plasmid miniprep kit according to the manufacturer's protocol.

Cloning of *bap* gene into a cloning host (*E. coli* Stbl2)

A primer pair, BFNd1 and BRXh1 (Table 1) with the NdeI-XhoI sites (underlined) was used to amplify full *bap* gene from the published sequence of *bap* of *S. aureus* V329 (GenBank accession no. AY220730.1). and cloned upstream from the His tag sequence in the pET-21b vector (Novagen). Amplification was carried out on a Master cycler (Eppendorf, Hamburg, Germany) using Qiagen LongRange PCR Kit with a reaction mixture of 50 µl. The PCR programme included an initial denaturation at 93°C for 5 min followed by 35 cycles of denaturation (93°C for 15 sec), annealing (56°C for 30 s) and extension (68°C for 8 min). Final extension was carried out at 68°C for 15 min. The PCR product was characterized by gel electrophoresis on 1% agarose gel with pre-stained ethidium bromide (10 mg/ml) in 0.5 M Tris–EDTA electrophoresis buffer. The DNA fragment of interest was excised from the gel and extracted from the gel using QIA quick gel extraction kit (Qiagen, Germany). Purified product as well as pET21b (+) (Novagen, Madison, WI) was kept for overnight digestion with restriction enzymes NdeI and XhoI (New England Biolabs, India) to generate sticky ends. Digested DNA fragment and pET21b (+) were mixed (using 3:1 molar ratio) in the ligation reaction mix (2 µl T4 DNA ligase buffer, 1 µl T4 DNA Ligase; 30 ng of pET21b (+) and 100 ng of PCR product in 20 µl reaction mixture) and incubated at room temperature for overnight. Next day, this ligated product was used for transformation using competent *E. coli* (Stbl2, Invitrogen) cells by the classical heat shock method.

Confirmation of cloning of *bap* gene by colony PCR and PCR using *bap* internal primers

Recombinant colonies were randomly picked up from the plate, resuspended in 200 µl of 50 mM NaOH and boiled for 5 min, then quickly placed to ice for 2 min and 32 µl of 1 M Tris-Cl was added. Sample was centrifuged at 10000 RPM for 5 min. 2 µl of supernatant was used for PCR reaction. To carry out PCR with *bap* internal primers, a single colony was picked up from the plate and inoculated in LB broth containing ampicillin (50 µg ml^{-1}) and incubated overnight at 30°C and 150 rpm. The plasmid was extracted using Qiagen Plasmid Miniprep kit following manufacturer's protocol. Extracted plasmid was used as template to carry out PCR reactions.

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 s) 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^{-1}) in 0.5 M TAE electrophoresis buffer. A GeneRuler™ 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^{-1}) at 30°C. Overnight grown cultures were diluted 1:100 into fresh 500 mL LB broth supplemented with carbenicillin (50 µl ml^{-1}) and incubated at 30°C with shaking at 150 rpm to optical density (OD_{600}) 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^{-1} 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 a 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	CCCTATATCGAAGGTGTAGAATTGCAC
sab R	GCTGTTGAAGTTAATACTGTACCTGC
BFNd1	GAGGTGAGTA**CATATG**GGAAATAAACAAGGTTTTTTACC
BRXh1	CAATAATTTAAA**CTCGAG**TTTTTTATCATTTTCTTTTCT ACGACG

Table 1: List of primers used in the study.

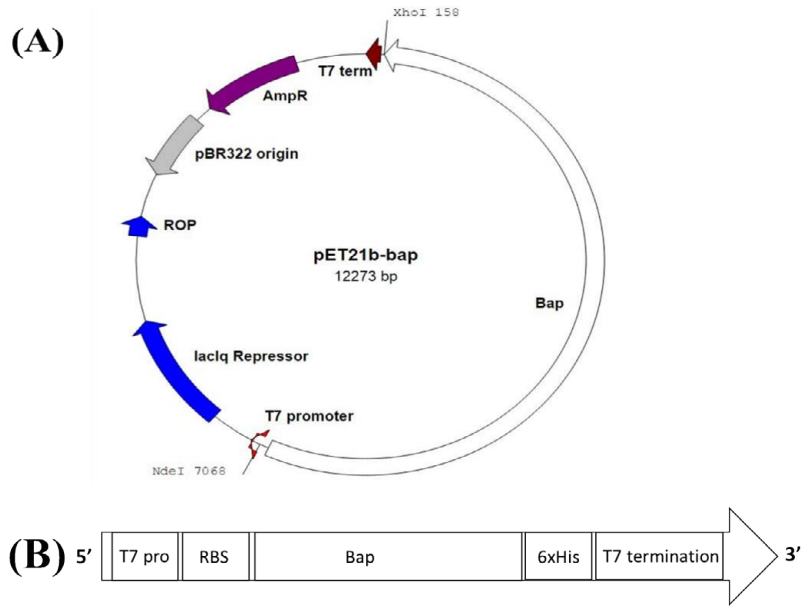

Figure 1: Expression plasmid pET21b (+) used for Biofilm associate protein (Bap) expression. **(A)** Schematic representation of recombinant pET21b-*bap* expression vector. **(B)** Diagram shows the essential elements of the construct for *E. coli* expression. T7 promoter; ribosome binding site, *bap* gene, 6x His Tag and T7 termination element.

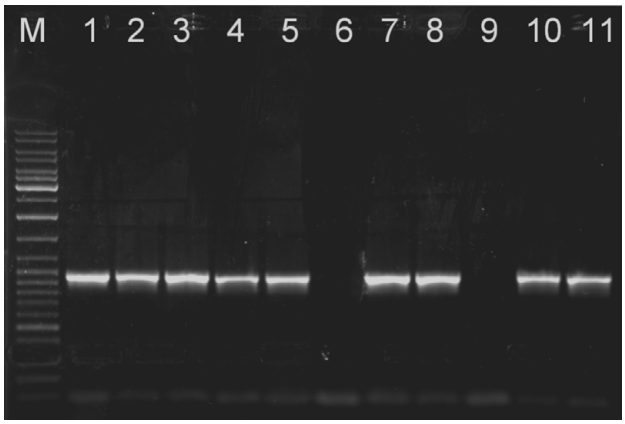

Figure 2: Confirmation of positive selection of transformed cells by Colony PCR. Lane M: DNA marker; Lane 1 to 11: 971 bp long PCR products amplified from cell lysate.

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 (C-term) 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).

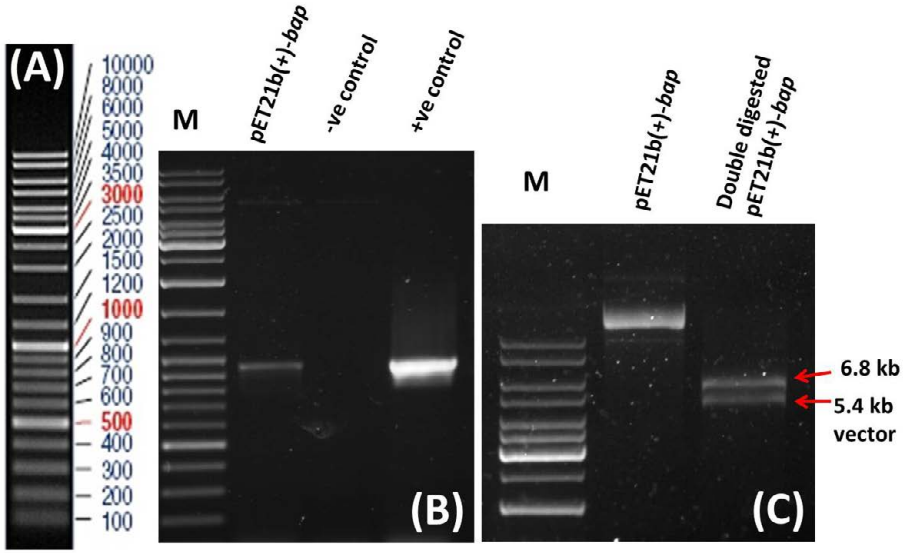

Figure 3: (A) DNA ladder showing size of respective bands. **(B)** Detection 971 bp *bap* fragment: lane 1- DNA ladder; lane 2- pET-*bap*; lane 3- negative control i.e. empty vector pET21b(+); lane 4- +ve control i.e. genomic DNA of *S. aureus* V329. **(C)** Confirmation of cloning of *bap* by release of *bap* gene from recombinant pET21b(+)-*bap* expression vector by RE digestion with NdeI and XhoI. Lane M- DNA marker; Lane 2- pET21b(+)-*bap*; Lane empty expression vector pET21b(+) (5.4 kb fragment) releasing *bap* gene insert (6.8 kb fragment).

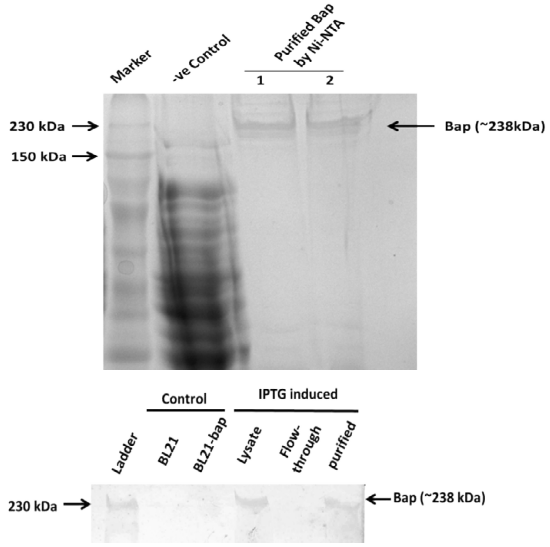

Figure 4: Analysis of expression, purification and identification of the Bap protein with C-terminal fused His-tag by 10% SDS–PAGE and Western blotting. **(A)** The SDS–PAGE analysis of pET21b-*bap* expression in BL21 (DE3) induced by 1 mM IPTG. Lane 1, molecular weight marker; lane 2, uninduced *E. coli* BL21 (DE3)/pET21b-bap; lane 3, induced *E. coli* BL21(DE3)/pET21b-*bap*; lane 3 & 4, purified fused Bap protein with His_6 tag with Ni-NTA **(B)** Western blotting analysis of the Bap. Over-expressed Bap with c-terminal His_6-tag was detected in *E. coli* BL21(DE3)/pET21b-*bap* lysate and purified sample by Ni-NTA column with anti-His_6-Tag antibodies.

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_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_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 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* DH5-α 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-direct 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-His_6-Tag antibodies substantiated the above speculation (Figure 2B). Therefore, *E. coli* stbl2 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 Stbl2 cell culture at lower temperature (at 30°C) the presence of full *bap* gene was 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 supresses 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_6 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 SaPIbov2, 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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