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Molecular Analysis of the Acinetobacter baumannii Biofilm-Associated Protein

H. M. Sharon Goh,* Scott A. Beatson,* Makrina Totsika,* Danilo G. Moriel,* Minh-Duy Phan,* Jan Szubert,* Naomi Runnegar,* Hanna E. Sidjabat,† David L. Paterson,#,#,#,# Graeme R. Nimmo,** Jeffrey Lipman,** Mark A. Schembri*

Acinetobacter baumannii is a multidrug-resistant pathogen associated with hospital outbreaks of infection across the globe, particularly in the intensive care unit. The ability of A. baumannii to survive in the hospital environment for long periods is linked to antibiotic resistance and its capacity to form biofilms. Here we studied the prevalence, expression, and function of the A. baumannii biofilm-associated protein (Bap) in 24 carbapenem-resistant A. baumannii ST92 strains isolated from a single institution over a 10-year period. The bap gene was highly prevalent, with 22/24 strains being positive for bap by PCR. Partial sequencing of bap was performed on the index case strain MS1968 and revealed it to be a large and highly repetitive gene approximately 16 kb in size. Phylogenetic analysis employing a 1,948-amino-acid region corresponding to the C terminus of Bap showed that BapMS1968 clusters with Bap sequences from clonal complex 2 (CC2) strains ACICU, TCDC-AB0715, and 1656-2 and is distinct from Bap in CC1 strains. By using overlapping PCR, the bapMS1968 gene was cloned, and its expression in a recombinant Escherichia coli strain resulted in increased biofilm formation. A Bap-specific antibody was generated, and Western blot analysis showed that the majority of A. baumannii strains expressed an ~200-kDa Bap protein. Further analysis of three Bap-positive A. baumannii strains demonstrated that Bap is expressed at the cell surface and is associated with biofilm formation. Finally, biofilm formation by these Bap-positive strains could be inhibited by affinity-purified Bap antibodies, demonstrating the direct contribution of Bap to biofilm growth by A. baumannii clinical isolates.

Acinetobacter baumannii is a Gram-negative bacterial pathogen associated with multidrug resistance and hospital outbreaks of infection, particularly in the intensive care unit (1). A. baumannii accounts for almost 80% of all reported Acinetobacter infections, including ventilator-associated pneumonia, bacteremia, meningitis, peritonitis, urinary tract infections, and wound infections (2, 3). The rapid emergence of multidrug-resistant A. baumannii strains has resulted in limited treatment options, with most strains being resistant to clinically useful antibiotics, such as aminoglycosides, fluoroquinolones, β-lactams (including carbapenems), tetracyclines, and trimethoprim-sulfamethoxazole (4, 5).

In addition to antibiotic resistance, the ability to form biofilms represents an important factor associated with A. baumannii virulence. Biofilms are sessile bacterial communities enclosed in a matrix comprised of extracellular material that can include polysaccharide, protein, and DNA (6). Biofilm formation by bacterial pathogens is associated with enhanced tolerance to host immune defenses, disinfectants, and antimicrobials (7, 8). A. baumannii strains readily form biofilms in vitro, and some of the molecular mechanisms associated with this phenotype have been studied; genes associated with biofilm formation include the csu locus (encoding the chaperone-usher Csu fimbriae), the pga locus (encoding the polysaccharide poly-N-acetylglucosamine [PNAG]), ompA (encoding the outer membrane protein OmpA), and bap (encoding the biofilm-associated protein [Bap]) (9–15).

A. baumannii Bap (Bapba) is a cell surface protein associated with biofilm formation. In the A. baumannii bloodstream isolate 307-0294, Bapba307-0294 is a large (854-kDa) protein comprised of multiple copies of repeat elements (13). Mutation of bap in A. baumannii 307-0294 resulted in decreased biofilm growth and decreased adherence to human bronchial epithelial and neonatal keratinocyte cells (13, 16). Bap homologues have also been identified and characterized in other bacteria, including members of other genera typically associated with hospital-acquired infection, such as Staphylococcus (17), Enterococcus (18, 19), and Pseudomonas (20, 21). Staphylococcus aureus Bap (BapSa) has been well characterized and is an important virulence factor that contributes to initial attachment, intercellular adhesion, and biofilm maturation (17, 22). Bap proteins from other organisms contribute to different stages of biofilm formation and adhesion to eukaryotic host cells (17, 22).

We previously assessed the molecular epidemiology of A. baumannii within a single, large institution and showed that A. baumannii strains from sequence type 92 (ST92) were dominant over a 10-year period (5). In this study, we examined the role of Bap in these A. baumannii ST92 strains. We show that almost all A. baumannii ST92 strains express Bap and that its expression is strongly associated with biofilm formation. This is the first analysis of Bap...
The A. baumannii bapenem-resistant ST92 clinical isolates were selected from a collection of bacterial strains, plasmids, and growth conditions.

**MATERIALS AND METHODS**

**Determined by Western blot analysis.**

**Determined by PCR.**

**Strain designation reported in reference 5.**

function in A. baumannii ST92 strains associated with hospital infection outbreaks.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Twenty-four carbapenem-resistant ST92 clinical isolates were selected from a collection of A. baumannii (isolated between 1999 and 2009) that caused sporadic and outbreak cases at the Royal Brisbane and Women’s Hospital, Brisbane, Australia (Table 1), some of which have been described previously (5). The Escherichia coli strains MS2989 (DH10B containing plasmid pSG25; bapMS1968 in pBR322) and MS3640 (DH10B containing the vector control plasmid pBR322) were used. A. baumannii strains were routinely grown at 28°C in tryptic soy broth (TSB; Becton, Dickinson) supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) as required. E. coli strains were cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) as required.

**DNA manipulations and genetic techniques.** Chromosomal DNA was extracted from A. baumannii strains by previously described methods (23). PCR was performed using either Taq polymerase (New England BioLabs) or an Expand long-template PCR system (Roche) according to the manufacturer’s instructions. PCR products were purified using a QIAquick PCR purification kit or a QIAquick gel extraction kit with spin columns according to the manufacturer’s instructions (Qiagen). Standard cloning techniques were employed to construct recombinant plasmids (24); plasmid DNA was isolated using a QIAprep spin miniprep or midiprep kit (Qiagen). DNA sequencing reactions were carried out with an ABI BigDye terminator sequencing kit (version 3.1) (Applied Biosystems).

**PCR screening of the bap gene.** The 24 ST92 A. baumannii clinical isolates were screened for the presence of the bap gene by using primers 1415F (5’-TTATTGCTCAG) and 1416R (5’-TTATTGCTCAG). This gene region corresponded to the region selected for anti-Bap serum production.

**Size determination and cloning of the bap gene.** In order to ascertain the exact size of the MS1968 bap gene, a long-range PCR was performed using Expand long-template PCR system 1 (primers 1649F [5’-CTAGCC AACCATGCTATGCAAAAT] and 1652R [5’-GCCGGGATCCCGCGACATTCTAAGCAAAATGCGT]). Amplification products were then resolved on a low-percent-agarose gel using the lambda DNA/HindIII marker (Fermentas) as a reference, and the product size was estimated using Bio-Rad Image Lab software (Bio-Rad). For cloning bap into pBR322, the bap gene of MS1968 was amplified in two sections: the 5’ fragment (primers 1649F and 1650R [GGCCGGGATCCCGCGACATTCTAAGCAAAATGCGT]) and the 3’ fragment (1651F [5’-CTTGGTAGGCGGAGCAGTAG] and 1652R). The 5’ fragment was digested with BntI and ligated into the BntI/BamHI sites of pBR322 to generate plasmid pSG24. Screening primers 1415F and 1416R were used to verify the presence of the 5’ bap fragment on pSG24, and primers 831F (5’-GC GCTCATGTCATCTGCTCTC) and 1161R (5’-CCCTATAGGCGTACTCCGC), which target the plasmid at the function sites, were used to verify the cojoining plasmid-insert region by sequencing. The 3’ fragment was digested with BsrGI/BamHI and ligated into the BsrGI/BamHI sites of pSG24 to generate pSG25. This plasmid was verified by sequencing the 5’ and 3’ joining sites. The confirmed clone (MS2989) was then tested for Bap expression and biofilm formation.

**DNA sequencing, assembly and bioinformatics.** The sequence of the bapMS1968 gene in pSG25 was determined by primer walking and Sanger sequencing, and sequence reads were manually assembled using Vector NTI Advance software (Life Technologies). The assembled DNA sequence of bapMS1968 was compared against bap_AB307-0294 using Easyfig (25). The C-terminal sequence of BapMS1968 (1,948 amino acids) was determined using the BLAST program (NCBI) and aligned with Bap homologues obtained from the NCBI database using Vector NTI Advance and ClustalW2 (26). The alignment generated using Vector NTI Advance was used to determine the region within Bap homologues that corresponded with the C-terminal sequence of BapMS1968. A neighbor-joining tree was generated using MEGA5 (27) by comparing 1,948 amino acids from the C-terminal sequence of BapMS1968 against amino acid sequences of Bap homologues identified in the NCBI database.

**Generation of Bap polyclonal antiserum, affinity purification, and immunoblotting.** A polyclonal antibody against Bap_MS1968 was prepared by amplifying a 1,254-bp segment of the bap_MS1968 gene using primers 1415F and 1416R with ligation-independent cloning (LIC) overhangs flanking both ends of the primers to enable cloning into the pMCSG7-6-histidine N-terminally tagged expression vector (28). The resultant plasmid (pSG13) contained base pairs 132 to 1,386 of bap_MS1968 fused to an N-terminal 6×His-encoding sequence. This bap sequence corresponds to amino acid residues 45 to 462 (418 amino acids) of the Bap_MS1968 sequence. E. coli BL21 was transformed with plasmid pSG13, and the desired clones were confirmed by PCR and sequencing using primers 1508F (5’-TAATACGACTCCTATAGGG) and 1509R (5’-TATGCTGTTATGCTGTC). MS2788 (BL21 + pSG13) was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the resultant fusion protein was purified using a Qiagen nickel-nitrioltriacetic acid (Ni-NTA) spin kit according to the manufacturer’s instructions. Protein was assessed for purity by SDS-PAGE analysis and quantified using a bicinchoninic acid kit (Sigma-Aldrich) (29). A polyclonal anti-Bap serum was raised in rabbits at the Institute of Medical and Veterinary Sciences (Adelaide, South Australia, Australia).

Affinity chromatography was used to purify Bap-specific antibodies from the rabbit polyclonal anti-Bap serum as follows. A 30-ml culture of MS2788 was grown at 37°C to an optical density of 600 nm (OD600) of 0.6 in the presence of 1 mM IPTG. Cells were pelleted via centrifugation and resuspended in chilled sonication buffer (25 mM Tris, 150 mM NaCl, pH 7.0). The suspension was sonicated three times with a 30-s burst and a 2-min incubation on ice between bursts. The cell extract was centrifuged.
at 12,000 × g for 20 min at 4°C. The column was prepared using a 1-ml bed volume of Talon cobalt metal affinity resin (Clontech) and equili- 

brated using 5 ml of equilibration/wash buffer (25 mM Tris, 10 mM NaCl [pH 7.0]). The MS2788 cell lysate was applied to the column, and non-

specific proteins were removed using 20 ml of equilibration/wash buffer. An aliquot of the polyclonal Bap antiserum was diluted with an equal volume of Tris-buffered saline (TBS) buffer (pH 7.2 to 7.4) and applied to the affinity column. Nonspecific proteins were removed using equilibration/wash buffer. Bap-specific antibodies bound on the column were eluted using a gentle antigen-antibody (Ag/Ab) elution buffer, pH 6.6 (Pierce). A 30-kDa desalting column (Millipore) was used to concentrate

eluted using a gentle antigen-antibody (Ag/Ab) elution buffer, pH 6.6 and exchange the purified antibodies into TBS buffer. Flowthrough frac-

tions were collected at every step of purification for SDS-PAGE and im-

munoblotting analysis. Immunoblotting was performed as previously de-

scribed (29). A 1:200 dilution of affinity-purified Bap-specific antibodies was used as primary serum, and the secondary antibody was alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich).

Extracellular matrix (ECM) protein binding assays. ECM protein binding by A. baumannii to MaxGel human ECM (Sigma-Aldrich) was performed as described previously, with the exception that wells that were washed with phosphate-buffered saline (PBS) and quenched with 2% bovine serum albumin (BSA) in PBS for 1 h, and overnight bacterial cultures were standardized to an OD_{600} of 1.0 (30). For negative-control wells, PBS was added instead of bacteria. Instead of an enzyme-linked immunosor-

bent assay (ELISA), adherent cells were stained with 0.01% crystal violet for 30 min at room temperature. Wells were washed twice with PBS and incubated with 200 μl ethanol/acetone (80:20) for 1 h at room tempera-

ture with gentle agitation. Absorbance measurements were obtained at 595 nm, and results were analyzed by analysis of variance (ANOVA) (GraphPad Prism 5 software).

Biofilm study. Biofilm formation by A. baumannii on 96-well poly-

styrene plates (Iwaki) was performed by previously described protocols, except that strains were grown at 28°C in TSB under static conditions (31). Biofilm formation by DH10β was performed as described above, except that cells were grown with shaking in polyvinylchloride (PVC) microtiter plates containing M9 supplemented with 0.3% Casamino Acids. Briefly, strains were grown as shaking cultures at 250 rpm for 20 h at 28°C in the appropriate culture medium supplemented with antibiotics, inoculated into microtiter plates with fresh medium, and incubated for 24 h at 28°C; wells were washed to remove unbound cells and subsequently stained with 0.01% crystal violet. Bound cells were quantified by addition of ethanol-

acetone (80:20) and measurement of the solubilized stain at an optical density of 595 nm using a Spectramax 250 microtiter plate reader with SOFTmax Pro v2.2.1 software (Molecular Devices). Readings obtained were analyzed by ANOVA (GraphPad Prism 5 software). These experiments were performed in eight replicates. Inhibition of biofilm formation using Bap affinity-purified antibody was performed using the microtiter plate biofilm protocol mentioned for A. baumannii, except that Bap-spe-

specific antibodies were added to a final concentration of 1:10 before addition of bacteria to the polystyrene plate. Readings obtained were analyzed by ANOVA. This experiment was performed in quadruplicate. Flow chamber biofilm experiments were performed as previously described (32), except that cells were grown in TSB supplemented with ampicillin and detected using 0.1 μM BacLight green fluorescent stain (Molecular Probes). Briefly, biofilms were allowed to form on glass surfaces in a mul-
tichannel flow system that permitted online monitoring of community structures. Flow cells were inoculated with standardized overnight cultures grown in TSB. Biofilm development was monitored by confocal laser scanning microscopy (CLSM) from 19 to 48 h postinoculation. This experiment was performed in duplicate.

Microscopy and image analysis. An anti-Bap serum was used for immunofluorescence microscopy as previously described (33), with modifications where strains were grown in TSB and a 1:5 dilution of the primary antibody was used followed by goat anti-rabbit IgG anti-

body conjugated to fluorescein isothiocyanate (FITC) (1:500) as the secondary antibody. Microscopic observation of biofilms and image acquisition was performed on a confocal laser scanning microscope (LSM510 Meta; Zeiss). Vertical cross sections through the biofilms were visualized using the Zeiss LSM image examiner, and the z stacks were analyzed using COMSTAT software (34). Results were analyzed by ANOVA (Minitab Statistical Software). Images were further pro-

cessed for display by using Photoshop software (Adobe Systems).

Protein sequence accession number. The sequence of Bap_MS1968 has been submitted to the GenBank database under accession numbers AGM37925.

RESULTS

The bap gene is highly prevalent in A. baumannii ST92 strains. Twenty-four carbapenem-resistant A. baumannii ST92 strains isolated from a single institution during a 10-year period from 1999 to 2009 were examined for the presence of the bap gene. Initially, a draft genome sequence of one strain, MS1968, was de-

termined, and this provided a partial sequence for bap, albeit with gaps in the large repeat regions. Based on this sequence, primers 1415F and 1416R were designed to amplify a 1,225-bp segment of bap_MS1968 from a nonrepetitive region. PCR analysis was per-

formed on all 24 A. baumannii ST92 strains, and a product of the correct size was detected in 91.7% (22/24) of the strains, demonstrat-

ing that the bap gene is highly prevalent in our collection (Table 1).

Cloning of the bap gene from A. baumannii MS1968. Based on the draft genome sequence of A. baumannii MS1968 and pre-
liminary PCR assays, the size of bap_MS1968 was estimated to be approximately 16 kb (data not shown). In order to clone bap_MS1968 two overlapping PCR amplicons were generated (a 12,144-bp fragment containing the 5’ region and a 4,170-bp frag-

ment containing the 3’ region). These fragments were cloned into plasmid pBR322 in a two-step process to generate plasmid pSG25, which contained the full-length bap_MS1968 gene.

Sequencing of bap_MS1968 and comparative analysis with other bap genes. In order to close the gap within the bap_MS1968 gene from the draft genome sequence, the sequence of bap_MS1968 was determined from plasmid pSG25 using a primer walking strategy. Approximately 9.5 kb of bap_MS1968, including 3,783 bp of the 5’ region and 5,847 bp of the 3’ region, was sequenced, leaving an estimated 5,500-bp gap that could not be closed by this method (Fig. 1). A nucleotide sequence alignment using ClustalW2 indicated that bap_MS1968 and bap_{AB307-0294} share approximately 50% sequence identity (Fig. 1). The ~5,500-bp unsequenced region of bap_MS1968 is most likely made up of the core repeat module D, thus causing the eventual sequencing problems.

Analysis of the 5,847-bp segment corresponding to the 3’ region of bap_MS1968 revealed an in-frame translated sequence comprising 1,948 amino acids. An amino acid sequence alignment using Clust-

alW2 indicated that this region of Bap_MS1968 shares 37% sequence identity with the corresponding region of Bap_{AB307-0294} (residues 6,669 to 8,620). The amino acid sequence similarity of Bap_MS1968 with other Bap proteins was evaluated using MEGAS5 (27). Figure 2 illustrates a neighbor-joining tree constructed using aligned Bap amino acid sequences from 26 bacterial strains. A consensus tree of 1,000 bootstrap replicates revealed two major clades. The two clades separate the majority of the Gram-negative and Gram-pos-

itive Bap proteins (with the exception of Bordetella bronchiseptica, Pseudomonas fluorescens, and Pseudomonas putida). The predicted Bap protein homologues of A. baumannii cluster within the large clade of the Gram-negative Bap homologues. A scheme for classi-
Figs. 1-5. Physical representation of the nucleotide sequence alignment between bapMS1968 (GenBank accession no. KC811110) and bapAB307-0294 (EU117203) (13). The size of bapMS1968 was determined by PCR (~16 kb), and the sequence was obtained by primer walking. The black bar indicates the region (5,500 bp) that could not be sequenced using primer walking. The yellow arrow indicates the region (1,254 bp) cloned and expressed for antibody production. The magenta bar indicates the region (5,847 bp) selected for phylogenetic analysis (Fig. 3). This figure was generated using EasyFig (http://easyfig.sourceforge.net/) with nucleotide sequence comparison (BLASTn) (25). The level of nucleotide identity is shown in the gradient scale.

Expression of Bap by E. coli harboring pSG25 results in increased biofilm formation. To demonstrate functional expression of Bap from plasmid pSG25 in E. coli, a polyclonal antibody was generated against a conserved, nonrepetitive region within BapMS1968. SDS-PAGE and Western blot analysis of whole-cell lysates of MS2989 (E. coli DH10B containing pSG25) grown in LB broth identified an ~200-kDa protein that reacted with the Bap-specific antiserum (data not shown). A microtiter plate biofilm assay demonstrated that expression of the bap gene by DH10B resulted in significantly increased biofilm formation by MS2989 compared to the vector control strain (MS3640) (Fig. 4). Thus, Bap can be expressed by E. coli, and its expression leads to increased biofilm formation.

Bap is expressed by most A. baumannii ST92 isolates. To investigate the expression of Bap in our collection of 24 A. baumannii ST92 strains, whole-cell lysates were prepared from each strain following overnight shaking growth in TSB and examined by Western blot analysis using the Bap-specific antibody described above. A strong Bap-specific cross-reacting band was detected at ~200 kDa in all but one of the 24 strains tested (95.8%; Table 1). This analysis identified inconsistencies with respect to the PCR prevalence assay; strains MS1976 and MS3003 expressed Bap but were negative in the PCR screen for the bap gene, while MS3007 failed to express Bap but was positive in the PCR screen. Out of the 24 ST92 strains, four strains were selected for further analysis of Bap expression and function, three strains positive for Bap expression (MS3007) (Fig. 5A).

Bap is located at the cell surface. The cellular localization of
Bap in *A. baumannii* ST92 strains was investigated by immunofluorescence microscopy employing our affinity-purified Bap antibody. Consistent with the Western blot analysis (Fig. 5A), the Bap antiserum reacted with MS3009, MS3011, and MS3014. In contrast, no reaction was observed for MS3007 (Fig. 5B). Thus, Bap is effectively expressed and is localized on the cell surfaces of *A. baumannii* strains MS3009, MS3011, and MS3014.

Bap does not mediate binding to ECM molecules. The four strains selected for Bap characterization (MS3007, MS3009, MS3011, and MS3014) were tested for their ability to bind MaxGel, a commercially available mixture of human ECM components, including collagens, laminin, fibronectin, tenascin, elastin, and a number of proteoglycans and glycosaminoglycans. None of the strains displayed significant binding to MaxGel in this assay (data not shown), suggesting that Bap expression by MS3009, MS3011, and MS3014 does not lead to adherence to ECM components under the conditions used in this experiment.

Expression of Bap is associated with strong biofilm formation. Biofilm formation by *A. baumannii* was examined using dynamic and static biofilm assays. The continuous flow chamber method was used to test the ability of Bap to promote biofilm formation under dynamic conditions, which permits monitoring of the bacterial distribution within an evolving biofilm at the single-cell level using scanning confocal laser microscopy. In this assay, the Bap-positive strains MS3009, MS3011, and MS3014 produced a strong biofilm compared to the Bap-negative strain MS3007 (Fig. 7). Taken together, these results demonstrate that the Bap-expressing *A. baumannii* strains MS3009, MS3011, and MS3014 can form strong biofilm.
films, while MS3007, which does not express Bap, does not form a significant biofilm.

**Bap is required for biofilm formation in vitro.** To further characterize the role of Bap in biofilm formation by *A. baumannii* MS3009, MS3011, and MS3014, we performed microtiter plate biofilm assays in the presence of affinity-purified Bap-specific antibody. In these assays, the addition of 1:10-diluted Bap antibody inhibited biofilm formation by all three strains (P < 0.0001) (Fig. 7). These results provide compelling evidence that Bap plays an important role in biofilm formation by *A. baumannii* ST92 strains associated with hospital infection outbreaks.

**DISCUSSION**

*A. baumannii* strains from ST92 and the associated CC92 (also known as European clone 2 or worldwide clone 2) represent the most sampled and widespread *A. baumannii* sequence type across the globe. Antibiotic susceptibility within ST92 is variable, suggesting a role for mechanisms other than antibiotic resistance in its successful dissemination. In this study, we examined the prevalence, sequence, and function of Bap from a collection of *A. baumannii* ST92 strains isolated from a single institution over a 10-year period.

Bap was first detected in *S. aureus* strains that cause bovine mastitis (42). Subsequently, more Bap homologues have been identified and characterized from a range of Gram-positive and Gram-negative bacteria, including *A. baumannii* (13, 17–22,

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**FIG 3** Genome context of the *bap* gene in *Acinetobacter*. (A) Genomic analysis of different *Acinetobacter* species indicates that *bap* is located at the same chromosomal position in all strains examined. The genome orientation was reversed for some strains to facilitate visualization (→). Also indicated are the respective core (black) and variable (green) regions flanking the *bap* gene. Orange triangles indicate the locations of sequence repeats. Genome alignments were performed using Easyfig (25). (B) Alignment of palindromic repeats localized upstream and downstream of the *bap* gene in *Acinetobacter*. The axis is indicated by a gray arrow.

**FIG 4** Microtiter plate biofilm formation by MS2989 in comparison to MS3640. Strains were grown under shaking conditions at 28°C for 24 h in polyvinyl chloride (PVC) microtiter plates containing M9 supplemented with 0.3% Casamino Acids. Plates were washed to remove nonadherent cells and stained with 0.01% crystal violet. Biofilm formation was quantified by solubilizing the crystal violet stain retained by adherent cells with ethanol-acetone (80:20) and measuring the absorbance at 595 nm. Results are the means for eight replicates per strain (± standard deviation). Mean values for MS3640 (0.4604) and MS2989 (0.7425) were calculated using GraphPad Prism 5 software (P < 0.001).
43–51). Common features of Bap in all of these organisms include its large size, the presence of multiple tandem repeats, its cell surface location and its role in biofilm formation. In *Pseudomonas fluorescens*, a large-repeat Bap-like protein referred to as LapA contributes to surface attachment and biofilm formation (21). LapA is translocated to the cell surface by an ABC transporter encoded by the adjacent *lapEBC* genes (52). Similarly, in *Salmonella enterica* serovar Enteritidis, BapA is secreted by a type I protein secretion system (BapBCD) situated downstream of the *bapA* gene (46). Examination of the genetic location of *bap* in *A. baumannii* did not reveal any evidence of a system that could mediate its translocation. Thus, the mechanism by which Bap is transported to the surface of *A. baumannii* remains to be elucidated. We note that a small but significant increase in biofilm formation was observed in the recombinant *E. coli* MS2989 strain expressing Bap, indicating that there may be some level of redundancy in its mode of export. However, we were unable to definitively detect Bap expression on the surface of *E. coli* MS2989 by immunofluorescence microscopy, suggesting that the level of Bap was very low.

Our analysis revealed that the *bap* gene is highly prevalent in *A. baumannii* ST92 strains. All but one *A. baumannii* strain in our collection (i.e., MS3007) also expressed the Bap protein. The in-
consistencies between gene prevalence by PCR and protein expression are most likely due to sequence variation. It is possible that MS3007 harbors an incomplete or truncated \textit{bap} gene. Indeed, Loehfelm et al. previously reported the presence of short homologous regions of \textit{bap}_{\text{AB307-0294}} within the genome sequence of \textit{A. baylyi} and \textit{A. baumannii} ATCC 17978 (13).

The previously characterized \textit{A. baumannii} \textit{Bap}_{\text{AB307-0294}} is a high-molecular-mass (854-kDa) protein consisting of multiple repeat regions (13). In contrast, the \textit{A. baumannii} ST92 strains examined in this study all expressed a Bap protein of approximately 200 kDa. A partial sequence of the \textit{bap} gene was obtained from one strain, MS1968, which represented the index case isolate from a small outbreak in 2001. Given that the \textit{A. baumannii} MS1968 \textit{bap} gene is \textasciitilde{}16 kb, we expected it to encode a significantly larger protein. It is possible that Bap\textsubscript{1968} is degraded or even processed; however, this remains to be determined. The difference in the size of the \textit{bap} genes from \textit{A. baumannii} strains MS1968 (\textasciitilde{}16 kb) and AB307-0294 (25.863 kb), despite their similar genetic context, also demonstrates that there is significant variation in the \textit{bap} genes from different \textit{A. baumannii} strains. The \textit{A. baumannii} Bap protein contains a modular structure (53), and the presence of large, identical repeat sequences within module D of \textit{bap}_{\text{MS1968}} prevented us from generating a complete sequence of the gene. However, we did identify a nonrepetitive sequence that was used to examine the phylogeny of Bap from several species. In comparison to Bap\textsubscript{AB307-0294} (which clustered in CC1), Bap\textsubscript{MS1968} clustered in CC2. The two Bap sequences exhibited significant variation and displayed only 37% amino acid identity over this region. Further analysis of Bap from other CC1 and CC2 strains was consistent with this clustering, and suggests that the nonrepetitive sequence of Bap can differentiate between CC1 and CC2 strains. When analyzed in the context of Bap sequences from different organisms, all of the \textit{A. baumannii} Bap homologues clustered uniquely. It remains to be determined if this nonrepetitive region of Bap is representative of its phylogenetic distribution in comparison to the entire protein sequence. However, given the size and highly repetitive nature of Bap, this approach avoided the comparative analysis of regions that might potentially contain multiple sequence errors.

Several lines of evidence suggest that Bap contributes to biofilm formation by \textit{A. baumannii} ST92. First, Bap expression by three \textit{A. baumannii} strains was associated with strong biofilm growth, while the \textit{A. baumannii} ST92 strain MS3007, which did not express Bap, did not form a biofilm in microtiter plate- and flow cell-based assays. Additionally, affinity-purified Bap-specific antibodies blocked Bap-mediated biofilm formation by \textit{A. baumannii} strains MS3009, MS3011, and MS3014. Taken together, our results demonstrate a role for Bap in biofilm formation that is consistent with previous literature examining other \textit{A. baumannii} strains (13, 16). Our results should provide the basis for more detailed studies to examine the translocation and function of Bap in \textit{A. baumannii}, including other common multidrug-resistant sequence types associated with hospital infection outbreaks.

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Biofilm-Associated Protein of A. baumannii

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