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<th><strong>Title</strong></th>
<th>Molecular analysis of the acinetobacter baumannii biofilm-associated protein</th>
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<td><strong>Author(s)</strong></td>
<td>Goh, Hwee Mian Sharon; Beatson, Scott A.; Totsika, Makrina; Moriel, Danilo G.; Phan, Minh-Duy; Szubert, Jan; Runnegar, Naomi; Sidjabat, Hanna E.; Paterson, David L.; Nimmo, Graeme R.; Lipman, Jeffrey; Schembri, Mark A.</td>
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A. 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 multidrug-resistant pathogen associated with 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, ß-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 (BapAb) is a cell surface protein associated with biofilm formation. In the A. baumannii bloodstream isolate 307-0294, BapAb307-0294 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

Received 30 April 2013 Accepted 7 August 2013
Published ahead of print 16 August 2013
Address correspondence to Mark A. Schembri, m.schembri@uq.edu.au.
* Present address: H. M. Sharon Goh, Singapore Centre on Environmental Life Sciences Engineering, School of Biological Sciences, Nanyang Technological University, Singapore.
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TABLE 1 Prevalence and expression of bap in A. baumannii ST92 clinical isolates

<table>
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<tr>
<th>Isolate</th>
<th>bap gene</th>
<th>Bap expression</th>
<th>Yr of isolation</th>
<th>Previous designation</th>
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<tr>
<td>MS1962</td>
<td>+</td>
<td>+</td>
<td>2004</td>
<td>Q11</td>
</tr>
<tr>
<td>MS1966</td>
<td>+</td>
<td>+</td>
<td>2001</td>
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<td>2004</td>
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</tr>
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<tr>
<td>MS3014</td>
<td>+</td>
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* Determined by PCR.
* Determined by Western blot analysis.
* 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; bap$_{MS1968}$ 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’-TTATCCACTTCCATGATCAGCAACCAAACCGCT) and 1416R (5’-TTATCCACTTCCATGATCAGCAACCAAACCGCT) as required. 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 AACCATTGATGATCCAAAT] and 1652R [5’-GGCGGGAATCCCGCAT GAACCTCTTACAGTAGG]) and Amplification products were then resolved on a low-percentage-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 [GGCGGGAATCCCGCATGAACCTCTTACAGTAGG]) and the 3’ fragment (1651F [5’-CTTGGTAGCGGCGG AGCAGTAG] and 1652R). The 5’ fragment was digested with BcmII and ligated into the BcmII/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 GCTCATGCTCATCCT] and 1161R [5’-CCCTTATGCGACTCCT] target the plasmid at the junction 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 bap$_{MS1968}$ 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 bap$_{MS1968}$ was compared against bap$_{ABA367-0294}$ using Easyfig (25). The C-terminal sequence of Bap$_{MS1968}$ (1,948 amino acids) was determined using the BLASTp 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 Bap$_{MS1968}$. A neighbor-joining tree was generated using MEGA5 (27) by comparing 1,948 amino acids from the C-terminal sequence of Bap$_{MS1968}$ 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 vector (23). The confirmed clone (MS2989) was then tested for Bap expression and biofilm formation. 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 vector (23). The confirmed clone (MS2989) was then tested for Bap expression and biofilm formation.

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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 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 equilibrated 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 nonspecific 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 to 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 and exchange the purified antibodies into TBS buffer. Flowthrough fractions were collected at every step of purification for SDS-PAGE and immunoblotting analysis. Immunoblotting was performed as previously described (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 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 immunosorbent 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 temperature 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 polystyrene 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-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 multichannel 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 antibody 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 processed 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 AGM7925.

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 determined, and this provided a partial sequence for bap, albeit with gaps in the large repeat regions. Based on this sequence, primers 1413F and 1416R were designed to amplify a 1,225-bp segment of bap from a nonrepetitive region. PCR analysis was performed on all 24 A. baumannii ST92 strains, and a product of the correct size was detected in 91.7% (22/24) of the strains, demonstrating 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 preliminary 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 fragment 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 ClustalW2 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 MEGA5 (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-positive 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-
flying A. baumannii into clonal complexes (CC) was proposed by Diancourt et al. in 2010 and reported AB307-0294, AB0057, and AYE as representatives of CC1, whereas European clone II isolates ACICU, TCDC-AB0715, and 1656-2 represented CC2 (35–37). Consistent with this scheme, BapMS1968 clustered together with Bap from other CC2 strains. The separate clustering of CC1 and CC2 Bap proteins indicates the presence of Bap variants within A. baumannii.

We also examined the genetic context of bap in A. baumannii. Based on the draft genome sequence of MS1968, the chromosomal location 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, MS3011 and MS3014) and one strain negative for Bap expression (MS3009) (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.

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. 6). Taken together, these results demonstrate that the Bap-expressing A. baumannii strains MS3009, MS3011, and MS3014 can form strong biofilm-Associated Protein of A. baumannii

FIG 2 Neighbor-joining tree indicating sequence similarity of BapMS1968 (GenBank accession no. AGM37925) in relation to Bap homologues from A. baumannii strains (TCDC-AB0715 [accession number ADX93581]; ACICU [ACCS8250, ACCS8252 to ACCS8258]; 1669-2 [ADX40628 to ADX40634]; ATCC 19560 [EEX02997]; ATCC 17978 [ABO13109]; AB307-0294 [ABX00640]; AYE [CAM85746]; AB0057 [ACI41698]), Acinetobacter baylyi (CAG69594), Burkholderia cepacia (AAT36485), Salmonella enterica serovar Enteritidis (AB46037), Salmonella enterica serovar Typhi (NP_806534), Vibrio parahaemolyticus (NP_809463), Escherichia coli (ACB16711), Listeria monocytogenes (CAG85314), Staphylococcus aureus (AAT36485), Staphylococcus epidermidis (AAY28519 and AAK29746 [Bhp]), Bordetella bronchiseptica (AAG39411), Pseudomonas fluorescens (AAY95454), Pseudomonas putida (NP_743337), Enterococcus faecalis (AAD99588), Enterococcus faecium (EFH34349), Lactobacillus reuteri (EDX43426), and Streptococcus pyogenes (AAD39805). Sequences were aligned using ClustalW2, and the phylogenetic tree was generated in MEGA5 by comparing 1,948 amino acids from the C-terminal sequence of BapMS1968. Numbers at the branches indicate confidence values determined from 1,000 bootstrap replications. The A. baumannii Bap proteins cluster according to their CC designations; a CC has not been proposed for the ATCC strains. The red arrow indicates the most recent common ancestor shared by CC1 and CC2 Bap proteins. The two major clades demonstrate separate clustering of Gram-negative and Gram-positive Bap homologues (with the exception of L. monocytogenes and V. parahaemolyticus).
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*/H11021 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,

**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).
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-

**FIG 5** (A) Western blot obtained using Bap-specific antiserum showing expression of Bap (~200 kDa) in *A. baumannii* MS3009 (lane 3), MS3011 (lane 4), and MS3014 (lane 5) but not MS3007 (lane 2) from whole-cell lysates of overnight shaking cultures. Molecular mass markers (HiMark prestained protein standard) are indicated in lane 1. (B) Immunofluorescence microscopy demonstrating surface localization of Bap. Phase contrast (i) and fluorescence (ii) images of MS3007, MS3009, MS3011, and MS3014 cells following overnight growth with agitation at 28°C are shown. Bar, 5 μm.

**FIG 6** Flow chamber biofilm formation by MS3007 (A), MS3009 (B), MS3011 (C), and MS3014 (D). Biofilm development was monitored by CLSM 48 h postinoculation. Substratum coverage of each strain is as follows: MS3007, 29.96%; MS3009, 53.26%; MS3011, 60.7% and MS3014, 60.38% (P = 0.002). Micrographs represent horizontal sections. Depicted to the right of and below each panel are the *yz* plane and *xz* plane, respectively, at the positions indicated by the lines.
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 bap gene. Indeed, Loehfelm et al. previously reported the presence of short homologous regions of bap\textsubscript{AB307-0294} within the genome sequence of A. baylyi and A. baumannii ATCC 17978\textsubscript{e} (13).

The previously characterized A. baumannii Bap\textsubscript{AB307-0294} is a high-molecular-mass (854-kDa) protein consisting of multiple repeat regions (13). In contrast, the A. baumannii ST92 strains examined in this study all expressed a Bap protein of approximately 200 kDa. A partial sequence of the bap gene was obtained from one strain, MS1968, which represented the index case isolate from a small outbreak in 2001. Given that the A. baumannii MS1968 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 bap genes from A. baumannii strains MS1968 (~16 kb) and AB307-0294 (25.863 kb), despite their similar genetic context, also demonstrates that there is significant variation in the bap genes from different A. baumannii strains. The A. baumannii Bap protein contains a modular structure (53), and the presence of large, identical repeat sequences within module D of bap\textsubscript{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 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 A. baumannii ST92. First, Bap expression by three A. baumannii strains was associated with strong biofilm growth, while the 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 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 A. baumannii strains (13, 16). Our results should provide the basis for more detailed studies to examine the translocation and function of Bap in A. baumannii, including other common multidrug-resistant sequence types associated with hospital infection outbreaks.

ACKNOWLEDGMENTS
This work was supported by grants from the Australian National Health and Medical Research Council, The University of Queensland, the Royal Brisbane and Women’s Hospital, and the Royal Brisbane and Women’s Hospital Foundation. M.A.S. was supported by an Australian Research Council (ARC) Future Fellowship (FT100100662). M.T. was supported by an ARC Discovery Early Career Researcher Award (DE130101169).

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FIG 7 Microtiter plate assay demonstrating biofilm formation by MS3007, MS3009, MS3011, and MS3014. Biofilm inhibition was performed by supplementing respective wells with a 1:10 dilution of affinity-purified Bap antibodies in TSB to assess inhibitory effects on biofilm formation. Plates were incubated under static conditions at 28°C for 24 h then washed to remove nonadherent cells and subsequently 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 of quadruplicates for each strain (± standard deviations).


