Identification and Characterization of an *Acinetobacter baumannii* Biofilm-Associated Protein

**Running Title:** *A. baumannii* biofilm-associated protein

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Abstract

We have identified a homologue to the Staphylococcal biofilm-associated protein (Bap) in a bloodstream isolate of *Acinetobacter baumannii*. The fully-sequenced open reading frame is 25,863 bp and encodes a protein with a predicted molecular mass of 854 kDa. Analysis of the nucleotide sequence reveals a repetitive structure consistent with bacterial cell-surface adhesins. Bap-specific monoclonal antibody (mAb) 6E3 was generated to an epitope conserved among 41% of *A. baumannii* strains isolated during a recent outbreak in the US military health care system. Flow cytometry confirms that the mAb 6E3 epitope is surface-exposed. Random transposon mutagenesis was used to generate *A. baumannii bap1302::EZ-Tn5*, a mutant negative for surface-reactivity to mAb 6E3 in which the transposon disrupts the coding sequence of *bap*. Time-course confocal laser scanning microscopy and three-dimensional image analysis of actively growing biofilms demonstrates that this mutant is unable to sustain biofilm thickness and volume, suggesting a role for Bap in supporting the development of the mature biofilm structure. This is the first identification of a specific cell-surface protein directly involved in biofilm formation by *A. baumannii*, and suggests that Bap is involved in intercellular adhesion within the mature biofilm.
Introduction

Acinetobacter spp. are Gram-negative aerobic coccobacilli that are ubiquitous in nature, persistent in the hospital environment, and cause a variety of opportunistic nosocomial infections (1). A number of species of Acinetobacter are associated with human infection, including genomic species 3 and 13TU (8, 46), although A. baumannii is generally regarded as the major pathogen. A. baumannii is a causative agent of nosocomial pneumonia, bacteremia, meningitis, and urinary tract infection (1), and more recently has caused serious infections among American military personnel serving in Iraq and Afghanistan (12, 38). Because it is often multi- or pan-drug resistant, infections are difficult to treat (17), resulting in attributable mortalities of up to 23% for hospitalized patients and 43% for patients under intensive care (16). Indeed, the Antimicrobial Availability Task Force of the Infectious Diseases Society of America recently identified A. baumannii, along with Aspergillus spp., extended-spectrum β-lactamase-producing Enterobacteriaceae, vancomycin-resistant Enterococcus faecium, Pseudomonas aeruginosa, and methicillin-resistant Staphylococcus aureus, as “particularly problematic pathogens” for which there is a desperate need for new drug development (42). In the case of A. baumannii, there is an additional unmet need for an understanding of its basic pathogenesis.

Most A. baumannii research to date has focused on cataloging and understanding the variety of antimicrobial resistance genes and mechanisms found within the species (3, 30, 45, 50). An intriguing observation that ethanol stimulates virulence of A. baumannii (39) led to the identification of a number of genes affecting virulence towards Caenorhabditis elegans and Dicytostelium discoideum (40) that await further characterization. A well-characterized porin of A. baumannii, the 38kDa outer membrane protein A, has been shown to induce apoptosis of eukaryotic cells (9) and to activate dendritic cells leading to the differentiation of CD4+ T-cells.
towards a Th1 phenotype (26). Finally, it was noted that A. baumannii forms biofilms with enhanced antibiotic resistance (48, 49), and more recently that a chaperone-usher secretion system involved in pilus assembly affects biofilm formation (44).

Biofilms are highly-structured communities of bacteria attached to a surface (41), and are recognized as a common cause of human infection (10). It has been proposed that all bacterial biofilms have a number of functionally-conserved components in common, including the production of an extracellular polysaccharide matrix, GGDEF/EAL-domain-mediated intracellular signaling, and large surface adhesins homologous to the biofilm-associated protein (Bap) first identified in S. aureus (23). Bap family members are defined as high molecular weight proteins that are present on the bacterial surface, contain a core domain of tandem repeats, and confer on bacteria the ability to form a biofilm (24). Since the initial identification of Bap, homologues have been identified in at least 13 pathogenic species (24, 25) and the proteins generally share structural and functional similarities, though not necessarily primary sequence similarity.

In this study, we have identified and fully sequenced a gene encoding a Bap homologue in A. baumannii 307-0294. We have generated a transposon-insertion mutant deficient in Bap surface expression, and a specific monoclonal antibody (mAb) 6E3 recognizing Bap. The epitope recognized by mAb 6E3 is surface-accessible and conserved among 41% of A. baumannii isolates recovered during the US military health care system outbreak. Quantitative comparison of biofilms formed by a Bap-deficient mutant and wild-type bacteria demonstrates that the mutant is unable to sustain biovolume and biofilm thickness development.
Materials and Methods

Bacterial strains and culture conditions. Wild-type *A. baumannii* strain 307-0294 was isolated from the bloodstream of a patient in 1994. A library of 98 *Acinetobacter* strains was obtained from the Walter Reed Army Medical Center, including 76 isolates of *A. baumannii*, 13 of genome species 3, 5 of species 13TU, 1 of genome species 10, and 3 otherwise uncharacterized *Acinetobacter* spp. *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA) was used as the host strain for all plasmid manipulations.

All *Acinetobacter* strains were cultured in Mueller-Hinton medium (MH broth or agar), supplemented with kanamycin (50 μg/mL) and carbenicillin (200 μg/mL) when appropriate. For biofilm studies, FAB medium, (0.1mM CaCl₂, 0.15mM (NH₄)₂SO₄, 0.33mM Na₂HPO₄, 0.5mM NaCl, and 0.2mM KH₂PO₄) supplemented with 10mM sodium citrate and, unless otherwise indicated, 0.5% (w/v) casamino acids, (FAB-citrate) was used. Static culture biofilm experiments for time course confocal microscopy were incubated at 37°C in room air on a heated microscope stage; in all other cases, bacteria were grown at 35.5°C in 5% CO₂.

Plasmid pMU125, an *E. coli*-Acinetobacter shuttle vector conferring green fluorescent protein (GFP) expression (14), was generously provided by Luis Actis.

DNA and RNA manipulations. Routine DNA manipulations were performed using standard procedures (37). Chromosomal DNA was purified as previously described (36). Restriction endonucleases were supplied by New England Biolabs Inc. (Ipswich, MA) and Promega Corp. (Madison, WI); assays were performed as recommended by the manufacturer. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA).
Total RNA was isolated using the RNeasy® Mini kit (QIAGEN, Santa Clara, CA), and transcripational analysis was performed using the OneStep Reverse Transcriptase-PCR (RT-PCR) kit (QIAGEN) and the primers listed in Table 1.

**Monoclonal antibody development.** mAb 6E3, an IgG1 isotype that reacts to an epitope on Bap, was developed by injecting BALB/c mice intraperitoneally with live A. baumannii 307-0294 suspended in PBS, according to a previously described protocol (5). Hybridoma supernatants were screened by immunodot and Western blot assays for the presence of antibody reactive to whole bacteria, whole cell lysate, and proteinase K-digested whole cell lysate of A. baumannii 307-0294.

Hybridoma cell line 6E3 produced antibody reactive to a proteinase K-sensitive epitope on a high molecular weight antigen. This cell line was used to generate high-titer mouse ascites fluid and protein A affinity purified antibody at a stock concentration of 2.2 mg/mL (Rockland Immunochemicals, Gilbertsville, PA).

**Transposon mutagenesis.** EZ-Tn5™ <Kan-2> Transposomes (Epicentre, Madison, WI) were electroporated into electrocompetent A. baumannii 307-0294, prepared by thrice washing log-phase bacteria (OD_{600nm} ≈ 1) with ice-cold sterile ultrapure water and resuspending in ice-cold sterile 10% glycerol. Electrocompetent cells were stored in 50µL single-use aliquots at -80°C. Transformants were selected with kanamycin and screened for surface reactivity to mAb 6E3 by colony lift assay.

**Flow cytometry.** Wild-type A. baumannii 307-0294 and bap1302::EZ-Tn5 from late-log phase growth (optical density at 600nm [OD_{600nm}] ≈ 1.8) in MH broth cultures were diluted in sterile saline to an OD_{600nm} = 0.03. mAb 6E3 was labeled with the Zenon® Alexa Fluor® 488 Mouse IgG1 kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol. Blocking
antibody, supplied with the kit, was labeled separately as a control for non-specific binding. One microgram of labeled antibody was added to 100 µL of diluted bacteria and incubated at room temperature for 20 minutes. 900 µL of sterile saline was added to the samples and the cells were analyzed in a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) with the following settings: FSC voltage: E02 (log); SSC voltage: 582 (log); FL1 voltage: 665 (log); event threshold: FSC = 434; SSC = 380.

**DNA sequencing and computer analysis.** DNA sequence was determined by the dideoxy chain termination method, using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Biopolymer Resource facility at Roswell Park Cancer Institute (Buffalo, NY). PCR products were cloned into the pGEM T-easy vector (Promega), and genomic DNA into pUC18 after appropriate restriction endonuclease digestion. For sequencing the internal, highly repetitive region of *bap*, chromosomal DNA from nine transposon-insertion mutants, each with insertions in *bap*, was partially digested with Sau3AI, separated by 1.5% agarose gel electrophoresis to allow selection of specific fragment sizes, and ligated into BamHI-digested pUC18. In some cases, nested deletions of the resultant plasmids were generated using the Erase-a-Base system (Promega). Sequence of the genomic regions neighboring *bap* was determined by pyrophosphate-based sequencing-by-synthesis on a 454 Genome Sequencer 20™ DNA sequencing system (Roche Applied Science, Indianapolis, IN) at the New York State Center of Excellence in Bioinformatics and Life Sciences (Buffalo, NY). Sequence similarity searches were performed using the BLAST and BLASTP programs at the NCBI. All other sequence assembly and analysis was performed using the MacVector software package (ver. 7.2.3; MacVector, Inc., Cary, NC).
**SDS-PAGE, Western blot, and colony lift immunoassay.** SDS-PAGE, Western blot, and colony lift analyses were performed using standard procedures (37), with the exception that protein samples were not boiled prior to gel electrophoresis. Equal amounts of protein were loaded in each lane, and gels were stained with the Colloidal Blue Staining kit (Invitrogen). Western blots and colony lift immunoassays were probed with mAb 6E3 and detected colorimetrically with horseradish peroxidase-conjugated secondary antibody (KPL, Inc., Gaithersburg, MD). Whole cell protein extracts were prepared with BugBuster Protein Extraction reagent (Novagen) according to the manufacturer’s protocol. Zwittergent-extracted outer membrane proteins were prepared as described (6).

**Nanoscale liquid chromatography with tandem mass spectrometry.** The protein identification work was performed by ProtTech Inc. (Norristown, PA) using the NanoLC-MS/MS peptide sequencing technology. In brief, each protein gel band was destained, cleaned, and digested in-gel with sequencing-grade modified trypsin. The resultant peptide mixture was analyzed by an LC-MS/MS system, and the mass spectrometric data acquired were used to search the most recent non-redundant and patent protein databases with ProtTech’s proprietary software suite.

**Static culture biofilm growth for confocal microscopy.** For single time-point biofilm imaging, *A. baumannii* 307-0294 and *bap1302::EZ-Tn5* were resuspended from an overnight MH plate and inoculated into 35mm glass bottom Petri dishes (MatTek, Corp., Ashland, MA). The plates were incubated at 35.5°C for 48 hours, then stained with the LIVE/DEAD® BacLight® Bacterial Viability kit (Invitrogen) according to the manufacturer’s instructions. Image stacks were acquired by confocal laser scanning microscopy (CLSM) at 630X magnification on a Zeiss
Axiovert 200M inverted microscope with attached Zeiss LSM 510 Meta NLO imaging system. Images were produced from the raw LSM files using ImageJ, freely available from the NIH.

For time-course biofilm imaging, *A. baumannii* 307-0294 and *bap1302::EZ-Tn5* were transformed by electroporation with pMU125. The resultant GFP-expressing strains were incubated overnight at 35.5°C on MH agar with appropriate antibiotics. Cells were resuspended in FAB-citrate to an OD$_{600nm} = 0.4$, and 10µL was inoculated into 4-well chambered coverglass slides (Nunc; Lab-Tek catalog # 136420) containing 1mL of FAB-citrate. The slides were loaded onto the motorized preheated stage of the CLSM system, and image stacks were acquired every sixty minutes for fourteen hours from two non-overlapping fields-of-view at 400X magnification, covering a total slide surface area of $1 \times 10^5 \mu m^2$, in order to obtain a representative sample of the biofilm (21). The CLSM image stacks were manually edited to remove extraneous images (defined as any image including and below those containing reflections of the glass coverslip, and any images including and above the first image to contain no bright pixels representing bacterial cells) to minimize bias during quantitative image analysis (29). The manually edited image stacks were analyzed using the Image Structure Analyzer-3D program to calculate twenty parameters describing the three-dimensional biofilm structure (2), including biovolume and average thickness at each time point for each field-of-view. Statistical analyses were performed with Prism (version 4.0c; GraphPad Software, Inc.) by fitting the data with the best-fit fourth-order polynomial equation and performing an F test of the null hypothesis that the curves describing biofilm development by wild-type and mutant bacteria were best described by the same equation. 96-well polystyrene plate inverse biofilm experiments were performed as described (28).
Nucleotide sequence accession number. The *A. baumannii* 307-0294 *bap* locus, with each repeat unit annotated, has been deposited in GenBank under accession number EU117203.
Results

Characterization of mAb 6E3. Immunodot assay was initially used to identify mAb 6E3, which reacted to whole cell lysates of *A. baumannii* 307-0294 (data not shown). Western blot analysis demonstrated that the mAb 6E3 epitope is detected on a high molecular weight antigen present in whole cell lysate and outer membrane proteins of *A. baumannii* 307-0294 (Figure 1B). The epitope is sensitive to proteinase K digestion, and electrophoretic mobility of the antigen is modified by heat but not by sodium dodecyl sulfate or β-mercaptoethanol (data not shown).

The mAb 6E3 antigen contains surface-exposed epitopes. Flow cytometry was used to determine if the antigen recognized by mAb 6E3 was surface-accessible. Figure 2 shows that mAb 6E3 binds to an epitope expressed on the surface of wild-type *A. baumannii* 307-0294 (panel A), while the Bap-deficient mutant *bap*1302::EZ-Tn5 (described below) has lost reactivity to this antibody (panel B). These data confirm that the epitope on the high molecular weight antigen recognized by mAb 6E3 is surface-exposed.

The mAb 6E3 epitope is conserved among recent clinical isolates. A panel of 98 *Acinetobacter* strains, representing the 216 isolates recovered during an outbreak in the military health care system (15), was screened by colony lift assay to determine the level of conservation of the mAb 6E3 epitope. The results of these studies demonstrate that 43% of *Acinetobacter* isolates are reactive to mAb 6E3 (Table 2).

Peptide sequence from the mAb 6E3 antigen. The high molecular weight protein band reactive to mAb 6E3 was excised from a Coomassie blue-stained SDS-polyacrylamide gel and identified by liquid chromatography and tandem mass spectrometry-based peptide sequencing. Six peptides were sequenced (Table 3), and the sequences were concatenated and subjected to BLASTP analysis against the patented protein sequences database at NCBI, which identified...
sequence 5503 from US patent #6562958: “Nucleic acid and amino acid sequences relating to
Acinetobacter baumannii for diagnostics and therapeutics.” A similar search performed against
the non-redundant protein sequence database identified a small number of repetitive surface
proteins, as well as two predicted proteins from A. baumannii ATCC 17978: A1S_2724, a
putative hemaglutinin/hemolysin related protein, and A1S_2696, a hypothetical protein.

**Random transposon mutagenesis.** In order to identify the gene encoding the high molecular
weight proteinaceous antigen described above, we screened 550 kanamycin-resistant EZ-Tn5
insertional mutants and identified nine that lost surface reactivity to mAb 6E3. We obtained
DNA sequence flanking the transposon insertion site for each of the nine mutants that suggested
a common gene was disrupted.

The full-length coding sequence of the disrupted gene was assembled from sequence data
obtained as described in the Methods. BLASTP analysis against the non-redundant protein
sequences database at NCBI showed that this coding sequence was similar to biofilm-associated
proteins (Baps) from various bacterial species. The predicted full-length A. baumannii 307-0294
*bap* open reading frame is 25,863 bp and composed primarily of four similar, tandem repeated
modules (designated A-D), ranging in size from 237-315 bp (Figure 3A). The 3’ end of the
coding sequence contains additional repeat modules (designated E-G) that are not similar to A-D.

Each of the peptides identified by nanoLC-MS/MS (Table 3) is found within the
translated predicted *bap* open reading frame. As mentioned above, BLASTP analysis of the
predicted amino acid sequence against the non-redundant protein database at NCBI (most recent
search: 08/07) demonstrates regions of sequence similarity to the biofilm-associated proteins
from several staphylococcal species, including *S. hyicus* Bap (NCBI accession # AAY28520.1),
S. aureus Bap (NCBI accession # AAK38834.2), and S. epidermidis Bap (NCBI accession # AAY28519.1).

A. baumannii bap1302::EZ-Tn5 was selected from the nine available bap-insertional mutants because it contains a transposon insertion closest to the predicted bap start codon, preventing translation of the 95% of the gene that is 3’ to the insertion site. Whole cell lysates of wild-type A. baumannii 307-0294 and bap1302::EZ-Tn5 were analyzed by SDS-PAGE (Figure 1A), demonstrating that the wild-type contained a high molecular weight band (Figure 1C, lane 1) which was missing in the mutant (lane 2). In addition, Western blot analysis confirms that whole cell lysate and outer membrane proteins of A. baumannii bap1302::EZ-Tn5 lost reactivity to mAb 6E3 (Figure 1B, lanes 2 and 4).

**Characterization of bap transcription by reverse transcriptase PCR.** A. baumannii bap1302::EZ-Tn5 was further analyzed to determine if the transposon insertion resulted in down-stream polar effects. We performed RT-PCR to determine if bap and A1S_2695 were co-transcribed (see Figure 4 for the genetic arrangement of the bap locus in A. baumannii 307-0294). Oligonucleotide primers (Table 1) were designed to amplify regions of bap up- and down-stream of the transposon insertion site; primers were also designed to an internal region of A1S_2695, and to a region spanning the intergenic region linking bap to A1S_2695. The results in Figure 5 demonstrate that bap and A1S_2695 are not co-transcribed: therefore, disruption of bap does not appear to exert polar effects on neighboring genes.

**Structural features of Bap.** Bap is composed primarily of multiple copies of seven repeat units (designated A-G; see Figure 3A) there are 5 copies of repeat module A (54-99% amino acid sequence identity between copies), 22 copies of module B (72-100%), 21 copies of module C (73-100%), 28 copies of module D (78-100%), 2 copies of module E (62%), 2 copies of module
F (67%), and 3 copies of module G (36-51%). For the majority of the sequence (amino acids 188 to 7499, out of 8621), the repeat modules are directly in tandem, with no additional amino acids between consecutive repeats.

Analysis of the primary structure reveals the absence of Cys and an abundance of Thr (1389 residues; 16% of the total), Ala (1176; 14%), and Val (1109; 13%). In addition, Bap has a remarkably low isoelectric point (pI), estimated to be 2.9, placing it among the most acidic bacterial proteins yet described. The low pI may explain why the presence of SDS during electrophoresis has no apparent effect (data not shown), as very acidic proteins do not bind SDS under standard SDS-PAGE conditions (18), and is a reflection of an 11:1 imbalance in the number of acidic and basic amino acids: there are 1168 acidic (984 Asp + 184 Glu) and 105 basic residues (60 His + 34 Lys + 11 Arg). Since trypsin cleaves exclusively after Lys and Arg (33), the paucity of these amino acids explains why only a few peptides were sequenced following tryptic digest of Bap (Table 3).

BLASTP analysis of the ‘D’ repeat region, which in aggregate contains 35% of the total amino acids, identifies similarities to a putative outer membrane adhesin from *Shewanella* sp. ANA-3 (accession number YP_868031), while the similarity to the staphylococcal biofilm-associated proteins is limited to the ‘A-C’ repeats, which contain 48% of the total amino acids.

Clustal alignment of repeat units A-D from Bap<sub>A. baumannii</sub> and C from Bap<sub>S. aureus</sub> (Figure 3B) indicated that sequence similarities are limited to small stretches of amino acids (particularly in positions 2-6 [consensus ‘DTTAP’] and 95-103 [consensus ‘VTATDAAGN’]), which are separated from each other by longer stretches with more divergent sequence.

Comparison of the *bap* locus in *A. baumannii* 307-0294 with homologous loci in other *Acinetobacter* species. There are two publicly available *Acinetobacter* genomes: *A. baylyi*
ADP1 and *A. baumannii* 17978, and we have identified regions homologous to the *A. baumannii* 307-0294 *bap* locus in each (Figure 4). *A. baumannii* 307-0294 *bap* is flanked by *nhaP* (A1S_2723) upstream, and a putative glucosyltransferase (A1S_2695) downstream, with all three genes in the same orientation. *A. baylyi* ADP1 contains a single contiguous genomic region with a 5kb putative hemagglutinin in place of the 26kb *bap* open reading frame. The published genome for *A. baumannii* 17978 (40), surprisingly, does not contain a single homologous locus, but rather two loci separated by 30kb of unrelated sequence, and oriented in opposition relative to the organization in *A. baylyi* ADP1 and *A. baumannii* 307-0294. The non-repetitive 5' and 3' ends of *bap* are annotated as two distinct putative open reading frames in *A. baumannii* 17978, and the internal repeats are not found in the assembled genome. *A. baumannii* 17978 and *A. baylyi* ADP1 are not reactive to mAb 6E3 (data not shown).

**Disruption of bap diminishes biovolume and biofilm thickness.** Biofilms formed by *A. baumannii* 307-0294 and *bap1302::EZ-Tn5* after 48 hours of static culture in FAB-citrate supplemented with 0.05% (w/v) casamino acids were stained with a live/dead stain and imaged by CLSM (Figure 6). Upon examination of these confocal micrographs, the biofilm formed by wild-type bacteria was noticeably thicker than that formed by mutant bacteria. In order to more carefully analyze this difference, we designed an experiment that allowed us to quantify the dynamics of biofilm development over time.

Static cultures of GFP-expressing *A. baumannii* 307-0294 and *bap1302::EZ-Tn5* were imaged every hour for fourteen hours to visualize and quantify biofilm development on glass coverslips. The resultant image stacks were analyzed using the ISA-3D software program (2), and of the twenty calculated parameters, two followed significantly different trends over time for the mutant compared to wild-type biofilms: mean biofilm thickness and total biovolume (Figure
Specifically, biofilms formed by wild-type and mutant bacteria are distinguishable after hour six, when the mutant reaches a biofilm thickness plateau that is less than half that ultimately achieved by wild-type bacteria. The mutant also steadily loses biovolume for the remainder of the experiment, in contrast to the stable biovolume maintained by wild-type bacteria. It is important to note that these differences are specific to biofilm formation, as these strains are indistinguishable by growth curve analysis in shaking broth cultures in the same FAB-citrate media used in the static culture biofilm experiments (data not shown).

Semi-quantitative analysis of biofilms grown on polystyrene pins in 96-well plates is consistent with the results obtained from the confocal experiments: the biofilm formed by the \textit{bap1302::EZ-Tn5} retains less crystal violet stain, indicative of less accumulated biovolume, compared to wild-type (data not shown).
Discussion

*A. baumannii* is an opportunistic pathogen that is particularly successful at colonizing and persisting in the hospital environment, able to resist dessication (19, 20, 53) and survive on inanimate surfaces for months (22). It is among the most common causes of device-related nosocomial infection (13, 43, 52), which result when the organism is able to resist physical and chemical disinfection, often by forming a biofilm (7, 27, 34). We chose to study biofilm formation by *A. baumannii* in order to understand how this pathogen persists in the hospital environment to cause outbreaks worldwide (51).

Baps were first characterized in *S. aureus* (11) and have since been identified in a number of other Gram-positive and Gram-negative pathogenic bacteria (reviewed in (24, 25)). They are defined by shared structural and functional characteristics, and are essentially high molecular weight, repetitive surface proteins involved in biofilm formation (24). The prototypical Bap from *S. aureus* is involved in the primary attachment step of biofilm formation as well as in promoting intercellular adhesion and biofilm maturation (11); other Baps are involved in the various stages of biofilm formation and in adhesion to eukaryotic host cells (25).

We have identified a protein produced by *A. baumannii* 307-0294 that satisfies all of the criteria to be included in the Bap family: it is high molecular weight, with a predicted molecular mass of 854kDa; it is repetitive, composed of multiple copies of seven repeat modules (A-G); it is exposed on the surface; and it is involved in biofilm formation and development.

There are several conceptual, sequential stages in bacterial biofilm formation (reviewed in (41)): *i)* reversible primary attachment of individual cells to a surface; *ii)* progression to irreversible attachment mediated by extracellular polysaccharide; *iii)* early development of biofilm architecture; *iv)* maturation of biofilm architecture; and *v)* dispersal of single cells from...
the biofilm. Bap \textsubscript{A. baumannii} does not appear to be involved in the primary attachment of cells to glass or polystyrene, in contrast to Bap \textsubscript{S. aureus} (11). Our data suggests that Bap \textsubscript{A. baumannii} appears to be involved in maintaining the mature biofilm architecture (steps 3 and 4), as biofilms formed by the mutant are neither as thick nor as voluminous as those formed by the wild-type. There are at least three potential Bap-mediated interactions to explore in future studies: Bap \textsubscript{A. baumannii} may mediate direct intercellular adhesion from one bacterium to a surface receptor on a neighboring bacterium; or, this may be an example of auto-adhesion between Bap molecules on adjacent bacteria; or, cells may be linked indirectly via shared interactions with some extracellular biofilm matrix component. While these are all logical hypotheses, further studies are needed in order to carefully determine the actual function of Bap \textsubscript{A. baumannii}.

Bap is a remarkable protein: it contains 8,621 amino acids, making it one of the largest bacterial proteins yet described; it has a predicted pI of 2.9, making it one of the most acidic bacterial proteins; and it is composed of tandemly arranged repeat modules. The protein is divided almost equally in two parts: the first contains modules A-C, alternating one after the other; the second contains 28 direct tandem repeats of module D. Each repeat unit A-D appears to be related to the HYR domain (4) (PFAM accession # PF02494) based on the conserved amino acids ‘DTTAP’ and ‘VTATDAAGN’ (Figure 3), although the HYR seed alignment contains two conserved cysteines that are conspicuously absent from Bap. HYR was first identified in eukaryotic proteins involved in cellular adhesion, and is found in a number of large repetitive prokaryotic proteins as well, including several adhesins (4). Three-dimensional structure prediction using the PHYRE server (http://www.sbg.bio.ic.ac.uk/phyre/) suggests that each module A-D folds into a seven-stranded β-sandwich similar to proteins within the immunoglobulin-like fold superfamily (PFAM accession # CL0159, which includes the HYR
domain as a family member). This suggests Bap adopts a “beaded-filament” tertiary structure, and that each module might redundantly contribute to the overall function. Other bacterial proteins that adopt a tertiary structure similar to that predicted for Bap include invasin from *Yersinia pseudotuberculosis*, and intimin, FimH, and PapG from enteropathogenic *E. coli* (32).

The latter two are actually individual subunits that resemble an isolated repeat unit of Bap, and are assembled into filamentous pili that recapitulate in quaternary structure what Bap is predicted to accomplish in tertiary structure. The pattern of sequence similarity to Bap<sub>*S. aureus*</sub> (Figure 3B), with short stretches of highly conserved residues separated by longer regions of divergence, suggests that the conserved regions might function to maintain a common structural motif, while the divergent regions might confer unique functionality to the respective proteins. In this light, it is interesting that Bap<sub>*A. baumannii*</sub> contains four distinct modules (A-D) each based on the same HYR motif, while Bap<sub>*S. aureus*</sub> seems to contain multiple copies of only a single repeat type.

Using a previously reported typing scheme (15), *A. baumannii* 307-0294 is a sequence type ST15 (data not shown). ST15 is phylogenetically linked with European hospital clone I (15, 35), which is associated with epidemic behavior and multi-drug resistance (31, 35, 47).

Although at least 39 sequence types of *A. baumannii* were distinguished among the military outbreak strains (15), four of them (ST11, 14, 16, and 10) accounted for over half of the isolates, suggesting either that the outbreak was clonal in nature, or that particular sequence types of *A. baumannii* are more likely to cause human infection. It is intriguing that mAb 6E3 recognizes 14/15 (93%) of the representatives from these four sequence types, compared to 17/61 (28%) of the representatives from the other 35 sequence types (Table 2), although the significance of this observation is not clear. For example, we do not know whether those strains that failed to react to mAb 6E3 did not express a Bap homologue, or whether they expressed a variant that simply
lacked the recognized epitope. For these reasons, and because we do not have access to disease severity information for the patients involved in the military outbreak, we are unfortunately unable to comment on the contribution of Bap to disease severity. Nevertheless mAb 6E3 will be a useful tool for future studies designed to answer such questions.

In conclusion, we have identified a novel *A. baumannii* protein, Bap, expressed on the surface these bacteria that is involved in biofilm formation in static culture. Bap has a predicted structure similar to bacterial adhesins within the immunoglobulin-like fold superfamily and may function as an intercellular adhesin in such a way that supports the mature biofilm structure. Although more studies are needed to test this hypothesis, a better understanding of the contribution of this Bap to *A. baumannii* biofilm formation and maturation may help to explain why *Acinetobacter* remains a common cause of nosocomially-acquired device-related infection.
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Table 1. Sequences of oligonucleotide primers used for RT-PCR

Table 2. Survey of mAb 6E3 reactivity among a representative library of Acinetobacter strains isolated during an outbreak in the military health care system.

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Figure 2. Flow cytometric analysis of A. baumannii 307-0294 (A) and bap1302::EZ-Tn5 (B) demonstrates surface accessibility of the mAb 6E3 epitope. Cells from late-log phase cultures were probed with Alexa Fluor ® 488-conjugated control non-specific antibody (shaded histograms) or mAb 6E3 (unshaded). Each histogram shows the fluorescence intensity distribution of >50,000 flow cytometer events.

Figure 3. Graphical representation of bap demonstrating the repetitive structure (A), and a Clustal alignment (B) showing sequence similarity between the BapA baumannii and BapS aureus repeat units. (A) The 25,863 bp A. baumannii bap open reading frame is composed almost
entirely of tandemly arranged repeats, labeled A-D. The 3’ end of the coding sequence contains additional repetitive units E-G that are not similar to A-D. (B) A Clustal alignment of an example of each of the major repeat types found in Bap<sub>A. baumannii</sub> and of the ‘C’ repeat unit from Bap<sub>S. aureus</sub>. For both proteins, the start and end of each repeat, indicated in parentheses, was defined by comparison to the seed alignment of the HYR domain (PFAM accession number PF02494).

Figure 4. The publicly available Acinetobacter genomes <i>A. baumannii</i> 17978 (NCBI acc. # NC_009085.1) and <i>A. baylyi</i> ADP1 (NCBI acc. # NC_005966.1) contain regions homologous to the <i>bap</i> locus in <i>A. baumannii</i> 307-0294. The rulers indicate the size (<i>A. baumannii</i> 307-0294) and genomic coordinates (<i>A. baumannii</i> 17978 and <i>A. baylyi</i> ADP1) of the fragments. Percentage nucleotide identity is shown for the regions delimited by dashed lines. Predicted ORFs in <i>A. baumannii</i> 307-0294 are annotated with the identifier of the homologous ORF in <i>A. baumannii</i> 17978 (prefix A1S_, except for AS1_3829), and those in <i>A. baylyi</i> ADP1 are annotated with the gene symbol when available, and the serially numbered identifiers (prefix: ACIAD) otherwise.

Figure 5. RT-PCR and agarose gel electrophoresis demonstrates that <i>bap</i> and A1S_2695 are not co-transcribed, and that transcription of A1S_2695 is not disrupted in <i>A. baumannii</i> <i>bap1302</i>::EZ-Tn5. Each primer set amplifies a product from chromosomal DNA template (A). Pr1160-1330 (lane 3), however, fails to amplify a product from RNA template (C), indicating that <i>bap</i> and A1S_2695 are not present on the same fragment of RNA. The samples in lane 5 included RNA template but were not subject to the reverse-transcription step, as a control for DNA contamination, and hence were not applicable to the reaction sets containing either chromosomal DNA (A) or no nucleic acid template (B). Primers (Table 1) were designed to
target the following regions: Lane 1: 5' bap; lane 2: 3' bap; lane 3: 3' bap – A1S_2695; lane 4: A1S_2695; lane 5: 3' bap (no template control).

Figure 6. Confocal laser scanning micrographs of biofilms formed by A. baumannii 307-0294 (A) and bap1302::EZ-Tn5 (B) clearly demonstrate the decreased thickness achieved by the mutant after 48 hours in static culture. Biofilms were grown in 35mm glass-bottom Petri dishes for 48 hours and stained with the LIVE/DEAD® BacLight® Bacterial Viability kit, which stains live cells green with SYTO-9, and dead cells red with propidium iodide. The images show the average intensity projections through the confocal image stack, with the maximum intensity x-z and y-z projections shown along the bottom and side of each image. Images were produced in ImageJ.

Figure 7. Comparison of biovolume (A) and mean biofilm thickness (B) achieved by A. baumannii 307-0294 (■) and A. baumannii bap1302::EZ-Tn5 (▲) during static culture. The data points are the mean +/- standard deviation for two separate high power fields-of-view, representing a total slide surface area of 1 x 10^5 µm^2 per sample. The best-fit nonlinear regression curve is shown as a thin trend line with 95% confidence bands. Because the biofilms formed by wild-type and mutant bacteria cannot be described with the same curve (P<0.0001), we conclude that the biofilms differ significantly with respect to biovolume and mean biofilm thickness development over time.
Table 1. Sequences of oligonucleotide primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Position&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1124</td>
<td>TCATACGTCTGAAAAATGGCGAG</td>
<td>152-174</td>
<td>5’ region of bap</td>
</tr>
<tr>
<td>1125</td>
<td>CATCAAGTGCTACTGTGCGC</td>
<td>1151-1171&lt;sup&gt;b&lt;/sup&gt;</td>
<td>bap</td>
</tr>
<tr>
<td>1461</td>
<td>CAGATGTGCCCTATTGTCGG</td>
<td>25025-25045</td>
<td>3’ region of bap</td>
</tr>
<tr>
<td>1462</td>
<td>CCTGTATTCACTCTTGACCAGCAG</td>
<td>25259-25283&lt;sup&gt;c&lt;/sup&gt;</td>
<td>bap</td>
</tr>
<tr>
<td>1160</td>
<td>AAGGAGTGAATACAGGCGAAG</td>
<td>25268-25288</td>
<td>3’ bap to A1S_2695</td>
</tr>
<tr>
<td>1330</td>
<td>CATTTCATCGTAGCGACTCG</td>
<td>26572-26592&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A1S_2695</td>
</tr>
<tr>
<td>1437</td>
<td>ACCAAATAGAGCAGCAGGTTC</td>
<td>26278-26298</td>
<td>A1S_2695</td>
</tr>
<tr>
<td>1438</td>
<td>CTCCCTTTCTTACCATCAATC</td>
<td>26888-26909&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A1S_2695</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative to the predicted bap start codon. The predicted bap coding sequence is from position 1-25,863, and A1S_2695 from 26,160-27,665.

<sup)b</sup> (c) indicates that the primer anneals to the complementary DNA strand.
Table 2. Survey of mAb 6E3 reactivity among a representative library of *Acinetobacter* strains isolated during an outbreak in the military health care system.

<table>
<thead>
<tr>
<th><em>Acinetobacter</em> species</th>
<th>Sequence type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total # isolated</th>
<th># screened</th>
<th># reactive to mAb 6E3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>baumannii</em></td>
<td></td>
<td>189</td>
<td>76</td>
<td>31 (41)</td>
</tr>
<tr>
<td></td>
<td>ST11</td>
<td>51</td>
<td>5</td>
<td>4 (80)</td>
</tr>
<tr>
<td></td>
<td>ST14</td>
<td>22</td>
<td>6</td>
<td>6 (100)</td>
</tr>
<tr>
<td></td>
<td>ST16</td>
<td>13</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>ST10</td>
<td>12</td>
<td>2</td>
<td>2 (100)</td>
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<tr>
<td></td>
<td>other&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91</td>
<td>61</td>
<td>17 (28)</td>
</tr>
<tr>
<td>13TU</td>
<td></td>
<td>8</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>genome sp. 3</td>
<td></td>
<td>13</td>
<td>13</td>
<td>11 (85)</td>
</tr>
<tr>
<td>other&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>6</td>
<td>4</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>216</strong></td>
<td><strong>98</strong></td>
<td><strong>42 (43)</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Sequence type assignments were extracted from Figure 2 in reference (15).

<sup>b</sup> Includes strains representing 35 other sequence types, and two isolates of unknown sequence type.

<sup>c</sup> Includes one isolate of genome species 10, and three otherwise undescribed genomic species; additional single isolates of *A. ursingii* and *A. haemolyticus* were recovered during the outbreak, but these were not included in our screened library.
Table 3. Internal peptide fragments of Bap identified by NanoLC-MS/MS from a single band on a protein gel.

<table>
<thead>
<tr>
<th>Peptide Mass</th>
<th>Peptide Sequence</th>
<th>Start-end position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1141.64</td>
<td>VAASDVLVVNR</td>
<td>35-45</td>
</tr>
<tr>
<td>2630.35</td>
<td>NIPADAANTA TVVINGVTYNATVDK</td>
<td>228-253</td>
</tr>
<tr>
<td>1900.95</td>
<td>AAGTWTVSVGSLVADADK</td>
<td>254-273</td>
</tr>
<tr>
<td>2596.33</td>
<td>TVVADSSDTGVIDLLGIFGSEVQFK</td>
<td>8032-8056</td>
</tr>
<tr>
<td>1642.78</td>
<td>VDSFYTVSPVTGR</td>
<td>8181-8195</td>
</tr>
<tr>
<td>1690.84</td>
<td>PLDSAANATVDVIDYK</td>
<td>8444-8459</td>
</tr>
</tbody>
</table>
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Note that the rulers for the two *A. baumannii* 17978 fragments are not continuous, and not even in the same direction: the fragments in the assembled genome are in opposition relative to the arrangement in *A. baumannii* 307-0294 and *A. baylyi* ADP1, and are separated by over 30kb of intervening genomic DNA that is not similar to *bap*. 
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References


