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ORIGINAL ARTICLE

The sensor kinase BfmS mediates virulence in *Acinetobacter baumannii*

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Background/Purpose: BfmR, the response regulator component of the two-component system BfmRS, has important roles in biofilm formation and cellular morphology of *Acinetobacter baumannii*. Until now, the contribution of the sensor kinase BfmS to the virulence of this bacterium remains unknown. In this study, a *bfmS* knockout and complementation studies were performed to clarify the role of BfmS in *A. baumannii* virulence.
Methods: We constructed a *bfmS* knockout mutant in the *A. baumannii* 17978 type strain by transposon inactivation. To clarify the role of *bfmS* in *A. baumannii* virulence, the biofilm formation, adherence ability to eukaryotic cells, serum resistance, and antibiotic susceptibility tests were performed in *A. baumannii* 17978 and its derivative knockout and complementation strains.
Results: The *bfmS* knockout displayed a reduction in biofilm formation, loss of adherence to eukaryotic cells, and greater sensitivity to serum killing compared with the parent strain. Proteomic analysis of culture supernatants revealed that the release of outer membrane proteins (Omps), including CarO and outer membrane protein A (OmpA), was associated with the inactivation of BfmS in *A. baumannii*.

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Conclusion: This study is the first to demonstrate that the pathway regulated by the sensor kinase BfmS is associated with biofilm formation, adherence to biotic surfaces, serum resistance, and antibiotic susceptibility, which may be associated with the release of Omps in *A. baumannii*.

Introduction

Acinetobacter baumannii is an important nosocomial human pathogen.[1] The widespread virulence of *A. baumannii* strains is thought to depend on the expression of virulence factors that enable bacterial infection and the expression of antimicrobial-resistant determinants.[2,3] The remarkable resistance phenotype may be facilitated by the ability of *A. baumannii* strains, particularly those isolated from catheter-related urinary or bloodstream infections, to form biofilms that adhere to and persist on abiotic surfaces.[4] Moreover, a novel pilus assembly system,[5] a homologue of staphylococcal biofilm-associated protein (Bap),[6] and quorum sensing[7] are involved in *A. baumannii* biofilm formation. Similarly, adherence of *A. baumannii* to human epithelial cells could contribute to the initial step of colonization on biotic surfaces, which is enhanced by the presence and expression of the bla_{PER-1} gene.[8] Furthermore, resistance to serum killing could explain the high mortality in patients with an ultimately fatal underlying disease, septic shock at the onset of bacteremia, and mechanical ventilation.[9] Among the virulence factors reported in *A. baumannii*, outer membrane protein A (OmpA) plays a versatile role in *A. baumannii* pathogenic processes including biofilm formation on abiotic surfaces, adherence to eukaryotic cells, and the inhibition of complement via recruitment of factor H to the cell surface.[10] However, the mechanisms of OmpA-mediated pathogenesis and the regulatory processes controlling these mechanisms remain to be characterized.

Bacterial two-component systems (TCSs) are key factors in the ability of microorganisms to survive and grow in various environments.[11] TCSs have been studied in many bacteria, particularly in their control of the virulence mechanisms of those microorganisms.[12,13] However, much less is known about TCS-mediated virulence in *A. baumannii*. BfmRS is a TCS in *A. baumannii* ATCC 19606 that controls biofilm formation and cellular morphology.[14] This transcriptional regulatory system activates the expression of the usher–chaperone assembly system responsible for the production of pili needed for biofilm formation on polystyrene surfaces. TCSs such as PhoPQ are examples of regulatory systems that control antimicrobial peptide resistance, survival within macrophages, and virulence in animal models and human volunteers.[13,15] It is possible that *A. baumannii* BfmRS, in addition to its role in biofilm formation, could participate in virulence mechanisms such as cell adhesion and resistance to serum killing, which is associated with OmpA-mediated pathogenesis.[15] To test this hypothesis, we constructed a *bfmS* knockout mutant in the *A. baumannii* 17978 type strain by transposon inactivation. We show in this report that *bfmS* gene may play dual roles in *A. baumannii* virulence and antibiotic susceptibility via the loss or inactivation of Omps.

Table 1 Bacterial strains, plasmids and oligonucleotide primers used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
A. baumannii strains		
17978	Type strain	American Type Culture Collection (Manassas, VA)
bfmS::Km	*bfmS*::EZ-Tn5<R6Kγori/KAN-2>derivative of 17978	This study
bfmS::KmC	*bfmS*::Km harboring pAB0749	This study
E. coli strains		
DH5α	Used for recombinant DNA methods	Invitrogen
Plasmids		
pGEM-T-easy	High-copy-number cloning vector; Amp^r	Promega
pGEM-bfmS	pGEM-T vector containing *bfmS* gene	This study
pGEMT-bfmS::km	*bfmS*::EZ-Tn5<R6Kγori/KAN-2>derivative of pGEM-bfmS	This study
pWH1266	*E. coli–A. baumannii* shuttle vector Ap^rTc^r	Gaddy et al. [18]
pAB0749	pWH1266 harboring the 17978 *bfmS* allele; Amp^r	This study
Primers	5′ to 3′	
bfmS-FP	TTGCTCGAACTTCCAATTTATTATAC	This study
bfmS-RP	TTATGCAGGTGCTTTTTTATTGGTC	This study
bfmS-RTFP	TCGGCGGGTATTACCTTATTTAGCT	This study
bfmS-RTRP	GCCTCAATCAAACGCTGAATATGGT	This study
bfmR-FP	ATGTTGCCGGGTGCAGAT	This study
bfmR-RP	TTACAATCCATTGGTTTCTTTAACAA	This study

[a] Amp^r = ampicillin resistance; Km^r = kanamycin resistance; Tc^r = tetracycline resistance.

Materials and methods

Bacterial strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Bacteria were routinely cultured at 37°C in Luria–Bertani (LB) medium.

Insertion mutagenesis and genetic complementation

DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *Escherichia coli* were performed using standard protocols.[16] The bfmS::Km mutants were constructed using the method as described.[14] Briefly, a 1368-bp DNA fragment, corresponding to the *bfmS* DNA region, was amplified by polymerase chain reaction (PCR) using primers bfmS-FP and bfmS-RP. The amplified fragment was cloned into plasmid pGEM-T-easy to form pGEM-bfmS. A mutagenesis plasmid pGEM-bfmS::km was generated with the EZ-Tn5<R6Kγori/KAN-2>Insertion kit (Epicentre Inc., Madison, WI, USA). Plasmid pGEM-bfmS::km was transformed into *A. baumannii* ATCC 17978 by electroporation, and bacteria were selected on LB agar containing kanamycin. The bfmS::Km mutants were verified by PCR using primer set 1 (bfmR-FP and bfmS-RP) and set 2 (bfmS-FP and bfmS-RP) as shown in Table 1. In complementation experiment, the *bfmS* parental allele was PCR amplified using primers bfmS-FP and bfmS-RP and was ligated into *A. baumannii–E. coli* shuttle vector pWH1266 and transformed into *E. coli* Top10 cells. Recombinant plasmid DNA, named pAB0749, was electroporated into *A. baumannii* 17978 cells. The presence and stability of pAB0749 in the complemented strain was confirmed by restriction analysis.

Reverse transcriptase-PCR

Total RNA was isolated from all the parental and mutant strains using an acid–phenol extraction protocol.[16] The concentration and quality of the RNA in each sample were determined by measuring absorbance at 260 nm. All RNAs were adjusted to a concentration of 50 ng mL^{-1}. The reverse transcriptase (RT)-PCR protocol was carried out using the MMLV High Performance Reverse Transcriptase Kit (Epicentre). The expression of each target gene was then normalized to that of the 16S rRNA gene. The primers used for determination of *bfmS* and *bfmR* gene expression by RT-PCR are listed in Table 1.

Biofilm assays

Biofilm formation on polystyrene was assessed by crystal violet staining of cells cultured in LB broth as previously described.[17] Each experiment was performed in triplicate and repeated three times. The quantity of biofilm in each sample was normalized to that of the parental strain.

A549 cell adhesion assays

Cell adhesion assays were performed as previously described.[18] Briefly, A549 human alveolar epithelial cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA, USA) at 37°C in 5% CO_2. All cells were grown in 10-cm culture-coated Petri dishes and seeded to 70% confluence in six-well plates or on sterile coverslips placed in six-well plates for experiments. Bacterial cells (2×10^6 CFU) were added and co-cultured with A549 cells for 1 hour at 37°C. The infected monolayers were washed with phosphate-buffered saline (PBS) and lysed in 1 mL sterile deionized water. The lysates were plated on LB agar plates to determine the number of bacteria that attached to A549 cells. Each experiment was performed in triplicate and repeated three times. The number of adherent bacteria in each sample was normalized to that of the parental strain.

Serum bactericidal assay

Serum bactericidal assays were performed according to the procedure.[10] Briefly, normal human sera (NHS) were collected from five healthy donors. The pooled NHS was aliquotted and stored at −20°C until further use. *A. baumannii* overnight cultures were washed with PBS and adjusted to a final concentration of 1×10^9 CFU mL^{-1} with PBS. Then, 200 μL PBS containing 20% NHS was prepared and mixed with 10 μL bacterial suspension (1×10^7 cells) and incubated with shaking for 2 hours at 37°C. Next, 200 μL PBS without NHS was used as a positive control. The number of surviving bacteria in each sample was determined by serial dilution and plating on LB agar at 37°C for 18–24 h. The survival rate of bacteria was calculated as the number of surviving bacteria in 20% NHS divided by those without NHS.

Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy

The overnight cultures of bacteria cells were harvested by centrifugation, and supernatants were concentrated 20-fold using a Vivaspin concentrator (GE Healthcare) with 10 kDa MW cutoff. The purified proteins were analyzed using SDS-polyacrylamide gel (12%) electrophoresis. Gels were stained with Coomassie brilliant blue, and selected bands were manually excised from the gels and transferred to microcentrifuge tubes and subjected to in-gel trypsin digestion according to a standard protocol.[19] After digestion, the samples were analyzed using a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometer 4800 Proteomics Analyzer (Applied Biosystems). Peptide masses were searched against a comprehensive nonredundent protein database (NCBInr) using the mascot program for protein identification.[20]

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined with the microdilution method in accordance with the guidelines of

the Clinical and Laboratory Standards Institute.[21] The agents tested included ceftazidime, amikacin, ciprofloxacin, and imipenem. *E. coli* strain ATCC 25922 and *Pseudomonas aeruginosa* strain ATCC 27853 were used as references in the susceptibility testing. A more than twofold reduction or induction in the minimum inhibitory concentration (MIC) values in the bfmS::Km mutant compared to their parental strain was considered significant.

Statistical analysis

Differences were determined using the paired Student *t*-test. A p value <0.05 was considered statistically significant. Data entry and analyses were performed using the Statistical Package for the Social Sciences software version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Characterization of the bfmS locus in the bfmS::Km mutant

The insertion mutant bfmS::Km was constructed from *A. baumannii* ATCC 17978 (Fig. 1A). After the gene construction procedure, the electroporated pGEM-T-bfmS::km homologous recombined with the *bfmS* gene in *A. baumannii* ATCC 17978. PCR detection of the transposon insertion using primer set 1 (bfmR-FP and bfmS-RP) and set 2 (bfmS-FP and bfmS-RP) showed 4222-

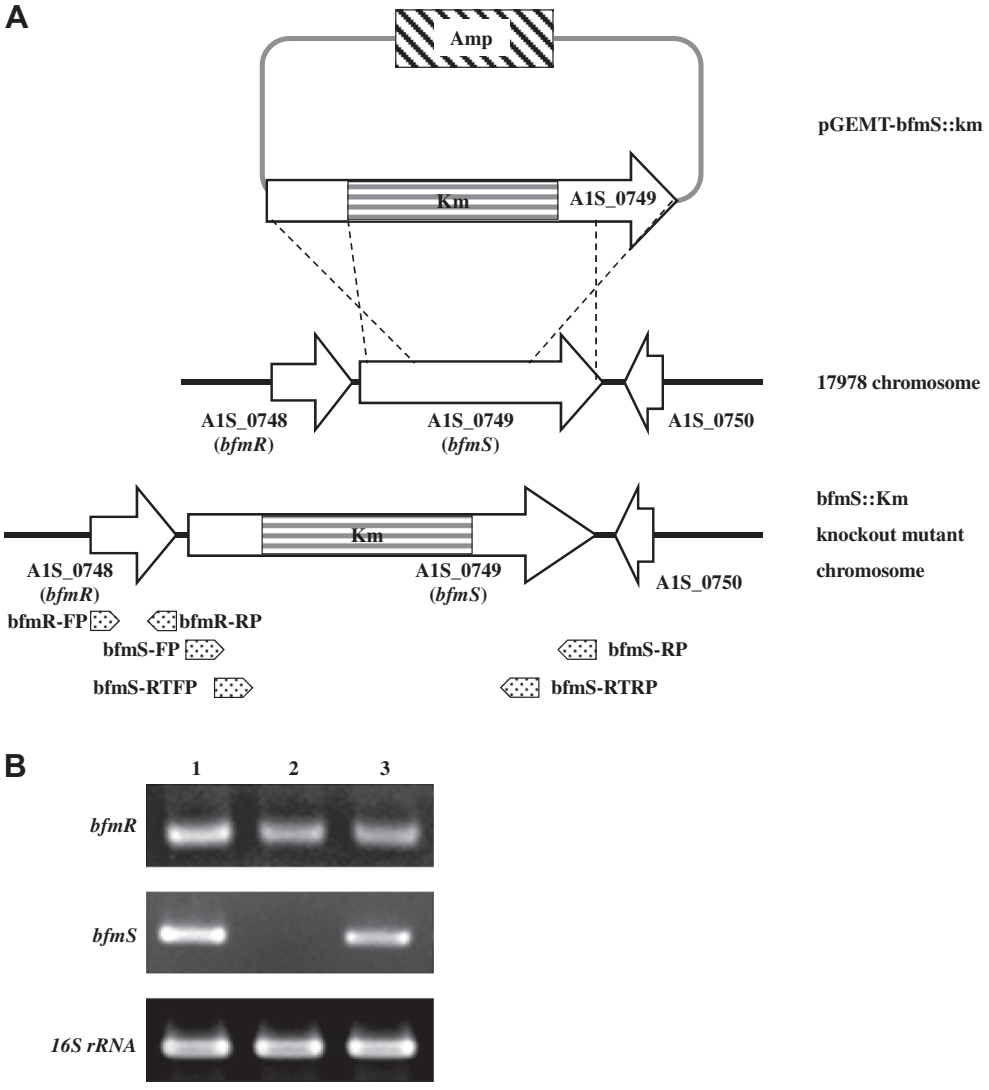

Figure 1. Construction and identification of the transposon insertion mutant bfmS::Km. (A) The orientations of open reading frames are represented by arrows. The dotted lines denote homologous recombination between the *bfmS* gene located in pGEMT-bfmS::km and the 17978 chromosome. Amp = ampicillin resistance cassette; Km = kanamycin resistance cassette. (B) RT-PCR analysis of total RNA isolated from 17978 (lane 1), bfmS::Km (lane 2), and bfmS::KmC (lane 3) cells cultured in LB broth. Primers used to analyze the transcription of *bfmS* and *bfmR* are listed in Table 1.

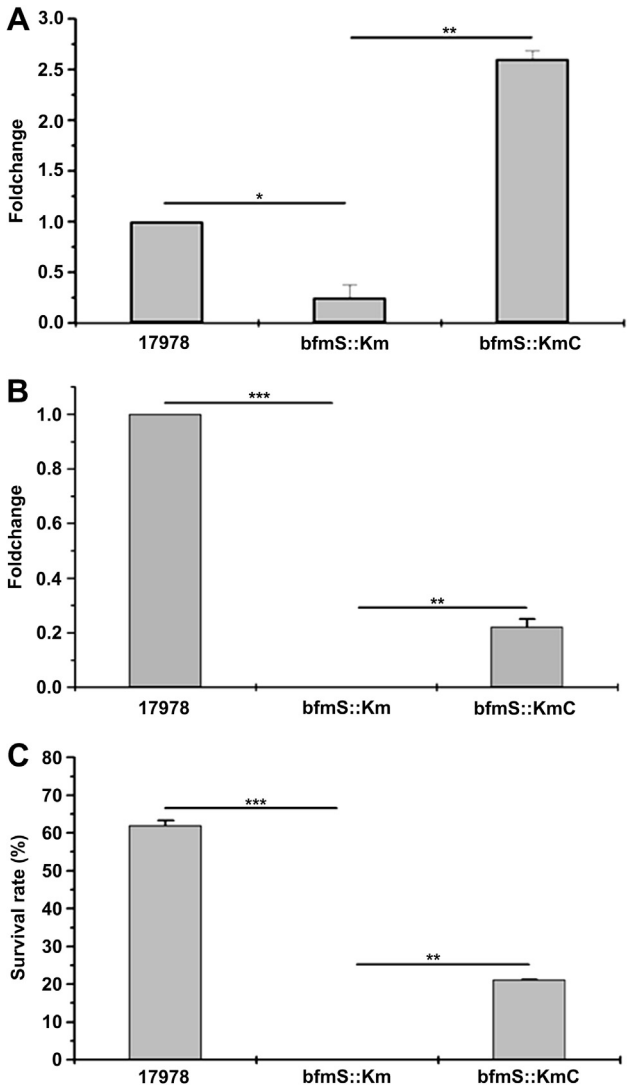

Figure 2. Virulence assay in 17978, bfmS::Km, and bfmS::KmC cells. (A) Quantitative biofilm assays. Fold change is determined as the quantity of biofilm in each sample normalized to that of 17978 strain. (B) Bacterial adhesion on A549 human alveolar epithelial cells. Fold change is defined as the number of adherent bacteria in each sample normalized to that of the 17978 strain. (C) Killing of bacteria in normal human serum. The data are normalized as the number of surviving bacteria in 20% NHS divided by the number of surviving bacteria incubated without NHS. Error bars, 1 standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and 3428-bp fragments respectively in bfmS::Km mutants. Fig. 1B shows the result of the mRNA expression of the *bfmR* and *bfmS* genes in the bfmS::Km, bfmS::KmC, and their parental strain. The mRNA levels of *bfmS* in bfmS::KmC were restored to levels similar to those of the parental strain. However, the mRNA levels of *bfmR* were not significantly reduced in the bfmS::Km strain relative to the parent strain despite an absence of *bfmS* mRNA expression.

Virulence assay

Fig. 2A shows the result of quantitative biofilm assays. The growth rate did not differ in bfmS::Km, bfmS::KmC, and their parental strain (data not shown). A significant reduction ($p < 0.05$) in biofilm formation in the bfmS::Km strain was observed compared to the 17978 type strain. In contrast, a recovery in biofilm formation was shown in the bfmS::KmC strain ($p < 0.01$). Fig. 2B shows the result of adherence to A549 cells by bfmS::Km, bfmS::KmC, and their parental strain. Quantitative adherence assays showed a significant reduction ($p < 0.01$) in adherence capability in bfmS::Km compared with the 17978 type strain. A partial recovery in adherence capability was observed in the bfmS::KmC strain ($p < 0.05$). Fig. 2C shows the survival rate of the ATCC 17978 type strain and bfmS::Km mutant cells incubated with 20% NHS. The 20% NHS concentration was selected for the serum bactericidal assay based on the study.[10] A significant reduction of the survival rate in bfmS::Km was observed compared to the 17978 type strain ($p < 0.001$). A partial reversal in serum resistance was observed in the bfmS::KmC strain ($p < 0.05$).

Characterization of supernatant proteins in bfmS::Km mutant

Fig. 3 shows the result of cellular and supernatant protein profiles in bfmS::Km, bfmS::KmC, and their parental strain. The cellular protein profiles were not different in the three strains. However, three protein bands were found in the bfmS::Km supernatant and were then identified as OmpA,

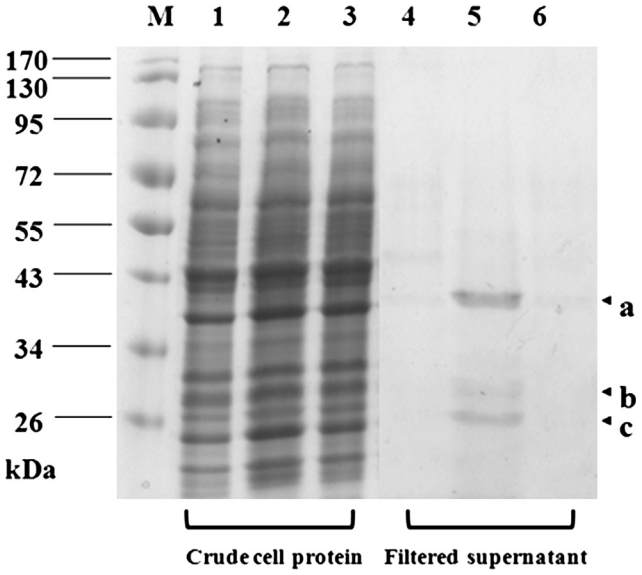

Figure 3. Inactivation of *bfmS* leads to increased release of proteins into culture supernatants. Total proteins from crude cell proteins and filtered supernatants were extracted from 17978 (lanes 1 and 4), bfmS::Km (lanes 2 and 5), and bfmS::KmC (lanes 3 and 6) cells were analyzed on 12.5% polyacrylamide gels. Three proteins released by bfmS::Km strain are indicated by arrows.

Table 2 Proteins identified by MALDI-TOF-MS-MS analysis of supernatants from bfmS::Km strains

Protein	Identity	Source species	GenBank accession number	Sequence coverage of matched peptides	Theoretical Mr/pI
a	Outer membrane protein A	*A. baumannii*	YP_001085848	42%	7279
b	Chain A, N276d Mutant Of *E. coli* Tem-1 β-Lactamase	*E. coli*	1CK3_A	89%	5418
c	CarO	*A. baumannii*	YP_001085557	51%	5190

MALDI-TOF = matrix-assisted laser desorption/ionization-time of flight; MS = mass spectrometry.

Tem-1 beta-Lactamase, and CarO by MALDI-TOF MS as shown in Table 2.

Antimicrobial susceptibility testing

The changes in secreted protein profiles suggest that the inactivation of *bfmS* in *A. baumannii* may lead to an increased resistance to antibiotics. Table 3 shows the susceptibility result of *A. baumannii* mutants and their parental strains. The increase of MIC to ciprofloxacin and the decrease of MIC to imipenem were observed in the bfmS::Km mutant. The partially restored MIC values to the two drugs were found in the bfmS::KmC strain.

Discussion

This study is the first to demonstrate that the insertional inactivation of *bfmS* in the *A. baumannii* ATCC 17978 type strain results in deficient biofilm formation on abiotic surfaces, reduction of adherence to eukaryotic cells, and sensitization to serum killing.

Omps play important roles in antimicrobial resistance and/or virulence in Gram-negative bacteria.[22] Among all the surface proteins reported to date, *A. baumannii* OmpA mediates interactions with epithelial cells and then targets the nucleus, resulting in cytotoxicity.[23] Moreover, this protein is also found in the outer membrane vesicles that could induce *A. baumannii* OmpA (AbOmpA)-dependent host cell death.[24] The mouse pneumonia model showed that the AbOmpA$^-$ mutant has some defects in serum resistance or the ability to disseminate into the blood.[23] All study results imply that OmpA has been implicated in virulence, both in cell culture and in animal models of *A. baumannii* pathogenesis, and was modeled to contribute to biofilm formation. To date, the regulatory processes controlling the OmpA-mediated virulence remain unclear. Our study shows the loss or secretion of AbOmpA into bfmS::Km culture supernatants, suggesting that the inactivation of *bfmS* may affect the regulatory process and/or translational modification of OmpA leading to loss of virulence in *A. baumannii* despite the fact that *ompA* mRNA levels were not different in the mutants and their parental strains (data not shown).

Table 3 Susceptibility of *A. baumannii* mutants and their parental strains

Antibiotic	MIC (mg/L) [a]		
	17978	bfmS::Km	bfmS::KmC
Ceftazidime	16	16	16
Ciprofloxacin	0.25	**2**	**1**
Amikacin	2	2	2
Imipenem	2	**<0.25**	1

[a] Significant change is indicated in bold.
MIC = minimum inhibitory concentration.

Loss of Omps is one of the important mechanisms that contribute to antimicrobial resistance in Gram-negative bacteria.[25,26] In *A. baumannii*, CarO was previously shown to form nonselective pores, and a loss or inactivation of this porin has been associated with increased resistance to carbapenems.[26] The upregulatory mechanism of CarO remains to be characterized; however, our result shows that inactivation of *bfmS* may affect the translational modification of CarO resulting in the secretion of CarO into culture medium. Moreover, the *carO* mRNA levels were significantly increased in the bfmS::Km mutants (data not shown), suggesting that *bfmS* may modulate the mRNA expression of *carO* in *A. baumannii*. The antimicrobial susceptibility result shows that the inactivation of *bfmS* could alter the MICs to ciprofloxacin and imipenem, suggesting that *bfmS* may play dual roles in antimicrobial resistance and virulence. Notably, the decrease in imipenem MIC found in *bfmS* knockout mutant was associated with the increased mRNA expression in that strain, implying that CarO may play an important role in the resistance to carbapenem in *A. baumannii*.[26]

There is one notable difference between the studies of Tomaras et al[14] and ours. Tomaras et al reported that the biofilm formation of the 19606 type strain depends on the production of Csu pili chaperone—usher assembly system, which is regulated by *bfmR*. Nevertheless, *bfmS* plays a less relevant role in biofilm formation. Our study shows that the inactivation of *bfmS* could reduce biofilm formation in *A. baumannii* ATCC 17978. Moreover, the deficiency of biofilm formation in the Km::bfmS mutant is associated with the repression of *csuA* mRNA expression (data not shown). One possible explanation for these results is that modulation of *bfmS* either at the chromosomal level or in phenotypic characterization might differ among strains. Detailed mechanisms for such differences remain to be elucidated. In conclusion, this report is the first demonstration that BfmS mediates multiple virulence processes in *A. baumannii*. The identification and characterization of the BfmS-mediated regulatory system will be required for a fuller understanding of the pathobiology of this bacterium.

Acknowledgments

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References

1. Giamarellou H, Antoniadou A, Kanellakopoulou K. *Acinetobacter baumannii*: a universal threat to public health? *Int J Antimicrob Agents* 2008;**32**:106—19.
2. Gordon NC, Wareham DW. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents* 2010;**35**:219—26.
3. Lo WT, Lin WJ, Chiuh TS, Lee SY, Wang CC, Lu JJ. Changing trends in antimicrobial resistance of major bacterial pathogens, 1985—2005: a study from a medical center in northern Taiwan. *J Microbiol Immunol Infect* 2011;**44**:131—8.
4. Rodríguez-Baño J, Martí S, Soto S, Fernández-Cuenca F, Cisneros JM, Pachón J, et al. Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications. *Clin Microbiol Infect* 2008;**14**:276—8.
5. Tomaras AP, Dorsey CW, Edelmann RE, Actis LA. Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone—usher pili assembly system. *Microbiology* 2003;**149**:3473—84.
6. Loehfelm TW, Luke NR, Campagnari AA. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J Bacteriol* 2008;**190**:1036—44.
7. Bhargava N, Sharma P, Capalash N. Quorum sensing in *Acinetobacter*: an emerging pathogen. *Crit Rev Microbiol* 2010;**36**: 349—60.
8. Lee HW, Koh YM, Kim J, Lee JC, Lee YC, Seol SY, et al. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. *Clin Microbiol Infect* 2008;**14**:49—54.
9. Seifert H, Strate A, Pulverer G. Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. *Medicine (Baltimore)* 1995;**74**: 340—9.
10. Kim SW, Choi CH, Moon DC, Jin JS, Lee JH, Shin JH, et al. Serum resistance of *Acinetobacter baumannii* through the binding of factor H to outer membrane proteins. *FEMS Microbiol Lett* 2009;**301**:224—31.
11. Beier D, Gross R. Regulation of bacterial virulence by two-component systems. *Curr Opin Microbiol* 2006;**9**:143—52.
12. Ashby MK. Survey of the number of two-component response regulator genes in the complete and annotated genome sequences of prokaryotes. *FEMS Microbiol Lett* 2004;**231**: 277—81.
13. Groisman EA. The pleiotropic two-component regulatory system PhoP—PhoQ. *J Bacteriol* 2001;**183**:1835—42.
14. Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology* 2008;**154**:3398—409.
15. Hohmann EL, Oletta CA, Killeen KP, Miller SI. phoP/phoQ-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J Infect Dis* 1996;**173**:1408—14.
16. Sambrook J, Russell DW. *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
17. Overhage J, Lewenza S, Marr AK, Hancock RE. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-lux mutant library. *J Bacteriol* 2007; **189**:2164—9.
18. Gaddy JA, Tomaras AP, Actis LA. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect Immun* 2009;**77**:3150—60.
19. Soares NC, Cabral MP, Parreira JR, Gayoso C, Barba MJ, Bou G. 2-DE analysis indicates that *Acinetobacter baumannii* displays a robust and versatile metabolism. *Proteome Sci* 2009;**7**:37.
20. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999;**20**: 3551—67.
21. Wikler MAClinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. *Clinical and Laboratory Standards Institute [Fulltext pdf.]*. 8th:[xvi, 65 p.]. Available from: http://www.clsi.org/source/orders/free/m07-a8.pdf; 2009.
22. Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* 2000;**37**: 219—25.
23. Choi CH, Lee JS, Lee YC, Park TI, Lee JC. *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC Microbiol* 2008;**8**:216.
24. Jin JS, Kwon SO, Moon DC, Gurung M, Lee JH, Kim SI, et al. *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. *PLoS One* 2011;**6**: e17027.
25. Clark RB. Imipenem resistance among *Acinetobacter baumannii*: association with reduced expression of a 33—36 kDa outer membrane protein. *J Antimicrob Chemother* 1996;**38**: 245—51.
26. Limansky AS, Mussi MA, Viale AM. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. *J Clin Microbiol* 2002; **40**:4776—8.