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Journal Title: Antimicrobial Agents and Chemotherapy

Volume: Volume 60, Number 6

Publisher: American Society for Microbiology | 2016-06-01, Pages 3751-3758

Type of Work: Article | Final Publisher PDF

Publisher DOI: 10.1128/AAC.00361-16

Permanent URL: <https://pid.emory.edu/ark:/25593/s0pzzq>

Final published version: <http://dx.doi.org/10.1128/AAC.00361-16>

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Accessed June 24, 2024 8:04 PM EDT

Mutations Decreasing Intrinsic β -Lactam Resistance Are Linked to Cell Division in the Nosocomial Pathogen *Acinetobacter baumannii*

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Transposon mutagenesis was used to identify novel determinants of intrinsic β -lactam resistance in *Acinetobacter baumannii*. An EZ-Tn5 transposon insertion in a gene corresponding to the A1S_0225 sequence resulted in a 4-fold decrease in resistance to ampicillin, cefotaxime, imipenem, and ceftriaxone but did not alter resistance to other classes of antibiotics. Based on this phenotype, the gene was designated *blhA* (β -lactam hypersusceptibility). The *blhA::EZ-Tn5* mutation conferred a similar phenotype in *A. baumannii* strain ATCC 17978. The wild-type *blhA* gene complemented the *blhA::EZ-Tn5* insertion and restored β -lactam resistance levels back to wild-type levels. The *blhA* mutation also increased β -lactam susceptibility in an *adeB adeJ* double mutant, indicating that the *blhA* mutation acted independently of these efflux systems to mediate susceptibility. In addition, mRNA levels for the *bla_{OXA}* and *bla_{ADC}* β -lactamase genes were not altered by the *blhA* mutation. The *blhA* mutation resulted in a prominent cell division and morphological defect, with cells exhibiting a highly elongated phenotype, combined with large bulges in some cells. The *blhA* gene is unique to *Acinetobacter* and likely represents a novel gene involved in cell division. Three additional mutations, in *zipA*, *zapA*, and *ftsK*, each of which encode predicted cell division proteins, also conferred increased β -lactam susceptibility, indicating a common link between cell division and intrinsic β -lactam resistance in *A. baumannii*.

The Gram-negative bacterium *Acinetobacter baumannii* is rapidly becoming an important human pathogen and is associated with infections of the lungs, skin and soft tissues, bloodstream, and urinary tract (1–5). Infections due to this bacterium are primarily health care associated, but the incidence of community-acquired infections is on the rise and may reflect the increased virulence of some strains (6–8). The importance of this bacterium has been recognized by the Infectious Diseases Society of America, who classified it as a member of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens due to the extensive antibiotic resistances that are present (9, 10). Treatment of this bacterium with antibiotics is becoming increasingly problematic, with decreasing therapeutic options, and in some cases isolates are resistant to all commonly used antibiotics.

Intrinsic resistance to the β -lactam class of antibiotics in *A. baumannii* is mediated by a number of mechanisms, including (i) chromosomally encoded β -lactamases; (ii) alterations in penicillin binding proteins (PBPs); (iii) efflux via members of the resistance-nodulation-division (RND) family, such as AdeABC and AdeIJK; and (iv) changes in outer membrane permeability (11–22). The chromosomally encoded β -lactamases include those encoded by the *bla_{ADC}* and *bla_{OXA}* genes (17–22). Resistance conferred by the *bla_{ADC}* and *bla_{OXA}* genes typically requires increased expression, and this can be mediated by an *ISAbal* element inserting upstream and driving overexpression via a strong outward-facing promoter (23–27).

In this study, we sought to identify genes that contribute to the intrinsic levels of β -lactam resistance in *A. baumannii*. The results of this analysis revealed a novel gene required for cell division in *A. baumannii*, and mutations in this gene reduced intrinsic β -lactam resistance. Moreover, the isolation of additional mutations in the cell division genes *zipA*, *zapA*, and *ftsK* indicates a common link

between the inhibition of cell division and increased susceptibility to β -lactams in *A. baumannii*.

MATERIALS AND METHODS

Strains and growth conditions. *A. baumannii* strains AB900 and ATCC 17978 were used in this study (28, 29). Bacteria were grown in lysogeny broth (LB) at 37°C. Antibiotics were used at the following concentrations for *Escherichia coli*: ampicillin, 150 μ g/ml; kanamycin, 20 μ g/ml; and chloramphenicol, 25 μ g/ml. For *A. baumannii*, ampicillin was used at 800 μ g/ml, and kanamycin was used at 40 μ g/ml for selection of transposon insertions and at 7.5 μ g/ml for recombineering. The strains and plasmids used for this study are described in Table 1.

Transposon mutagenesis. The transposon EZTn5<KAN-2>Tnp (Epicenter, Madison, WI) was used for all experiments. To create an insertional library, *A. baumannii* strain AB900 was grown to an optical density at 600 nm (OD₆₀₀) of 0.5 in 30 ml LB medium and pelleted by centrifugation. Cells were washed 3 times with cold 10% glycerol and resuspended at 1/100 the original volume. Cells (60 μ l) were electroporated with 1 μ l EZ::Tn5<KAN-2>Tnp, plated on LB agar plates with kanamycin (40 μ g/ml), and incubated overnight at 37°C. The resulting individual colonies were saved in 96-well microtiter plates containing 150 μ l of LB plus 20% glycerol and stored at –80°C until further use. Colonies

Received 17 February 2016 Returned for modification 15 March 2016

Accepted 3 April 2016

Accepted manuscript posted online 11 April 2016

Citation Knight D, Dimitrova DD, Rudin SD, Bonomo RA, Rather PN. 2016. Mutations decreasing intrinsic β -lactam resistance are linked to cell division in the nosocomial pathogen *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 60:3751–3758. doi:10.1128/AAC.00361-16.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00361-16>.

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TABLE 1 Strains and plasmids

Plasmid or strain	Relevant characteristics or genotype	Source or reference
Plasmids		
pBC.KS	High copy number; Cm ^r	Lab stock
pWH1266	<i>E. coli</i> - <i>A. baumannii</i> shuttle plasmid; Amp ^r Tc ^r	57
pACYC184	Cm ^r Tc ^r	30
pEX18Tc	Suicide plasmid; Tet ^r <i>sacB</i>	31
pBC.adeB	pBC.KS containing the cloned <i>adeB</i> gene	This study
pBC.adeJ	pBC.KS containing the cloned <i>adeJ</i> gene	This study
pDK1	pEX18Tc containing the <i>blhA</i> gene	This study
pAT02	IPTG-inducible RecET recombination system	32
pAT03	IPTG-inducible FLP recombinase	32
pWH.zipA	<i>zipA</i> gene cloned into the <i>ScaI</i> site of pWH1266	This study
pWH.zipA	<i>zapA</i> gene cloned into the <i>ScaI</i> site of pWH1266	This study
Strains		
AB900	Clinical isolate, β -lactam sensitive	28
ATCC 17978	Wild type, β -lactam sensitive	29
DK1	ATCC 17978 <i>blhA</i> ::EZ::Tn5<KAN-2>	This study
DK2	DK1/pDK1	This study
DD1	ATCC 17978 Δ <i>adeB</i> Δ <i>adeJ</i>	This study
DK3	DD1 <i>blhA</i> ::EZ::Tn5<KAN-2>	This study
DK4	DD1 <i>zipA</i> ::EZ::Tn5<KAN-2>	This study
DK5	DD1 <i>ftsK</i> ::EZ::Tn5<KAN-2>	This study
DK6	DD1 <i>zapA</i> ::EZ::Tn5<KAN-2>	This study
DK7	ATCC 17978 <i>zipA</i> ::EZ::Tn5<KAN-2>	This study
DK8	ATCC 17978 <i>ftsK</i> ::EZ::Tn5<KAN-2>	This study
DK9	ATCC 17978 <i>zapA</i> ::EZ::Tn5<KAN-2>	This study

were tested for ceftriaxone sensitivity by dropping 1 μ l of each cell suspension on LB plates containing ceftriaxone at either 1 μ g/ml or 2 μ g/ml.

Cloning and identification of EZ::Tn5<KAN-2> insertion sites.

Genomic DNAs from mutants were isolated and subjected to a partial *Sau3A1* digestion. Fragments in the 2- to 5-kb range were gel purified and ligated to pACYC184 (30) digested with *Bam*HI. The resulting ligation product was electroporated into EC100D competent cells (Epicentre, Madison, WI) and plated onto LB agar plates containing chloramphenicol (25 μ g/ml) and kanamycin (20 μ g/ml). Plasmids were sequenced with the FP1 and RP1 primers, which read outward from the transposon, and the chromosomal region disrupted by the transposon was determined by BLAST analysis.

Complementation of the A1S_0225 mutation. A DNA fragment containing the A1S_0225 gene and promoter region was amplified from ATCC 17978 by PCR using the primers 5'-CTGAGAAGGTCATTGCTCGCC-3' and 5'-GTTTGTAGTATAGAACCCTCGAACC-3'. The PCR fragment was purified and ligated into the suicide vector pEX18 (31), which was linearized with *Sma*I. The ligation product was electroporated into *E. coli* DH5 α cells and plated onto LB agar plates with tetracycline (5 μ g/ml) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). A plasmid with the correct insert was designated pDK1 and electroporated into ATCC 17978 *blhA*::*Km*, and resulting colonies were plated onto LB plus tetracycline (5 μ g/ml) to select for integration into the chromosome by a single crossover.

Complementation of the *zipA* and *zapA* mutations. The wild-type *zapA* and *zipA* genes were cloned by PCR using the following primers: *zapA*.for, 5'-ATGCTGAAGCTGGTGAGGAGAC-3'; *zapA*.rev, 5'-CTGACTGACTCGTTGGCAATC-3'; *zipA*.for, 5'-GACTTGTGGGTGTAACCATGCCT-3'; and *zipA*.rev, 5'-AAAAGGGTGGAAATTTCTCCAC

CC-3'. Each PCR product contained its native ribosome binding site and was cloned into the *ScaI* site of pWH1266 such that each gene was expressed from the promoter for the ampicillin resistance gene.

Construction of an *adeB adeJ* double mutant. For cloning of the *adeB* gene, the primers 307 *adeB*.for (5'-AAAAATATCAGCTTAAAAAG-3') and *adeB*.rev (5'-CATCGATTGGCTTAAAAATC-3') were used to PCR amplify the *adeB* gene from *A. baumannii* AB307 chromosomal DNA. The *adeB* fragment was then cloned into the *SmaI* site of pBC.KS. For cloning of *adeJ*, the primers *adeJ*.for (5'-TTCAGGCATCATACGGC TGC-3') and *adeJ*.rev (5'-ACCAAAATGGTGTAGCCGC-3') were used to amplify the *adeJ* gene from AB307 chromosomal DNA, and the resulting PCR product was cloned into the *SmaI* site of pBC.KS. For both plasmids, DNA sequence analysis was used to verify that the correct gene was cloned.

Plasmids pBC.adeB and pBC.adeJ were digested with *Hinc*II and *Nsi*I, respectively, and then ligated with a PCR-amplified kanamycin resistance cassette flanked by FRT recombination sites obtained from pKD4. To amplify a fragment containing *adeB*::*Km*, the primers AdeB-EF.For and AdeB-EF.Rev were used. For PCR amplification of *adeJ*::*Km*, the primers AdeJ-ET.For and AdeJ-ET.Rev were used. To create chromosomal disruptions, *A. baumannii* ATCC 17978 carrying pAT02 was inoculated into LB broth with ampicillin to maintain the plasmid, and IPTG (isopropyl- β -D-thiogalactopyranoside; Sigma-Aldrich) was added at an OD₆₀₀ of 0.1 to a final concentration of 5 mM. The culture was shaken for 3 h at 37°C and then harvested. The pelleted bacteria were washed three times in ice-cold 10% glycerol and concentrated 1,000-fold. The linear *adeB*::*Km* PCR product was used to electroporate 60 μ l of *A. baumannii* ATCC 17978/pAT02 at 2.5 kV. The cells were incubated for 30 min at 37°C without shaking in 1 ml LB broth containing 5 mM IPTG and then for 1 h with shaking at 37°C. The electroporation mixture was then plated on LB agar with 7.5 μ g/ml kanamycin. Colonies with the *adeB*::*Km* disruption were verified by PCR. The *adeB*::*Km* mutant was cured of pAT02 and then transformed with pAT03, expressing the FLP recombinase, and induced with 5 mM IPTG at an OD₆₀₀ of 0.5 for 3 h. The culture was plated on LB agar plates containing ampicillin (800 μ g/ml), and single colonies were checked for loss of the kanamycin cassette and then verified by PCR. The cells were then cured of the pAT03 plasmid by growth without antibiotic selection, and cells were retransformed with pAT02. A linear fragment containing the *adeJ*::*Km* disruption was electroporated as described above, and colonies with the *adeJ*::*Km* disruption were verified by PCR. The kanamycin resistance cassette in *adeJ* was excised as described above for *adeB*::*Km*, and a strain with the *adeB adeJ* double mutation was verified by PCR and cured of all plasmids. The *adeB* and *adeJ* mutants containing FLP scars had the same phenotypes as those when the genes were disrupted by the kanamycin resistance cassette (data not shown).

Construction of mutants by recombineering. Recombineering was used to move the *blhA*::EZ::Tn5 insertion into ATCC 17978 (32). First, the plasmid pAT02 was electroporated into either ATCC 17978 or ATCC 17978 Δ *adeB* Δ *adeJ*. A linear DNA fragment containing the *blhA*::EZ::Tn5 disruption was amplified from the AB900 background by PCR with the primers 5'-GTGGCATTAAATGTCGGGCAAG-3' and 5'-CTTCTTGAG CCGCAGCATGC-3'. The resulting product was ethanol precipitated and electroporated into either ATCC 17978 or ATCC 17978 Δ *adeB* Δ *adeJ* containing pAT02 that had first been induced with 3 mM IPTG for 3 h. Cells were plated onto LB plus kanamycin (10 μ g/ml) and incubated at 37°C. The correct disruption was verified by PCR using the above-described primers, and cells were cured of pAT02 as described above.

To move the *zipA*::*Km*, *ftsK*::*Km*, and *zapA*::*Km* mutations into a wild-type background, ATCC 17978/pAT02 was electroporated with PCR-generated linear fragments containing the coding region of each gene along with the EZ::Tn5<KAN-2> insertion. Putative recombinants were verified by PCR, and each strain was cured of pAT02 as described above.

Semiquantitative reverse transcription-PCR (RT-PCR) to examine mRNA levels from the *bla_{ADC}* and *bla_{OXA}* genes. RNAs from wild-type *A. baumannii* ATCC 17978 (wild type) and the *blhA::Km* mutant were prepared from 2-ml LB cultures grown to an OD₆₀₀ of 0.7 by use of a MasterPure RNA purification kit (Epicentre Biotechnologies) per the manufacturer's protocol. Contaminating DNA was removed using a Turbo DNA-free kit (Ambion, Inc.). Purified RNA was tested for the absence of DNA by PCR, and cDNA was prepared using an iScript Select cDNA synthesis kit (Bio-Rad). PCR amplification was performed using primers 16SqPCR.for (5'-GATCTTCGGACCTTGCGCTA-3') and 16SqPCR.rev (5'-GTGTCTCAGTCCCAGTGTGG-3') in 5- μ l aliquots of the PCR mixture collected at cycles 5, 10, and 15 in order to normalize cDNA samples to 16S rRNA gene expression. Once the amplification was equilibrated, *bla_{OXA}* expression was examined using primers *oxa-51qPCR.for* (5'-TCGTGCTTCGACCGAGTATG-3') and *oxa-51qPCR.rev* (5'-CTTATG GTGCTCAAGGCCGA-3') at cycles 20, 25, 30, and 35; *bla_{ADC}* expression was examined using primers *adc-7qPCR.for* (5'-ACCAGCGGTTTCGG AACATA-3') and *adc-7qPCR.rev* (5'-GCTGCCTTAATGCGCTCTTC-3') at cycles 30, 35, 40, and 45; and *clpX* expression was examined using primers *clpX.for* (5'-GCGTTTGAAGTTCGGGCAAT-3') and *clpX.rev* (5'-CCATTGCAAACGGCACATCT-3') at cycles 15, 20, 25, and 30.

MIC analysis. The strains of interest were freshly streaked out on Mueller-Hinton agar plates. The following day, cell suspensions at a 0.5 McFarland turbidity standard were prepared for each strain and inoculated onto fresh Mueller-Hinton agar plates. The suspensions were allowed to absorb into the plates for 15 to 20 min. Once the plates were dry, Etest strips were applied. The plates were incubated at 37°C for 16 to 20 h until an even lawn of growth was clearly visible before MIC determinations were made.

RESULTS

A novel intrinsic determinant of β -lactam resistance in *A. baumannii*. To identify genes contributing to intrinsic β -lactam resistance in *A. baumannii* strain AB900, a library of transposon insertions was screened for increased susceptibility to ceftriaxone (see Materials and Methods). Two insertions with this phenotype were isolated. One mutant contained an insertion in *adeI*, encoding a component of the AdeIJK efflux system previously shown to have a role in β -lactam resistance, and was not studied further (15). The second insertion was in a gene of unknown function that corresponded to the A1S_0225 gene of the ATCC 17978 genome. The insertion disrupted the gene at a position corresponding to amino acid 42 of the 211-amino-acid protein. The A1S_0225 gene appeared to be monocistronic, and the nearest downstream gene was 349 bp from the stop codon for A1S_0225. The product of the A1S_0225 gene was highly conserved in *Acinetobacter* and exhibited at least 89% amino acid identity to a protein encoded in a wide variety of non-*baumannii* *Acinetobacter* species (33), including *A. nosocomialis*, *A. calcoaceticus*, *A. pittii*, and *A. oleivorans*, and in *Acinetobacter* sp. strains SH024, WC-141, NIPH542, V2, and WC-487. The A1S_0225 gene product was also 60 to 75% identical to a protein encoded in *A. tjernbergiae*, *A. venetianus*, *A. haemolyticus*, *A. parvus*, *A. johnsonii*, *A. lwoffii*, *A. johnsonii*, and *A. baylyi*. However, the A1S_0225 gene product did not appear to have close homologs in other bacteria, with the exception of *Bacillus mycoides*, where an ortholog with 61% amino acid identity and 75% similarity was present (WP_044738812.10). A region of the A1S_0225-encoded protein from amino acids 17 to 110 exhibited limited homology with the NtpI ATPase family, but it did not possess any obvious transmembrane regions or signal sequences, suggesting a cytoplasmic location for the A1S_0225-encoded protein.

TABLE 2 MICs of antibiotics in this study

Antibiotic	MIC (μ g/ml) for strain ^a				
	ATCC 17978	<i>blhA::Km</i> mutant	<i>blhA::Km</i> <i>blhA</i> ⁺ strain	Δ <i>adeB</i> Δ <i>adeJ</i> mutant	Δ <i>adeB</i> Δ <i>adeJ</i> <i>blhA::Km</i> mutant
Ampicillin	24	6	48	8	3
Ceftriaxone	8	2	16	2	0.38
Cefotaxime	12	2	ND	1.5	0.38
Amikacin	3	3	ND	3	3
Chloramphenicol	256	256	ND	24	12
Gentamicin	0.75	0.5	ND	1	0.75
Ofloxacin	0.38	0.19	ND	0.047	0.023
Rifampin	8	8	ND	4	4
Tigecycline	0.19	0.19	ND	0.023	0.023
Trimethoprim	32	32	ND	8	3

^a ND, not determined.

The insertion in the A1S_0225 homolog in AB900 resulted in a 4-fold increase in susceptibility to ceftriaxone relative to that of wild-type AB900 (2 μ g/ml versus 8 μ g/ml). The levels of resistance to the β -lactams ampicillin and cefoxitin were reduced at least 4-fold, and that to imipenem was reduced 3-fold. However, the susceptibility to non- β -lactam antibiotics was either unchanged (for ofloxacin, amikacin, and tigecycline) or reduced slightly (for trimethoprim and rifampin) (see Table S1 in the supplemental material). Based on this resistance profile, the gene was designated *blhA* (β -lactam hypersusceptibility).

To confirm that the *blhA::Km* mutation conferred β -lactam sensitivity, we attempted to use recombineering in the AB900 wild-type strain to recreate this mutation (32). Despite repeated attempts, we were unable to construct this mutation in AB900. However, when recombineering was used in the ATCC 17978 background, this mutation was easily constructed, and all subsequent characterizations of the *blhA::Km* mutation were done in the ATCC 17978 background. The resistance profile of ATCC 17978 *blhA::Km* was determined for a panel of antibiotics, and this mutant also showed selective susceptibility to β -lactams, with 4- to 6-fold decreases in resistance to ampicillin, ceftriaxone, and cefotaxime (Table 2).

To demonstrate that the phenotype of the *blhA::Km* mutant was not due to polar effects on a downstream gene, complementation analysis was performed using the cloned *blhA* gene. Initially, when the *blhA* gene was cloned into the shuttle vector pWH1266, the resulting plasmid gave rise to very small colonies for *E. coli*. When the *blhA*-containing plasmid was then electroporated into either the *blhA::Km* mutant or wild-type *A. baumannii*, the transformation frequency was approximately 0.01% compared to that for the pWH1266 vector alone. In addition, the rare transformants that arose contained deletions in the cloned *blhA* gene. This indicated that the *blhA* gene in multicopy was toxic to *A. baumannii*. To circumvent this problem, the *blhA* gene was cloned into the suicide plasmid pEX18, resulting in pDK1, and integrated as a single copy at the *blhA* locus by homologous recombination. In this case, the *blhA::Km* mutation was fully complemented by a wild-type copy of *blhA* and restored the levels of ceftriaxone resistance to the levels seen for wild-type ATCC 17978 (Table 2).

The *blhA* mutation results in a cell division defect. The ATCC 17978 *blhA::Km* mutant formed colonies on agar plates that were

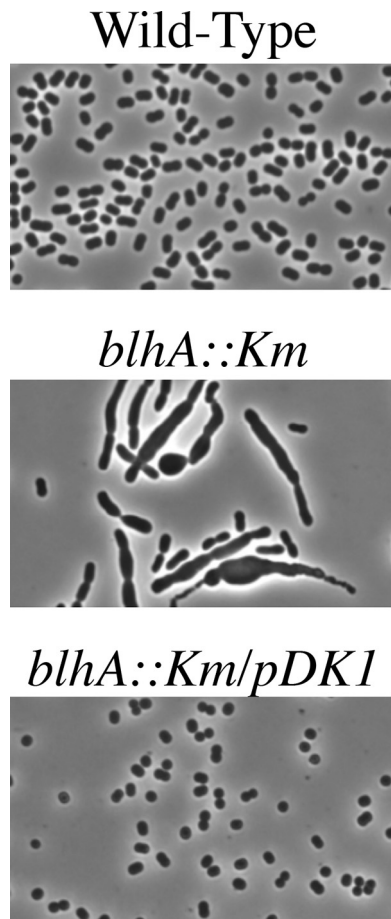


FIG 1 Cell morphologies of wild-type ATCC 17978, the *blhA::Km* mutant, and the *blhA::Km* mutant complemented with pDK1, containing the wild-type *blhA* gene in single copy. Cells were grown to mid-log phase in LB broth, pelleted, and resuspended in a 1/10 volume of LB broth. Images were taken by phase-contrast microscopy using an Olympus BX51 microscope and photographed with an Infinity 2-1 charge-coupled device (CCD) camera (Lumenera). Magnification, $\times 1,000$.

distinctly different from the wild-type colonies, suggesting that cell morphology might be altered. When the *blhA* mutant was examined by phase-contrast microscopy, irregular cells were observed that either (i) were highly elongated, (ii) formed chains of cells, or (iii) formed cells with a bloated/swollen appearance (Fig. 1). The appearance of these cells did not change significantly upon the addition of ceftriaxone at $0.5\times$ and $0.25\times$ MIC (data not shown). The altered cell division/morphology phenotype was corrected to wild-type when a wild-type copy of the *blhA* gene was introduced into the *blhA::Km* mutant (Fig. 1). A similar cell division defect was observed in the original AB900 *blhA::Km* mutant (see Fig. S1 in the supplemental material).

The *blhA* mutation does not act through the AdeABC or AdeIJK efflux system. Bioinformatic analysis of the BlhA protein did not give any clues to its function, and the mechanism by which it contributed to β -lactam resistance was unclear. To address the possibility that the *blhA* mutation reduced β -lactam resistance by inhibiting the AdeABC or AdeIJK efflux system, the *blhA::Km* mutation was introduced into an ATCC 17978 strain with null alleles of both the *adeB* and *adeJ* genes. The *blhA::Km* mutation in the

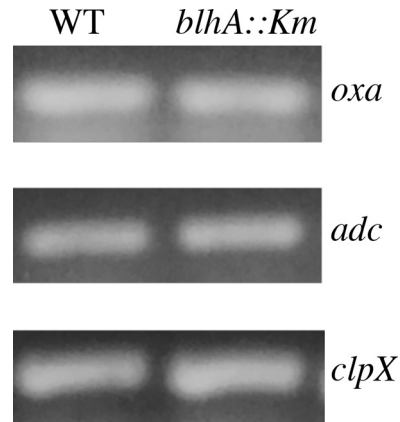


FIG 2 Semiquantitative RT-PCR analysis. Total RNAs were prepared from wild-type ATCC 17978 and the *blhA::Km* mutant, and contaminating DNA was removed by DNase treatment. The resulting RNAs were used to examine the accumulation of mRNAs from the *bla_{OXA}* and *bla_{ADC}* β -lactamase genes. The *clpX* gene was used as an internal control. The use of primers for the 16S rRNA gene also gave equal expression values for each sample (data not shown). The images shown are at cycle 30 for *bla_{OXA}*, at cycle 35 for *bla_{ADC}*, and at cycle 25 for *clpX*.

adeB adeJ double-mutant background exhibited a 3- to 5-fold reductions in the MICs of β -lactams, similar to the 4- to 6-fold decreases seen in the wild-type background (Table 2). This result indicated that the *blhA::Km* mutation acted independently of the AdeABC and AdeIJK efflux systems to reduce β -lactam resistance.

The *blhA* mutation does not alter *bla_{OXA}* or *bla_{ADC}* mRNA accumulation. The possibility that the *blhA::Km* mutation acted to decrease β -lactam resistance by reducing expression of the *bla_{OXA}* or *bla_{ADC}* β -lactamase gene was investigated by semiquantitative RT-PCR. The expression of each gene showed similar levels in both the wild-type and *blhA::Km* backgrounds (Fig. 2).

Identification of additional mutations that decrease intrinsic β -lactam resistance in a strain lacking the AdeABC and AdeIJK efflux systems. In the above search for mutations that reduced β -lactam resistance in a wild-type background, the contributions of the AdeABC and AdeIJK RND efflux systems to intrinsic β -lactam resistance may have limited the spectrum of mutations that were identified. For example, if the combined activity of AdeABC and AdeIJK provided most of the intrinsic levels of β -lactam resistance, then the role of other mechanisms may have been masked. To determine if additional intrinsic mechanisms were present, the previously described transposon screen was repeated in an ATCC 17978 background containing mutations in both *adeB* and *adeJ*. In this background, among 2,000 colonies screened, three additional insertions were identified that conferred β -lactam sensitivity (Table 3). These insertions were in *A. baumannii* homologs of the previously identified *E. coli* cell division genes *zipA*, *zapA*, and *ftsK* (34–37). The insertion in *zipA* disrupted the open reading frame at a position corresponding to amino acid 258 of the 345-amino-acid protein. The *A. baumannii* ZipA protein exhibited 26% identity and 48% similarity to amino acids 69 to 206 of the *E. coli* ZipA protein. In addition, the genetic organization in *A. baumannii* was similar to that in *E. coli*, with *zipA* being adjacent to the *ligA* gene, encoding DNA ligase. The insertion in *zapA* disrupted the gene region corresponding to amino acid 3 of the 94-amino-acid protein. The *A. baumannii*

TABLE 3 MICs for cell division mutants^a

Antibiotic	MIC ($\mu\text{g/ml}$) for strain			
	ATCC 17978 $\Delta\text{adeB } \Delta\text{adeJ}$	<i>zipA</i> mutant	<i>zapA</i> mutant	<i>ftsK</i> mutant
Ampicillin	4	2	2	1.5
Ceftriaxone	1.5	0.38	0.38	0.38
Rifampin	3	2	3	2
Tigecycline	0.094	0.125	0.094	0.094
Chloramphenicol	16	16	16	16
Ofloxacin	0.032	0.016	0.012	0.012
Trimethoprim	2	0.5	0.25	0.38

^a All mutants were constructed in the ATCC 17978 $\Delta\text{adeB } \Delta\text{adeJ}$ background.

ZapA protein exhibited 23% identity and 55% similarity to the ZapA protein from *E. coli*. The insertion in *ftsK* disrupted the gene at a position corresponding to amino acid 721 of the 1,010-amino-acid protein. *A. baumannii* FtsK amino acids 497 to 1,010 exhibited 56% identity and 69% similarity to FtsK from *E. coli*.

As expected, each of these insertions had a dramatic effect on cell division and morphology (Fig. 3). Both the *zipA* and *zapA* mutants had phenotypes similar to that of the *blhA* mutant, with a combination of elongated cells, bloated cells, and cell chaining. However, the *ftsK* mutant tended to produce a larger percentage of cells that existed in chains, although elongated and bloated cells could occasionally be seen (Fig. 3).

Introduction of the wild-type *zipA* and *zapA* genes cloned into pWH1266 into the respective mutants restored the levels of ceftriaxone resistance from 0.38 $\mu\text{g/ml}$ to 1.0 $\mu\text{g/ml}$, similar to the 1.5 $\mu\text{g/ml}$ seen for the *adeB adeJ* parent strain. In addition, the cell division defect was corrected in each mutant (data not shown). Despite repeated attempts, we were unable to clone the *ftsK* gene in multicopy. The basis for this is unclear, but overexpression of *ftsK* may be toxic to *E. coli*.

The phenotypes of the *zapA*, *ftsK*, and *zipA* mutants were not dependent on the *adeB adeJ* mutant background. When the mutations were moved into the wild-type ATCC 17978 background by recombineering, they exhibited the same morphological and cell division defects, and the MIC of ceftriaxone was reduced 4- to 5-fold for each mutant relative to that for wild-type ATCC 17978 (data not shown).

DISCUSSION

In this study, a novel *A. baumannii* gene, *blhA*, was identified based on the β -lactam susceptibility phenotype resulting from a *blhA::EZ::Tn5* mutation. The *blhA* mutation did not alter the levels of resistance to other classes of antibiotics (Table 2). The *blhA* mutation did not alter expression of the chromosomal *bla_{OXA}* or *bla_{ADC}* β -lactamase gene. In addition, the *blhA* mutation resulted in 3- to 5-fold reductions in β -lactam resistance in an *adeB adeJ* double mutant, similar to the levels of reduction observed for wild-type cells. This indicated that the *blhA* mutation acted independently of both the AdeABC and AdeIJK efflux systems and the *bla_{OXA}* or *bla_{ADC}* β -lactamase gene to reduce β -lactam resistance.

The function of the BlhA protein is currently unknown, and a bioinformatic analysis did not revealed obvious clues to its role in cell division. The BlhA protein does not possess an apparent signal sequence or membrane-spanning regions and appears to be a cytoplasmic protein. Loss of *blhA* resulted in a striking cell division defect associated with a variety of cell morphologies, including (i)

highly elongated cells; (ii) cells exhibiting a chaining phenotype; and (iii) extensive bloating, indicating a loss of cell wall integrity. These various cell division defects are difficult to reconcile with known cell division mutants. The defect in cell separation and the resulting chaining phenotype are similar to those seen in cells lacking peptidoglycan hydrolases that cleave septal peptidoglycan, such as AmiABC, or in the twin-arginine transport pathway required for secretion of AmiA and AmiC. However, *ami* or *tat* mutants typically form chains of cells and are not highly elongated and/or bloated (38–40). Moreover, none of the Ami proteins are cytoplasmic, suggesting that BlhA does not possess their functions. The bloating phenotype may result from a defect in peptidoglycan synthesis or from the enhanced activity of one or more peptidoglycan hydrolases. The putative cytoplasmic localization of BlhA may indicate a possible role in lipid II synthesis; however, it is unclear how this would result in the chaining phenotype. Regardless of the role of BlhA, it is intriguing that this protein is highly specific to *Acinetobacter* spp., which suggests that a unique aspect of cell division is present in this bacterium and thus represents a potential target for small molecules that may inhibit BlhA and sensitize *A. baumannii* to β -lactams. This study also suggests that small molecules capable of targeting ZipA, ZapA, or FtsK

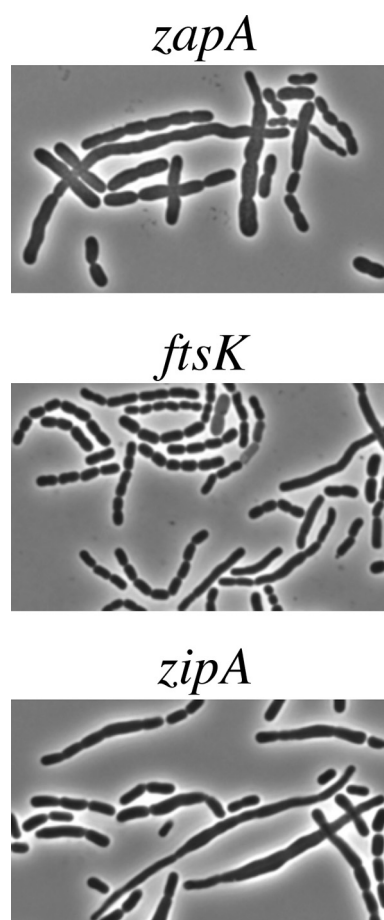


FIG 3 Morphologies of cell division mutants. Cells were grown to mid-log phase in LB broth, pelleted, and resuspended in a 1/10 volume of LB broth. Images were taken by phase-contrast microscopy using an Olympus BX51 microscope and photographed with an Infinity 2-1 CCD camera (Lumenera). Magnification, $\times 1,000$.

would also sensitize *A. baumannii* to β -lactams. In addition, targeting multiple cell division proteins simultaneously may have synergistic effects with respect to β -lactam susceptibility. This possibility is currently being investigated by constructing a strain with more than one cell division gene deleted.

The mechanism by which loss of BlhA results in β -lactam hypersusceptibility is currently unknown, but it seems to be related directly to the cell division defect. Consistent with this, mutations in genes encoding the predicted cell division proteins ZipA, ZapA, and FtsK also conferred β -lactam hypersusceptibility (Table 3). The ZipA, ZapA, and FtsK proteins colocalize at the division site (41–43). The ability to obtain a transposon insertion in *zipA* was unexpected because it is an essential gene in *E. coli* (35). In addition, there was no difficulty in moving the *zipA::Km* mutation into the wild-type ATCC 17978 background by recombineering, although the resulting colonies were small. In *E. coli*, a suppressor mutation that allows cells to survive in the absence of *zipA* maps to the cell division gene *ftsA* and results in an arginine-to-tryptophan (R286W) change in FtsA (44). The *A. baumannii* FtsA protein has a glutamine (Q) at this position, and this change together with additional differences in FtsA may allow for cells to be partially independent of ZipA.

The mechanism by which defects in cell division sensitize cells to β -lactams may be the result of a feedback mechanism that reduces peptidoglycan synthesis when cells are unable to divide correctly. There is a growing body of evidence linking cell division to peptidoglycan synthesis (45–50). If this is correct, the resulting decrease in peptidoglycan synthesis would then sensitize cells to agents that further disrupt peptidoglycan synthesis, such as β -lactams. The finding that a variety of mutations altering cell division and peptidoglycan synthesis lead to β -lactam sensitivity supports this hypothesis. However, it should also be pointed out that our model would be inconsistent with studies reporting that inhibition of cell division increased the levels of resistance to β -lactams, although this was dependent on where the block occurred (51, 52). In addition, if it is present, the nature of the hypothesized feedback checkpoint is unknown, although it may involve post-transcriptional changes, as a previous study with *E. coli* found no transcriptional changes in *E. coli* related to peptidoglycan synthesis when cell division was inhibited (53).

It has also been demonstrated that the activities of septal cell wall hydrolases are tightly regulated by cell division (54–56). Therefore, an alternative explanation for the increased β -lactam susceptibility is that septal murein hydrolases are more active in cell division mutants and that this increases the susceptibility to β -lactams. Regardless of the mechanism, the identification of second-site suppressors that restore β -lactam resistance to strains with mutations in the previously mentioned cell division genes will reveal if these phenotypes are genetically separable or if cell division defects are intimately tied to decreased β -lactam resistance. These studies are currently in progress.

ACKNOWLEDGMENTS

This work was supported by grant R01AI72219 from the National Institutes of Health to P.N.R. and R.A.B. P.N.R. is also supported by grants from the Merit Review program and by a Research Career Scientist Award, both from the Department of Veterans Affairs. Research reported in this publication was also supported in part by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (grants R01AI100560 and R01AI063517 to R.A.B.). This study was also supported

in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs (grant 1I01BX001974 to R.A.B.) and by the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center (grant VISN 10 to R.A.B.).

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We are grateful to Bryan Davies at the University of Texas at Austin for providing the *A. baumannii* recombineering strains and for advice on their use. We also thank Piet de Boer at Case Western Reserve University for comments on the manuscript.

FUNDING INFORMATION

This work, including the efforts of Robert A. Bonomo, was funded by HHS | National Institutes of Health (NIH) (R01AI063517). This work, including the efforts of Philip N. Rather and Robert A. Bonomo, was funded by HHS | National Institutes of Health (NIH) (R01AI72219). This work, including the efforts of Robert A. Bonomo, was funded by HHS | National Institutes of Health (NIH) (R01AI100560).

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