ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2010, p. 4971–4977 Vol. 54, No. 12
0066-4804/10/$12.00 doi:10.1128/AAC.00834-10

Colistin Resistance in *Acinetobacter baumannii* Is Mediated by Complete Loss of Lipopolysaccharide Production▽

Jennifer H. Moffatt,[1] Marina Harper,[1,2] Paul Harrison,[3] John D. F. Hale,[4] Evgeny Vinogradov,[5] Torsten Seemann,[3] Rebekah Henry,[1] Bethany Crane,[1] Frank St. Michael,[5] Andrew D. Cox,[5] Ben Adler,[1,2,3]* Roger L. Nation,[4] Jian Li,[4] and John D. Boyce[1,2]

Department of Microbiology, Monash University, Clayton, Victoria, Australia[1]; Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Victoria, Australia[2]; Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia[3]; Facility for Anti-Infective Drug Development and Innovation, Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Clayton, Victoria, Australia[4]; and Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada[5]

Received 18 June 2010/Returned for modification 30 July 2010/Accepted 10 September 2010

Infections caused by multidrug-resistant (MDR) Gram-negative bacteria represent a major global health problem. Polymyxin antibiotics such as colistin have resurfaced as effective last-resort antimicrobials for use against MDR Gram-negative pathogens, including *Acinetobacter baumannii*. Here we show that *A. baumannii* can rapidly develop resistance to polymyxin antibiotics by complete loss of the initial binding target, the lipid A component of lipopolysaccharide (LPS), which has long been considered to be essential for the viability of Gram-negative bacteria. We characterized 13 independent colistin-resistant derivatives of *A. baumannii* type strain ATCC 19606 and showed that all contained mutations within one of the first three genes of the lipid A biosynthesis pathway: *lpxA*, *lpxC*, and *lpxD*. All of these mutations resulted in the complete loss of LPS production. Furthermore, we showed that loss of LPS occurs in a colistin-resistant clinical isolate of *A. baumannii*. This is the first report of a spontaneously occurring, lipopolysaccharide-deficient, Gram-negative bacterium.

Acinetobacter baumannii is an emerging, opportunistic, Gram-negative bacterial pathogen (19). It is associated with a range of nosocomial infections, including bacteremia, pneumonia, meningitis, and urinary tract infections. Outbreaks, especially in intensive care unit settings, have been identified in numerous countries around the world (23). The treatment of these infections is hampered by the rapid rise in prevalence of *A. baumannii* strains that are resistant to almost all available antibiotics, including β-lactams, fluoroquinolones, tetracyclines, and aminoglycosides (23). In these multidrug-resistant (MDR) strains, colistin (also known as polymyxin E) is often the only remaining treatment (15), although colistin-resistant clinical isolates have already been reported (7, 10, 21). Intriguingly, some *A. baumannii* isolates have been shown to display heteroresistance to colistin, where an apparently colistin-susceptible strain (based upon the MIC) harbors a small proportion of colistin-resistant cells (9, 16). Under selective pressure both *in vitro* (33) and *in vivo* (10), heteroresistant *A. baumannii* strains can rapidly give rise to strains with high-level colistin resistance.

Colistin is a cationic polypeptide antibiotic that is composed of a cyclic decapeptide linked by an α-amide linkage to a fatty acyl chain (15). Its structure differs from that of polymyxin B by only a single amino acid; the two antibiotics demonstrate comparable activities against a range of Gram-negative bacteria (6). Polymyxins are proposed to exert their antibacterial effect on Gram-negative bacteria via a two-step mechanism comprising initial binding to and permeabilization of the outer membrane, followed by destabilization of the cytoplasmic membrane (37). While the exact mechanism of bacterial killing is not clearly defined, a critical first step in the action of polymyxins is the electrostatic interaction between the positively charged peptide and the negatively charged lipid A, the endotoxic component of lipopolysaccharide (LPS) (3). It has been proposed that because polymyxins target the bacterial outer membrane lipid bilayer, resistance against these antimicrobial peptides is rare (3). However, polymyxin-resistant bacteria have been identified, and the characterized mechanisms of resistance generally involve modifications to lipid A that reduce or abolish this initial charge-based interaction with polymyxins. In many Gram-negative bacteria, modifications of lipid A by addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn) act to reduce the net LPS negative charge, thereby increasing resistance to polymyxins. The expression of the L-Ara4N and PEtn transferases in *Escherichia coli* and *Salmonella enterica* is regulated by the two-component regulatory system PmrA/PmrB, which responds to pH, Fe^{3+} and Mg^{2+} concentrations, as well as the presence of polymyxins (26). While the mechanism(s) of polymyxin resistance in *A. baumannii* is currently unknown, recent work has indicated that mutations in *pmrA* and *pmrB* may be linked to colistin resistance (1). Here we show that in *A. baumannii* type strain ATCC 19606, colistin-resistant variants contain mutations within genes essential for lipid A biosynthesis (either *lpxA*, *lpxC*, or *lpxD*) and that these strains have lost the ability to produce lipid A and therefore LPS. Furthermore, we show

* Corresponding author. Mailing address: Department of Microbiology, Building 76, Monash University, Clayton, Victoria 3800, Australia. Phone: 61 3 9902 9177. Fax: 61 3 9902 9222. E-mail: ben.adler@monash.edu.

▽ Published ahead of print on 20 September 2010.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant description	Source or reference
Strains		
ATCC 19606	*A. baumannii* type strain	American Type Culture Collection
19606R	Colistin-resistant derivative of ATCC 19606	This study
19606R+*lpxA*	19606R containing pAL840	This study
19606R+V	19606R containing pWH1266	This study
FADDI008	*A. baumannii* colistin-heteroresistant clinical isolate	16
FADDI008R	Colistin-resistant derivative of FADDI008	This study
B0707-070	*A. baumannii* colistin-resistant clinical isolate	22
Plasmids		
pWH1266	*Acinetobacter-E. coli* shuttle vector, Amp^r Tet^r	11
pAL840	*lpxA* cloned into the pWH1266 tetracycline resistance gene, Amp^r	This study
Primers		
BAP6205	ACGCCAGGATCCGGTTCATTATTCCTGTTTGCT; forward primer for amplification of *lpxA*, contains a BamHI site for cloning	This study
BAP6206	ATTCAAGGATCCCACCTCGAGCATTGTACCA; reverse primer for amplification of *lpxA*, contains a BamHI site for cloning	This study
BAP6098	TGAAGCATTAGCTCAAGTTT; forward primer for amplification and sequencing of *lpxA*	This study
BAP6099	GTCAGCAAATCAATACAAGA; reverse primer for amplification and sequencing of *lpxA*	This study
BAP6197	CAAAGTATGAATACAACTTTTGAG; forward primer for amplification and sequencing of *lpxD*	This study
BAP6198	GTCAATGGCACATCTGCTAAT; reverse primer for amplification and sequencing of *lpxD*	This study
BAP6402	TGAAGATGACGTTCCTGCAA; forward primer for amplification and sequencing of *lpxC*	This study
BAP6403	TGGTGAAAATCAGGCAATGA; reverse primer for amplification and sequencing of *lpxC*	This study

that loss of lipid A leading to colistin resistance is observed in other *A. baumannii* strains, including a colistin-resistant clinical isolate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *A. baumannii* type strain ATCC 19606 was obtained from the American Type Culture Collection, while strain FADDI008 was obtained from the bronchoalveolar lavage fluid of an intensive care unit patient at the Alfred Hospital, Melbourne, Victoria, Australia. Colistin-resistant variants of ATCC 19606 (designated 19606R) and FADDI008 (designated FADDI008R) were isolated by direct plating of these parent strains onto Mueller-Hinton agar (Oxoid) containing 10 μg/ml of colistin sulfate (Sigma); colistin-resistant colonies were identified (at a frequency of between 1 in 10^8 and 1 in 10^9) following a single round of selection. Colistin-resistant clinical isolate B0707-070 was obtained from Kwan Soo Ko, Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, South Korea. All *A. baumannii* strains were maintained on Mueller-Hinton agar or cultured in cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid) at 37°C, with the addition of 10 μg/ml of colistin sulfate where appropriate. Plasmid pWH1266 was obtained from the American Type Culture Collection.

Isolation and manipulation of DNA. *A. baumannii* genomic DNA was prepared using a Genomic DNA extraction minikit (blood/bacterial; RBC Bioscience), according to the manufacturer's instructions. Plasmid DNA was isolated using QIAprep spin miniprep columns (Qiagen), according to the manufacturer's instructions. Standard methods were used for the digestion, ligation, and analysis of plasmid and genomic DNA and PCR products (28). All enzymes used were obtained from Roche or New England Biolabs and were used according to the manufacturer's instructions.

Analysis of LPS by PAGE. LPS was purified from overnight cultures of *A. baumannii* using the previously described method of Westphal and Jann (35). LPS preparations (derived from approximately 3×10^7 cells) and proteinase K-treated whole-cell lysates (equivalent to approximately 3×10^6 cells) were separated on a Bio-Rad mini-protean gel apparatus using polyacrylamide gel electrophoresis (PAGE), as described previously (13). To visualize LPS, 12.5% polyacrylamide gels were fixed in a 30% ethanol–10% acetic acid solution, followed by oxidation in a 0.3% periodic acid–30% ethanol–10% acetic acid solution. The gels were then stained using a SilverSNAP stain kit (Pierce Biotechnology), according to the manufacturer's instructions.

Complementation of strain 19606R. The full-length *lpxA* gene was amplified by PCR from parent strain ATCC 19606 using primers BAP6205 and BAP6206 (Table 1), both of which contained BamHI sites. The amplified 1,176-bp fragment was cloned into the unique BamHI site located within the tetracycline resistance gene of the *E. coli*/*Acinetobacter* shuttle vector pWH1266 (11), generating plasmid pAL840 (Table 1). Complementing plasmid pAL840 and the vector only, pWH1266, were separately introduced into the colistin-resistant derivative of *A. baumannii* ATCC 19606 (19606R) by electroporation, as described previously (5). Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with 100 μg/ml of ampicillin (Sigma). *A. baumannii* transformants containing either pAL840 (designated 19606R+*lpxA*) or vector only (designated 19606R+V) were confirmed by restriction digest and nucleotide sequencing of the isolated plasmid.

LAL assay. *Limulus* amebocyte lysate (LAL) assays were performed on triplicate biological samples using an E-Toxate kit (Sigma), according to the manufacturer's instructions. For sample preparation, overnight cultures of the *A. baumannii* strains were washed once in sterile CAMHB and serially diluted in sterile, pyrogen-free water.

Structural analysis of LPS. O-deacylated LPS was prepared from purified LPS as described previously (2). Sugars were determined as their alditol acetate derivatives by gas-liquid chromatography mass spectrometry (GC-MS) as described previously (32). Capillary electrophoresis electrospray mass spectrometry (CE-ES-MS) experiments were performed as described previously (32). For the determination of fatty acids, 1 ml of methanol was added to 1 mg of purified LPS, and the mixture was cooled in dry ice, followed by the addition of 0.1 ml of acetyl chloride. Samples were then incubated for 3 h at 120°C in Teflon-lined screw-cap vials, air dried, and extracted with 2 ml of dichloromethane. The extracted samples were dried and then acetylated with 0.5 ml acetic anhydride and 0.5 ml pyridine for 30 min at 100°C. Samples were then dried and analyzed by GC-MS on a DB17 column together with the appropriate standards.

Determination of antibiotic MICs. MICs were determined by broth microdilution in CAMHB, according to the Clinical and Laboratory Standards Institute protocol (4).

Thin section transmission electron microscopy. Overnight cultures of parent strain ATCC 19606 and colistin-resistant, LPS-deficient strain 19606R, grown in CAMHB at 37°C with agitation, were used to inoculate (1/50) 5 ml of fresh CAMHB medium. Colistin-resistant strain 19606R was consistently grown in the presence of 10 μg/ml colistin. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.6, and 1 ml of each culture was harvested by centrifugation at

TABLE 2. Antibiotic sensitivity profiles of *A. baumannii* strains

Strain	MIC (μg/ml)				
	Colistin	Polymyxin B	Cefepime	Teicoplanin	Azithromycin
ATCC 19606 parent	1	0.5	16	>128	64
19606R	>128	>128	0.125	0.5	2
19606R+*lpxA*	1	0.5	16	>128	64
19606R+V	>128	>128	0.125	1	2

4,000 × *g* for 10 min at room temperature. Cell pellets were resuspended in 2.5% glutaraldehyde in NaCl and incubated for 2 h at room temperature. Suspensions were submitted to Monash Micro Imaging (Monash University, Clayton, Victoria, Australia) for agarose embedding and thin section preparation. Sections were placed on Formvar carbon-coated grids and viewed in a Hitachi H7500 120-kV transmission electron microscope.

Genome sequencing. The genome sequences of strains ATCC 19606 and 19606R were determined using 36-bp paired-end sequencing chemistry on an Illumina Genome Analyzer II apparatus (Illumina) at the Micromon Sequencing Facility (Monash University). Raw sequence data from both strains were independently aligned to the published strain ACICU genome sequence (12) using the SHRiMP program (27) (average read depth, ~110), as described previously (31). The raw read data were also assembled *de novo* using Velvet software (39) and a CLC genomics workbench (CLCbio, Denmark) to confirm the mutation.

Amplification of lipid A biosynthesis genes. Lipid A biosynthesis genes *lpxA*, *lpxC*, and *lpxD* were amplified from *A. baumannii* genomic DNA by PCR using the primers listed in Table 1. The nucleotide sequences of the amplified fragments were determined using an ABI BigDye Terminator mixture, and the products were separated on an ABI 3730S genetic analyzer.

Outer membrane integrity assay. Overnight cultures of each strain, grown in CAMHB at 37°C with agitation (100 rpm), were used to inoculate (1/100) 10 ml of fresh medium for each experiment. The ATCC 19606 parent strain was grown in plain CAMHB, while the other strains were grown in CAMHB with the following antibiotic additions: 19606R, 10 μg/ml colistin; 19606R+V, 10 μg/ml colistin and 100 μg/ml ampicillin; and 19606R+*lpxA*, ampicillin 100 μg/ml. To maintain selective pressure, overnight cultures of colistin-resistant strains were grown in the presence of colistin, but no antibiotic selection was used during subculture immediately prior to the assays. Cells were grown to an OD_{600} of 0.5 and harvested by centrifugation at 1,000 × *g* for 10 min at room temperature, washed once in buffer 1 (5 mM HEPES containing 5 mM sodium azide, pH 7.4), and resuspended in buffer 1 to an OD_{600} of 0.5. Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc.). A 1-ml aliquot of cell suspension was placed in a quartz cuvette (Sigma), and the baseline fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Following a stable baseline reading of 30 s, 10 μM (final concentration) 1-*N*-phenylnaphthylamine (NPN) was added and monitoring was continued until the fluorescence stabilized for a minimum of 30 s. The change in fluorescence was calculated as the average stable fluorescence reading after addition of NPN less the average initial reading.

RESULTS AND DISCUSSION

Colistin resistance can result from mutation of *lpxA*. To elucidate the mechanism(s) of colistin resistance in *A. baumannii*, we isolated a colistin-resistant derivative from type strain ATCC 19606 by direct selection on colistin-containing medium (10 μg/ml). The colistin-resistant variant was designated 19606R and displayed a colistin MIC of >128 μg/ml, whereas the colistin MIC was 1 μg/ml for the parent strain (Table 2). The growth rate in broth of colistin-resistant strain 19606R was indistinguishable from that of the parent strain (doubling time for ATCC 19606 = 48 min ± 5.5 min, doubling time for 19606R = 55.2 min ± 8.1 min; $n = 3$). However, it displayed a smaller colony morphology on Mueller-Hinton agar. To determine whether resistance to colistin in 19606R was due to a mutation, we determined the entire genome sequence of parent strain ATCC 19606 and its colistin-resistant variant, 19606R, using Illumina short-read sequencing (average read depth, >110×). Reference assemblies were generated separately for both strains using the published *A. baumannii* ACICU genome sequence as a scaffold (12), and these assemblies were used to identify nucleotide changes unique to 19606R. Comparison of the genome sequences of ATCC 19606 and 19606R revealed no mutations in either *pmrA* or *pmrB*; this finding was confirmed by directed Sanger sequencing. Indeed, their genome sequences were identical except for a single mutation, a deletion of nucleotide 90 within *lpxA*, which would result in premature termination of LpxA translation at amino acid 34. LpxA is predicted to encode the UDP-*N*-acetylglucosamine acyltransferase that catalyzes the first step in the biosynthesis of lipid A. There are nine lipid A biosynthetic genes, including *lpxA*, that are considered to be essential for the growth of most species of Gram-negative bacteria (25). Stable directed mutations of *lpxA* have been constructed in only two species, *Neisseria meningitidis* (30) and *Moraxella catarrhalis* (24), but this is the first report of a spontaneously occurring *lpxA* mutant.

To confirm that inactivation of *lpxA* was responsible for the observed resistance to colistin, the full-length intact *lpxA* gene from parent strain ATCC 19606 was amplified by PCR using primers BAP6205 and BAP6206 (Table 1) and cloned into *Acinetobacter-E. coli* shuttle vector pWH1266 (11) to generate pAL840 (Table 1). The complementing plasmid, pAL840, and the empty vector, pWH1266, were introduced separately into colistin-resistant *lpxA* mutant strain 19606R to generate strains 19606R+*lpxA* and 19606R+V, respectively. Strain 19606R complemented with intact *lpxA* (19606R+*lpxA*) was returned to colistin sensitivity, with the MIC (1 μg/ml) being identical to that of the parent strain (Table 2). Strain 19606R harboring the vector only (19606R+V) remained highly resistant to colistin (MIC > 128 μg/ml; Table 2). Therefore, mutation of *lpxA* is directly responsible for the colistin resistance observed in strain 19606R.

Mutation of *lpxA* results in complete loss of lipopolysaccharide. The *lpxA* gene is essential for the biosynthesis of lipid A and therefore LPS (26). To assess the ability of the *A. baumannii* wild-type, *lpxA* mutant, and complemented *lpxA* mutant strains to produce LPS, we purified carbohydrate from each strain using the hot phenol LPS extraction method of Westphal and Jann (36) and analyzed the purified material using PAGE, followed by carbohydrate-specific silver staining (Fig. 1A). Both ATCC 19606 and 19606R+*lpxA* produced observable amounts of LPS, with the strong bands visible between 6 and 10 kDa likely corresponding to lipid A plus core oligosaccharide. However, no observable LPS was produced by either the *lpxA* mutant strain (19606R) or the *lpxA* mutant strain containing the empty vector (19606R+V) (Fig. 1A). To refute the possibility that there had been a loss of LPS during the purification process, we analyzed the carbohydrates produced by each strain by PAGE of proteinase K-treated whole-cell lysates. While a range of carbohydrates was produced by all strains (Fig. 1B), only the ATCC 19606 parent strain and strain 19606R complemented with *lpxA* (19606R+*lpxA*) produced a carbohydrate that migrated at a molecular mass of between 6 and 10 kDa, consistent with the hypothesis that only the colistin-susceptible strains produce LPS. Finally, carbohydrate structural analyses were performed on LPS preparations

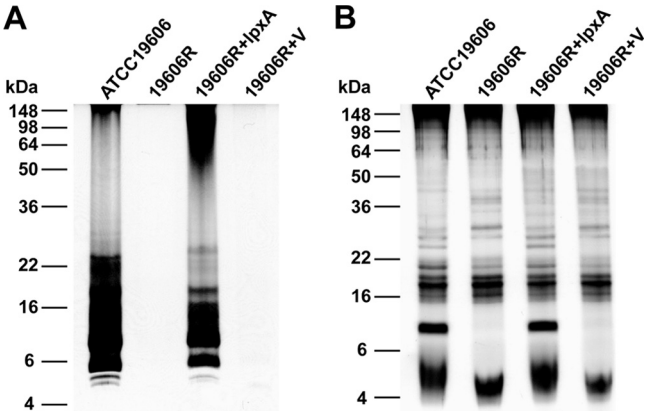

FIG. 1. Colistin-resistant *A. baumannii* strains do not produce LPS. PAGE separation and carbohydrate-specific silver staining of purified LPS (A) and proteinase K-treated whole-cell lysates (B) of colistin-susceptible parent strain ATCC 19606, colistin-resistant variant 19606R, 19606R complemented with *lpxA* (19606R+*lpxA*), or 19606R containing vector only (19606R+V) were performed. The positions of standard molecular mass markers are shown on the left.

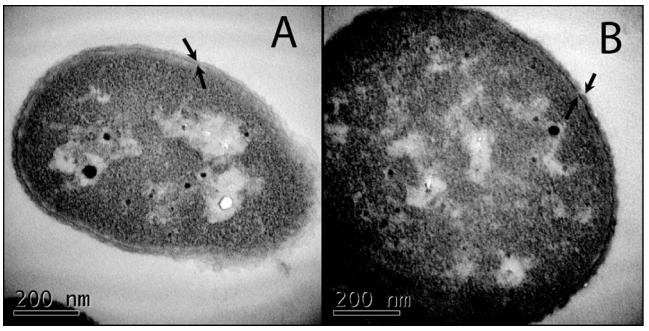

FIG. 2. *A. baumannii* elaborates an outer membrane in the absence of LPS. Transmission electron micrographs of thin sections of colistin-susceptible parent strain ATCC 19606 (A) and colistin-resistant *lpxA* mutant 19606R (B) are shown. Arrows indicate two distinct membranes present in both the ATCC 19606 and 19606R strains.

isolated from each strain. MS analyses on O-deacylated LPS revealed the expected 4 Hex, 2 HexN, HexNAcA, and 3-deoxy-D-manno-octulosonic acid glycoform for parent strain ATCC 19606, consistent with the known LPS structure (35), whereas no interpretable spectra were obtained for the colistin-resistant *lpxA* mutant (19606R) or the *lpxA* mutant containing empty vector. A spectrum consistent with the wild-type glycoform was observed for the strain complemented with *lpxA* (19606R+*lpxA*). Fatty acid analysis of the products obtained from the parent and mutant strains showed that the parent strain contained 3-OH-C_{12}, 3-OH-C_{14}, C_{16}, and C_{18} acids, characteristic for LPS, whereas no identifiable fatty acids were found in the material isolated from the *lpxA* mutant or vector-only strain. Taken together, these data show unequivocally that colistin-resistant *lpxA* mutant strains 19606R and 19606R+V do not produce LPS.

To confirm that no lipid A was produced by the *lpxA* mutant strains, we analyzed intact cells of each strain using the LAL assay, which directly detects lipid A (endotoxin). Parent strain ATCC 19606 and strain 19606R complemented with *lpxA* produced significant endotoxin (6×10^5 endotoxin units [EU]/ml; $n = 3$). In contrast, colistin-resistant strains 19606R and 19606R+V produced levels of endotoxin identical to the level measured in growth medium alone (0.06 EU/ml; $n = 3$). Therefore, the lack of any measurable LPS or lipid A in 19606R followed by the restoration of lipid A production and colistin sensitivity when *lpxA* is provided in *trans* demonstrates that the colistin resistance in this strain results from mutation of *lpxA*, which in turn leads to complete loss of lipid A (endotoxin) and LPS production. To our knowledge, this is the first example of polymyxin resistance due to loss of LPS, the initial binding target of colistin.

Colistin resistance and loss of LPS from the outer membrane result in a decrease in membrane integrity. LPS forms the outer leaflet of the outer membrane of Gram-negative bacteria, creating a permeability barrier that prevents large molecules and hydrophobic compounds from freely entering the cell (20). To determine whether an outer membrane was still present in the colistin-resistant *A. baumannii* strain, we visualized the ATCC 19606 parent strain and the LPS-deficient strain, 19606R, by transmission electron microscopy. Both strains possessed a distinct outer membrane (Fig. 2), indicating that *A. baumannii* can elaborate an outer membrane, even in the absence of LPS. The outer membrane of each strain was further examined using the hydrophobic fluorescent probe NPN as a marker for membrane integrity (17) (Fig. 3). NPN fluoresces more strongly in hydrophobic environments than in aqueous environments. NPN fluorescence was significantly higher ($P < 0.001$) in colistin-resistant, LPS-deficient strains 19606R and 19606R+V than in the colistin-susceptible parent strain and the complemented *lpxA* mutant (19606R+*lpxA*) (Fig. 3). Therefore, NPN can more readily gain access to the hydrophobic membrane environment in the colistin-resistant strain. These data clearly show that there are substantial differences in the integrity of the outer membrane between the

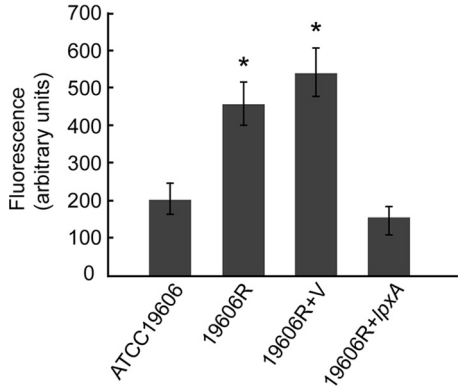

FIG. 3. Loss of LPS alters outer membrane integrity. Changes in fluorescence following the addition of the hydrophobic fluorescent probe NPN for *A. baumannii* strains ATCC 19606 parent ($n = 14$), 19606R ($n = 9$), 19606R+V ($n = 14$), and 19606R+*lpxA* ($n = 13$) are shown. Bars show the mean change in fluorescence ± 1 standard deviation. Both 19606R and 19606R+V show increased fluorescence uptake (*, $P < 0.001$) compared with the uptake by both the parent strain (ATCC 19606) and the complemented *lpxA* mutant strain (19606R+*lpxA*), as determined by analysis of variance with the Bonferroni *post hoc* method.

colistin-resistant and -susceptible strains and that this is most likely due to altered permeability of the outer membrane in the colistin-resistant strains resulting from the absence of LPS.

Loss of LPS from the outer membrane alters the antibiotic resistance profile. Colistin-resistant *A. baumannii* strains have previously been shown to display increased sensitivity to a range of other antibiotics (14). To determine if this increased sensitivity was associated with loss of LPS, we determined the MICs of three clinically relevant antibiotics, alongside colistin and polymyxin B, for the parent, mutant, and complemented strains (Table 2). While the *lpxA* mutant strain (19606R) was highly resistant to both colistin and polymyxin B, it displayed a significant increase in sensitivity to the three other antibiotics tested, including teicoplanin, an antibiotic routinely used to treat Gram-positive bacterial infections (Table 2). The increased sensitivity to nonpolymyxin antibiotics was also observed for the *lpxA* mutant strain transformed with the vector only (19606R+V). However, the same strain complemented with *lpxA* in *trans* (19606R+*lpxA*) showed an antibiotic resistance profile identical to that observed for the parent strain. One of the major functions of LPS in the outer membrane of Gram-negative bacteria is to provide a highly selective permeability barrier, in part due to the ability of adjacent LPS molecules to form strong lateral interactions via the bridging action of divalent cations (20). Therefore, we propose that the increased sensitivity of the colistin-resistant strains to nonpolymyxin antibiotics is a direct result of the lack of LPS in the outer membrane and the concomitant increased permeability of the outer membrane to the antibiotics. Indeed, directed *M. catarrhalis* and temperature-sensitive *E. coli lpxA* mutants also demonstrate an increase in sensitivity to a variety of antibiotics (8, 24). However, *M. catarrhalis lpxA* mutants showed an apparently increased sensitivity to polymyxin B (24), suggesting that the mechanism of polymyxin action may be different in this species.

Colistin resistance in *A. baumannii* can result from mutation of *lpxA*, *lpxC*, or *lpxD*. In *E. coli*, the biosynthesis of the lipid A component of LPS involves nine enzymatic steps requiring the enzymes LpxA, LpxC, LpxD, LpxH, LpxB, LpxK, KdtA, LpxL, and LpxM (25). Recent genome sequences of *A. baumannii* have shown that the arrangement of *lpxA* and *lpxD* is similar to that found in *E. coli*, with *lpxA* being the final gene of a putative operon that also contains *lpxD* and *fabZ*, a dehydratase involved in fatty acid elongation. Other genes involved in lipid A biosynthesis are located elsewhere on the *A. baumannii* genome (12, 29). To further investigate the phenomenon of heteroresistance and to assess whether colistin resistance in *A. baumannii* can result from mutation of a lipid A biosynthesis gene other than *lpxA*, we isolated and screened a further 12 colistin-resistant derivatives of ATCC 19606. All colistin-resistant strains appeared to be LPS deficient by carbohydrate silver staining of their whole-cell lysates (data not shown). Each of the first three genes in the lipid A biosynthesis pathway, *lpxA*, *lpxC*, and *lpxD*, was amplified by PCR (with the primers listed in Table 1), and the nucleotide sequences of the fragments were determined. Analysis of the sequences showed that in each LPS-deficient strain analyzed there was a mutation in one of the genes, *lpxA*, *lpxC*, or *lpxD*; the enzymes encoded by these genes catalyze the first three steps in the lipid A biosynthesis pathway (Table 3). A number of different mutations were observed, ranging from single point mutations to large deletions (up to 445 nucleotides [nt]; Table 3). The mutations involving single amino acid substitutions identified in these colistin-resistant derivatives (AL1833, AL1844, AL1846, and AL1848; Table 3) indicate that P30 in LpxC and H159, G68, and Q72 in LpxA are critical residues for the function of LpxC and LpxA, respectively. Indeed, all three mutated LpxA residues identified here are highly conserved across all bacterial LpxA proteins (34). Furthermore, H160 in the *E. coli* LpxA (corresponding to H159 in the *A. baumannii* LpxA) has been shown to be critical for enzyme activity, and both H159 and Q72 are predicted to be adjacent to amino acids involved in substrate binding (34, 38).

TABLE 3. Mutations identified in the lipid A biosynthesis genes of colistin-resistant *A. baumannii* ATCC 19606 derivatives

Strain	Gene	Nucleotide change	Effect
AL1833	*lpxC*	C → T substitution at nt 89	Amino acid change P30L
AL1834	*lpxC*	Single base deletion at nt 135	Frameshift after D45
AL1842	*lpxC*	84-bp deletion at nt 858	Frameshift after T285
AL1843	*lpxC*	C → T substitution at nt 89	Amino acid change P30L
AL1844	*lpxA*	C → G substitution at nt 475	Amino acid change H159D
AL1845	*lpxA*	C → T substitution at nt 700	Truncation after D233
AL1846	*lpxA*	G → A substitution at nt 203	Amino acid change G68D
AL1847	*lpxA*	30-bp deletion at nt 391	Frameshift after D130
AL1848	*lpxA*	C → A substitution at nt 214	Amino acid change Q72K
AL1849	*lpxA*	2-bp deletion at nt 76	Frameshift after I25
AL1851	*lpxA*	445-bp deletion at nt 364	Frameshift after H121
AL1852	*lpxD*	Single base deletion at nt 952	Frameshift after K317

Taken together, these data clearly show that the principal mechanism of colistin resistance in the ATCC 19606 strain is loss of LPS due to mutation in one of the first three genes involved in lipid A biosynthesis and that the phenomenon of colistin heteroresistance previously described for ATCC 19606 is due to the presence of cells with random mutations in these key genes.

Colistin resistance due to loss of LPS occurs in clinical isolates of *A. baumannii*. While we have shown that the principal mechanism of colistin resistance in the ATCC 19606 strain is loss of LPS, we sought to determine if a similar mechanism occurred in clinical isolates of *A. baumannii*. To examine the potential for clinical isolates to develop colistin resistance due to loss of LPS, we first selected colistin-resistant derivatives of an *A. baumannii* strain isolated from a human bronchoalveolar lavage fluid sample (strain FADDI008, originally designated isolate 8) (16) by direct selection on Mueller-Hinton agar containing 10 μg/ml of colistin sulfate. A colistin-resistant colony (MIC > 128 μg/ml) was isolated and designated FADDI008R. PAGE analysis of proteinase K-treated whole-cell lysates indicated that the parent strain elaborated a carbohydrate band which migrated at approximately 8 kDa, but this band was absent in the FADDI008R strain (Fig. 4). LAL assays confirmed that the colistin-susceptible parent, FADDI008, expressed high levels of endotoxin (6×10^5 EU/ml; $n = 3$) but that the resistant strain, FADDI008R, did not (0.06 EU/ml; $n = 3$). We amplified the *lpxA*, *lpxC*, and *lpxD* genes by PCR from both the colistin-susceptible and colistin-resistant FADDI008 strains and determined the nucleotide sequences of each gene from both strains. The colistin-susceptible parent strain encoded an intact *lpxA* gene, but the *lpxA* gene of FADDI008R contained a single base deletion at nu-

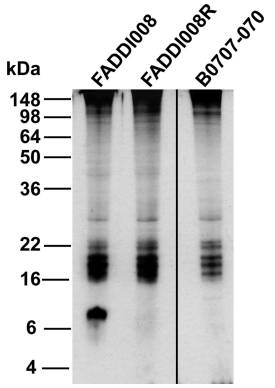

FIG. 4. Colistin-resistant clinical isolates of *A. baumannii* do not produce LPS. Carbohydrate-specific silver stain of proteinase K-treated whole-cell lysates of colistin-susceptible clinical isolate FADDI008, its colistin-resistant derivative (FADDI008R), and colistin-resistant clinical isolate B0707-070. The positions of standard molecular mass markers are shown on the left.

cleotide 459, resulting in premature termination of LpxA translation at amino acid 154. This finding confirmed that mutation of *lpxA* and loss of LPS in *A. baumannii* is not limited to the ATCC 19606 strain and that clinical isolates of *A. baumannii* have the potential to develop colistin resistance as a result of loss of LPS.

Next, we analyzed a colistin-resistant clinical isolate, B0707-070, isolated from a hospital patient in South Korea (22). Analysis of the whole-cell lysate of this strain by PAGE and carbohydrate-specific silver staining revealed an apparently LPS-deficient profile (Fig. 4), which was confirmed by LAL assay (0.06 EU/ml; $n = 3$). Sequencing analysis of the *lpxA*, *lpxC*, and *lpxD* genes revealed that *lpxD* was interrupted after 42 nucleotides by a novel 873-bp insertion sequence (IS) element, flanked by 12-bp inverted repeats. Sequence comparison revealed similarity to the IS*4* family IS element IS*X03* from the environmental organism *Xanthomonas oryzae.* Interestingly, this novel *A. baumannii* IS element appears to contain two distinct open reading frames which show significant identity to the 5′ and 3′ regions, respectively, of a putative transposase encoded by a *Xanthomonas campestris* IS element. It is possible that these open reading frames are translated independently, but further analysis of the sequence revealed that there is a polytract consisting of eight adenosines at the 3′ end of the first open reading frame. This suggests that translational frameshifting may occur in this IS element, which would allow full expression of the transposase and thus transposition. Translational frameshifting has previously been described for the IS element IS*Aba1* from *A. baumannii*, which is involved in resistance to a variety of other antibiotics (18).

Taken together, these data confirm that loss of LPS due to the mutation of key lipid A biosynthesis genes is a mechanism of colistin resistance in a range of *A. baumannii* strains, including a recently described clinical isolate.

In conclusion, here we report for the first time spontaneously occurring, lipid A-deficient, Gram-negative bacterial mutants. Moreover, we show the first evidence that loss of LPS can lead to polymyxin resistance. We have shown that loss of LPS production in *A. baumannii* can result from inactivation of a lipid A biosynthesis gene, *lpxA*, *lpxC*, or *lpxD*, and that colistin resistance via loss of lipid A can occur in clinical isolates. Loss of LPS production in each of the strains tested resulted in high-level colistin resistance, clearly indicating that the interaction of colistin with LPS is critical for the bactericidal action of colistin against *A. baumannii*. The clinical significance of colistin-resistant strains has recently been highlighted with the report of the emergence of colistin resistance after colistin treatment of an infection caused by a heteroresistant *A. baumannii* strain (10). Here, we demonstrate in two independent strains of *A. baumannii* that selection of colistin-resistant strains from within an apparently susceptible population is due to the selection of lipid A biosynthesis mutants and that these mutants devoid of LPS are highly resistant to colistin. Furthermore, we show that a colistin-resistant clinical isolate also lacks lipid A and LPS, and we are currently investigating the *in vivo* fitness of these LPS-deficient strains. We predict that these strains will have altered susceptibility to a range of host defenses, including antimicrobial peptides and, as LPS is a critical stimulator of innate immunity via Toll-like receptor signaling, may invoke a very different host response.

Importantly, this study clearly demonstrates that colistin resistance mediated by loss of LPS results in increased sensitivity to other clinically relevant antibiotics and that this could be exploited in combination antibiotic treatment regimens comprising colistin and a second antibiotic effective against colistin-resistant, LPS-deficient cells. Such a strategy may ultimately prolong the use of colistin as an effective antimicrobial against MDR *A. baumannii*.

ACKNOWLEDGMENTS

We thank Perry Fleming for cell growth, Jacek Stupak for mass spectrometry, and Anima Poudyal and Heidi Yu for MIC assays. We also thank Dennis Spelman (Alfred Hospital, Melbourne, Victoria, Australia) and Kwan Soo Ko (Department of Molecular Cell Biology, Sungkyunkwan University) for providing the clinical isolates.

This work was supported by an Australian National Health and Medical Research Council project grant. J.H.M. is supported by an Australian Postgraduate Award scholarship. J.L. is an Australian National Health and Medical Research Council R. Douglas Wright Research Fellow.

REFERENCES

1. **Adams, M. D., G. C. Nickel, S. Bajaksouzian, H. Lavender, A. R. Murthy, M. R. Jacobs, and R. A. Bonomo.** 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. Antimicrob. Agents Chemother. **53:**3628–3634.
2. **Boyce, J. D., M. Harper, F. St. Michael, M. John, A. Aubry, H. Parnas, S. M. Logan, I. W. Wilkie, M. Ford, A. D. Cox, and B. Adler.** 2009. Identification of novel glycosyltransferases required for assembly of the *Pasteurella multocida* A:1 lipopolysaccharide and their involvement in virulence. Infect. Immun. **77:**1532–1542.
3. **Clausell, A., M. Garcia-Subirats, M. Pujol, M. A. Busquets, F. Rabanal, and Y. Cajal.** 2007. Gram-negative outer and inner membrane models: insertion of cyclic cationic lipopeptides. J. Phys. Chem. B **111:**551–563.
4. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing; 18th informational supplement. M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Dorsey, C. W., A. P. Tomaras, and L. A. Actis.** 2002. Genetic and phenotypic analysis of *Acinetobacter baumannii* insertion derivatives generated with a transposome system. Appl. Environ. Microbiol. **68:**6353–6360.
6. **Evans, M. E., D. J. Feola, and R. P. Rapp.** 1999. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria. Ann. Pharmacother. **33:**960–967.
7. **Gales, A. C., R. N. Jones, and H. S. Sader.** 2006. Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of Gram-negative bacilli: report from the SENTRY Antimicrobial Surveillance Programme (2001-2004). Clin. Microbiol. Infect. **12:**315–321.

8. **Galloway, S. M., and C. R. Raetz.** 1990. A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. J. Biol. Chem. **265:**6394–6402.
9. **Hawley, J. S., C. K. Murray, and J. H. Jorgensen.** 2008. Colistin heteroresistance in *Acinetobacter* and its association with previous colistin therapy. Antimicrob. Agents Chemother. **52:**351–352.
10. **Hernan, R. C., B. Karina, G. Gabriela, N. Marcela, V. Carlos, and F. Angela.** 2009. Selection of colistin-resistant *Acinetobacter baumannii* isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagn. Microbiol. Infect. Dis. **65:**188–191.
11. **Hunger, M., R. Schmucker, V. Kishan, and W. Hillen.** 1990. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. Gene **87:**45–51.
12. **Iacono, M., L. Villa, D. Fortini, R. Bordoni, F. Imperi, R. J. Bonnal, T. Sicheritz-Ponten, G. De Bellis, P. Visca, A. Cassone, and A. Carattoli.** 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. Antimicrob. Agents Chemother. **52:**2616–2625.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
14. **Li, J., R. L. Nation, R. J. Owen, S. Wong, D. Spelman, and C. Franklin.** 2007. Antibiograms of multidrug-resistant clinical *Acinetobacter baumannii*: promising therapeutic options for treatment of infection with colistin-resistant strains. Clin. Infect. Dis. **45:**594–598.
15. **Li, J., R. L. Nation, J. D. Turnidge, R. W. Milne, K. Coulthard, C. R. Rayner, and D. L. Paterson.** 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect. Dis. **6:**589–601.
16. **Li, J., C. R. Rayner, R. L. Nation, R. J. Owen, D. Spelman, K. E. Tan, and L. Liolios.** 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **50:**2946–2950.
17. **Loh, B., C. Grant, and R. E. Hancock.** 1984. Use of the fluorescent probe 1-N-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**546–551.
18. **Mugnier, P. D., L. Poirel, and P. Nordmann.** 2009. Functional analysis of insertion sequence IS*Aba1*, responsible for genomic plasticity of *Acinetobacter baumannii*. J. Bacteriol. **191:**2414–2418.
19. **Munoz-Price, L. S., and R. A. Weinstein.** 2008. *Acinetobacter* infection. N. Engl. J. Med. **358:**1271–1281.
20. **Nikaido, H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **67:**593–656.
21. **Park, Y. K., J. Y. Choi, S. I. Jung, K. H. Park, H. Lee, D. S. Jung, S. T. Heo, S. W. Kim, H. H. Chang, H. S. Cheong, D. R. Chung, K. R. Peck, J. H. Song, and K. S. Ko.** 2009. Two distinct clones of carbapenem-resistant *Acinetobacter baumannii* isolates from Korean hospitals. Diagn. Microbiol. Infect. Dis. **64:**389–395.
22. **Park, Y. K., S. I. Jung, K. H. Park, H. S. Cheong, K. R. Peck, J. H. Song, and K. S. Ko.** 2009. Independent emergence of colistin-resistant *Acinetobacter* spp. isolates from Korea. Diagn. Microbiol. Infect. Dis. **64:**43–51.
23. **Peleg, A. Y., H. Seifert, and D. L. Paterson.** 2008. *Acinetobacter baumannii*: emergence of a successful pathogen. Clin. Microbiol. Rev. **21:**538–582.
24. **Peng, D., W. Hong, B. P. Choudhury, R. W. Carlson, and X. X. Gu.** 2005. *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. Infect. Immun. **73:**7569–7577.
25. **Raetz, C. R., Z. Guan, B. O. Ingram, D. A. Six, F. Song, X. Wang, and J. Zhao.** 2009. Discovery of new biosynthetic pathways: the lipid A story. J. Lipid Res. **50**(Suppl.)**:**S103–S108.
26. **Raetz, C. R., C. M. Reynolds, M. S. Trent, and R. E. Bishop.** 2007. Lipid A modification systems in gram-negative bacteria. Annu. Rev. Biochem. **76:**295–329.
27. **Rumble, S. M., P. Lacroute, A. V. Dalca, M. Fiume, A. Sidow, and M. Brudno.** 2009. SHRiMP: accurate mapping of short color-space reads. PLoS Comput. Biol. **5:**e1000386.
28. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. **Smith, M. G., T. A. Gianoulis, S. Pukatzki, J. J. Mekalanos, L. N. Ornston, M. Gerstein, and M. Snyder.** 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes Dev. **21:**601–614.
30. **Steeghs, L., R. den Hartog, A. den Boer, B. Zomer, P. Roholl, and P. van der Ley.** 1998. Meningitis bacterium is viable without endotoxin. Nature **392:**449–450.
31. **Steen, J. A., J. A. Steen, P. Harrison, T. Seemann, I. Wilkie, M. Harper, B. Adler, and J. D. Boyce.** 2010. Fis is essential for capsule production in *Pasteurella multocida* and regulates expression of other important virulence factors. PLoS Pathog. **6:**e1000750.
32. **St. Michael, F., J. Li, E. Vinogradov, S. Larocque, M. Harper, and A. D. Cox.** 2005. Structural analysis of the lipopolysaccharide of *Pasteurella multocida* strain VP161: identification of both Kdo-P and Kdo-Kdo species in the lipopolysaccharide. Carbohydr. Res. **340:**59–68.
33. **Tan, C. H., J. Li, and R. L. Nation.** 2007. Activity of colistin against heteroresistant *Acinetobacter baumannii* and emergence of resistance in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob. Agents Chemother. **51:**3413–3415.
34. **Ulaganathan, V., L. Buetow, and W. N. Hunter.** 2007. Nucleotide substrate recognition by UDP-N-acetylglucosamine acyltransferase (LpxA) in the first step of lipid A biosynthesis. J. Mol. Biol. **369:**305–312.
35. **Vinogradov, E. V., J. Ø. Duus, H. Brade, and O. Holst.** 2002. The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter baumannii* strain ATCC 19606. Eur. J. Biochem. **269:**422–430.
36. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure. Methods Carbohydr. Chem. **5:**81–91.
37. **Wiese, A., T. Gutsmann, and U. Seydel.** 2003. Towards antibacterial strategies: studies on the mechanisms of interaction between antibacterial peptides and model membranes. J. Endotoxin Res. **9:**67–84.
38. **Williams, A. H., and C. R. Raetz.** 2007. Structural basis for the acyl chain selectivity and mechanism of UDP-N-acetylglucosamine acyltransferase. Proc. Natl. Acad. Sci. U. S. A. **104:**13543–13550.
39. **Zerbino, D. R., and E. Birney.** 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. **18:**821–829.