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Phosphoethanolamine Modification of Lipid A in Colistin-Resistant Variants of *Acinetobacter baumannii* Mediated by the *pmrAB* Two-Component Regulatory System▽

Alejandro Beceiro,[1,2,3]* Enrique Llobet,[4,5] Jesús Aranda,[2] José Antonio Bengoechea,[4,5,6] Michel Doumith,[1] Michael Hornsey,[1,7] Hiran Dhanji,[1] Henrik Chart,[1] Germán Bou,[2] David M. Livermore,[1] and Neil Woodford[1]

Health Protection Agency, Microbiology Services-Colindale, London, United Kingdom[1]; Complexo Hospitalario Universitario A Coruña-INIBIC, A Coruña, Spain[2]; Hospital Son Espases–Universitat de les Illes Balears, Instituto Universitario de Investigación en Ciencias de la Salud (IUNICS), Palma de Mallorca, Spain[3]; Program Infection and Immunity, Fundación Caubet-CIMERA Illes Balears, Bunyola, Spain[4]; Centro Investigación Biomédica en Red Enfermedades Respiratorias (CIBERES), Bunyola, Spain[5]; Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain[6]; and Blizard Institute, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom[7]

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Colistin resistance is rare in *Acinetobacter baumannii*, and little is known about its mechanism. We investigated the role of PmrCAB in this trait, using (i) resistant and susceptible clinical strains, (ii) laboratory-selected mutants of the type strain ATCC 19606 and of the clinical isolate ABRIM, and (iii) a susceptible/resistant pair of isogenic clinical isolates, Ab15/133 and Ab15/132, isolated from the same patient. *pmrAB* sequences in all the colistin-susceptible isolates were identical to reference sequences, whereas resistant clinical isolates harbored one or two amino acid replacements variously located in PmrB. Single substitutions in PmrB were also found in resistant mutants of strains ATCC 19606 and ABRIM and in the resistant clinical isolate Ab15/132. No mutations in PmrA or PmrC were found. Reverse transcriptase (RT)-PCR identified increased expression of *pmrA* (4- to 13-fold), *pmrB* (2- to 7-fold), and *pmrC* (1- to 3-fold) in resistant versus susceptible organisms. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry showed the addition of phosphoethanolamine to the hepta-acylated form of lipid A in the resistant variants and in strain ATCC 19606 grown under low-Mg^{2+} induction conditions. *pmrB* gene knockout mutants of the colistin-resistant ATCC 19606 derivative showed >100-fold increased susceptibility to colistin and 5-fold decreased expression of *pmrC*; they also lacked the addition of phosphoethanolamine to lipid A. We conclude that the development of a moderate level of colistin resistance in *A. baumannii* requires distinct genetic events, including (i) at least one point mutation in *pmrB*, (ii) upregulation of *pmrAB*, and (iii) expression of *pmrC*, which lead to addition of phosphoethanolamine to lipid A.

Acinetobacter baumannii is a Gram-negative pathogen often associated with nosocomial infections and outbreaks (8). It has a great capacity to acquire antibiotic resistance, and this has necessitated numerous successive changes to therapeutic strategies. The increasingly frequent isolation of strains resistant to antimicrobials, such as imipenem, sulbactam, rifampin, or tigecycline (18, 48), has recently driven the use of polymyxins as treatment. These antibiotics, colistin and polymyxin B, were used in the 1960s and 1970s but then largely abandoned owing to reports of toxicity, though recent studies have demonstrated that revised dosing regimens can minimize this problem (25).

Colistin and polymyxin B are rapidly bactericidal for Gram-negative bacteria, interacting with the lipid A moiety of lipopolysaccharide (LPS) to cause disorganization of the outer membrane (16). Although they remain active against most otherwise extremely resistant *A. baumannii* isolates, this activity is not universal, and colistin heteroresistance has been described in *A. baumannii* (24), as has the development of full resistance both *in vitro* and *in vivo* (17, 41). Outbreaks of polymyxin-resistant *A. baumannii* isolates have been reported (12, 35, 47). Acquired polymyxin resistance in Gram-negative bacteria is most often mediated by replacement of lipid A with aminoarabinose, which requires the products of the *ugd* and *pbg* loci and ethanolamine, mediated by *pmrC*. These modifications remove negative charges, lowering the affinity of LPS for polymyxins (21, 34, 44). In this context, it is known that the two-component response regulator and sensor kinase PmrA/B, which allows bacteria to sense and respond to various environmental conditions, including pH or Fe^{3+} and Mg^{2+} levels, also affects expression of genes implicated in lipid A modification and thereby influences susceptibility to colistin (13).

Point mutations in the PmrA/B two-component system in colistin-resistant *A. baumannii*, *Pseudomonas aeruginosa*, and *Salmonella* have been reported (2, 31, 32, 40), and also, increased expression of *pmrA* in this event in *A. baumannii* seems to be implicated (2). Modifications in the PhoP/Q two-component regulatory system in the resistance to polymyxins due to modifications in LPS in *P. aeruginosa* (7) and *Salmonella* (14, 15) are implicated, but this system appears to be absent from

* Corresponding author. Mailing address: Servicio de Microbiología-Unidad de Investigación, Complexo Hospitalario Universitario A Coruña, As Xubias, s/n, 15006 A Coruña, Spain. Phone: 0034-981176087. Fax: 0034-981176097. E-mail: Alejandro.Beceiro.Casas@sergas.es.
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A. baumannii genomes (3). An *A. baumannii* ATCC 19606 colistin-resistant laboratory derivate with mutations in PmrB showed remodeling of the bacterial outer membrane and a decreased membrane permeability compared with those of its susceptible counterpart, highlighting the importance of this gene in colistin resistance.

Recently, Moffatt et al. have reported resistance to colistin in this species mediated by loss of LPS production (30). Laboratory derivates carrying mutations in the genes implicated in lipid A biosynthesis, *lpxA*, *lpxC*, and *lpxD*, showed increased MICs of colistin (30). An LPS-deficient colistin-resistant LpxA mutant showed changes in the membrane potential probably due to outer membrane modification and loss of affinity by colistin (38). Indeed, colistin exposure causes differences in the cell morphology of *A. baumannii*, particularly a more spherical appearance and increased cell surface roughness, leading to outer membrane damage (39). Although the precise molecular basis of polymyxin resistance remains unknown, it seems clear that LPS modifications and loss of affinity for anionic polymyxins are necessary to the development of resistance to these antibiotics in *A. baumannii* (26).

In the present study, we investigated the role of PmrA/B in colistin resistance in clinical isolates and laboratory mutants of *A. baumannii* and analyzed the lipid A moiety among these organisms.

MATERIALS AND METHODS

Clinical isolates and mutants. Isolates of *A. baumannii* were identified by phenotype (API 20NE; bioMérieux, La Balme-les-Grottes, France) and PCR for $bla_{\text{OXA-51}}$ (46). They included five colistin-resistant and five colistin-susceptible clinical isolates (Table 1). The five susceptible isolates and three of the resistant isolates were collected in United Kingdom between 2006 and 2009, one resistant isolate was collected during an outbreak in Spain in 2000 (47), and the remaining resistant isolate was collected in Saudi Arabia in 2009. One resistant/susceptible pair of clinical isolates (Ab15/132 and Ab15/133, respectively) was from the same patient. Colistin-resistant mutants were selected from susceptible type strain ATCC 19606 (12) and from susceptible clinical isolate ABRIM (9) by serial passage in LB broth with increasing concentrations of colistin (1, 2, 4, and 8 μg/ml).

Characterization of isolates. Pulsed-field gel electrophoresis (PFGE) was performed on ApaI-digested genomic DNA as described previously (42). MICs of colistin were determined by agar dilution (27) and Etest (AB Biodisk, Solna, Sweden) and interpreted using British Society for Antimicrobial Chemotherapy (BSAC) criteria (4).

Sequencing of *pmrCAB*. Based on the reference sequence of *A. baumannii* ATCC 17978 (GenBank accession number CP000521.1), primers Full pmrCAB-F and Full pmrCAB-R (Sigma-Genosys Ltd., Cambridge, United Kingdom) were designed to amplify a 3,699-bp product containing the *pmrCAB* coding region (Table 2). Primers pmrC-F, pmrC-R, pmrC2-F, pmrC2-R, pmrA-F, pmrA-R, pmrB-F, pmrB-R, pmrB2-F, and pmrB2-R were used for sequencing (Table 2). The Expand high-fidelity PCR system (Roche Diagnostics, Lewes, United Kingdom) was used for amplification, and the resulting amplicons were purified with the GeneClean Turbo kit (MD Biomedicals, Strasbourg, France). Sequences were determined using an ABI genetic analyzer capillary platform 3130XL automatic DNA sequencer (ABI, Warrington, United Kingdom).

Insertional inactivation of *pmrB* in *A. baumannii* ATCC 19606 ColR and ColS. Gene inactivation was carried out as previously described by Aranda et al. (5). Briefly, the target gene was amplified by PCR with the oligonucleotide pair pmrBintFW-pmrBintRV, and genomic DNA from *A. baumannii* ATCC 19606 was used as the template. The 539-bp PCR product obtained was cloned into the kanamycin and zeocin resistance plasmid pCR-BluntII-TOPO (Invitrogen Ltd., Paisley, United Kingdom), which is unable to replicate in *A. baumannii*, and propagated in *Escherichia coli* strain TG1. The recombinant plasmid (0.1 μg) was introduced into *A. baumannii* ATCC 19606 ColR and ColS, which are susceptible to kanamycin, by electroporation. Candidate transformant clones with Δ*pmrB*

TABLE 1. *A. baumannii* strains used

Strain	Colistin MIC (μg/ml)[a]	Colistin susceptibility[b]	Pulsed-field profile	Source (country, year, type of infection)[c]	Parent strain[d]	*pmrB* genotype
Ab14/144	4	R	OXA-24 European clone II	UK, 2008, wound infection	N/A	Met145Lys
Ab804	16	R	Unique	Saudi Arabia, 2009, blood infection	N/A	Pro233Ser
Ab15/132	32	R	Strain ac-1	UK, 2009, respiratory infection	Same clone as Ab15/133	Leu87Phe
Ab10/30	128	R	NW strain	UK, 2006, blood infection	N/A	Ser14Leu
Ab208628	32	R	Unique	Spain, 2009 (47)	N/A	Phe387Tyr Ser403Phe
Ab15/133	1	S	Strain ac-1	UK, 2009, respiratory infection	Same clone as Ab15/132	Wild type
Ab1/137	1	S	OXA 23 clone 1	UK, 2009, colonization	N/A	Wild type
Ab15/88	0.5	S	Unique	UK, 2009, wound infection	N/A	Wild type
Ab15/24	0.5	S	Strain 00AC-2	UK, 2008, colonization	N/A	Wild type
Ab15/120	0.5	S	Unique	UK, 2009, urinary infection	N/A	Wild type
ATCC 19606 ColS	1	S	Unique	2009 (12)	N/A	Wild type
ATCC 19606 ColR	32	R		2009 (12)	ATCC 19606	Ala227Val
ATCC 19606 ColS Δ*pmrB*	0.125	S		This study	ATCC 19606 ColS	Δ*pmrB*
ATCC 19606 ColR Δ*pmrB*	0.125	S		This study	ATCC 19606 ColR	Δ*pmrB*
ABRIM ColS	0.5	S	Unique	Spain, 2000 (9)	N/A	Wild type
ABRIM ColR	16	R		This study	ABRIM	Asn353Tyr

[a] MICs were obtained by the agar dilution method and by Etest.
[b] R, resistant; S, susceptible.
[c] UK, United Kingdom.
[d] N/A, not applicable.

TABLE 2. DNA sequences of oligonucleotides used in real-time RT-PCR in this study

Oligonucleotide[a]	Sequence	Reference
Full pmrCAB-F	5′-GCATCATAAAAAGATTGTAGTCAC-3′	This study
Full pmrCAB-R	5′-GCGATTTGTATTCATCGTTTTGAG-3′	This study
pmrA-F	5′-ATGACAAAAATCTTGATGATTGAAGAT-3′	This study
pmrA-R	5′-TTATGATTGCCCCAAACGGTAG-3′	This study
pmrB-F	5′-GTGCATTATTCATTAAAAAAAC-3′	This study
pmrB-R	5′-TCACGCTCTTGTTTCATGTA-3′	This study
pmrB2-F	5′-GGTTCGTGAAGCTTTCG-3′	This study
pmrB2-R	5′-CCTAAATCGATTTCTTTTTG-3′	This study
pmrC-F	5′-ATGTTTAATCTCATTATAGCCA-3′	This study
pmrC-R	5′-TTAGTTTACATGGGCACAA-3′	This study
pmrC2-F	5′-GGTTGTTATTGAAGAAAGTAT-3′	This study
pmrC2-R	5′-TCAATCCAAGTCACTTGGTAAC-3′	This study
PMRBINTFW	5′-CGCTCAAGGTATAGTCAG-3′	This study
PMRBINTRV	5′-CAGTAGGCTCGACCATAC-3′	This study
PMRBEXTFW	5′-CAGTGTCATCTTAGGTTG-3′	This study
PMRBEXTRV	5′-CGTAGTGATCTGGATCGG-3′	This study
RT-PCR pmrA-F	Same as that of pmrA-F	This study
RT-PCR pmrA-R	5′-CCATCATAGGCAATCCTAAATCCA-3′	This study
RT-PCR pmrB-F	5′-GAACAGCTGAGCACCCTTTAA-3′	This study
RT-PCR pmrB-R	5′-ACAGGTGGAACCAGCAAATG-3′	This study
RT-PCR pmrC-F	5′-CTCTTTACGCTTTGTTTTATGGAC-3′	This study
RT-PCR pmrC-R	5′-GTAAAAAGTAAAACACCGACCA-3′	This study
RT-PCR 16S rRNA-F	5′-TCAGCTCGTGTCGTGAGATG-3′	19
RT-PCR 16S rRNA-R	5′-CGTAAGGGCCATGATG-3′	19
$bla_{\text{OXA-51like}}$-F	5′-TAATGCTTTGATCGGCCTTG-3′	46
$bla_{\text{OXA-51like}}$-R	5′-TGGATTGCACTTCATCTTGG-3′	46

[a] F, forward oligonucleotide; R, reverse oligonucleotide.

were then selected on kanamycin-containing plates (50 μg/ml) and confirmed by sequencing of the PCR products obtained using primers pmrBextFW-T7 and pmrBextRV-SP6 and genomic DNA from *A. baumannii* mutants.

Real-time RT-PCR of *pmrCAB* expression. Real-time reverse transcriptase (RT)-PCR was used to monitor the expression of *pmrCAB*. Bacteria were grown on LB broth to an optical density at 600 nm (OD_{600}) of 0.4 at 37°C, and total RNA was extracted with an RNeasy minikit (Qiagen, West Sussex, United Kingdom). This RNA was treated with RNase-free DNase (Invitrogen) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Winsford, United Kingdom). The QuantiFast SYBR green RT-PCR kit (Qiagen) was used for analysis, with 30 ng of total RNA and the primers shown in Table 2. The 16S rRNA gene was used as an internal control for quantification of relative gene expression (19), which was described as the mean of three independent experiments. Negative controls without reverse transcriptase were included to detect DNA contamination.

Real-time RT-PCR was used to monitor the expression of *pmrCAB* under induction conditions in the presence of Fe^{3+} and low Mg^{2+} (20). Bacterial RNA was extracted from cells grown in LB with 2 mM ferric chloride and without ferric chloride (2), and also, RNA was extracted from cells grown in *Acinetobacter* minimal medium (AMM) supplemented with 2 μM and 2 mM magnesium sulfate (36). RNA extraction and RT-PCR were performed as described above.

Analysis of lipid A structure. Lipid A was extracted by an ammonium hydroxide-isobutyric acid method and subjected to negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry analysis (10, 28). Briefly, lyophilized crude cells (10 mg) were resuspended in 400 μl isobutyric acid-1 M ammonium hydroxide (5:3, vol/vol) and incubated in a screw-cap test tube at 100°C for 2 h, with occasional vortexing. Samples were then cooled in ice water and centrifuged (2,000 × *g* for 15 min), with supernatants transferred to new tubes, diluted with equal volumes of water, and lyophilized. These samples were then washed twice with 400 μl methanol and centrifuged (2,000 × *g* for 15 min) to pellet the insoluble lipid A, which subsequently was solubilized in 100 to 200 μl chloroform-methanol-water (3:1.5:0.25, vol/vol/vol). Analyses were performed on a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Coventry, United Kingdom) in negative reflective mode with delayed extraction. Each spectrum was an average of 300 shots. The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (Sigma Chemical Co., St. Louis, MO) was used as a matrix. A few microliters of lipid A suspension (1 mg/ml) was desalted with a few grains of ion-exchange resin (H^+; Dowex 50WX8) in an Eppendorf tube, and 1 μl was deposited on the target and covered with the same amount of the matrix suspended at 10 mg/ml in 0.1 M citric acid. Different ratios between the samples and dihydroxybenzoic acid were used when necessary. A peptide calibration standard (Bruker Daltonics) was used to calibrate the MALDI-TOF mass spectrometry. Further calibration was performed using lipid A extracted from *Escherichia coli* strain MG1655 grown in LB at 37°C. Detailed mass spectrometry analysis of *E. coli* lipid A has been described previously (50).

Nucleotide sequence accession numbers. The nucleotide sequences presented in this study have been submitted to the GenBank database, as follows: *pmrCAB* genes, GenBank accession numbers HM134921 (ABRIM ColR), HM134922 (ABRIM ColS), HM149342 (Ab15/132 ColR), HM149343 (Ab15/133 ColS), HM149344 (ATCC 19606 ColS), and HM149345 (ATCC 19606 ColR); *pmrAB* genes, GenBank accession numbers HM138195 (Ab208628 ColR), HM138196 (Ab804 ColR), HM138197 (Ab14/144 ColR), and HM138198 (Ab10/30 ColR).

RESULTS

Isolate characterization. All clinical isolates had distinct PFGE banding profiles, except isolates Ab15/133 and Ab15/132, which were both from the same patient and were confirmed to be identical.

The MICs for the colistin-resistant isolates ranged from 4 to 128 μg/ml, while those for the susceptible isolates were ≤1 μg/ml; those for the paired isolates Ab15/133 and Ab15/132 were 1 and 32 μg/ml, respectively. The polymyxin-resistant mutants of *A. baumannii* strains ATCC 19606 and ABRIM showed 32-fold increases in colistin MICs, which rose from 1 to 32 and 0.5 to 16 μg/ml, respectively. The pandrug-resistant *A. baumannii* Ab208628 clinical strain showed a resistant phenotype to colistin (64 μg/ml) (47) (Table 1).

Analysis of *pmrCAB* nucleotide sequences. The PmrAB system is implicated in the resistance to colistin in *A. baumannii* (2), and the *pmrCAB* operon is implicated in *Salmonella* (21);

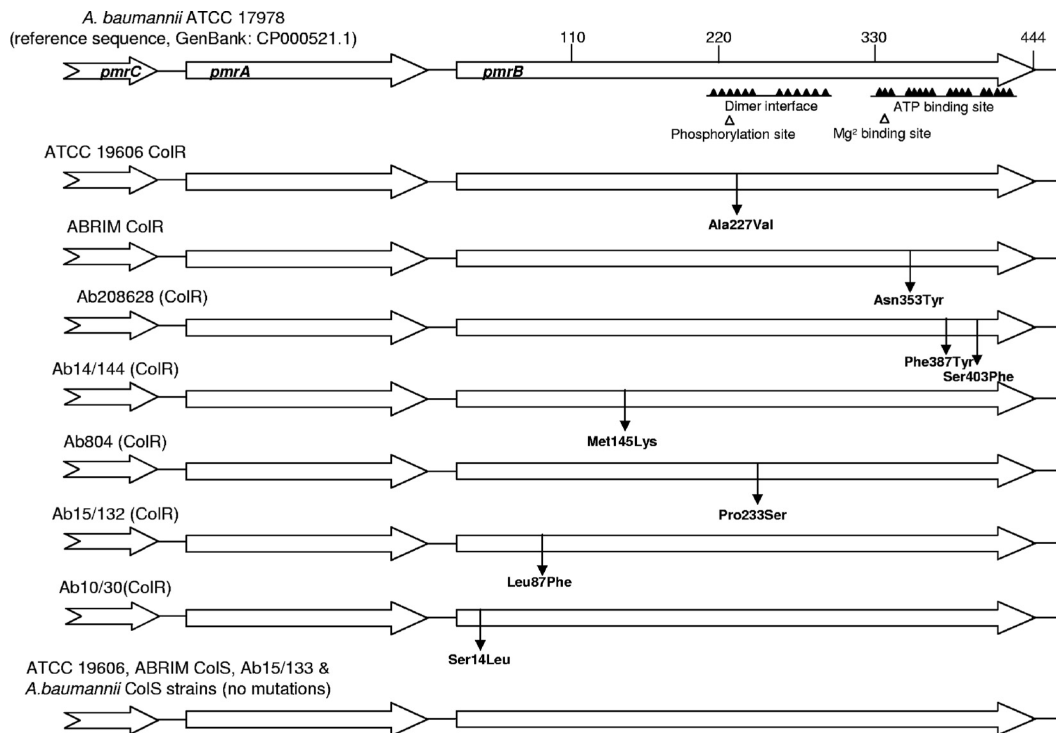

FIG. 1. Schematic representation of the *pmrCAB* genes. No amino acid changes were found in PmrA or PmrC. Amino acid replacements predicted in PmrB are identified with the arrows. No substitutions were found in these proteins of ATCC 19606 ColS, ABRIM ColS, and Ab15/133 or in the colistin-susceptible *A. baumannii* strains. The main domains in PmrB are identified, specifically, (i) the dimerization/phosphoacceptor domain, with the conserved histidine 228, and (ii) the ATP binding site.

so, the *pmrCAB* genes of the colistin-resistant and -susceptible isolates and mutant pairs, and the *pmrAB* genes of the other isolates, were amplified and sequenced on both strands. The *pmrB* sequences of all the colistin-susceptible isolates were identical to that of a reference sequence (GenBank accession number CP000521.1), whereas all the colistin-resistant isolates harbored mutations that resulted in at least one substitution in PmrB (Table 1). Three isolates had single amino acid changes (Ser14Leu, Met145Lys, or Pro233Ser), and one had changes at two amino acids (Phe387Tyr and Ser403Phe). Isolate Ab15/132 harbored a Leu87Phe change as opposed to its colistin-susceptible counterpart, Ab15/133. Single replacements in PmrB were likewise found in the two laboratory-derived colistin-resistant mutants, Ala227Val in ATCC 19606 ColR and Asn353Tyr in ABRIM ColR, compared with their susceptible parent strains. No differences were found in the *pmrA* or *pmrC* gene of any of the isolates (Table 1; Fig. 1).

Expression of *pmrCAB*. Expression of the *pmr* genes was investigated to determine whether differential expression was associated with colistin resistance. RT-PCR identified upregulation of *pmrA* and *pmrB* in all the resistant clinical isolates, compared with that in the susceptible ones: mean increases were 12.4-fold for *pmrA* expression and 6.8-fold for *pmrB* expression in colistin-resistant versus -susceptible isolates, whereas expression of *pmrC* was not significantly changed (mean increase of 1.1-fold) (Fig. 2). The polymyxin-resistant mutants of ATCC 19606 and ABRIM and clinical isolate Ab15/132 had increased expression of *pmrA* (4.7-, 12.7-, and 7.0-fold, respectively) and *pmrB* (2.6-, 6.0-, and 6.8-fold, respectively) compared with that of their colistin-susceptible counterparts, whereas increases in *pmrC* expression were present but smaller (2.6-, 2.1-, and 2.8-fold, respectively). Such differences were seen regardless of whether RNA was prepared from cells grown in the presence or absence of 2 μg/ml colistin. No significant differences of expression in the *pmrA*, *pmrB*, or *pmrC* gene were identified by RT-PCR in the ATCC 19606 ColS and ATCC 19606 ColS Δ*pmrB* strains in the presence of high Fe^{3+}. However, low upregulation of *pmrC* and *pmrB* (1.77- and 1.46-fold, respectively) was observed when the strain was grown in AMM supplemented with 2 μM magnesium sulfate compared with that when the strain grown in the presence of 2 mM magnesium sulfate. No significant differences in *pmrA* expression under these conditions were found; likewise, neither *pmrA* nor *pmrC* of the ATCC 19606 ColS Δ*pmrB* strain showed expression differences (0.72- and 1.02-fold, respectively).

Analysis of lipid A composition. Modification of lipid A has been associated with polymyxin resistance in other genera (21, 44). We therefore characterized and compared by MALDI-TOF mass spectrometry the lipid A moieties of three colistin-susceptible and -resistant pairs of isolates, as follows: Ab15/133 and Ab15/132, ATCC19606 ColS and ColR, and ABRIM ColS and ColR (Fig. 3). Previous studies have unambiguously shown that the lipid A disaccharide backbone of *Acinetobacter* spp. is composed of two β-(1-6)-linked 2-amino-2-deoxyglucose residues (GlcNI and GlcNII) phosphorylated at positions 1 and 4′ (22, 23, 49). The lipid A fraction of *A. baumannii* contains two major species which may correspond to a hepta-acylated lipid A with two 2-amino-2-deoxyglucose residues, two phosphates,

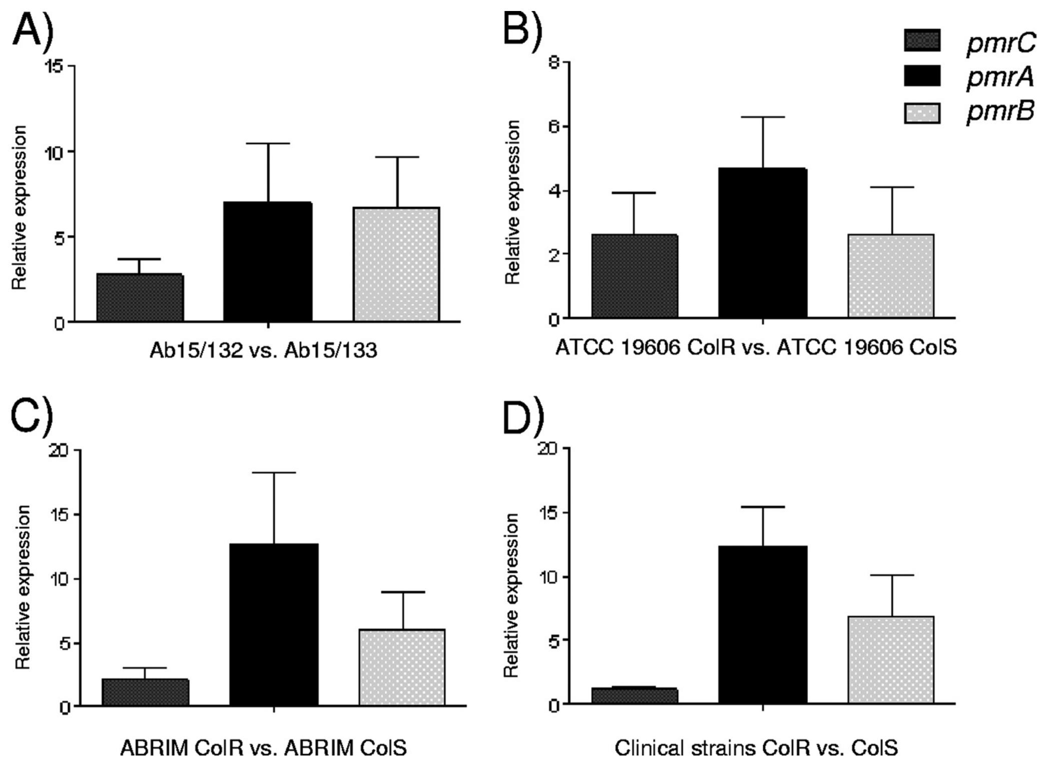

FIG. 2. Relative expression of the *pmrCAB* genes in the colistin-resistant and -susceptible strains (expression level of *pmrCAB* in the ColS strains equal to 1). (A) Ab15/132 ColR compared with Ab15/133 ColS; (B) ATCC 19606 ColR compared with ATCC 19606 ColS; (C) ABRIM ColR compared with ABRIM ColS; (D) means for five clinical ColR strains compared with those for five clinical ColS strains. Values are the means and the standard deviations from three independent experiments.

three 12:0(3-OH) fatty acids, two 14:0(3-OH) fatty acids, and two 12:0 (*m*/*z* 1,910) fatty acids attached or to a hexa-acylated lipid A with two 2-amino-2-deoxyglucose residues, two phosphates, and three 12:0(3-OH) fatty acids, two 14:0(3-OH) fatty acids, and one 12:0 (*m*/*z* 1,728) fatty acid attached (22) (Fig. 4). The colistin-susceptible Ab15/133, ABRIM, and ATCC 19606 ColS isolates all contained both of these (Fig. 3A to C; Table 3). Other ion peaks detected were *m*/*z* 1,530, also previously found (28), which may represent a penta-acylated lipid A lacking one 12:0 fatty acid moiety, and *m*/*z* 1,404, which may correspond to a tetra-acyl lipid A with three 12:0(3-OH) fatty acids and one 14:0(3-OH) fatty acid attached to the phosphorylated disaccharide (Fig. 4). In the colistin-resistant isolates, only three ion peaks were detected, corresponding to *m*/*z* 1404, 1,910, and 2,034. Critically, the latter species is consistent with the addition of phosphoethanolamine (theoretical *m*/*z* 124) to the hepta-acylated form of lipid A (*m*/*z* 1,910).

Role of *pmrB* in colistin resistance. To examine whether the PmrA/B two-component system was implicated in the development of colistin resistance, we generated Δ*pmrB* mutants of the ATCC 19606 ColR and ColS strains. These proved to be more susceptible than their corresponding parents (MICs of 0.19 μg/ml versus 32 μg/ml in ATCC 19606 ColR derivatives and MICs of 0.125 μg/ml versus 1 μg/ml in ATCC 19606 ColS derivatives), showing the importance of this gene. In addition, 5.3-fold downregulation of *pmrC* was observed by RT-PCR in the ATCC 19606 ColR Δ*pmrB* mutant compared with that in its parent. These results accord with the similar *pmrAB*-regulated PmrC model proposed for *Salmonella* by Lee et al., in which a Δ*pmrC* mutant and a null *pmrA* mutant resulted in a lipid A species without the addition of phosphoethanolamine (21).

Lipid A from the Δ*pmrB* mutant of ATCC 19606 ColR contained the major molecular species with *m*/*z* 1,910, 1,728, and 1,404 but lacked the ion peak (*m*/*z* 2,034), seen in its parent and consistent with the addition of phosphoethanolamine (theoretical *m*/*z* 124) to hepta-acylated lipid A (*m*/*z* 1,910) (Fig. 3D). Lipid A from the ATCC 19606 ColS Δ*pmrB* strain was identical to those of the ATCC 19606 wild type and ATCC 19606 ColR Δ*pmrB* (Fig. 3C and D).

Lipid A analysis of ATCC 19606 grown in low Mg^{2+} (Fig. 5A) revealed the presence of the ion peak (*m*/*z* 2,034), which was absent in lipid A from the ATCC 19606 ColS Δ*pmrB* strain grown under the same conditions (Fig. 5B). Ion peaks (*m*/*z* 1,404, 1,728, and 1,910) were found in strain ATCC 19606 grown in LB (Fig. 3), whereas another ion peak (*m*/*z* 1,530) was identified in the Ab15/133 and ABRIM ColS strains. Molecular species with *m*/*z* 1,348 may correspond to *m*/*z* 1,530 lacking one 12:0 fatty acid moiety. The absence of molecular species with *m*/*z* 1,530 and 1,348 in bacteria grown in LB (Fig. 3C) but presence in lipid A from bacteria grown in minimal medium with either low or high Mg^{2+} (Fig. 5) suggests that they are dependent on the growth medium and not on the cation content.

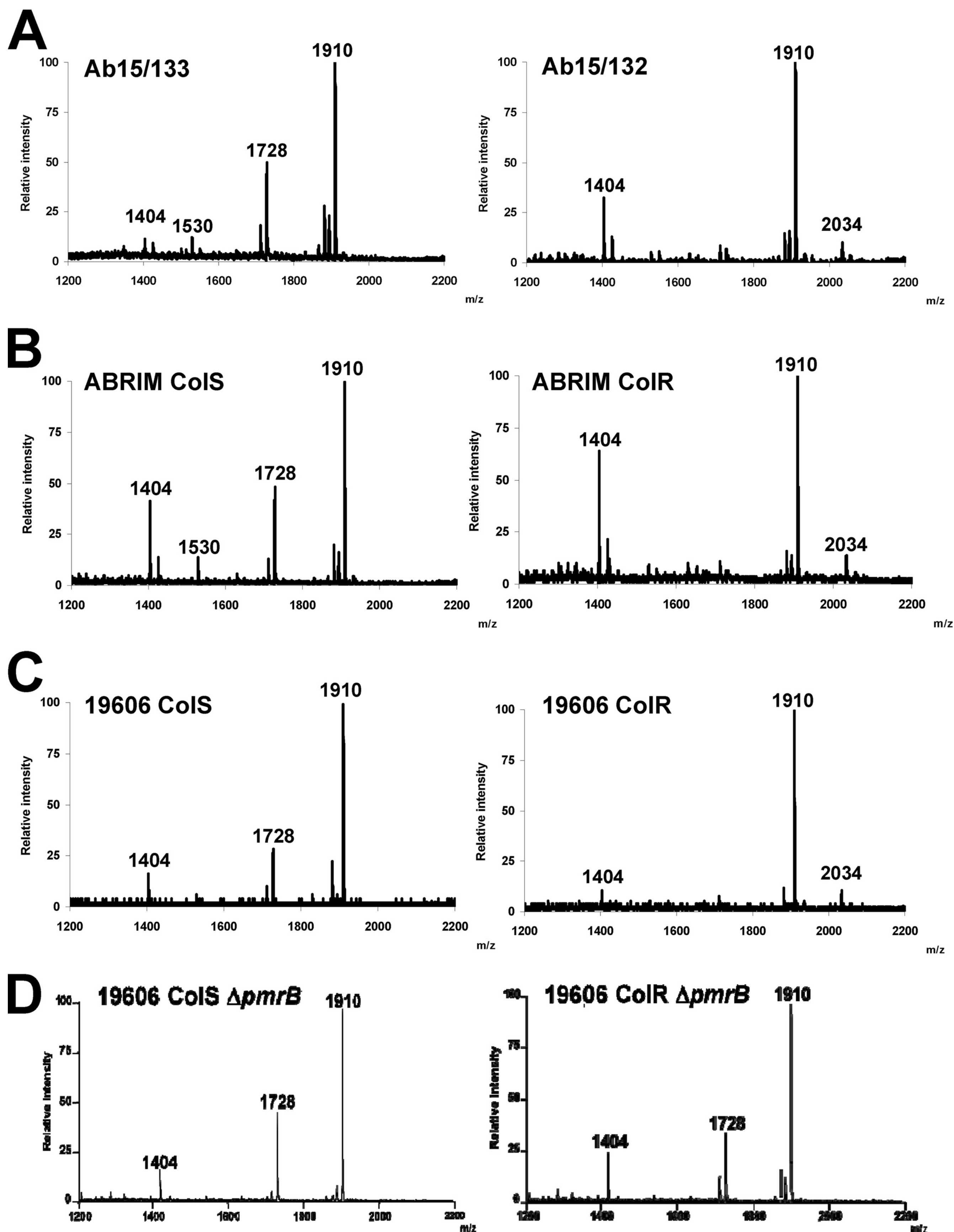

FIG. 3. Negative-ion MALDI-TOF mass spectra of lipid A isolated from colistin-resistant and -susceptible *A. baumannii* isolates. (A) Ab15/133 and Ab15/132; (B) ABRIM ColS and ABRIM ColR; (C) ATCC 19606 ColS and ATCC 19606 ColR; (D) ATCC 19606 ColS Δ*pmrB* and ATCC 19606 ColR Δ*pmrB*.

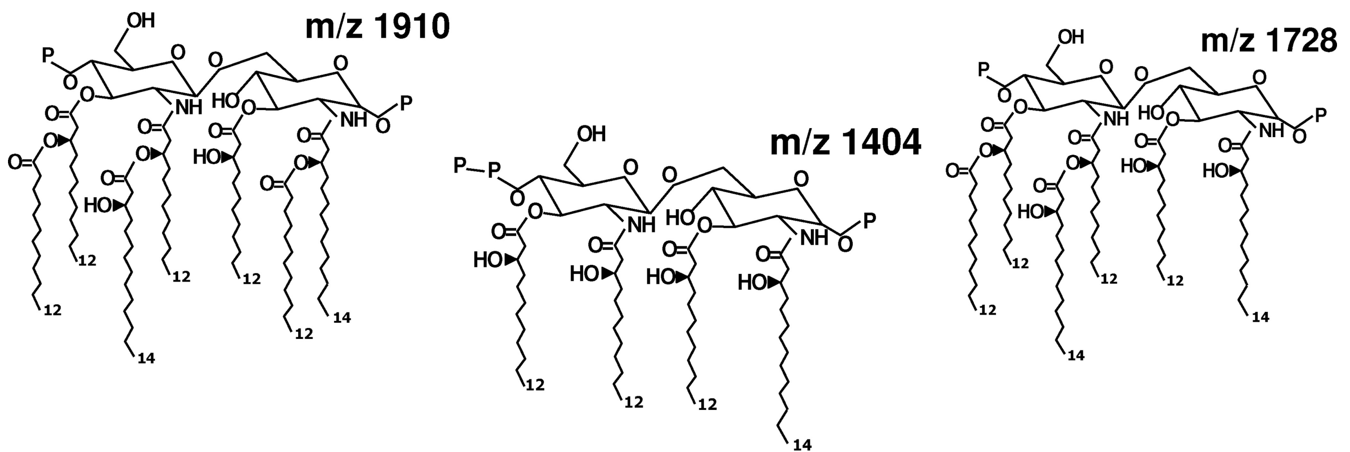

FIG. 4. Proposed structures of the main molecular lipid A species.

DISCUSSION

Colistin and tigecycline are often the only treatment options for multiresistant *A. baumannii* infections, but resistance to both agents has recently been described, with colistin resistance scattered worldwide. *A. baumannii* Ab208628, used here (Table 1), was resistant to all antibiotics tested and was associated with a nosocomial outbreak (47). We sought to define the mechanism(s) of colistin resistance in this species. Our data show that at least one mutation in PmrB and *pmrAB* upregulation seems necessary to confer this resistance.

Several mutations were found in PmrB, located at widely scattered sites. Ala227Val lies adjacent to the conserved histidine at the site of phosphorylation (His228), and Pro233Ser lies at an essential position for dimer formation (43). Both of these were found previously in colistin-resistant clinical strains or laboratory mutants (2), and the positions seem critical for the phosphatase activity. Mutations Asn353Tyr, Phe387Tyr, and Ser403Phe lie inside the ATP binding site and may have an effect on the phosphorylation of His228 of PmrB and thereby the phosphorylation levels of Asp52 of PmrA, the response regulator, which is the final acceptor of the phosphate group, affecting the expression levels of target genes, such as *pmrC* (45). The other mutations found in the nonconserved N-terminal domains of PmrB (Table 1) may be responsible for regulating the activity of the C-terminal kinase-phosphatase conserved domain, typical of the two-component regulatory systems (6, 33). Single substitutions in *pmrB* of *P. aeruginosa* at positions 243, 248, and 292, affect the function of PmrB, possibly by increasing phosphatase activity, thereby activating the PmrA regulon and increasing the resistance to colistin by LPS modifications (1, 11). Similar effects in *A. baumannii* may apply.

We observed increased expression of *pmrA* and *pmrB* in all the colistin-resistant clinical isolates and laboratory mutants. Adams et al. also reported increased *pmrA* expression in laboratory-selected colistin-resistant mutants of *A. baumannii* (2). The *pmrA* and *pmrB* genes seem to interact as an operon, as they showed similar expression levels in the colistin-susceptible strains, whereas inactivation of *pmrB* led to clear downregulation of *pmrC*, also highlighting the interaction between these components and the possible implication of *pmrB* in colistin resistance.

Negative-ion MALDI-TOF mass spectra revealed the presence of phosphoethanolamine (*m*/*z* 2,034) in lipid A isolated from the three colistin-resistant strains examined, a change previously associated with resistance to polymyxins in *Salmonella* (13). This phosphoethanolamine was lost when *pmrB* was inactivated in a colistin-resistant strain, confirming its role. Analysis also revealed the absence of hexa- and penta-acylated lipid A species in colistin-resistant strains. This is in good agreement with the idea that increased lipid A acylation (in the dominant hepta-acylated material) is associated with antimicrobial peptide resistance (11). An interesting finding is the relative increase in the levels of the tetra-acylated lipid A species (*m*/*z* 1,404) in colistin-resistant clinical isolates (Fig. 3A and B). This type of lipid A is associated with the lowest level of activation of the human immune system (37), leading to the tantalizing hypothesis that colistin-resistant *Acinetobacter* strains may lead to less of an inflammatory response than a colistin-susceptible one. Studies are ongoing to test this speculation.

Low-Mg^{2+} conditions in *Pseudomonas aeruginosa* can regulate the resistance to colistin in response to environmental conditions (29). In this study, reverse transcriptase (RT)-PCR showed a slight increase of expression in *pmrC*, and MALDI-TOF mass spectra revealed lipid A modification; thus, in a similar way, the development of a moderate level of colistin resistance in *A. baumannii* seems to be induced by the environmental conditions.

Our results indicate a different mechanism of colistin resistance in *A. baumannii* from that described by Moffatt et al. (30), who noted the total loss of LPS production via inactivation of the biosynthesis pathway genes *lpxA*, *lpxC*, and *lpxD*. Differences in selection methods may explain the development of different mechanisms: our mutants were selected in LB broth supplemented with stepwise increased colistin concentrations from 1 to 8 mg/liter, whereas Moffat et al. selected with colistin at a fixed concentration of 10 μg/ml in agar. These LPS-negative mutants became susceptible to cefepime, teicoplanin, and azithromycin, apparently reflecting defects to mem-

TABLE 3. Composition of lipid A in colistin-resistant and -susceptible *A. baumannii* clinical pair Ab15/133 and Ab15/32 and in ATCC 19606 and ABRIM and their derivatives

Peak (*m/z*)	Chemical inference	Chemical composition[a]	Presence/absence of chemical interference in:							
			Ab15/133 (colistin susceptible)	Ab15/132 (colistin resistant)	ABRIM ColS (colistin susceptible)	ABRIM ColR (colistin resistant)	ATCC 19606 ColS (colistin susceptible)	ATCC 19606 ColR (colistin resistant)	ATCC 19606 ColS Δ*pmrB* (colistin susceptible)	ATCC 19606 ColR Δ*pmrB* (colistin susceptible)
1,404	Tetra-acylated lipid A	2 glucose residues 2 phosphates 3 acyl groups, 12:0(3-OH) 1 acyl group, 14:0(3-OH)	+	+	+	+	+	+	+	+
1,530	Penta-acylated lipid A	2 glucose residues 2 phosphates 2 acyl groups, 12:0(3-OH) 2 acyl groups, 14:0(3-OH) 1 acyl group, 12:0	+	−	+	−	−	−	−	−
1,728	Hexa-acylated lipid A	2 glucose residues 2 phosphates 3 acyl groups, 12:0(3-OH) 2 acyl groups, 14:0(3-OH) 1 acyl group, 12:0	+	−	+	−	+	−	+	+
1,910	Hepta-acylated lipid A	2 glucose residues 2 phosphates 3 acyl groups, 12:0(3-OH) 2 acyl groups, 14:0(3-OH) 2 acyl groups, 12:0	+	+	+	+	+	+	+	+
2,034	Phosphoethanolamine-modified hepta-acylated lipid A	2 glucose residues 2 phosphates 3 acyl groups, 12:0(3-OH) 2 acyl groups, 14:0(3-OH) 2 acyl group, 12:0 1 phosphoethanolamine	−	+	−	+	−	+	−	−

[a] Fig. 4.

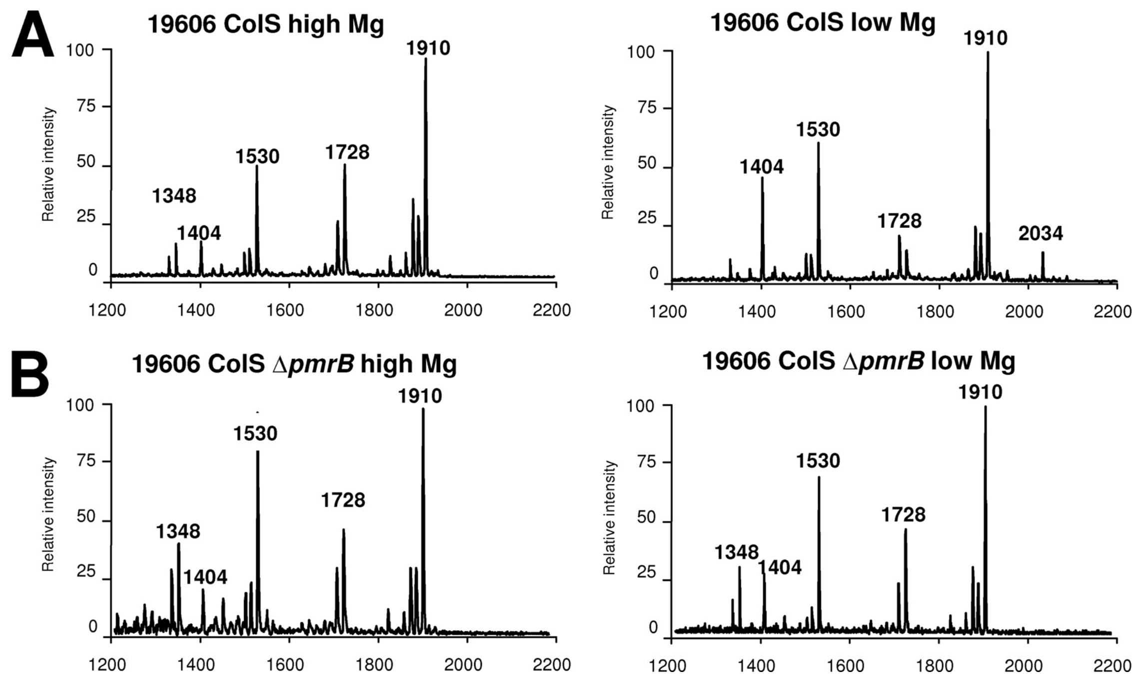

FIG. 5. Negative-ion MALDI-TOF mass spectra of lipid A isolated from strains grown under high-Mg^{2+} conditions (2 mM magnesium sulfate) and low-Mg^{2+} conditions (2 μM magnesium sulfate). (A) ATCC 19606 ColS wild type; (B) ATCC 19606 ColS Δ*pmrB*.

brane integrity. No such traits in the present isolates and mutants were seen, which retained LPS.

In summary, we report the analysis of *pmrCAB* in a diverse collection of clinical isolates and laboratory mutants of *A. baumannii* and its relationship with colistin resistance. We also analyzed the composition of lipid A from resistant and susceptible isolates. Our data suggest that resistance to colistin in *A. baumannii* requires at least two distinct genetic events, as follows: (i) at least one amino acid change in PmrB, although these changes were diverse and not localized to a specific domain, and (ii) upregulated expression of *pmrA* and *pmrB*. The precise genetic events that cause *pmrAB* upregulation remain to be defined. These genetic changes lead, in turn, to the addition of phosphoethanolamine to hepta-acylated lipid A, leading to the LPS modifications that directly confer the colistin resistance.

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