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Resistance to Colistin in *Acinetobacter baumannii* Associated with Mutations in the PmrAB Two-Component System▽

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The mechanism of colistin resistance (Col^r) in *Acinetobacter baumannii* was studied by selecting in vitro Col^r derivatives of the multidrug-resistant *A. baumannii* isolate AB0057 and the drug-susceptible strain ATCC 17978, using escalating concentrations of colistin in liquid culture. DNA sequencing identified mutations in genes encoding the two-component system proteins PmrA and/or PmrB in each strain and in a Col^r clinical isolate. A colistin-susceptible revertant of one Col^r mutant strain, obtained following serial passage in the absence of colistin selection, carried a partial deletion of *pmrB*. Growth of AB0057 and ATCC 17978 at pH 5.5 increased the colistin MIC and conferred protection from killing by colistin in a 1-hour survival assay. Growth in ferric chloride [Fe(III)] conferred a small protective effect. Expression of *pmrA* was increased in Col^r mutants, but not at a low pH, suggesting that additional regulatory factors remain to be discovered.

Among gram-negative pathogens that are reported as "multidrug resistant" (MDR), *Acinetobacter baumannii* is rapidly becoming a focus of significant attention (1, 7, 25, 32, 38, 39, 46, 51). In intensive care units, up to 30% of *A. baumannii* clinical isolates are resistant to at least three classes of antibiotics, often including fluoroquinolones and carbapenems (25).

The emergence of MDR gram-negative pathogens, including *A. baumannii*, has prompted increased reliance on the cationic peptide antibiotic colistin (12). Regrettably, increasing colistin use has led to the discovery of resistant strains (10, 11, 22, 26). For example, in a recent study, 12% of carbapenemase-producing *Enterobacteriaceae* were found to be colistin resistant (Col^r) (6). Although still uncommon, *A. baumannii* isolates resistant to all available antimicrobial agents have been reported (26, 45) and are of enormous concern, given their potential to spread in the critical care environment.

Colistin and other polymyxins are cyclic cationic peptides produced by the soil bacterium *Bacillus polymyxa* that act by disrupting the negatively charged outer membranes of gram-negative bacteria (37, 50). The following three distinct mechanisms that give rise to colistin resistance are known: (i) specific modification of the lipid A component of the outer membrane lipopolysaccharide, resulting in a reduction of the net negative charge of the outer membrane; (ii) proteolytic cleavage of the drug; and (iii) activation of a broad-spectrum efflux pump (13, 14, 49). The mechanism of colistin resistance in *Acinetobacter* spp. is not yet known. Heteroresistance to colistin in *A. baumannii* has been described (17, 24), but it is uncertain whether the basis for this resistance is the presence of a genetically distinct population of cells or whether variation in the regulatory program among genetically identical cells may be sufficient for the expression of resistance.

In *Salmonella enterica*, the two-component signaling systems PmrAB and PhoPQ are involved in sensing environmental pH, Fe^{3+}, and Mg^{2+} levels, leading to altered expression of a set of genes involved in lipid A modification (14, 43, 53). A small adapter protein, PmrD, serves as an interface between the two-component systems by stabilizing the activated form of PmrA in *S. enterica* (19), but other mechanisms of coordinated regulation are described for other species (52). Mutations causing constitutive activation of PmrA and PmrB are associated with colistin resistance (31, 33). Interestingly, the *phoPQ* and *pmrD* genes do not appear to be present in *Acinetobacter* spp., based on computational analysis of the genome sequences (2).

PmrA-regulated resistance to colistin in *S. enterica* and *P. aeruginosa* results from modification of lipid A with 4-deoxy-aminoarabinose (Ara4N) or phosphoethanolamine via activation of *ugd*, the *pmrF* (or *pbgP*) operon, and *pmrC*, which encode UDP-glucose dehydrogenase (the first step in Ara4N biosynthesis), Ara4N biosynthetic enzymes, and lipid A phosphoethanolamine transferase, respectively (8, 15, 21, 41, 48). The Ara4N biosynthesis and attachment genes are not present in *A. baumannii* or *Neisseria meningitidis* (36, 47). *N. meningitidis* is intrinsically resistant to polymyxins, demonstrating that Ara4N modification of lipid A is not required for resistance. Mutations in the *pmrC* ortholog *lptA*, encoding the lipid A phosphoethanolamine transferase, reduce colistin resistance in *N. meningitidis*, suggesting that this modification alone may be sufficient for conferring colistin resistance (49). Here we show that the PmrAB system is involved in regulating colistin resistance in *A. baumannii* by identification of mutations in resistant isolates that exhibit constitutive expression of *pmrA*.

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TABLE 1. Strains used in this study[a]

Strain	Parent	Colistin susceptibility	*pmr* genotype	Reference
ATCC 17978*	NA	S	Wild type	44
AB0057*	NA	S	Wild type	2, 18
MAC101	AB0057	R	PmrA P102H, PmrB A262P	This study
MAC102	AB0057	R	PmrB A227V	This study
MAC103	MAC101	S	Same as MAC101	This study
MAC201	ATCC 17978	R	PmrB T13N, PmrB P233S	This study
MAC203	MAC201	S	PmrB T13N, PmrB P233S, PmrB Δ411-444	This study
ACCA152*	NA	R	PmrB P233T	
AB060*	NA	S	PmrB FS209 (frameshift)	18
ATCC 27853	NA	S	ND	28

[a] All strains are *A. baumannii*, except for *P. aeruginosa* ATCC 27853. *, clinical isolate. NA, not applicable; R, resistant; S, susceptible.

TABLE 2. Primer sequences used for *pmrCAB* sequencing and quantitative PCR

Primer	Sequence
pmrBAC_L	GCGAGGAGCACATTTCCTAA
pmrBAC_R	TGTAGTCACTCACGATGCTGAA
pmrBAC_L2	TTAAAGTTACATCTTGCTTTGCC
pmrBAC_L2c	GGCAAAGCAAGATGTAACTTTAA
pmrBAC_R2	TCGATGAAATTCTAGATACTCAAATG
pmrBAC_L3	CCCAAATATCGATAAACAGATCTTC
pmrBAC_R3	TTGAAGCAGATCCGTCAAAG
pmrBAC_L4	TGCACCCAAATTTAAACCATC
pmrBAC_R4	CCGACTTGTGATACGAATGC
pmrBAC_L5	TCATTTGGCTTAATACATGGTCTG
pmrBAC_R5	GGATGATTTCACCGCAAAATAG
pmrBAC_R5c	CTATTTTGCGGTGAAATCATCC
pmrA_1	GGTGTTGCTGCTCTTTGACG
pmrA_2	GGTGGAATGGGTCAATAACG
16S_rRNA_1	CAGCTCGTGTCGTGAGATGT
16S_rRNA_2	CGTAAGGGCCATGATGACTT

MATERIALS AND METHODS

Strains of *A. baumannii*. AB060 and AB0057 are colistin-susceptible MDR strains isolated from patients at Walter Reed Army Medical Center in 2004, and ATCC 17978 is a largely drug-susceptible strain isolated from a patient in the late 1950s in New York City (2, 18, 44). The clinical isolate ACCA152 is a Col^r strain isolated from the respiratory tract of a patient at Cedars-Sinai Medical Center. Identification as *A. baumannii* was confirmed with API 20NE kits (bioMérieux, Durham, NC). Relevant features of each strain are given in Table 1.

Susceptibility testing. MICs were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (MHB) according to Clinical and Laboratory Standards Institute methods (34), using in-house trays for colistin (Sigma-Aldrich, St. Louis, MO) and commercial trays (Sensititre [Trek Diagnostics, Cleveland, OH] and MicroScan [Siemens Healthcare Diagnostics, West Sacramento, CA]) for other agents. MICs were determined for ampicillin, piperacillin-tazobactam, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, cefoxitin, meropenem, imipenem, levofloxacin, ciprofloxacin, gentamicin, amikacin, and tetracycline. Colistin MICs were also determined by Etest (bioMérieux, Durham, NC) on Mueller-Hinton agar plates (BBL, Sparks, MD). *Pseudomonas aeruginosa* ATCC 27853 was used for quality control on each day of testing. All assays used colistin sulfate (referred to as "colistin" hereafter; Sigma-Aldrich, Inc.), and concentrations are reported as the concentration of colistin base, after adjusting for potency based on information from the manufacturer.

Selection of Col^r mutants. Col^r derivative clones were obtained by growth in liquid culture (lysogeny broth [LB]) with colistin at 1.0 μg/ml, which is above the MIC. Successive passage in increasing colistin concentrations demonstrated a high level of resistance in each derivative. Mutation detection was performed by PCR amplification of the entire *pmrCAB* operon, followed by DNA sequencing using primers distributed approximately every 500 bases; both strands were completely sequenced. Mutations were confirmed to be specific to the Col^r derivatives by PCR and sequencing of selected regions of the parental isolates. Primer sequences are reported in Table 2.

Survival analysis assays were performed by growth in LB to an optical density of ~0.6, followed by dilution into medium containing colistin. After 1 hour at 37°C, cultures were diluted and plated on LB plates without colistin. The percentage of surviving cells was determined. Population analysis profiles were determined by plating dilutions of a mid-log-phase culture (optical density, ~0.6) onto LB plates, with and without the addition of 7 μg/ml colistin; the percentage of Col^r cells in the original culture was determined based on the ratio of the number of colonies on colistin plates to that on LB plates without colistin.

Quantitative PCR. Total RNA was isolated from bacterial cells grown in LB to mid-log phase (A_{600} = 0.5). Colistin (7 μg/ml) or ferric chloride (1 mM) was added as appropriate. Bacterial RNA was stabilized using RNAprotect bacterial reagent (Qiagen) and purified using an RNeasy kit (Qiagen) and nuclease-free water. The RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Before reverse transcription (RT), RNA samples were treated with Turbo DNase (Ambion) to digest genomic DNA, followed by treatment with RNaseOUT recombinant RNase inhibitor (Invitrogen) to prevent RNA degradation. The RT reaction was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Negative control reactions were performed using equal concentrations of RNA without RT reagents.

Primers for RT-PCR were designed using Primer3 and are listed in Table 2. Specificity was evaluated by melting curve analysis, and only artifact-free primers were used for RT-PCR. 16S rRNA was used as a housekeeping gene for normalization.

Real-time PCR amplification was carried out on a Chromo4 continuous fluorescence detector (MJ Research), using Power SYBR green PCR master mix (Applied Biosystems) as directed by the manufacturer. In each run, a blank sample (distilled water) and a no-reverse-transcriptase control were run to evaluate DNA contamination. The critical cycle threshold was determined by Opticon Monitor software, version 2.03 (MJ Research). Relative gene expression differences were calculated using the standard curve method. The quantity of the target transcript, *pmrA*, was determined from the standard curve of the 16S rRNA housekeeping gene, and the appropriate wild-type strain (AB0057 or ATCC 17978) was used as the calibrator sample to which differences were compared. For each sample, at least three biological replicates (from separate initial cultures) were performed, and the expression level in each replicate was measured a minimum of six times.

RESULTS

Survival analysis of strains with reference genome sequences. The origins and genotypes of the parent strains and derived mutants used in this study are shown in Table 1 and Fig. 1, and the colistin susceptibility of these isolates is shown in Table 3. We tested two clinical isolates of *A. baumannii* (AB0057 and ATCC 17978) for colistin susceptibility in rich media (LB and MHB). Using colistin in a survival analysis assay, the MDR isolate AB0057 was killed in a concentration- and time-dependent manner (Fig. 2). Fewer than 1% of cells

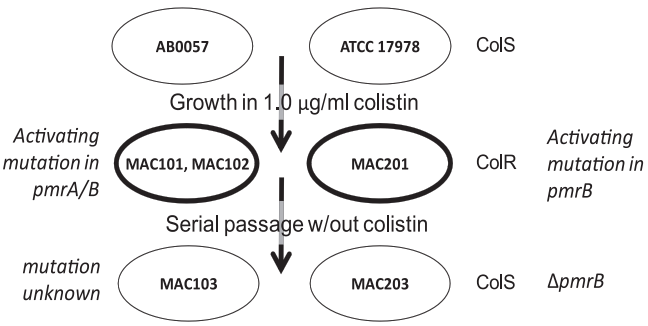

FIG. 1. Selection of Col^r and Col^s mutants. The scheme for selection or isolation of Col^r mutants and revertants of each mutant is shown. Col^r strains are indicated by bold circles.

TABLE 3. Antimicrobial susceptibilities of parent and mutant strains of *A. baumannii*

Isolate	Colistin MIC (μg/ml)	
	Etest[a]	Broth microdilution
AB0057	1.5	1–2
MAC101	32	>128
MAC102	4–8	64
MAC103	0.5	1
ATCC 17978	0.75	2
MAC201	64	128
MAC203	0.5	2
ACCA152	24	>128
AB060	0.5	1

[a] For Etest, susceptible MICs were ≤2 μg/ml and resistant MICs were ≥4 μg/ml.

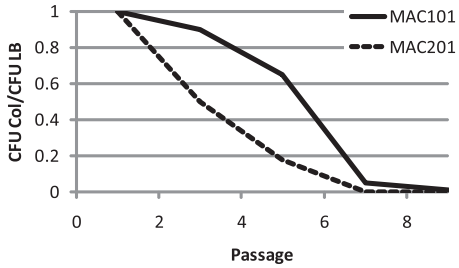

FIG. 3. Reversion of colistin resistance. Each clone was passaged in successive overnight and daily cultures. Each daily culture was plated on LB agar with and without 10 μg/ml colistin. The ratio of CFU from the two plates is plotted.

survived a 1-hour incubation in 1.4 μg/ml colistin. Bactericidal activity was time dependent, with a half-life of approximately 5 minutes at 1.4 μg/ml colistin. A population analysis profile showed that within a log-phase culture, <0.00001% of AB0057 cells were able to grow on LB agar containing 1.4 μg/ml colistin. Similar results were obtained with *A. baumannii* ATCC 17978 (data not shown).

Selection of Col^r derivatives of isolates with completely sequenced genomes. In order to define genetic loci responsible for colistin resistance, we selected Col^r derivatives of *A. baumannii* isolates AB0057 and ATCC 17978, for which complete genome sequences are available (2, 44). Overnight cultures were diluted 1:100 in LB containing 0, 0.1, 0.5, 1.0, 2.0, and 4.0 μg/ml colistin. The cells in cultures containing 0, 0.1, and 0.5 μg/ml colistin (i.e., below the MIC) demonstrated robust growth, while the cells in cultures containing ≥1 μg/ml colistin did not grow. Cultures containing higher concentrations of colistin were left to grow for 18 to 24 h. At 24 h, the cultures with 1.0 μg/ml colistin demonstrated growth. These cultures were plated onto LB agar containing 7 μg/ml colistin, and individual colonies were propagated for further analysis. These cells were confirmed to be *A. baumannii* by PCR, using several primer pairs that had been used for gap closure during genome sequencing (2), and by biochemical identification on API 20NE panels. Two independent Col^r derivatives of AB0057 (MAC101 and MAC102) and one of ATCC 17978 (MAC201) were obtained.

Col^r derivative strains were tested for resistance to increasing concentrations of colistin and were able to grow in up to 64 μg/ml colistin in liquid culture. Colistin MICs determined by Etest for the Col^r derivative strains ranged from 8 to 64 μg/ml (Table 3). Cells were routinely grown in and maintained on LB agar or agar containing 7 μg/ml colistin. The antibiotic susceptibility profiles of the parental and Col^r derivative strains showed no changes for any of the agents tested other than colistin (data not shown). In contrast to the results of other studies (23), acquisition of colistin resistance did not increase susceptibility to other antibiotics.

The proportion of resistant cells declined when Col^r strains were grown in the absence of colistin. Col^r strains were passaged in LB every 12 hours. By the ninth passage, only about 1% of cells were able to grow on plates containing 7 μg/ml colistin (Fig. 3). Thus, Col^r strains exhibit a modest growth disadvantage compared with their respective Col^s parents. A single colony from an LB plate at the ninth passage was isolated and maintained as an example of a revertant for each parental strain (MAC103 and MAC203). These revertant clones were Col^s (Table 3).

Mutational analysis of Col^r derivative strains and revertants. The two-component system proteins PmrB (sensor kinase) and PmrA (response regulator) are implicated in polymyxin resistance in several genera (31, 42, 52). The *A. baumannii* orthologs of these genes are not obvious based solely on pairwise alignments with the *P. aeruginosa* PmrB and PmrA sequences. AB57_3174 is a clear ortholog of PmrC, however, strongly impli-

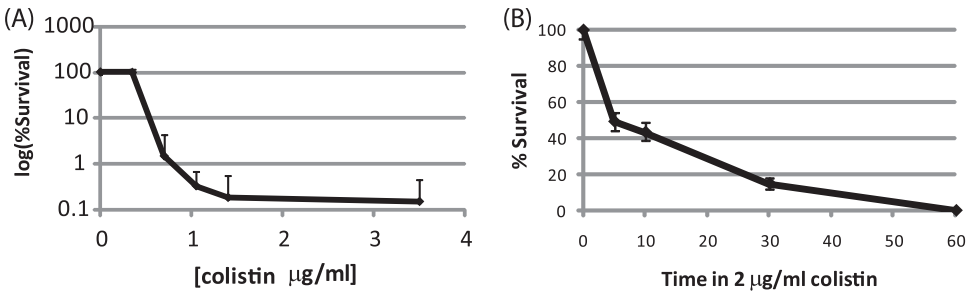

FIG. 2. Survival of strain AB0057 in colistin. (A) Mid-log-phase cultures of AB0057 in LB were treated with the indicated concentration of colistin for 1 h at 37°C, and then quantitative counts of live cells were determined by plating 0.1-ml samples of serial 10-fold dilutions on LB agar plates. (B) Cells were grown in LB for 4 hours, and then colistin was added at the indicated concentration. Aliquots were removed at the given intervals, and quantitative counts were performed as described above. "Percent survival" corresponds to 100 × the ratio of the number of CFU in the presence of colistin to the number of CFU in the absence of colistin at each time/concentration point.

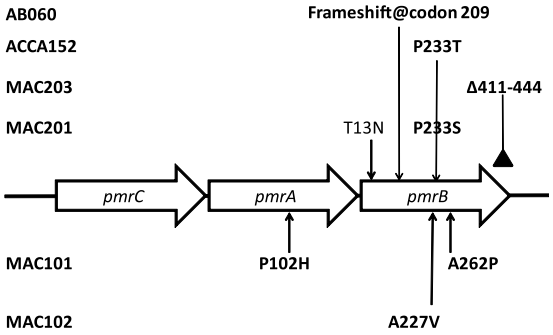

FIG. 4. Mutational analysis of the *pmr* operon. Mutations in bold affect conserved residues in functional domains. All strains are Col^r, except for AB060 and MAC203, which are Col^s.

cating the adjacent genes, encoding the two-component system proteins AB57_3173 and AB57_3172, as PmrA and PmrB, respectively. The ATCC 17978 proteins encoded by these genes are A1S_2752, A1S_2751, and A1S_2750, respectively.

In order to understand the genetic basis for this phenotype, the complete coding region and promoter of the *pmrCAB* operon were sequenced for MAC101, MAC102, and MAC201. Mutations in *pmrB* and *pmrA* that are not present in the parental isolates were found in the derivative strains (Fig. 4). MAC101 has mutations in both *pmrA* and *pmrB*, and MAC201 has two point mutations in *pmrB*. In each case, at least one of the mutations falls within a conserved functional domain. Supporting the importance of the *pmrAB* system in clinically relevant resistance to colistin, the Col^r *A. baumannii* isolate ACCA152 carries the *pmrB* mutation P233T. A mutation at the same codon was found in MAC201.

Sequencing of the *pmr* operon in MAC203 revealed a deletion in *pmrB* that removes the C-terminal 32 amino acids, including a portion of the histidine kinase domain. The sequence of the *pmrCAB* region in the revertant MAC103 is 100% identical to the MAC101 sequence, so the suppressing mutation maps outside the *pmrCAB* locus.

Expression analysis of *pmrA*. The identification of several independent mutations in *pmrB* and *pmrA* demonstrates the importance of these genes as regulators of colistin resistance in *A. baumannii*. Based on prior analysis of PmrA function, we hypothesized that these mutations result in activation of PmrA (33). To evaluate the effects of these mutations in the Col^r strains, quantitative RT-PCR was performed using primers designed to target the *pmrA* transcript. The Col^r strains (MAC101, MAC102, and MAC201) exhibited 5- to 40-fold increased expression of *pmrA* (Fig. 5A). This is consistent with activating mutations and PmrA auto-regulation (27). Growth in the presence of colistin was not necessary for high-level *pmrA* expression (data not shown). In contrast, expression levels in the Δ*pmrB* revertant strain MAC203 were similar to those in the wild-type parental strain. Interestingly, *pmrA* expression in the Col^s revertant MAC103 was also at approximately baseline levels, despite the observation that the *pmr* locus is identical in sequence to that in MAC101. Further analysis will be necessary to identify the mutation in this strain and to explain the reduction in *pmr* expression.

Induction of colistin resistance by environmental factors. Growth of the two wild-type *A. baumannii* strains at pH 5.5

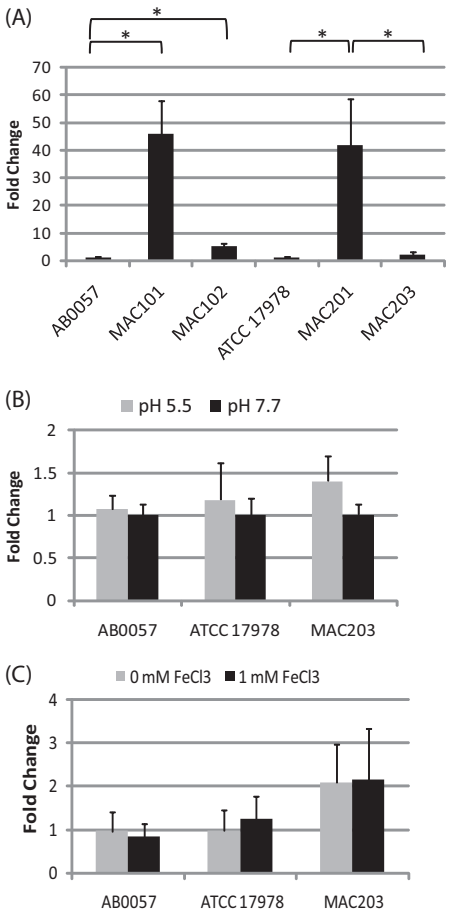

FIG. 5. Gene expression analysis of *pmrA*. The level of expression of *pmrA* was measured by real-time quantitative RT-PCR. Expression levels were normalized to 16S rRNA. (A) Expression analysis of wild-type and mutant strains. Changes with respect to wild-type expression levels are shown. (B) Expression analysis at pH 5.5 and 7.7. Changes are expressed with respect to expression levels measured at pH 7.7. (C) Expression analysis in the presence of 1 mM $FeCl_3$. Changes are expressed with respect to expression levels of the wild-type strain grown without iron. Each value represents the mean ± standard deviation for at least three independent cultures. An asterisk indicates that the difference is significant ($P < 0.01$) by the *t* test.

resulted in a remarkable increase in the MIC for colistin, to ≥64 μg/ml (Table 4). The MIC was significantly attenuated for the *pmrB* deletion mutant MAC203, suggesting that PmrB is required for the induction of colistin resistance, as seen with *S. enterica* (40). A second clinical isolate (AB060), harboring a frameshift mutation at codon 209 of PmrB, was also unable to grow in colistin-containing medium at pH 5.5. Thus, PmrB seems to be required for acid pH-induced colistin resistance. Contrary to expectations, the level of expression of *pmrA* was unchanged at pH 5.5 compared with that at pH 7.7 (Fig. 5B). The broth microdilution assay measures the effect of chronic exposure of cells to colistin. We also performed a 1-hour survival analysis assay, as described above, to assess the short-term effect of colistin on cells grown at acid pH. In this assay, MAC203 exhibited a level of protection from the bactericidal activity of colistin that was equivalent to that of both wild-type strains (Table 4).

TABLE 4. Effects of acid pH on colistin susceptibility

Strain	Broth microdilution colistin MIC (μg/ml) (MHB)		% Survival following 1-h colistin challenge (LB)[a]	
	pH 5.5	pH 7.7	pH 5.5	pH 7.7
AB0057	64	1	70 ± 11	<1
ATCC 17978	64	1–2	75 ± 26	<1
MAC103	128	1	ND	ND
MAC203	4	1	68 ± 52	<1
AB060	1	0.25	ND	ND
P. aeruginosa ATCC 27853	1	2	ND	ND

[a] Values are means for at least two independent cultures ± standard deviations. ND, not done.

TABLE 5. Effects of Fe^{3+} on colistin susceptibility in LB

Strain	Broth microdilution colistin MIC (μg/ml)		% Survival following 1-h colistin challenge[a]	
	0 mM $FeCl_3$	1 mM $FeCl_3$	0 mM $FeCl_3$	1 mM $FeCl_3$
AB0057	0.25	0.5	1.6 ± 0.2	16 ± 8
ATCC 17978	0.25	0.5	0.2 ± 0.4	56 ± 65
MAC203	0.5	4	0.1 ± 0.05	15 ± 10
MAC201	16	128	ND	ND
P. aeruginosa ATCC 27853	0.25	0.25	ND	ND

[a] Values are means for at least two independent cultures ± standard deviations. ND, not done.

The addition of 1 mM ferric chloride [iron(III)] increased the MIC for colistin ~2- to 4-fold (Table 5). Ferric chloride precipitates in MHB at this concentration, so assays were performed in LB. AB0057 and ATCC 17978 cells grown in LB in the presence of 1 mM $FeCl_3$ were able to resist lysis by colistin during a 1-hour survival assay better than cells grown without iron (Table 5), although the effect was variable. Fe^{3+}-induced colistin resistance is rapid: pretreatment of cells for 1 hour resulted in essentially the same level of resistance as that observed in cells grown overnight in LB supplemented with 1 mM $FeCl_3$ (data not shown). *pmrA* expression was unaffected by Fe^{3+} exposure (Fig. 5C). MAC203 cells, carrying a partial deletion of *pmrB*, exhibited protection from colistin in the presence of ferric chloride equivalent to that of the wild-type parent. In addition, the Col^r strain MAC201 showed an increase in colistin resistance in the presence of iron. Taken together, these observations suggest that the Pmr system may not be the only contributor to regulation of colistin resistance.

DISCUSSION

We show that mutations in the genes encoding the two-component signaling proteins PmrB and PmrA are linked to colistin resistance in *A. baumannii*. In the Col^r mutants, expression of *pmrA* is increased, suggesting that the mutations cause constitutive activation of PmrA, which in turn autoregulates the *pmrCAB* promoter. A partial deletion of *pmrB* in a Col^r background results in reversion to a colistin-susceptible phenotype, reinforcing the importance of the PmrAB system in regulating colistin resistance in *A. baumannii*. In addition, colistin resistance can be induced by acid pH and, to a lesser extent, Fe^{3+} supplementation. This induction is rapid. Thus, it is likely to involve solely changes in the cellular regulatory program rather than selection of rare spontaneous mutations. Acid pH- and Fe^{3+}-induced resistance appears to be at least partially independent of PmrA. Expression of *pmrA* is unchanged during growth at acid pH or in ferric chloride. The Δ*pmrB* strains MAC203 and AB060 exhibit markedly reduced pH-induced resistance but no difference in Fe^{3+}-induced resistance. It is possible that PmrB interacts with other response regulator proteins (3). This would explain both the lack of *pmrA* upregulation and the ability of Δ*pmrB* cells to respond to Fe^{3+} treatment.

The downstream targets of PmrA that are responsible for colistin resistance still remain to be determined. Autoregulation of the *pmrCAB* operon is observed in *S. enterica* and *P. aeruginosa*, suggesting that increased PmrC-mediated transfer of phosphoethanolamine to lipid A may be involved. A recent study of proteins that are differentially expressed in a Col^r derivative of *A. baumannii* ATCC 19606 found ~35 protein expression changes but not PmrC (9). The *pmr* operon was not sequenced for this mutant, and it remains to be determined whether these proteins may be encoded by PmrA target genes. Knowledge of the mechanism(s) of resistance in *A. baumannii* will be informative for consideration of the development of strategies to prevent and combat it. For example, inhibition of Ara4N biosynthesis and/or attachment to lipid A has been proposed as a means of disabling polymyxin resistance (20), but this approach would likely be completely ineffective for *Acinetobacter* spp., which do not contain the target enzymes.

The regulation of colistin resistance observed in *A. baumannii* differs in substantive ways from that in *P. aeruginosa*, the closest relative that has been studied in detail. Unlike the case in *A. baumannii*, there is no evidence of acid pH- or Fe^{3+}-induced colistin resistance in *P. aeruginosa*, and this was confirmed by the use of a *P. aeruginosa* control strain in the analyses presented here. The role of a limiting Mg^{2+} concentration in the regulation of colistin resistance in *A. baumannii* has not yet been evaluated. Regulation of colistin resistance has been studied most extensively in *S. enterica*, where low magnesium, high iron, and low pH can induce resistance, mediated by the PhoPQ (Mg^{2+}) and PmrAB (Fe^{3+} and pH) two-component systems (16, 43). Fe^{3+}-mediated changes in lipid A are PmrA dependent and are the same as those associated with colistin resistance (35). Thus, both *S. enterica* and *P. aeruginosa* exhibit regulation of colistin resistance in response to environmental factors, although in different ways (27).

In addition to regulating resistance to cationic antimicrobial peptides, the PmrAB two-component system is linked to the control of genes associated with virulence in *S. enterica* and *Legionella pneumophila* (3, 14). The role, if any, of the *A. baumannii* PmrAB system in other aspects of its pathology remains to be determined. The Col^r isolates exhibited differences in protein expression compared to their Col^s progenitors (data not shown), and identification of these differences may prove useful for dissecting the genetic and biochemical features of colistin resistance.

Heteroresistance to colistin was previously observed in *A. baumannii*, with the percentage of Col^r cells ranging from 0.00001% to 0.0002% (17, 24). Heteroresistance has been defined as "the presence of one or several bacterial subpopulations at a frequency of 10^{-7} to 10^{-3}, which can grow at higher antibiotic concentrations than predicted by the MIC for the majority of cells" (30). These subpopulations may reflect genetic heterogeneity in a mixed population or genetically identical cells that express different gene sets in response to divergent regulatory programs (4, 5, 29). The clinical significance of colistin heteroresistance in *A. baumannii* is not yet known, but it is quite worrisome because (i) it is possible that selection of resistant strains could lead to treatment failure and (ii) the inadvertent transmission of Col^r strains of *Acinetobacter* spp. could have a significant impact by necessitating additional isolation and decontamination regimens. We have shown that growth conditions can modulate the MIC and affect the percentage of cells that are able to survive a 1-hour colistin challenge. Stable colistin resistance was correlated with the presence of genetic mutations, but high-level resistance could also be induced by growth at pH 5.5.

If colistin resistance can readily arise based on regulatory changes in response to environmental factors or selection of a rare mutation in the presence of colistin, the phenotype of colistin susceptibility will be subject to significant changes over the course of an infection. Rapid determination of the presence of a subpopulation of Col^r cells within a sample from an infected site, for example, by enzyme-linked immunosorbent assay or genetic assay, could assist in treatment decisions and infection control.

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