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Acinetobacter baumannii Increases Tolerance to Antibiotics in Response to Monovalent Cations▽†

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Acinetobacter baumannii* is well adapted to the hospital environment, where infections caused by this organism are associated with significant morbidity and mortality. Genetic determinants of antimicrobial resistance have been described extensively, yet the mechanisms by which *A. baumannii* regulates antibiotic resistance have not been defined. We sought to identify signals encountered within the hospital setting or human host that alter the resistance phenotype of *A. baumannii*. In this regard, we have identified NaCl as being an important signal that induces significant tolerance to aminoglycosides, carbapenems, quinolones, and colistin upon the culturing of *A. baumannii* cells in physiological NaCl concentrations. Proteomic analyses of *A. baumannii* culture supernatants revealed the release of outer membrane proteins in high NaCl, including two porins (CarO and a 33- to 36-kDa protein) whose loss or inactivation is associated with antibiotic resistance. To determine if NaCl affected expression at the transcriptional level, the transcriptional response to NaCl was determined by microarray analyses. These analyses highlighted 18 genes encoding putative efflux transporters that are significantly upregulated in response to NaCl. Consistent with this, the effect of NaCl on the tolerance to levofloxacin and amikacin was significantly reduced upon the treatment of *A. baumannii* with an efflux pump inhibitor. The effect of physiological concentrations of NaCl on colistin resistance was conserved in a panel of multidrug-resistant isolates of *A. baumannii*, underscoring the clinical significance of these observations. Taken together, these data demonstrate that *A. baumannii* sets in motion a global regulatory cascade in response to physiological NaCl concentrations, resulting in broad-spectrum tolerance to antibiotics.

Acinetobacter baumannii has become a species of increasing clinical importance over the course of the last 3 decades. *A. baumannii* has established itself within the hospital niche, where it is responsible for approximately 6% of Gram-negative infections in the intensive care setting in the United States (28). Of particular concern is the high rate of antibiotic resistance observed for *A. baumannii* isolates. In the United States, multidrug resistance (MDR) in *Acinetobacter* spp. has increased dramatically, soaring from 6.7% in 1993 to 29.9% in 2004, a level more than twice that observed for any other Gram-negative bacillus causing infections in intensive care units (28). Despite these increasing rates of multidrug resistance and reports of panresistance emerging, there have been no new drugs developed to treat infections caused by MDR Gram-negative bacilli such as *A. baumannii* (5). Acquiring in-depth knowledge of the basic physiology of *A. baumannii* as well as identifying the specific factors that enable this bacterium to persist within the hospital environment and the human host are therefore imperative in the quest to identify novel drug targets.

Armed with its arsenal of antibiotic resistance determinants and its ability to persist for long periods on dry surfaces, *A. baumannii* is poised for survival in the hospital niche (4, 21, 35). Antibiotic resistance has been investigated extensively at the genetic level, revealing specific mechanisms of resistance to a number of antibiotics. These resistance mechanisms include antibiotic efflux (e.g., AdeABC and AbeM), enzymatic inactivation (e.g., AmpC and OXA-like β-lactamases), and a decreased permeability of the outer membrane (e.g., a loss of CarO and a 33- to 36-kDa Omp) (13, 19, 27, 30, 31, 38, 39, 41, 44; for a comprehensive review, see references 4, 36, and 47). In addition, whole-genome sequencing approaches comparing MDR *A. baumannii* strains with susceptible strains have highlighted additional genetic features that potentially contribute to antibiotic resistance. One feature common to MDR *A. baumannii* strains is the presence of one or more large resistance islands containing up to 90 genes associated with antibiotic resistance (1, 20, 46). The size of these islands makes them prominent features in the genomes sequenced to date. However, the size and composition of resistance islands vary considerably among MDR *A. baumannii* strains, and many antibiotic resistance genes present in MDR strains do not reside within a discrete resistance island (1). These studies highlight the diversity of antibiotic resistance mechanisms encoded at the genetic level in *A. baumannii* and the complexity of antibiotic resistance in this nosocomial pathogen.

Although considerable progress has been made toward identifying genes associated with resistance, few studies have investigated the mechanisms regulating resistance in *A. baumannii* (2, 13, 19, 31, 38). In particular, little is known about whether resistance phenotypes are constitutive and therefore static or whether resistance is modulated in response to exter-

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nal signals. Genomic comparisons between pathogenic *A. baumannii* and nonpathogenic *Acinetobacter baylyi* strain ADP1 have highlighted a subset of 475 genes, referred to as the pan-*A. baumannii* accessory genes, that are conserved among pathogenic strains but absent from ADP1 (1). As noted previously by Adams et al., approximately 12% of the pan-*A. baumannii* genes encode predicted transcription factors, suggesting that *A. baumannii* has acquired an extensive regulatory capacity as a consequence of growth in association with the human host (1). Further underscoring its regulatory needs, *A. baumannii* can survive under a wide variety of environmental conditions, which is highlighted by an ability to survive desiccation for long periods of time, resist antimicrobials, and utilize a broad range of nutrient sources (21, 43, 46). In keeping with this, we hypothesized that *A. baumannii* must possess mechanisms to sense and respond to the external environment and that the associated regulatory systems may contribute to antibiotic resistance in this organism. In an attempt to identify the regulatory mechanisms governing resistance in *A. baumannii*, we first sought to identify environmental signals encountered within the hospital environment or the human host that contribute to antibiotic resistance. We examined sodium chloride (NaCl) specifically, as NaCl is ubiquitous within the hospital environment and within the human host and is found in various concentrations in drug formulations, wound dressings, intravenous fluids, and body fluids and on the surface of the skin, among other sites. Through proteomic and transcriptional analyses our work establishes that NaCl and more broadly monovalent cations are important environmental signals sensed by *A. baumannii*. Specifically, NaCl exposure induces a regulatory cascade that ultimately results in a decreased susceptibility to antibiotics of distinct classes. Our data further demonstrate that this response to NaCl is conserved among MDR clinical isolates and that NaCl-induced antibiotic tolerance is likely multifactorial, being mediated through both the transcriptional and posttranslational regulation of cell envelope composition. Taken together, these data demonstrate that *A. baumannii* regulates its intrinsic antibiotic resistance profile in response to a commonly encountered environmental signal, underscoring the adaptability of this organism to growth within the hospital environment and within its host.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics. Reference strain ATCC 17978 and sequenced clinical strain AYE were obtained from the American Type Culture Collection (Manassas, VA). Clinical isolates were obtained from the University of Nebraska Medical Center (Omaha, NE). *A. baumannii* strain AB0057 was a gift from Robert Bonomo (Case Western Reserve University, Cleveland, OH), and *A. baumannii* strains AB900 and AB307-0294 were gifts from Anthony Campagnari (State University at Buffalo, Buffalo, NY). All experiments were performed using reference strain ATCC 17978 unless otherwise specified. Bacteria were routinely maintained on Mueller-Hinton agar (MHA) or Mueller-Hinton broth (MHB). All antibiotics and the efflux pump inhibitor phenylarginine β-naphthylamide (PAβN) were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of antibiotics were made in water, stored at −80°C, and thawed on ice prior to use.

SDS-PAGE analysis of supernatant proteins. Cultures of *A. baumannii* ATCC 17978 cells grown overnight were diluted 1:100 in Luria broth (LB) with or without 200 mM NaCl or MHB (without NaCl) and MHB supplemented with NaCl or KCl to a final concentration of 50, 90, 150, or 300 mM and incubated at 37°C with shaking at 180 rpm. Bacteria were harvested by centrifugation, and supernatants were collected and filtered through 0.22-μm syringe filters (Millipore Corporation, Billerica, MA) to remove residual cells. Proteins were precipitated from the supernatants by the addition of cold trichloroacetic acid (TCA) to a final concentration of 20% (vol/vol), and the samples were incubated at 4°C overnight. Precipitated proteins were pelleted by centrifugation (20 min at 10,500 × *g*), washed once with cold ethanol (100%), and resuspended in Laemmli sample buffer (62.5 mM Tris, 10% [vol/vol] glycerol, 2% [wt/vol] sodium dodecyl sulfate, 5% [vol/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromophenol blue) (24). Proteins were resolved by SDS-PAGE in 15% polyacrylamide gels and visualized by staining with Coomassie brilliant blue (Pierce, Rockford, IL).

Protein sample preparation for proteomic analysis. For proteomic analyses, *A. baumannii* cells were cultured as described above in LB with or without 200 mM NaCl. Proteins were precipitated from filtered supernatants by the addition of ammonium sulfate to 80% saturation and incubation with constant mixing at 4°C for 4 h. Precipitated proteins were pelleted by centrifugation (10,500 × *g* for 20 min) and resuspended in 600 μl Tris-buffered saline (150 mM NaCl, 10 mM Tris [pH 7.6]). The samples were dialyzed into Tris buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.02% sodium azide) overnight, mixed with Laemmli sample buffer, and electrophoresed approximately 2 cm into a 15% polyacrylamide gel. Gels were stained with colloidal blue and destained with water, and the entire protein-containing region was excised and subjected to in-gel trypsin digestion according to a standard protocol (18). Briefly, the gel regions were washed with 100 mM ammonium bicarbonate for 15 min, and the proteins were reduced with 5 mM dithiothreitol [DTT] in fresh ammonium bicarbonate for 20 min at 55°C. After cooling to room temperature, iodoacetamide was added to a 10 mM final concentration and placed in the dark for 20 min at room temperature. The solution was discarded, and the gel pieces were washed with 50% acetonitrile–50 mM ammonium bicarbonate for 20 min, followed by dehydration with 100% acetonitrile. The liquid was removed, and the gel pieces were completely dried, reswelled with 0.8 μg of modified trypsin (Promega, Madison, WI) in 100 mM NH_4HCO_3, and digested overnight at 37°C. Peptides were extracted by three changes of 60% acetonitrile–0.1% trifluoroacetic acid, and all extracts were combined and dried *in vacuo*. Samples were reconstituted in 30 μl of 0.1% formic acid for liquid chromatography (LC)-tandem mass spectrometry (MS-MS) analysis.

LC-MS-MS analysis and protein identification. The resulting peptides were analyzed by using a Thermo Finnigan LTQ ion trap instrument equipped with a Thermo MicroAS autosampler and Thermo Surveyor high-performance liquid chromatography (HPLC) pump, Nanospray source, and Xcalibur 2.0 SR2 instrument control. Peptides were separated on a packed capillary tip (100 μm by 11 cm; Polymicro Technologies) with Jupiter C_{18} resin (5 μm, 300 Å; Phenomenex) by using an in-line solid-phase extraction column (100 μm by 6 cm) packed with the same C_{18} resin (using a frit generated with liquid silicate Kasil 1 [12]), which was similar to that described previously (25). The flow from the HPLC pump was split prior to the injection valve to achieve flow rates of 700 nl/min to 1,000 μl/min at the column tip. Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 0.1% formic acid in acetonitrile. A 95-min gradient was performed with a 15-min washing period (100% mobile phase A for the first 10 min followed by a gradient to 98% mobile phase A at 15 min) to allow for solid-phase extraction and the removal of any residual salts. Following the washing period, the gradient was increased to 25% mobile phase B by 50 min, followed by an increase to 90% mobile phase B by 65 min, and held for 9 min before being returned to the initial conditions. Tandem spectra were acquired by using a data-dependent scanning mode in which one full mass spectrometry scan (*m*/*z* 400 to 2000) was followed by 9 MS-MS scans. Tandem spectra were searched against the *Acinetobacter* subset of the UniRef100 database using the SEQUEST algorithm. The database was concatenated with the reverse sequences of all proteins in the database to allow the determination of false-positive rates. Protein matches were preliminarily filtered by using the following criteria: cross-correlation (X_{corr}) values of ≥1.0 for singly charged ions, ≥1.8 for doubly charged ions, and ≥2.5 for triply charged ions. A ranking of primary score (RSp) of ≤5 and a preliminary score (Sp) of ≥350 were also required for positive peptide identifications (IDs). Once filtered based on these scores, all proteins identified by less than two peptides were eliminated, resulting in false-positive rates of <1%. The SEQUEST output was then filtered by using IDPicker with a false-positive ID threshold (the default is 0.05, or 5% false-positive results) based on reverse sequence hits in the database. Protein reassembly from the identified peptide sequences was done with the aid of a parsimony method recently described by Zhang et al., which identifies indiscernible proteins (protein groups) that can account for the identified peptides (50). Only proteins present in each of three independent samples were considered in subsequent analyses. The relative abundance of each protein was estimated by counting total spectra corresponding to each protein ID and normalizing first to the size of the predicted protein and subsequently to spectral counts for EF-Tu. EF-Tu was selected for sample normalization because this protein is a highly abundant cyto-

plasmic protein that is constitutively expressed under a wide range of tested conditions, and its abundance in culture supernatants was not expected to change in response to NaCl (our unpublished data). Data from three independent samples were averaged for each condition (low and high NaCl), and statistically significant differences were determined by a Student's *t* test ($P \leq 0.05$).

Growth conditions for bacterial RNA isolation. Cultures of *A. baumannii* strain ATCC 17978 grown overnight were diluted 1:100 in fresh medium (LB for microarray only and tryptic soy broth [TSB] or MHB for quantitative reverse transcriptase PCR [RT-PCR]) or medium supplemented with 150 or 200 mM NaCl. Cultures were grown at 37°C to early exponential and stationary phases and then mixed with an equal volume of ice-cold ethanol-acetone (1:1) or with 2 volumes of RNA Protect bacterial reagent and stored at −80°C. For RNA isolation, mixtures were thawed on ice, and cells were collected by centrifugation. Cells were disrupted either mechanically or by enzymatic lysis. For mechanical disruption, cell pellets were washed once and suspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]). Cell suspensions were transferred into lysing matrix B tubes (MP Biomedicals, Solon, OH) and were lysed by two cycles of mechanical disruption in an FP120 shaker (Thermo Scientific, Waltham, MA) at settings 5.0 and 4.5 for 20 s. Cell debris was removed by centrifugation at 16,000 × *g* at 4°C for 10 min. For enzymatic lysis, bacterial pellets were suspended in TE buffer containing 15 mg/ml lysozyme and 20 mg/ml proteinase K (Qiagen, Valencia, CA) and incubated at 37°C for 1 h. Following mechanical or enzymatic lysis total RNA was isolated from cell lysates using Qiagen RNeasy minicolumns according to the manufacturer's recommendations for prokaryotic RNA purification (Qiagen, Valencia, CA). RNA concentrations were determined spectrophotometrically (40 μg/ml for an optical density at 260 nm [OD_{260}] of 1).

GeneChip analyses. Ten micrograms of each RNA sample was reverse transcribed, fragmented, 3′ biotinylated, and hybridized to an *A. baumannii* GeneChip according to the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix, Santa Clara, CA). The GeneChips used in this study, PMDACBA1, are custom-made microarrays that were developed based on the genomic sequence of *A. baumannii* strain ATCC 17978 and all additional unique *A. baumannii* GenBank entries that were available at the time of design (42). In total, 3,731 predicted *A. baumannii* open reading frames and 3,892 ATCC 17978 intergenic regions greater than 50 bp in length are represented on PMDACBA1. GeneChip data for biological replicates were normalized, averaged, and analyzed by using GeneSpring GX 7.3.1 Analysis Platform software (Agilent Technologies; Redwood City, CA). Genes that were considered differentially expressed in response to NaCl exhibited a ≥2-fold increase or decrease in transcript titer in comparison to mock-treated cells, were determined to be "present" by Affymetrix algorithms during the induced condition, and demonstrated a significant change in levels of expression ($P \leq 0.05$) as determined by a Student's *t* test. Transcripts demonstrating significant changes were divided based on whether they were upregulated or downregulated and organized according to the Cluster of Orthologous Groups (COG) functional classifications (see Tables S2 and S3 in the supplemental material).

Quantitative RT-PCR confirmation of microarray results. To validate the results of the microarray analyses, five predicted transporters and two transcriptional regulators for which levels of transcripts were increased in response to NaCl were selected for confirmation by quantitative real-time reverse transcriptase PCR. To confirm that NaCl exerted similar changes in gene expression in MHB, we examined the expressions of representative transporters that were upregulated in high NaCl in the array as well as *carO*, which was downregulated in NaCl. Reverse transcription was carried out with 2 μg total RNA by using 200 units Moloney murine leukemia virus (M-MLV) reverse transcriptase and 1 μg random hexamers according to the manufacturer's protocol (Promega, Madison, WI). Real-time PCR was performed by using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Each 20-μl reaction mixture contained 10 μl SuperMix, 200 nM primers, and 10 ng cDNA template (0.01 ng template for 16S rRNA). Primers for real-time PCR are listed in Table S1 in the supplemental material. The efficiency of each primer pair was determined by carrying out RT-PCR on serial dilutions of cDNA, and the specificity was verified by melting-curve analyses (95°C for 1 min followed by melting at 1°C decrements for 10 s from 95°C to 35°C). Following the verification of primer efficiency and specificity, RT-PCR analyses were routinely carried out with an iQ5 real-time PCR detection system (Bio-Rad) according to the following amplification protocol: 50°C for 2 min (UDG incubation), followed by 95°C for 2 min and 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Data were analyzed by using iQ5 Optical System software, version 2.0 (Bio-Rad), and the relative quantification was determined by the $\Delta\Delta C_T$ method normalizing to 16S rRNA.

Growth curve and MIC analyses of antimicrobial resistance. Cultures of *A. baumannii* cells grown overnight were diluted 1:100 in MHB without NaCl and grown to an OD_{600} of 0.4. The cultures were then diluted to a final cell density of 10^5 CFU/ml in 100 μl MHB or MHB supplemented with NaCl (150 mM) or KCl (150 mM) with or without the following antibiotics: amikacin (4.5 mg/liter), gentamicin (1.125 mg/liter), colistin (0.75 or 1.5 mg/liter), imipenem (0.0625 mg/liter), or levofloxacin (0.09 mg/liter). These antibiotic concentrations were selected because they were at or just below the inhibitory concentration for *A. baumannii* strain ATCC 17978 grown without NaCl. For efflux inhibition assays, *A. baumannii* cells were incubated with the efflux pump inhibitor PAβN (60 mg/liter) for 30 min at room temperature prior to the addition of antibiotics. The growth curves were performed in triplicate by using 96-well, round-bottom plates (Corning Inc., Corning, NY), incubating the cultures for 12 h at 37°C with shaking at 180 rpm. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm at 2-h intervals. MICs were determined by broth microdilution according to CLSI standards except that medium (MHB) was supplemented with 150 mM NaCl where indicated (11).

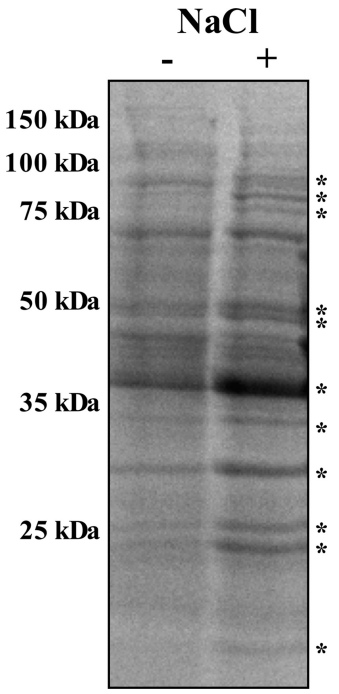

FIG. 1. NaCl induces increased release of proteins into culture supernatants. Total protein was precipitated with trichloroacetic acid from filtered supernatants of *A. baumannii* cells grown to stationary phase in LB (−) or LB supplemented with 200 mM NaCl (+) and resolved by SDS-PAGE in 15% polyacrylamide gels. *, bands that increased in high NaCl.

RESULTS

***A. baumannii* secretes antibiotic resistance determinants and virulence factors in response to NaCl.** To investigate the response of *A. baumannii* to external signals that may be encountered within the hospital environment or upon infection of the human host, we first examined proteins released into culture supernatants upon the exposure of *A. baumannii* to a variety of conditions. Tested conditions included the use of several types of rich growth medium (TSB, Luria broth [LB], brain infusion broth, and brain heart infusion broth), iron limitation in rich or minimal medium, pH ranging from 5.5 to 8.5, and high concentrations of NaCl (Fig. 1 and data not shown). Of these conditions, we noted the most striking difference in supernatant protein profiles when *A. baumannii* cells were cultured in high NaCl. Specifically, supplementation

with 200 mM NaCl produces an overall increase in the amount of proteins released into the culture medium (Fig. 1).

To rule out the possibility that the increased abundance of protein in culture media was the result of increased cell lysis or a disruption of the bacterial membrane, we assessed membrane damage upon NaCl exposure. Propidium iodide staining of cells taken at several time points upon culture in low or high NaCl followed by flow cytometric analyses showed no significant difference in the proportion of stained cells (membrane compromised) to unstained cells (live and membrane intact) (data not shown). These results suggest that the increased release of proteins into the extracellular environment upon culture in NaCl is independent of cell lysis or membrane damage. To determine if this response involved a subset of proteins or represented a global increase in the level of protein secretion, we identified secreted proteins from *A. baumannii* cells grown in LB with or without 200 mM NaCl by liquid chromatography-tandem mass spectrometry (LC-MS-MS). The searching of resultant tandem spectra against *Acinetobacter* sequences led to the positive identification of approximately 60 proteins in *A. baumannii* supernatants (Table 1; for complete experimental details and data analysis, see Materials and Methods). These proteins were comprised predominantly of membrane and periplasmic proteins, with few predicted cytoplasmic proteins, further confirming that the increased abundance of proteins upon NaCl exposure is not due to increased cell lysis. Notably, a large number of the identified proteins were differentially secreted in response to NaCl (Table 1). Outer membrane proteins were overrepresented among proteins with levels that increased significantly in high NaCl, while intracellular proteins involved in metabolism and protein folding generally showed few changes between the two conditions. We predict that the lack of change in intracellular proteins between the two conditions reflects the fact that these proteins are not likely secreted. Rather, the identification of these proteins in supernatants likely results from a limited amount of cell lysis that occurs during normal bacterial growth under both conditions. Interestingly, levels of proteins associated with antibiotic resistance also increased significantly in high NaCl. These proteins were CarO and the 33- to 36-kDa outer membrane protein (Omp), which are two porins with predicted roles in antibiotic transport through the outer membrane. CarO was previously shown to form nonselective pores, and a loss or inactivation of this porin has been associated with increased resistance to carbapenems (27, 32, 44). The 33- to 36-kDa Omp has not been characterized as extensively as CarO; however, the loss of this predicted porin has also been associated with resistance to carbapenems in *A. baumannii* (10). In addition to these two porins, we observed a significant increase in the abundance of two chromosomally encoded beta-lactamases (AmpC and OXA-95) and outer membrane protein A (OmpA). OmpA has been implicated in virulence, both in cell culture and in animal models of *A. baumannii* pathogenesis, and was modeled to contribute to biofilm formation (7–9, 17). These changes in protein abundance in culture supernatants suggest that *A. baumannii* regulates the expression and/or secretion of specific proteins in response to NaCl. Furthermore, the large number of outer membrane proteins and proteins associated with antibiotic resistance suggests that the response to NaCl in *A. baumannii* may have an impact on antibiotic resistance.

NaCl induces the upregulation of putative efflux transporters. The results of our proteomic analyses of supernatant proteins suggest that *A. baumannii* orchestrates the release of proteins into the culture medium upon exposure to high concentrations of NaCl. To determine if these changes are transcriptionally mediated, we sought to determine the global transcriptional response to NaCl by microarray analyses. *A. baumannii* cells were cultured as described above in LB with or without 200 mM NaCl. RNA was isolated from stationary-phase bacteria and analyzed by hybridization onto Affymetrix GeneChip arrays. Over 150 genes were found to be significantly upregulated in response to NaCl (see Table S2 in the supplemental material). Genes involved in inorganic ion transport and metabolism; secondary metabolite biosynthesis, transport, and catabolism; and transcriptional regulation were among those most highly represented in the upregulated transcripts. We also observed an upregulation of several genes associated with pilus formation and a cluster of genes involved in the biosynthesis and transport of the siderophore acinetobactin, which is involved in resistance to iron starvation (14). Interestingly, we observed a downregulation of *carO* and the 33- to 36-kDa Omp (6.4-fold and 2.7-fold, respectively) (see Table S3 in the supplemental material) and the constitutive expression of *ompA*, *ampC*, and *oxa-95*, all of which were increased in abundance in the proteomic analyses of culture supernatants. These results suggest that the increased presence of these proteins in culture media is not likely controlled at the transcriptional level. Given that many of the proteins that increased in abundance in culture supernatants showed decreased transcript levels, it is possible that the release of these proteins represents a posttranslational mechanism for downregulating their membrane abundance.

One class of genes of which many members were upregulated is that of putative efflux transporters. Approximately 20% of the upregulated transcripts belong to genes encoding putative transport proteins (Table 2 and Fig. 2A). The overrepresentation of transporter genes in the upregulated transcripts is striking and, to our knowledge, has not been observed for other bacteria for which global transcriptional responses to NaCl or osmotic stress have been investigated (3, 48). Of the 33 transcripts encoding components of 25 distinct transporters that increased in abundance in high NaCl, 18 (14 distinct transporters) belong to families in which members have been associated with the transport of antibiotics or other toxic compounds (30, 33, 44). These include the resistance-nodulation-division (RND) family, the drug/metabolite transporter (DMT) family, the ATP-binding cassette (ABC) family, and the major facilitator superfamily (MFS). In addition, two genes (A1S_2141 and A1S_1814) are predicted to encode transporters for K^+ and Na^+, respectively (Fig. 2A). To validate the microarray results, we selected a subset of genes for confirmation by quantitative RT-PCR. Specifically, we selected five transporters with predicted roles in antibiotic resistance as well as two TetR family transcriptional regulators that were significantly upregulated under conditions of high NaCl (see Table S2 in the supplemental material). Representative quantitative PCR (qPCR) results are given in Fig. 2B, which confirmed that the levels of the tested transcripts were increased under high-NaCl compared to low-NaCl conditions. Notably, five transcripts displayed a dose-response to NaCl with a further increase in

TABLE 1. Proteins identified by LC-MS-MS analysis of supernatants from *A. baumannii* cells cultured in LB or LB plus 200 mM NaCl

Protein function and GenBank accession no.	Description[a]	Avg spectral count[a]		*P* value[b]	Predicted molecular mass (kDa)
		High NaCl	Low NaCl		
Outer membrane					
YP_001085848	**Outer membrane protein A**	1.605	0.355	0.033	38.4
YP_001085848	**Outer membrane protein A**	6.282	1.091	0.022	38.4
YP_001085848	**Outer membrane protein A**	14.415	2.104	0.018	38.4
YP_001085848	**Outer membrane protein A**	2.380	0.465	0.008	38.4
YP_001086308	**Putative outer membrane protein**	6.629	1.083	0.044	25.6
YP_001085614	**Peptidoglycan-associated lipoprotein**	1.564	0.209	0.032	20.8
YP_001083918	**Putative outer membrane protein**	3.332	0.863	0.018	22.5
YP_001085744	**Outer membrane lipoprotein carrier protein**	1.581	0.393	0.042	25.1
YP_001084998	Putative outer membrane protein	0.161	0.036	0.100	91.9
YP_001084997	**Putative outer membrane protein**	1.866	0.908	0.005	8.2
YP_001085452	Lipoprotein	0.274	0.110	0.191	29.9
Antibiotic resistance					
YP_001083108	Putative RND-type efflux pump involved in aminoglycoside resistance	2.764	0.881	0.101	36.7
YP_001086288	**33- to 36-kDa outer membrane protein; associated with carbapenem resistance**	1.753	0.338	0.033	34.0
YP_001085388	β-Lactamase (AmpC)				
YP_001083548	Carboxy-terminal protease for penicillin-binding protein	0.453	0.273	0.304	80.4
YP_001085752	RND family drug transporter (AdeK)	0.160	0.082	0.115	52.9
YP_001085752	**RND family drug transporter (AdeK)**	0.445	0.082	0.022	52.9
YP_001084546	**β-Lactamase OXA-95**	0.739	0.166	0.021	31.4
YP_001085557	**29-kDa outer membrane protein (CarO)**	1.132	0.263	0.036	29.0
YP_001085388	**β-Lactamase (AmpC)**	0.271	0.071	0.041	46.4
Hypothetical					
YP_001085392	**Hypothetical protein**	1.399	0.308	0.019	44.6
YP_001084326	Hypothetical protein	2.091	1.128	0.169	18.8
YP_001084552	**Putative signal peptide**	1.078	0.358	0.008	20.8
YP_001085962	Putative signal peptide, metallo-β-lactamase superfamily	0.385	0.309	0.410	31.9
YP_001084993	**Putative signal peptide**	0.398	0.197	0.037	30.9
YP_001084084	**Putative signal peptide (contains the OsmY region)**	0.665	0.133	0.048	24.7
YP_001083928	Outer membrane lipoprotein	1.100	0.269	0.152	12.2
YP_001083365	Hypothetical protein	0.666	0.223	0.053	14.7
Protein synthesis/chaperone					
YP_001083352	Protein chain elongation factor EF-Tu	1.000	1.000		44.5
YP_001083902	Elongation factor G	0.212	0.195	0.781	78.8
YP_001085682	60-kDa chaperonin	0.340	0.336	0.912	57.2
YP_001084601	30S ribosomal protein S1	0.179	0.205	0.761	61.1
YP_001085965	Chaperone protein DnaK	0.138	0.152	0.871	69.5
YP_001084218	ATP-dependent protease, Hsp100	0.057	0.081	0.575	95.3
YP_001083134	Thiol:disulfide interchange protein	0.460	0.142	0.155	23.2
YP_001086079	50S ribosomal protein L3	0.317	0.193	0.213	22.5
Bacterial programmed cell death					
YP_001085817	Bacteriolytic lipoprotein entericidin B	3.731	0.661	0.176	5.0
YP_001085544	Putative serine protease	0.416	0.127	0.200	50.2
Cell wall biosynthesis					
YP_001086026	Putative lytic murein transglycosylase, soluble	1.048	0.832	0.234	76.5
YP_001085967	Putative membrane-bound lytic murein transglycosylase	0.526	0.430	0.706	47.9
YP_001085340	**Membrane-bound lytic murein transglycosylase B**	0.481	0.183	0.036	36.9
Glucose metabolism					
YP_001083999	Aldose 1-epimerase precursor	4.167	2.989	0.236	41.5
YP_001084980	**Glucose dehydrogenase**	2.795	1.313	0.031	14.8
YP_001084980	**Quinoprotein glucose dehydrogenase B precursor**	0.979	0.487	0.023	52.8
YP_001084927	**Enolase**	0.118	0.183	0.025	46.4
TCA cycle					
YP_001085734	Succinyl-CoA ligase (ADP-forming) subunit alpha	0.179	0.403	0.067	30.7
YP_001085733	Succinyl-CoA synthetase beta chain	0.192	0.329	0.383	41.4

Continued on following page

TABLE 1—*Continued*

Protein function and GenBank accession no.	Description[a]	Avg spectral count[a]		*P* value[b]	Predicted molecular mass (kDa)
		High NaCl	Low NaCl		
YP_001085497	Isocitrate dehydrogenase	0.116	0.077	0.551	82.5
YP_001083613	Aconitate hydratase 1	0.055	0.060	0.896	100.3
Electron transport chain					
YP_001084520	**Glutamate/aspartate transport protein**	4.596	0.855	0.025	32.1
YP_001083240	**ATP synthase subunit beta**	0.109	0.190	0.023	50.3
Q6FAL6	**Glutaminase-asparaginase**	0.613	0.122	0.050	37.9
YP_001086024	Malate dehydrogenase	0.239	0.294	0.442	35.4
YP_001083238	ATP synthase subunit alpha	0.098	0.121	0.453	56.0
Antioxidant					
YP_001084237	**Alkyl hydroperoxide reductase, C22 subunit**	0.264	0.410	0.025	20.7
Other					
YP_001085613	Protein TolB precursor	1.085	0.648	0.705	46.4
YP_001085247	CsuA/CsuB	0.922	0.860	0.774	18.7
YP_001084991	RecA protein	0.145	0.232	0.301	37.8
Q6F9W2	Host factor I for bacteriophage Q beta replication	0.321	0.317	0.956	17.1

[a] Spectral counts from three independent replicates were averaged after normalization first to the size of the expected protein and subsequently to an internal constitutively expressed protein (EF-Tu).
[b] *P* values were determined by a Student's *t* test.
[c] Boldface type indicates proteins that exhibit a statistically significant difference in abundance between the low- and high-NaCl samples. CoA, coenzyme A.

expression at 260 mM NaCl compared to 200 mM NaCl. Taken together, these results demonstrate that NaCl induces significant changes in gene expression in *A. baumannii*. Furthermore, these results demonstrate the extensive regulation of efflux transporters upon NaCl exposure, which may contribute to antibiotic resistance.

NaCl induces tolerance to distinct classes of antibiotics. The changes observed for gene expression and secreted protein profiles suggest that the response of *A. baumannii* to NaCl may lead to an increased resistance to antibiotics. MHB is the recommended medium for antibiotic susceptibility testing; therefore, we first sought to confirm that NaCl induces changes in gene expression and protein secretion in MHB similar to those observed with LB (11). Furthermore, since MHB is formulated without NaCl, this medium permits the improved titration of NaCl concentrations and, therefore, a better resolution of the dose-response to NaCl. Quantitative RT-PCR results demonstrated that NaCl induces an increased level of expression of representative transporter genes as well as the downregulation of the transcript for CarO, further supporting data from the microarray analyses (Fig. 2C). *A. baumannii* cells were cultured in MHB or MHB supplemented with NaCl at concentrations between 50 and 300 mM, and the resulting supernatant proteins were examined by SDS-PAGE. Consistent with the results obtained with LB, there was a significant increase in the total abundance of supernatant proteins upon exposure to 300 mM NaCl (Fig. 2D). Furthermore, the amount of protein released into culture supernatants increased in a dose-dependent manner with increasing concentrations of NaCl. These data confirm that NaCl induces transcriptional and posttranslational regulation of membrane proteins with MHB similar to that with LB, providing the foundation for examining NaCl effects on antibiotic resistance in this medium. Moreover, these data expand upon previously reported results by demonstrating that the secretion of proteins into culture medium increases in a dose-dependent manner with increasing NaCl concentrations.

To determine whether NaCl impacts antibiotic resistance, we determined MICs for antibiotics from several distinct classes. These assays revealed modest increases in the MICs of amikacin, levofloxacin, and colistin (3-, 1.5-, and 2-fold increases, respectively). These changes were not sufficient to raise the MIC above clinical breakpoints for resistance to any of the drugs tested. However, the incremental increase in resistance nonetheless supported the hypothesis that the adaptive response to NaCl impacts susceptibility to antibiotics and suggested that NaCl may induce a tolerant phenotype in *A. baumannii*. To assess tolerance to antibiotics, we monitored the growth of *A. baumannii* cells challenged with sublethal concentrations of several classes of antibiotics in the presence or absence of NaCl. Growth curve analyses demonstrated that in the presence of physiological NaCl concentrations (150 mM), *A. baumannii* displays a significant increase in its ability to resist inhibition by antibiotics from four distinct classes: aminoglycosides (amikacin and gentamicin), quinolones (levofloxacin), carbapenems (imipenem), and polypeptides (colistin) (Fig. 3). Given that growth is reduced slightly by NaCl alone, the effect of NaCl on antibiotic resistance is even more striking. The protective effect of NaCl is more apparent at late time points, which supports a model in which *A. baumannii* must first adapt to NaCl, and this adaptive response results in increased tolerance to antibiotics. Together, data from the growth curve analyses demonstrate that NaCl induces tolerance to clinically relevant antibiotics.

To determine whether other cations similarly impact tolerance, we performed growth curve analyses as described above by using KCl in the place of NaCl. KCl induces significant tolerance to amikacin, colistin, and levofloxacin that is comparable to the effect observed with NaCl (Fig. 4A). Examination of supernatant proteins from *A. baumannii* cells cultured with

TABLE 2. Predicted transporters that were found to be significantly upregulated in response to NaCl by microarray analysis

Locus tag	Description[b]	Fold induction[a]
A1S_1769	**Putative RND family drug transporter**	2.9
A1S_2304	**Putative RND family drug transporter**	2.9
A1S_2932	**Heavy metal efflux pump (CzcA)**	2.6
A1S_2934	**Heavy metal RND efflux outer membrane protein (CzcC)**	16.0
A1S_3445	**Putative RND family cation/multidrug efflux pump**	3.2
A1S_0565	**DMT family permease**	2.1
A1S_1323	**DMT family permease**	3.4
A1S_1992	**DMT family permease**	2.6
A1S_1284	ABC-type nitrate/sulfonate/bicarbonate transport systems	2.3
A1S_1286	ABC-type nitrate/sulfonate/bicarbonate transport systems	10.1
A1S_1287	ABC nitrate/sulfonate/bicarbonate family transporter	2.0
A1S_1361	ABC-type spermidine/putrescine transport system	2.2
A1S_1362	ABC-type Fe^{3+} transport system	9.4
A1S_1722	Putative ATP-binding component of ABC transporter	2.0
A1S_2378	Putative ABC transporter	14.3
A1S_2388	Putative ferric acinetobactin transport system	4.0
A1S_2389	Putative ferric acinetobactin transport system	23.2
A1S_1751	**$AdeA_2$ membrane fusion protein**	25.9
A1S_1752	**$AdeA_1$ membrane fusion protein**	8.7
A1S_2376	**Putative ABC-type antimicrobial peptide transport system**	11.8
A1S_2377	**Putative ABC-type multidrug transport system**	3.6
A1S_3420	**MATE family drug transporter**	12.1
A1S_0596	**Putative transporter**	2.2
A1S_0915	**Putative MFS transporter**	3.2
A1S_1331	Major facilitator superfamily	2.7
A1S_1739	Major facilitator superfamily	4.1
A1S_2198	**Putative multidrug resistance protein**	2.4
A1S_3146	**Multidrug efflux transport protein**	17.6
A1S_1209	Putative benzoate transport porin (BenP)	16.2
A1S_1814	Predicted Na^+-dependent transporter	2.5
A1S_1956	Putative amino acid permease	2.0
A1S_2141	Potassium-transporting ATPase A chain	2.4
A1S_3251	Transporter, LysE family	3.9

[a] Fold induction in transcript level in LB with 200 mM NaCl relative to LB without NaCl supplementation.

[b] Boldface type indicates transporters with predicted roles in the extrusion of antibiotics or other toxic compounds from the cell.

KCl concentrations ranging from 50 to 300 mM revealed that KCl exposure results in an increased abundance of proteins in culture supernatants in a pattern similar to that observed for NaCl (Fig. 4B). We did not observe the same trend in supernatant protein profiles or increased antibiotic resistance upon the treatment of *A. baumannii* cells with high concentrations of sucrose (data not shown). These results suggest that *A. baumannii* may respond to increased concentrations of monovalent ions rather than to NaCl specifically or osmotic stress more generally.

Inhibition of efflux reduces NaCl-induced resistance to amikacin and levofloxacin. The effects of NaCl on resistance to aminoglycosides have been described for several species of both Gram-negative and Gram-positive bacteria (6, 44). While it was proposed in previous studies that the observed NaCl-induced increase in antibiotic resistance may be the result of the passive inhibition of antibiotic uptake by elevated external salt concentrations, this has not been conclusively demonstrated (6, 45). In addition, the possibility that a regulated response to NaCl mediates antibiotic resistance has not previously been investigated. The observation that NaCl induces an increased level of expression of efflux pumps introduces the intriguing possibility that the effect of NaCl on antibiotic resistance or tolerance is a regulated process rather than a passive effect on antibiotic uptake. Therefore, to determine the contribution of efflux to NaCl-induced antibiotic tolerance, we tested whether the efflux pump inhibitor PAβN prevents the NaCl-induced response. PAβN is active against a broad spectrum of efflux pumps, and its mechanism of action is thought to involve competitive inhibition (29). We pretreated *A. baumannii* cells with 60 mg/liter PAβN for 30 min in medium with or without NaCl and then challenged them as described above with amikacin, colistin, levofloxacin, or imipenem. NaCl-induced tolerances to levofloxacin and amikacin were significantly reduced upon the pretreatment of *A. baumannii* with PAβN (Fig. 5). However, we did not observe a difference in the effect of NaCl on the tolerance to imipenem in the presence or absence of the efflux pump inhibitor (data not shown). It is possible that the decreased permeation of imipenem into the cell, through the loss or decreased level of expression of CarO and the 33- to 36-kDa Omp, is a more important mechanism in mediating tolerance to imipenem in response to NaCl. Paradoxically, the level of tolerance to colistin is significantly increased in the presence of PAβN (Fig. 5). At colistin concentrations of 1.5 mg/liter, PAβN induces resistance to colistin regardless of the NaCl content of the medium, restoring growth to approximately 75% of that observed in the absence of colistin (Fig. 5). Importantly, NaCl alone was not sufficient to induce the protection of *A. baumannii* cells against challenge with 1.5 mg/liter of colistin (Fig. 5). NaCl induced a significant resistance to colistin at lower concentrations (0.75 mg/liter), preventing the assessment of the effect of PAβN on colistin resistance below 1.5 mg/liter (data not shown). Taken together, these data demonstrate that antibiotic efflux contributes significantly to NaCl-induced tolerance to levofloxacin and amikacin, while alternative mechanisms are necessary for mediating tolerance to imipenem and colistin.

NaCl induces tolerance to colistin in multidrug-resistant clinical isolates of *A. baumannii*. The strain used in the experiments described above is a drug-susceptible strain of *A. baumannii* isolated in 1951 (37). Given that recently isolated clinical strains of *A. baumannii* are resistant to most available antibiotics, we sought to determine whether NaCl impacts resistance in recently isolated MDR *A. baumannii*. We specifically investigated whether NaCl induces tolerance to drugs to which the clinical isolates were otherwise susceptible. MDR clinical isolates of *A. baumannii* were obtained from the University of Nebraska Medical Center (Table 3). Drug susceptibility profiles were reported by the University of Nebraska Medical Center clinical microbiology laboratory. The MDR phenotype was defined as resistance to three or more of the following antibiotic classes: β-lactam–β-lactamase inhibitor combinations, antipseudomonal cephalosporins (ceftazidime

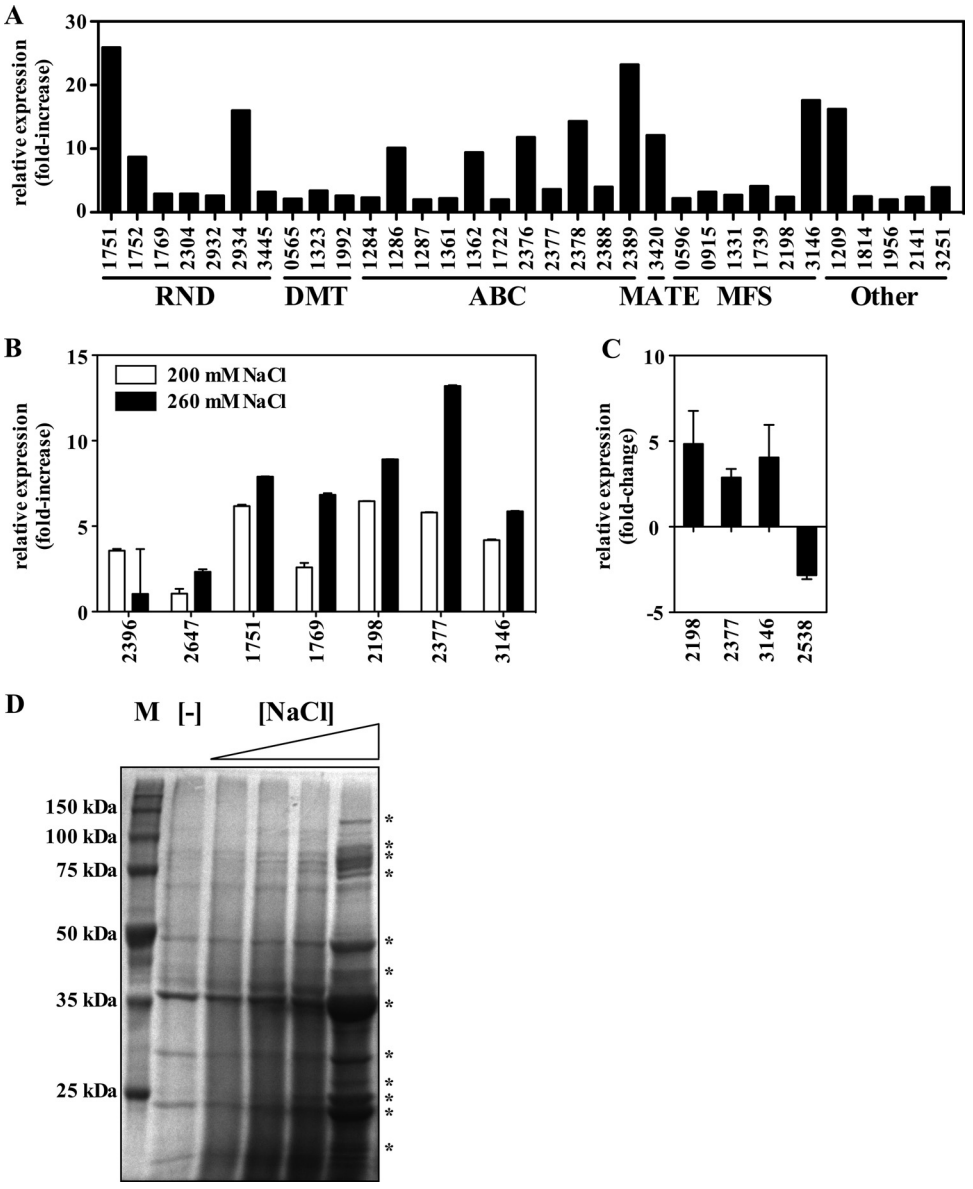

FIG. 2. *A. baumannii* upregulates putative efflux transporters upon culture in high NaCl medium. (A) RNA was extracted from *A. baumannii* cells grown to stationary phase in LB or LB supplemented with 200 mM NaCl. The fold changes in transcript levels determined by microarray analyses are shown for putative transporters with levels that increased significantly in *A. baumannii* cells upon culture in LB supplemented with 200 mM NaCl relative to *A. baumannii* cells cultured without NaCl supplementation. RND, resistance-nodulation-division; DMT, drug/metabolite transporter; MFS, major facilitator superfamily; MATE, multidrug and toxic compound extrusion; ABC, ATP-binding cassette. (B) Fold changes in transcript levels of selected transporters and transcriptional regulatory genes in 200 mM and 260 mM NaCl compared to medium alone as determined by real-time PCR. The fold change in expression was determined by using the $\Delta\Delta C_T$ method. Error bars represent 1 standard deviation (SD) from the mean and in some cases are too small to be seen. The data are representative of at least three independent biological replicates. (C) Fold change in transcript levels of selected genes in MHB with 150 mM NaCl relative to MHB without NaCl as determined by real-time PCR. Error bars represent the means ± SD. Each bar represents the average of data for three independent biological replicates. Expression changes comparing MHB with NaCl to MHB alone were statistically significant ($P < 0.05$ by a Student's t test) for each gene tested. (D) SDS-PAGE analyses of proteins released into culture supernatants. Total protein was precipitated with trichloroacetic acid from filtered supernatants of *A. baumannii* cells grown to stationary phase in MHB (−) or MHB supplemented with NaCl to final concentrations of 50 mM, 90 mM, 150 mM, and 300 mM and resolved by SDS-PAGE in 15% polyacrylamide gels. M, molecular mass marker (in kDa); ⋆, bands that increased with increasing NaCl concentrations.

of cefepime), aminoglycosides (gentamicin, amikacin, or tobramycin), quinolones (ciprofloxacin or levofloxacin), and carbapenems (imipenem or meropenem). Although MIC data were not available for colistin, resistance to colistin has been reported for only a few cases in the literature, and colistin is increasingly being used for the treatment of MDR *A. baumannii* infections (22, 35). We therefore sought to determine if NaCl could induce tolerance to colistin in each of the MDR clinical isolates. Growth curves performed with MHB with or without NaCl demonstrated that all of the isolates showed

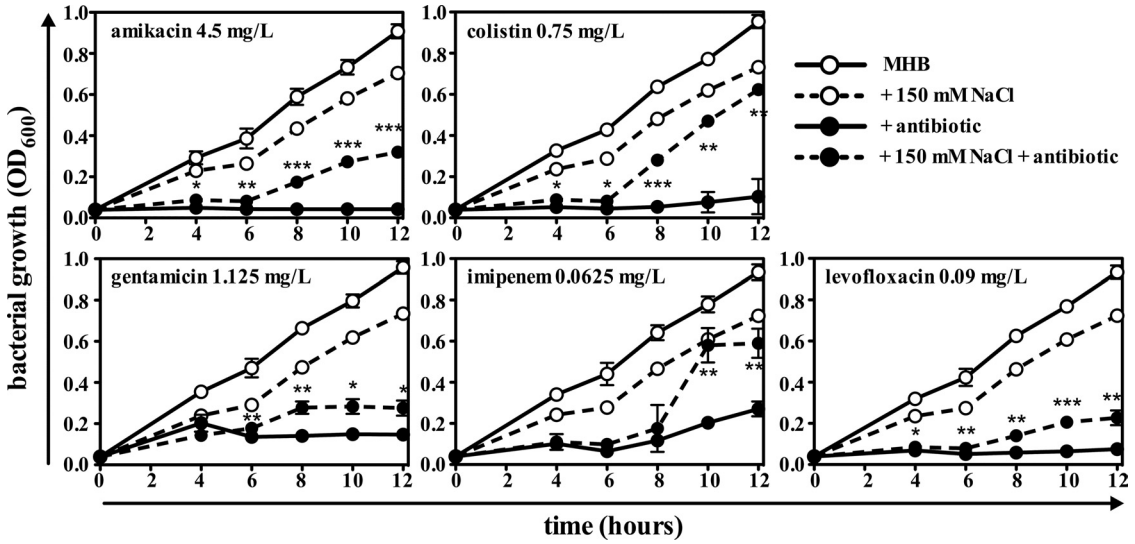

FIG. 3. NaCl induces increased tolerance to distinct classes of antibiotics in *A. baumannii*. *A. baumannii* strain ATCC 17978 cells were challenged with amikacin (4.5 mg/liter), colistin (0.75 mg/liter), gentamicin (1.125 mg/liter), imipenem (0.0625 mg/liter), or levofloxacin (0.09 mg/liter) with (dashed lines) or without (solid lines) NaCl supplementation of the culture medium to a final concentration of 150 mM. Bacterial growth was monitored by measuring the optical density of the cultures at 600 nm, with each point representing the mean ± SD for at least three cultures (error bars may be too small to be seen). Asterisks indicate statistically significant changes in growth upon antibiotic challenge in medium containing NaCl compared to medium lacking NaCl as determined by a Student's *t* test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

susceptibilities to colistin in the absence of NaCl similar to that observed with the reference strain. Likewise, all of the *A. baumannii* isolates were protected against 0.75 mg/liter colistin in the presence of 150 mM NaCl (Fig. 6). Similar to the results with the reference strain, the MIC of colistin was increased up to 2-fold in the presence of NaCl for the majority of the clinical isolates tested (Table 4). Interestingly, UNMC 4860 showed a more rapid tolerance to colistin in the presence of NaCl, as demonstrated by the growth curve analyses, but the MIC (determined following 24 h of exposure to drug) actually decreased slightly. To confirm that the conservation of the NaCl-induced response extended to clinical strains from distinct geographic locations, we also performed MIC assays for two recently sequenced MDR isolates (AYE and AB0057) and two susceptible isolates (AB307-0294 and AB900), all of which showed similar increases in the colistin MIC in response to

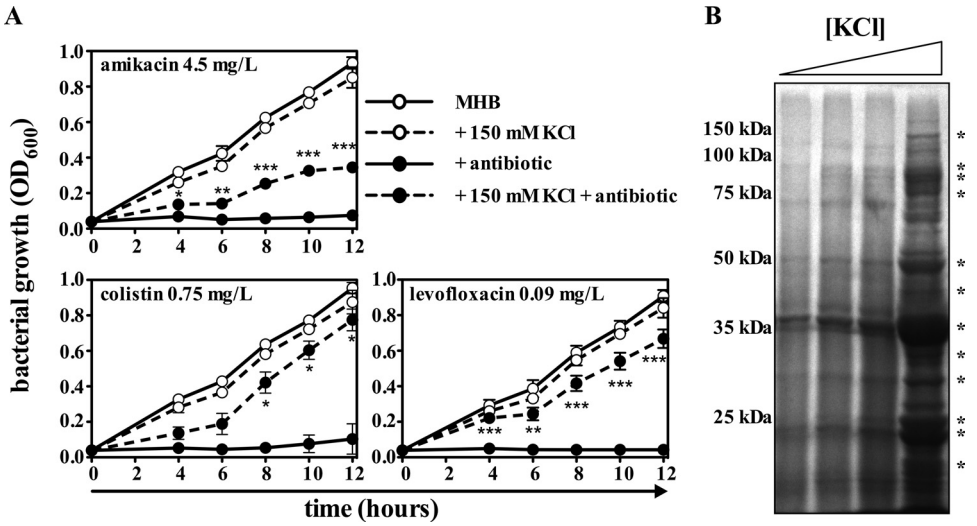

FIG. 4. Effects of KCl on resistance to antibiotics and on release of proteins into culture medium. (A) Growth curve analyses of *A. baumannii* cells challenged with antibiotics in MHB (solid lines) or MHB supplemented with 150 mM KCl (dashed lines) at the indicated concentrations. Error bars represent the mean ± 1 SD and may be obscured by the symbol in some cases. Asterisks indicate statistically significant changes in growth upon antibiotic challenge in medium containing KCl compared to medium lacking KCl as determined by a Student's *t* test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). (B) SDS-PAGE of TCA-precipitated proteins from *A. baumannii* culture supernatants. *A. baumannii* cells were grown to stationary phase in MHB supplemented with 50 mM, 86 mM, 154 mM, or 308 mM KCl. *, bands that increased with increasing concentrations of KCl.

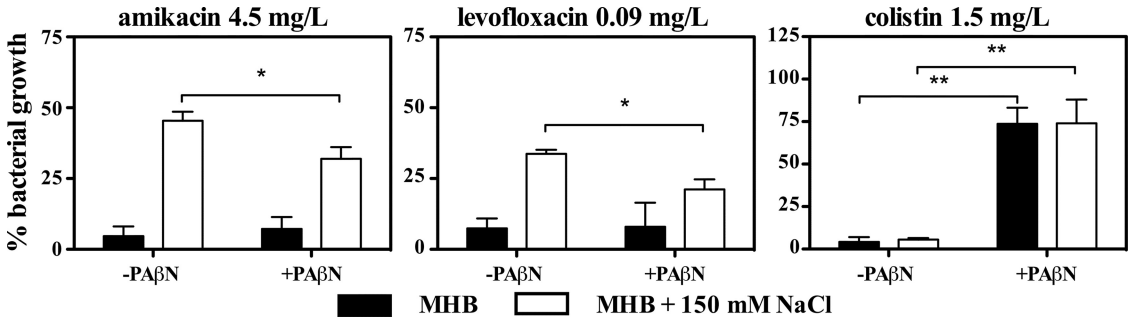

FIG. 5. NaCl-induced resistance to levofloxacin and amikacin is due in part to increased antibiotic efflux. *A. baumannii* cells were treated with amikacin, levofloxacin, or colistin in MHB (filled bars) or MHB with 150 mM NaCl (white bars) in the presence or absence of 30 mg/liter of the efflux pump inhibitor PAβN. Bacterial growth was monitored for 12 h, and the optical densities of the cultures were normalized to the respective untreated (i.e., antibiotic-free) controls and expressed as the percent bacterial growth. Statistical analysis was performed by a Student's *t* test comparing PAβN-treated with the respective PAβN-untreated controls. Error bars are ±1 SD from the mean. *, $P < 0.05$; **, $P < 0.005$.

NaCl (data not shown). These results demonstrate that NaCl-induced colistin tolerance is conserved among recently isolated clinical strains of MDR *A. baumannii*.

DISCUSSION

In this study we have taken a multifaceted approach to demonstrate that *A. baumannii* responds to extracellular NaCl by regulating membrane proteins through transcriptional and posttranslational mechanisms. Furthermore, the response to NaCl results in tolerance to four distinct classes of antibiotics. Both NaCl and KCl were found to induce similar changes in protein secretion and antibiotic resistance, suggesting that the signal sensed by *A. baumannii* is not NaCl itself but may be monovalent cations or anions. We have not tested directly whether it is the cationic or anionic component that is required for the NaCl/KCl-induced response; however, it was reported previously that Na^+ and K^+, but not Cl^-, induce resistance to tobramycin in *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* (45). We therefore hypothesize that monovalent cations are the relevant signal for *A. baumannii*.

Our data demonstrate that NaCl affects the expression of a large number of genes; however, the transcriptional response does not support a model whereby NaCl induces an osmotic stress response at the concentrations evaluated. The upregulation of a choline dehydrogenase (A1S_0925) may have a role in osmoprotection; however, apart from A1S_0925, the transcriptional changes observed did not resemble the osmotic stress responses of *P. aeruginosa* or *Escherichia coli* (3, 48). In particular, the upregulation of 18 genes encoding components of putative drug efflux pumps was distinctive. The transcriptional response to NaCl in *A. baumannii* therefore appears to be unique as a direct response to NaCl. Interestingly, the quality of the NaCl response in *A. baumannii* bore similarity with the transcriptional changes described previously for *B. cenocepacia* upon culturing in sputum from patients with cystic fibrosis, including the upregulation of genes encoding putative efflux transporters, oxidoreductases, and iron acquisition systems (15). It is unclear to what extent the NaCl concentration in sputum may have been responsible for inducing the transcriptional response in *B. cenocepacia*. It is interesting, however, that both responses appear to involve resistance to antibiotics as well as physiological stress conditions such as oxidative stress and iron limitation.

Previous studies investigating the effect of NaCl on antibiotic resistance have shown that NaCl and KCl induce an increased resistance to aminoglycosides, while other antibiotics have not been evaluated (45). The mechanisms proposed for mediating NaCl-induced resistance involve passive effects of

TABLE 3. Antibiotic susceptibility profiles of clinical isolates obtained from the University of Nebraska Medical Center compared to reference strain ATCC 17978[c]

Isolate[a]	Source	Site of isolation	Antibiotic susceptibility															
			SAM	TZP	TIM	ATM	FEP	CTX	CAZ	CRO	AMK	GEN	TOB	CIP	LVX	IPM	MEM	SXT	TET
ATCC 17978[b]	ATCC	CSF	S	S	ND	R	S	ND	ND	I	S	S	S	S	ND	S	S	R	ND
510	UNMC	Sputum	R	R	R	R	R	R	R	R	R	R	R	R	I	S	S	R	ND
2824	UNMC	Urine	S	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R
2898	UNMC	Ankle	R	R	R	R	R	R	R	R	R	R	R	R	I	S	S	R	ND
4860	UNMC	Urine	R	ND	R	R	R	R	R	R	R	R	R	R	R	S	ND	R	R
5191	UNMC	Urine	R	ND	R	R	R	R	R	R	R	R	R	R	R	S	ND	R	R

[a] Strains in boldface type are classified as being multidrug resistant.
[b] Susceptibility data for reference strain ATCC 17978 were reported previously (1).
[c] ATCC, American Type Culture Collection; UNMC, University of Nebraska Medical Center; R, resistant; S, susceptible; ND, not determined; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; TIM, ticarcillin-clavulanate; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; LVX, levofloxacin; IPM, imipenem; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; CSF, cerebrospinal fluid.

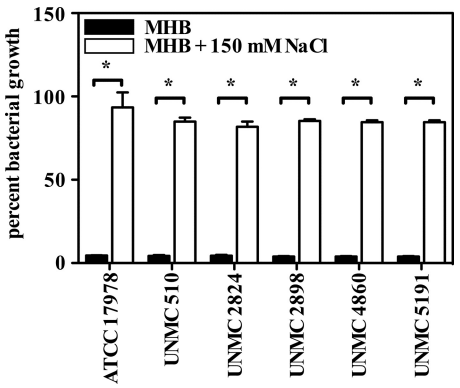

FIG. 6. NaCl-induced resistance to colistin is conserved among drug-susceptible and multidrug-resistant *A. baumannii* strains. *A. baumannii* ATCC 17978 and nine clinical isolates were treated with colistin (0.75 mg/liter) with (solid bars) or without (open bars) NaCl supplementation of the culture medium to a final concentration of 150 mM. Bacterial growth was monitored for 12 h, and the optical densities of the cultures were normalized to growth in the absence of colistin in the respective media (i.e., with or without NaCl). Statistically significant differences comparing medium alone to medium supplemented with NaCl were determined by a Student's *t* test. Error bars are ±1 SD from the mean. *, $P < 0.005$.

NaCl such as interference with antibiotic uptake. Our data reveal that NaCl-induced antibiotic tolerance may involve an active response mediated through a regulated transcriptional program as well as the posttranscriptional or posttranslational regulation of membrane protein expression and/or secretion.

As described above, our microarray analyses highlighted the upregulation of 33 genes representing 25 putative transporters, 12 of which are annotated as putative drug transporters. These include the membrane fusion component of the AdeABC efflux pump (AdeA) as well as the major facilitator superfamily transporter CraA. AdeABC mediates resistance to a number of antibiotics in *A. baumannii*, although the function and regulation of this pump have not been investigated for *A. baumannii* strain ATCC 17978 (31). It was recently determined that CraA is conserved among *A. baumannii* strains sequenced to date and mediates intrinsic chloramphenicol resistance (40). Given the number of putative efflux pumps that are upregulated in response to NaCl, it is possible that the antibiotic tolerance observed is the result of the combined action of several or all of these pumps.

The contribution of efflux to antibiotic tolerance in response to NaCl is supported by the fact that tolerance to levofloxacin and amikacin can be partially reversed upon the addition of a nonselective efflux pump inhibitor. The failure to observe a complete reversal of the resistance phenotype may be due to an inability to achieve complete efflux inhibition at the PAβN concentrations used; however, an increase in the concentration of PAβN leads to growth inhibition independent of antibiotic treatment. An alternative explanation for the failure to completely reverse NaCl-induced antibiotic resistance is that resistance to levofloxacin and amikacin may be mediated by multiple overlapping mechanisms, which is supported by our data that suggest that NaCl has a broad effect on gene expression.

Proteomic analyses of *A. baumannii* culture supernatants highlighted an increased abundance of several porins for which a loss or inactivation has been associated with resistance to carbapenems. Based on the known functions of the putative 29-kDa porin (CarO) and the 33- to 36-kDa porin, their release in response to NaCl may result in a decreased permeability to antibiotics. These proteins were downregulated at the transcriptional level, suggesting that *A. baumannii* may shed these proteins to decrease their membrane abundance. Similarly, it was recently reported that *A. baumannii* downregulates the membrane expression of $OmpA_{32}$ (33- to 36-kDa Omp), $OmpA_{38}$, CarO, and OmpW in response to sub-MIC tetracycline without alterations in transcript levels for the corresponding genes (49). The increased abundance of these Omp proteins in culture supernatants suggested that downregulation occurs through selective release from the membrane, and those authors proposed that this might be related to tetracycline resistance, although resistance was not evaluated directly (49). Consistent with the proposed model, we have demonstrated that *A. baumannii* is more resistant to several classes of antibiotics upon exposure to NaCl, including imipenem, which would be predicted based on the known role of CarO and the 33- to 36-kDa Omp in resistance to carbapenems (10, 27, 32, 42). In addition, that same group recently published the results of proteomic analyses of outer membrane vesicles (OMVs) produced by *A. baumannii*. Proteins enriched in OMVs included homologues of CarO, OmpA, and AmpC (23). The overlap between our proteomic results and the results for the OMV proteome suggests that monovalent cations may induce increased levels of membrane vesicle production, but this remains to be determined.

The finding that *A. baumannii* increases resistance to colistin upon exposure to NaCl is of considerable clinical interest, as colistin is currently the last-resort agent against MDR *A. baumannii* strains. Colistin resistance is uncommon clinically, with only a few case reports emerging from Asia (22, 35). The mechanisms involved in mediating resistance to colistin have not been fully elucidated for *A. baumannii*. Proteomic profiles have been determined for *in vitro*-generated colistin-resistant *A. baumannii* strains; however, these strains developed gross perturbations in general metabolic functions, complicating interpretations of the results (16). A recent advancement in our understanding of colistin resistance in *A. baumannii* was the finding that mutations in the two-component system PmrAB lead to colistin resistance (2). The mechanism of PmrB-dependent colistin resistance is thought to result from an increased

TABLE 4. Colistin MICs determined with MHB or MHB supplemented with 150 mM NaCl

Isolate[a]	Source	Site of isolation	MIC (μg/ml)[c]	
			−NaCl	+NaCl
ATCC 17978[b]	ATCC	CSF	0.75	1.5
510	UNMC	Sputum	0.75	1.5
2824	UNMC	Urine	0.75	1.5
2898	UNMC	Ankle	1	1.5
4860	UNMC	Urine	2	1.5
5191	UNMC	Urine	0.75	1.5

[a] Strains in boldface type are classified as being multidrug resistant.
[b] Susceptibility data for reference strain ATCC 17978 were reported previously (1).
[c] −NaCl, MHB; +NaCl, MHB supplemented with 150 mM NaCl.

level of expression of the lipid A-modifying phosphoethanolamine transferase, PmrC. In addition, low pH and ferric iron induce colistin resistance, yet this was not associated with an increased level of expression of the *pmrCAB* operon, suggesting that additional mechanisms exist for mediating colistin resistance in *A. baumannii* (2). Data from studies of *E. coli* suggested that there may be a relationship between osmotic stress responses and responses to polymyxin antibiotics (26, 34). While the transcriptional response reported herein does not support a typical osmotic stress response, it is possible that the intersection of osmotic adaptation and polymyxin resistance may converge on an as-yet-unidentified pathway in *A. baumannii*. Notably, we did not observe an increased tolerance to colistin in the presence of NaCl for *E. coli* (data not shown), further suggesting that the response to NaCl in *A. baumannii* may be distinct. Perhaps more intriguing is the observation that the efflux pump inhibitor PAβN induced significant resistance to colistin over and above that induced by NaCl. Importantly, we have determined that the combined effect of PAβN and NaCl results in an increase in the MIC to ≥4 mg/liter, which is the breakpoint for colistin resistance in *A. baumannii* (data not shown). The mechanism of PAβN-induced colistin resistance remains elusive. It is possible that the interaction of PAβN with efflux transporters promotes the stability of the outer membrane or that the inhibition of efflux itself stabilizes the flux of solutes and water that would otherwise contribute to colistin-mediated cell death. Alternatively, PAβN may induce additional transcriptional changes that confer protection against colistin. Further elucidation of the mechanisms of both cation-induced antibiotic tolerance as well as PAβN-mediated colistin resistance may provide insight into the normal resistance response to colistin in *A. baumannii*.

Clinical implications of cation-induced antibiotic tolerance. NaCl is ubiquitous within the hospital environment, suggesting that *A. baumannii* encounters this signal within its hospital niche. The finding that *A. baumannii* becomes more resistant to clinically relevant antibiotics in response to NaCl concentrations encountered within the human host highlights intriguing questions regarding the implications of these findings for the clinical setting. Our data suggest that MICs determined *in vitro* might be discrepant with the inducible tolerance of *A. baumannii* when exposed to NaCl or KCl within the human body. Although the magnitude of the change in resistance to antibiotics was not as large as that described for the constitutive overexpression of a broad-specificity efflux pump, the incremental increase in resistance may be sufficient to promote tolerance among otherwise susceptible isolates within the body. In this way, the response to NaCl might enhance the bacterium's ability to persist until conditions are more favorable for growth or until additional resistance determinants can be accumulated. In addition, local concentrations of electrolytes such as NaCl or KCl in specific tissues such as the urinary tract may exceed serum concentrations and may complicate the eradication of *A. baumannii* from these sites. Finally, given that NaCl induces tolerance to a broad spectrum of antibiotics and that the response to colistin appears to be conserved among multidrug-resistant clinical isolates, targeting the regulatory systems responsible for mediating antibiotic resistance in response to NaCl may have therapeutic potential in combination with conventional antibiotics. This strategy might be particularly beneficial in combination with drugs, such as colistin, that possess narrow therapeutic windows by decreasing the dose required for efficacy, thus limiting the associated toxicity.

This work demonstrates that an extracellular signal encountered by *A. baumannii* results in increased antibiotic tolerance. This extends previously reported observations regarding the effects of NaCl on aminoglycoside resistance by demonstrating that in *A. baumannii*, NaCl and KCl induce tolerance not only to aminoglycosides but also to levofloxacin, imipenem (NaCl), and colistin. We have also demonstrated that *A. baumannii* regulates membrane protein expression and secretion at the transcriptional and posttranslational levels in response to a ubiquitous, nonantibiotic signal, and we demonstrate that this response results in increased antibiotic resistance. Further work to identify the systems responsible for sensing and adapting to NaCl or other monovalent cations and translating this signal into an increased-resistance phenotype will provide valuable insight into the intrinsic mechanisms of adaptation and resistance in *A. baumannii* and holds promise in identifying novel therapeutic targets.

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