

# A PmrB-Regulated Deacetylase Required for Lipid A Modification and Polymyxin Resistance in *Acinetobacter baumannii*

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**Emerging resistance to “last-resort” polymyxin antibiotics in Gram-negative bacteria is a significant threat to public health. We identified the *Acinetobacter baumannii* NaxD deacetylase as a critical mediator of lipid A modification resulting in polymyxin resistance and demonstrated that *naxD* is regulated by the sensor kinase PmrB. This represents the first description of a specific PmrB-regulated gene contributing to polymyxin resistance in *A. baumannii* and highlights NaxD as a putative drug target to reverse polymyxin resistance.**

Multidrug antibiotic resistance is a rapidly growing threat to global public health. In the United States alone, the Centers for Disease Control and Prevention estimate that at least 2 million infections are caused by antibiotic-resistant pathogens each year, leading to roughly 25,000 deaths (1). A growing number of bacterial isolates are now resistant to most or all classes of antibiotics, rendering some infections untreatable. Polymyxin antibiotics, including polymyxin B and colistin (polymyxin E), are increasingly used as the “last resort” to treat highly drug-resistant Gram-negative bacterial pathogens. These cationic antimicrobial peptide antibiotics bind to the negatively charged lipid A moiety of lipopolysaccharide (LPS) through electrostatic interactions that lead to the disruption of the outer and inner bacterial membranes (2–4). Unfortunately, polymyxin resistance has emerged and is increasing globally (2, 5, 6), undermining the utility of these “last-line” antimicrobials. This clinical challenge is exemplified by *Acinetobacter baumannii*, a Gram-negative bacterium that accounts for up to 12,000 infections per year in the United States, 63% of which are multidrug resistant and some of which are polymyxin resistant (1).

Polymyxin resistance can emerge in *A. baumannii* via modifications to lipid A (4, 5). In particular, modifications of the highly anionic lipid A phosphate groups with positively charged moieties, such as phosphoethanolamine (pEtN), lead to increased bacterial surface charge and repulsion of cationic polymyxins (2, 7). In *Acinetobacter* spp., polymyxin resistance is often controlled by the PmrA/PmrB (PmrAB) two-component regulatory system, in which PmrB is a membrane-localized sensor kinase that phosphorylates and activates PmrA, the cytosolic DNA-binding response regulator. Point mutation(s) in PmrB (commonly within the histidine kinase domain) and/or constitutive expression of *pmrAB* has been observed in polymyxin-resistant *A. baumannii* strains (8–10). *A. baumannii* lipid A has been shown to be modified with pEtN (9, 10, 11), and recently, Pelletier et al. (11) first described a galactosamine (GalN) modification in polymyxin-resistant strains that have activating mutations in PmrB. However, it has remained unclear how PmrB exerts its effects on lipid A modification and polymyxin resistance since PmrB-regulated genes mediating these functions have not yet been identified in *A. baumannii*.

We previously identified NaxD, a deacetylase belonging to the YdjC superfamily, as being required for the GalN modification of

*Francisella tularensis* lipid A (12). Specifically, NaxD deacetylates *N*-acetylgalactosamine (linked to the lipid carrier undecaprenyl phosphate) to galactosamine, a step required for the subsequent addition of galactosamine to lipid A. Protein sequence analysis revealed a YdjC-like hypothetical protein with 32% identity to *F. tularensis* NaxD in the *A. baumannii* ATCC 17978 (17978 strain) genome (annotated as A1S\_2623; see Fig. S1 in the supplemental material). As a first step to determine whether *naxD/A1S\_2623* might be a gene involved in lipid A modification in *A. baumannii*, we measured *naxD* expression by quantitative real-time PCR (qRT-PCR) in the polymyxin-susceptible *A. baumannii* 17978 strain and a polymyxin-resistant derivative (R2 [10]). We observed that *naxD* was highly overexpressed in R2, compared to its expression level in the parental wild-type *A. baumannii* 17978 strain (Fig. 1). Since R2 has an activating mutation in PmrB (10), we tested whether PmrB was responsible for the overexpression of *naxD*. Indeed, *naxD* expression was reduced to a baseline level in an isogenic *pmrB* deletion mutant of R2 and restored to the wild-type level in the R2 *pmrB*-complemented strain, indicating that *naxD* expression was dependent on PmrB. Furthermore, the promoter region of *naxD* contains a consensus PmrA binding site (13–16) (see Fig. S2 in the supplemental material). These data clearly demonstrate PmrB-mediated regulation of *naxD* in *A. baumannii*.

We next set out to directly determine whether NaxD plays a role in *A. baumannii* lipid A modification by generating a *naxD* deletion mutant in the R2 strain and comparing the lipid A pro-

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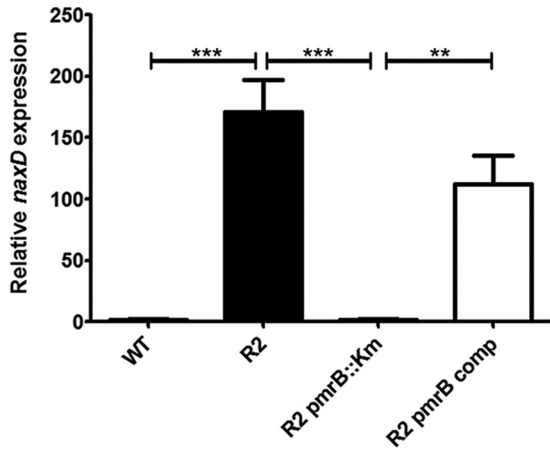


FIG 1 PmrB-dependent overexpression of *naxD* in a polymyxin-resistant strain of *A. baumannii*. The wild-type polymyxin-sensitive strain 17978 (WT), the polymyxin-resistant derivative R2, the isogenic R2 *pmrB* deletion mutant, and the *pmrB*-complemented (R2 *pmrB* comp) strains were grown in lysogeny broth (LB) supplemented with 1 mM MgCl<sub>2</sub> to an optical density at 600 nm of 0.6. RNA was harvested and used for quantitative real-time PCR analysis of *naxD* expression in relation to the housekeeping 16S rRNA. \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ .

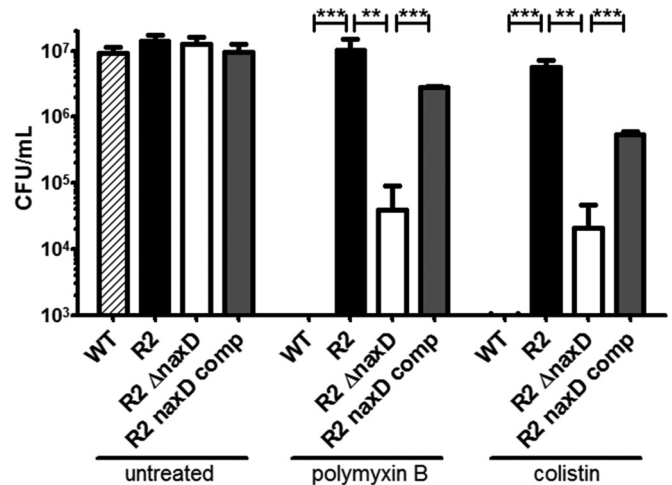


FIG 3 *A. baumannii* NaxD is required for polymyxin resistance. The *A. baumannii* wild-type strain, polymyxin-susceptible *A. baumannii* strain 17978 (WT), polymyxin-resistant strain R2, the R2 $\Delta$ *naxD* mutant, and the *naxD*-complemented strain (R2 $\Delta$ *naxD* comp) were incubated with 7  $\mu$ g/ml polymyxin B or 10  $\mu$ g/ml colistin for 2 h, and CFU were enumerated after plating. \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ .

files of the parental and mutant strains by mass spectrometry. Lipid A was isolated as described previously (11) and analyzed using a Bruker Microflex matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrophotometer in the negative ion mode (11). Mass spectra of lipid A isolated from the

polymyxin-resistant R2 strain revealed pEtN ( $m/z$  2,033;  $\Delta m/z$  123) and GalN ( $m/z$  2,072;  $\Delta m/z$  161) additions to the terminal phosphates of the hexa-acylated lipid A structure ( $m/z$  1,910) (Fig. 2). This was in contrast to lipid A isolated from the parental polymyxin-susceptible 17978 strain (Fig. 2), which did not contain

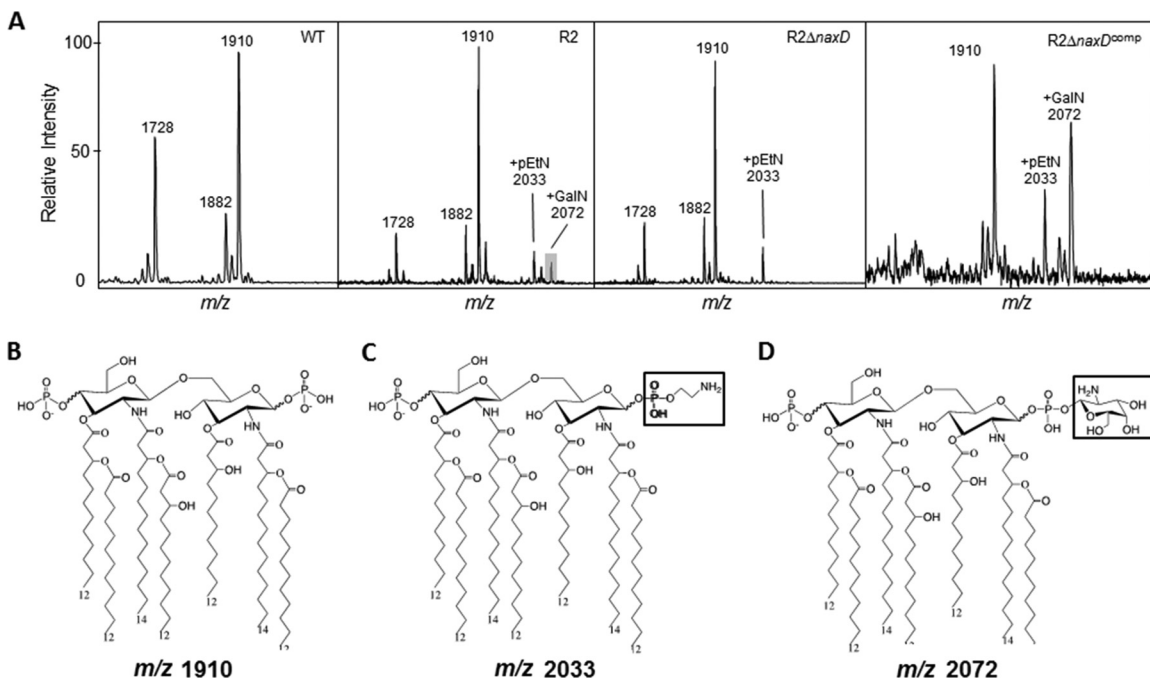
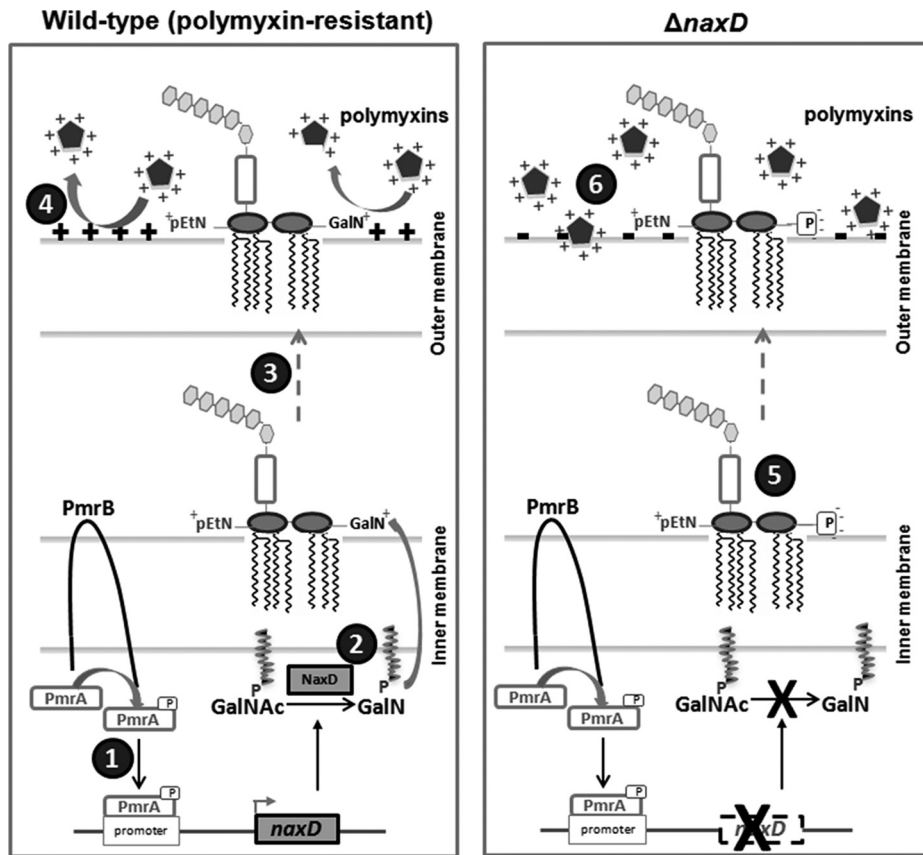


FIG 2 NaxD is required for the galactosamine modification of *A. baumannii* lipid A. (A) Quantification of lipids in the wild-type polymyxin-susceptible *A. baumannii* 17978 (WT) strain, the polymyxin-resistant derivative R2, the isogenic *naxD* deletion mutant strain (R2 $\Delta$ *naxD*), and the *naxD*-complemented (R2  $\Delta$ *naxD*<sup>comp</sup>) strain. (B to D) The peaks corresponding to bis-phosphorylated hepta-acylated lipid A ( $m/z$  1,910) (B), to that with phosphoethanolamine ( $m/z$  2,033) (C), and to that with galactosamine ( $m/z$  2,072) (D) are present in R2, but the galactosamine is absent in the R2 $\Delta$ *naxD* mutant. For each spectrum, the  $m/z$  range between 1,600 and 2,300 is shown. Note that phosphate additions can be at either the 1 or the 4' position. Ion peaks  $m/z$  1,728 and  $m/z$  1,882 correspond to bis-phosphorylated hepta-acylated lipid A ( $m/z$  1,910) with the loss of a C<sub>12</sub> fatty acid and C<sub>2</sub>H<sub>4</sub>, respectively.



**FIG 4** Model of the role of *A. baumannii* NaxD in resistance to polymyxins. Schematics of polymyxin-resistant wild-type *A. baumannii* and *naxD* ( $\Delta naxD$ ) mutant bacteria are shown. (1) PmrB phosphorylates and activates PmrA upon sensing of environmental stimuli (or when rendered constitutively active due to an amino acid mutation, often in the kinase domain). PmrA subsequently binds the *naxD* promoter, facilitating transcription. (2) NaxD deacetylates *N*-acetylgalactosamine (GalNAc) to galactosamine (GalN), which is attached to a lipid carrier and transported from the cytosolic to the periplasmic side of the inner membrane. GalN is subsequently added to the 1 position phosphate of lipid A. The lipid A is also modified with phosphoethanolamine (pEtN) through a NaxD-independent process. (3) Lipopolysaccharide (LPS), composed of lipid A, core, and O antigen, is then transported to the outer leaflet of the outer membrane. (4) GalN and pEtN are positively charged, and these modifications to lipid A increase the surface charge of the bacteria such that positively charged polymyxin antibiotics (pentagons) are repelled. (5) In *naxD* mutant bacteria, LPS lacks the lipid A GalN modification. (6) Therefore, the bacterial surface charge is decreased, allowing polymyxins to interact with and disrupt the bacterial membranes.

either pEtN or GalN additions, confirming that these lipid A modifications are associated with polymyxin resistance in *A. baumannii* as previously reported (11). However, while the pEtN modification was not affected, the GalN modification was absent in the lipid A from the *naxD* deletion mutant. Furthermore, the GalN modification was restored when the *naxD* mutant was complemented with *naxD* in *trans* (Fig. 2). These data indicate that *naxD* plays a critical role in the modification of lipid A with GalN in *A. baumannii*.

We next tested whether NaxD, and the GalN lipid A modification that it controls, was required for *A. baumannii* polymyxin resistance. Briefly, overnight cultures were grown from frozen stock in lysogeny broth (LB) (BD Biosciences, Sparks, MD) at 37°C with aeration and then diluted to a final concentration of  $10^7$  CFU/ml in 30% LB. Bacteria were treated with polymyxin B (7  $\mu$ g/ml) (USB Affymetrix, OH) or colistin sulfate (10  $\mu$ g/ml) (referred to as “colistin” here; Sigma-Aldrich, St. Louis, MO) and incubated at 37°C with aeration for 2 h. The parental wild-type 17978 strain was highly susceptible to both polymyxin and colistin, while the R2 strain was resistant (Fig. 3). This resistance was partially dependent on NaxD, since the viability of the *naxD* dele-

tion mutant was significantly reduced after polymyxin B or colistin treatment compared to the R2 strain (Fig. 3). Furthermore, this susceptibility was reversed in the *naxD*-complemented strain. These data demonstrate the important contribution of NaxD to *A. baumannii* polymyxin resistance and are the first description of a specific PmrB-regulated gene that mediates polymyxin resistance in this clinically important nosocomial pathogen.

Taken together, the data presented here demonstrate that an activating mutation in PmrB can lead to overexpression of *naxD*. NaxD then mediates modification of *A. baumannii* lipid A with positively charged GalN, thus lowering the affinity for cationic antibiotics and leading to increased resistance (Fig. 4). In addition to the NaxD-dependent GalN modification, the NaxD-independent pEtN modification is also likely important for this resistance (9, 10, 11). This work reveals the first identification of a two-component regulatory system controlling *naxD* expression and highlights NaxD as a potential drug target for inhibitors that reverse polymyxin resistance. Since NaxD is conserved in several pathogenic Gram-negative bacteria (see Fig. S1 in the supplemental material), such an approach could have significant utility in combating antibiotic-resistant bacteria.

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