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Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system

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Summary

Gram-negative bacteria survive harmful environmental stressors by modifying their outer membrane. Much of this protection is afforded upon remodeling of the lipid A region of the major surface molecule lipopolysaccharide (LPS). For example, the addition of cationic substituents, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoehthanolamine (pEtN) at the lipid A phosphate groups is often induced in response to specific environmental flux stabilizing the outer membrane. The work herein represents the first report of pEtN addition to *P. aeruginosa* lipid A. We have identified the key pEtN transferase which we named $EptA_{Pa}$ and characterized its strict activity on only one position of lipid A, contrasting from previously studied EptA enzymes. We further show that transcription of $eptA_{Pa}$ is regulated by zinc via the ColRS two-component system instead of the PmrAB system responsible for *eptA* regulation in *E. coli* and *S. enterica*. Further, although L-Ara4N is readily added to the same position of lipid A as pEtN under certain environmental conditions, ColR specifically induces pEtN addition to lipid A in lieu of L-Ara4N when Zn^{2+} is present. The unique, specific regulation of $eptA_{Pa}$ transcription and enzymatic activity described in this work demonstrates the tight yet inducible control over LPS modification in *P. aeruginosa*.

Keywords

lipid A; EptA; phosphoethanolamine; ColRS; *Pseudomonas*; outer membrane; lipopolysaccharide; LPS

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Introduction

Pseudomonas aeruginosa ubiquitously inhabits soil and water sources and is known for its intrinsic tolerance to potentially toxic contaminants such as heavy metals (Caille *et al*., 2007). It is also a formidable opportunistic pathogen frequently acquired in healthcare facilities due to its persistence in sinks, showers and many non-aquatic abiotic surfaces (Lyczak *et al*., 2000; Kerr and Snelling, 2009; Chai *et al*., 2014). Once inside a human host *P. aeruginosa* thrives in a variety of tissue types resulting in acute skin, eye, and burn wound infections (Lyczak *et al*., 2000). Perhaps most notably, however, are chronic *P. aeruginosa* infections that persist within the lungs of cystic fibrosis patients for years and are recalcitrant to most antimicrobial treatment (Kerr and Snelling, 2009; Moskowitz and Ernst, 2010).

The outer membrane of Gram-negative bacteria like *P. aeruginosa* acts as a protective barrier to prevent binding and uptake of toxic molecules. The major component of the outer leaflet, lipopolysaccharide, interfaces with the environment and is often remodeled to protect the cell from environmental stressors. Lipopolysaccharide is composed of three distinct domains: a lipid A anchor, a core sugar region, and an outer polysaccharide known as O-antigen (Whitfield and Trent, 2014). The lipid A domain is a potent immunostimulant, hence its other name, “endotoxin” (Needham *et al*., 2013). Furthermore, the negatively charged lipid A molecule is a prime target of cationic antimicrobial peptides that destabilize the outer membrane resulting in cell lysis (Vaara and Vaara, 1981; Needham and Trent, 2013).

Modification of the canonical, hexa-acylated, *bis*-phosphorylated lipid A molecule produced in Gram-negatives (Fig 1A; black) alter its chemical properties to bolster membrane integrity. A repertoire of modification enzymes is responsible for the dynamic structure of *P. aeruginosa* lipid A. Previous work from our laboratory recently revealed that *P. aeruginosa* has an LpxT lipid A kinase that adds an additional phosphate group to the 1- or the 4' position under standard laboratory growth conditions (Fig. 1A; brown)(Nowicki *et al*., 2014). Hydroxylation of the secondary acyl chains can also occur by one of two LpxO enzymes, although the purpose for this modification remains to be elucidated (Fig. 1B; orange) (King *et al*., 2009). In addition to these modifications, the toxicity of *P. aeruginosa* lipid A can be affected by altering the acylation pattern due to activity of the PagL deacylase or the PagP palmitoyltransferase (Fig. 1B; pink and green) (Ernst, 1999; Ernst *et al*., 2003; Ernst *et al*., 2006; Thaipisuttikul *et al*., 2014). Aside from influencing endotoxicity, lipid A modifications can contribute to antimicrobial peptide resistance (Needham and Trent, 2013). Addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to either phosphate group of *P. aeruginosa* lipid A by the enzyme ArnT is one such strategy (Fig. 1B; blue)(Bhat *et al*., 1990; Fernández *et al*., 2013). Palmitoylation has also been shown to increase antimicrobial resistance (Thaipisuttikul *et al*., 2014).

Transcription of lipid A modification enzymes is often induced through two-component system signaling (Needham and Trent, 2013). Signal transduction occurs when a bacterial sensor kinase autophosphorylates in response to an external stimulus and transfers this phosphate group to a response regulator protein. The resulting conformation in the response regulator promotes DNA binding at target promoters to alter gene expression (Rodrigue *et*

al., 2000). The two well-conserved two-component systems PhoPQ and PmrAB play a major role in modulating lipid A modification enzyme expression in *P. aeruginosa*. PhoPQ activates *pagP* and *arnT* transcription in response to limiting Mg^{2+} (McPhee *et al*., 2006), while PmrAB induces *arnT* transcription upon sensing either limiting Mg^{2+} or subinhibitory cationic antimicrobial peptide concentrations (McPhee *et al*., 2003; McPhee *et al*., 2006).

Aside from PhoPQ and PmrAB, three additional two-component systems in *P. aeruginosa* are involved in lipid A modification and resistance to the cationic antimicrobial peptide polymyxin. These include ParRS and CprRS, which are both activated by various antimicrobial peptides (Fernandez *et al*., 2010; Fernandez *et al*., 2012), and ColRS, which remains largely unstudied in *P. aeruginosa* but plays a role in heavy metal tolerance and overall membrane stability in *P. putida* and *P. fluorescens* (de Weert *et al*., 2006; Hu and Zhao, 2007; Ainsaar *et al*., 2014). A recent study investigated the role of CprRS and ColRS in *P. aeruginosa* polymyxin resistance. This report revealed that these two systems contribute to the elevated polymyxin resistance observed in *P. aeruginosa phoQ* mutants, and also suggested that ColRS and CprRS regulate additional unknown factors required for this resistance (Miller *et al*., 2011; Gutu *et al*., 2013). Given the complexities of two-component systems in *P. aeruginosa*, there are still significant questions regarding LPS modifications and their impact on antimicrobial resistance.

In Gram-negatives such as *E. coli*, *Helicobacter pylori*, *Campylobacter jejuni*, and *Neisseria gonorrhoeae* addition of phosphoethanolamine (pEtN) groups to lipid A by the enzyme EptA also promote polymyxin resistance and virulence (Tran *et al*., 2006; Cullen and Trent, 2010; Herrera *et al*., 2010; Cullen *et al*., 2011; Cullen *et al*., 2013; Hobbs *et al*., 2013). Although *P. aeruginosa* has pEtN transferase orthologs, pEtN addition has not been observed in *P. aeruginosa* lipid A prepared from cells grown under conditions that induce this modification in other organisms or from *P. aeruginosa* clinical isolates. We investigated whether or not *P. aeruginosa* lipid A could be modified with pEtN, and if so, under what conditions. Here we report that *P. aeruginosa* gene PA14_39020 is a functional pEtN transferase which we have named $EptA_{Pa}$ that strictly modifes the 4' phosphate group of lipid A. We also demonstrate that zinc acts as a signal to induce $eptA_{Pa}$ transcription via the ColRS two-component system (Fig. 7). While transcription of $eptA_{Pa}$ is upregulated in response to Zn^{2+}, *arnT* transcription is downregulated, suggesting that mechanisms are in place to mediate strict control over specific lipid A modifications. The existence of $eptA_{Pa}$ reveals the potential for greater diversity in *Pseudomonas* lipid A structure and the versatility of the outer membrane.

Results

P. aeruginosa *has a functional EptA enzyme*

In silico analysis identified three *P. aeruginosa eptA* orthologs with significant identity to the *S. enterica eptA* (*pmrC*) ortholog (Lee *et al*., 2004). These include PA14_58610 (24% identity, E-value 3e-28), PA14_21210 (43%, E-value 1e-149) and PA14_39020 (43%, E-value 6e-149). Since pEtN-modified *P. aeruginosa* lipid A has not been previously reported, we tested whether these orthologs could function as a lipid A pEtN transferase by expressing

each gene *in trans* in the *E. coli eptA* mutant (W3110Δ*eptA*$_{Ec}$). ^{32}P-labeled lipid A was prepared and separated by TLC from these and relevant control strains including wild-type strain W3110 and W3110Δ*eptA*$_{Ec}$+empty vector.

While no pEtN is detected in lipid A prepared from W3110 or W3110Δ*eptA*$_{Ec}$ + empty vector (Fig. S1A, lanes 1 and 2), expression of both *eptA*$_{Ec}$ and PA14_39020 resulted in pEtN-modified lipid A (Fig. S1A, lanes 3 and 6). MALDI-TOF mass spectrometry (MS) analysis also confirmed that while W3110Δ*eptA*$_{Ec}$ expressing empty vector had no pEtN (Fig. S1B), lipid A prepared from W3110Δ*eptA*$_{Ec}$+PA14_39020 (p*eptA*$_{Pa}$) was modified with pEtN, as evidenced by the ion of m/z 1920.4 (Fig. S1C; predicted [M-H]- at m/z 1920.2).

Since EptA$_{Pa}$ can add pEtN to *E. coli* lipid A, we next determined whether EptA$_{Pa}$ modified *P. aeruginosa* lipid A. ^{32}P-labeled lipid A from wild-type strain PA14, PA14+empty vector or PA14+*eptA*$_{Pa}$ was analyzed. TLC separation of lipid A clearly demonstrated an altered profile of PA14 expressing p*eptA*$_{Pa}$ (Fig. 2A, lane 3) relative to PA14 or PA14+empty vector (Fig. 2A, lanes 1 and 2). Penta-acylated, palmitoylated and L-Ara4N-modified lipid A species were observed by MALDI-TOF MS analysis of lipid A isolated from PA14+empty vector (Fig. 2B). Whereas expression of *eptA*$_{Pa}$ in PA14 resulted in abundant ions of m/z 1489.0 and 1727.3 which correspond to the pEtN-modified lipid A species (Fig. 2C; predicted [M-H]- at m/z 1489.9 and 1728.1, respectively). These results reveal that *P. aeruginosa* has a functional lipid A pEtN-transferase enzyme capable of modifying both *E. coli* and *P. aeruginosa* LPS.

EptA$_{Pa}$ adds pEtN strictly to the lipid A 4' phosphate group

Previous work from our laboratory has demonstrated that in *P. aeruginosa, S. enterica* and *E. coli* the position of lipid A modification can be important due to potential competition with other modification groups. For example in *S. enterica* and *E. coli* EptA preferentially adds pEtN to the lipid A 1-phosphate group, which is the sole site of phosphorylation by the kinase LpxT (Herrera *et al*., 2010). Environmental conditions that activate *eptA* transcription simultaneously inhibit LpxT activity to prevent competition (Herrera *et al*., 2010; Kato *et al*., 2012). However, since both LpxT and ArnT enzymes in *P. aeruginosa* can act on either lipid A phosphate group (Bhat *et al*., 1990; Nowicki *et al*., 2014) we questioned whether EptA$_{Pa}$ also has dual positional activity. To determine this we removed the 1- or the 4'-phosphate group of lipid A by heterologous expression of *Fransicella novicida* LpxE or LpxF phosphatases (Wang *et al*., 2004; Wang *et al*., 2006), respectively, and tested the ability of EptA$_{Pa}$ to modify lipid A. This experiment was done in *E. coli* strain BN2 (Needham *et al*., 2013) since its lipid A is penta-acylated, and LpxF can only act on penta-acylated lipid A (Wang *et al*., 2006). BN2 also lacks some lipid A modification machinery including LpxT to facilitate easier analysis of the lipid A profiles.

Expression of *eptA*$_{Pa}$ resulted in a pEtN-modified lipid A species that migrated below the unmodified *bis*-phosphorylated species (Fig. 3A, lane 2). Expression of either *lpxE* or *lpxF* caused a marked increase of mono-phosphorylated lipid A, which migrates near the top of the TLC plate (Fig. 3A, lanes 3 and 5). Simultaneous expression of *eptA*$_{Pa}$ and *lpxE* resulted

in the appearance of a species that migrated a distance between that of unmodified *bis*-phosphorylated lipid A and pEtN-modified lipid A (Fig. 3A, lane 4). MALDI-TOF MS confirmed this species to be pEtN-modified mono-phosphorylated lipid A (Fig. 3B). This same pEtN-modified species was not detected when LpxF was expressed with *eptA*$_{Pa}$ (Fig 3A, lane 6). The lack of pEtN addition to 4'-dephosphorylated lipid A was further confirmed by MALDI-TOF MS as simultaneous expression of EptA$_{Pa}$ and LpxF resulted in only a single ion of m/z 1506.1, corresponding to mono-phosphorylated lipid A (Fig. 3C; predicted [M-H]- at m/z 1507.1).

EptA$_{Pa}$-dependent addition of pEtN to the 4' phosphate group of BN2 and PA14 lipid A was corroborated by ultraviolet photodissociation (UVPD) tandem MS (Figs. S2, S3 and S4). For all UVPD mass spectra, cleavage sites (7) and (8) provided evidence of the presence of a pEtN group. In addition, the glycosidic and cross-ring cleavages at cleavage sites (10) and (11) in the fragmentation map shown in Figure S2, sites (9)-(12) in Figure S3, and sites (9)-(12) and (17)-(21) in Figure S4 further support the location of the pEtN modification at the 4' phosphate group of each lipid A species. Taken together, these results demonstrate that EptA$_{Pa}$ functions strictly at the 4' phosphate group, unlike any previously characterized EptA enzyme.

Extracellular zinc induces pEtN addition to lipid A

In *S. enterica*, modification of lipid A with pEtN is induced via the PmrAB two-component system in response to mildly acidic pH (Perez and Groisman, 2007), and indirectly via the PhoPQ system when Mg^{2+} is limiting or cationic antimicrobial peptides are present (Kox *et al*., 2000; Bader *et al*., 2005). Since none of these signals induced pEtN addition to *P. aeruginosa* lipid A (data not shown), we next tested transition metals including Fe^{3+} and Zn^{2+}, which activate PmrAB in *E. coli* (Hagiwara *et al*., 2004; Lee *et al*., 2005). We also tested Ga^{3+} due to its chemical similarity to Fe^{3+}, and Cd^{2+}, which is closely related to Zn^{2+} (Laddaga and Silver, 1985; Kaneko *et al*., 2007). Transition metals Co^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+} were also tested for their ability to induce pEtN modification of lipid A since they are associated with biological catalysts and are commonly found in the environment (Andreini *et al*., 2008; Mathiyazhagan and Natarajan, 2011).

Lipid A was isolated from ^{32}P-labeled *P. aeruginosa* grown in LB alone or supplemented with metal. Addition of extracellular Zn^{2+} but no other metal tested resulted in modified lipid A (Fig. 4A, lane 3). Zn^{2+}-dependent modification was abolished when *eptA*$_{Pa}$ was deleted from the genome and restored upon complementation with the native *eptA*$_{Pa}$ promoter (Figs. 4A, lanes 4 and 5), suggesting that the changes observed were due to EptA$_{Pa}$ activity. To determine whether Zn^{2+} induced transcription of *eptA*$_{Pa}$, cDNA was prepared from cells grown in the presence or absence of Zn^{2+}. As shown by both quantitative and semi-quantitative reverse-transcriptase (RT) PCR, *eptA*$_{Pa}$ gene expression is induced when Zn^{2+} is added to the growth media (Figs. 4B and S5). These results indicate that transcription of *eptA*$_{Pa}$ is dependent on extracellular Zn^{2+}

To confirm whether Zn^{2+}-dependent induction of EptA$_{Pa}$ synthesis resulted in pEtN addition to *P. aeruginosa* lipid A, MALDI-TOF MS analysis of lipid A isolated from cells grown with or without Zn^{2+} was performed. Wild-type *P. aeruginosa* lipid A was modified with

pEtN in the presence of Zn^{2+} (Fig. 4C), while lipid A prepared from the PA14Δ*eptA*$_{Pa}$ mutant showed no pEtN modification when Zn^{2+} was added to the media (Fig. 4D). Complementation of PA14Δ*eptA*$_{Pa}$ using the native *eptA*$_{Pa}$ promoter restored Zn^{2+}-dependent pEtN addition to the lipid A (Fig. 4E). MS analysis revealed that in addition to pEtN addition, L-Ara4N-modified lipid A was present in PA14 grown with Zn^{2+} (Fig. 4C). We were therefore curious as to how Zn^{2+} might influence L-Ara4N addition to lipid A. Since Zn^{2+} affected the transcription of *eptA*$_{Pa}$, we assessed whether Zn^{2+} altered *arnT* gene expression by performing quantitative RT-PCR. While *eptA*$_{Pa}$ transcription increased by 21-fold in the presence of 2mM Zn^{2+}, *arnT* transcription was downregulated >4-fold (Fig. 4B). This result indicates that pEtN modification is selected for in the presence of Zn^{2+} while *arnT* expression, and thus L-Ara4N modification of lipid A, is downregulated.

The ColRS two-component system induces pEtN addition to lipid A

Since inducible lipid A modification genes like *eptA* are commonly regulated by two-component systems, our next goal was to determine the system responsible for *eptA*$_{Pa}$ transcriptional activation in response to Zn^{2+}. The ColRS system has recently been shown to respond to transition metals including Zn^{2+} (Ainsaar *et al*., 2014). Our first approach was therefore to investigate the presence of potential ColR binding sites in the *eptA*$_{Pa}$ promoter. A consensus ColR binding site has been determined for promoters of genes within the ColR regulon in *P. putida* (Kivistik *et al*., 2009). Using the Virtual Footprint online analysis tool (Münch *et al*., 2005), we found three potential ColR binding sites within the *eptA*$_{Pa}$ promoter region with close agreement to this consensus sequence (Fig 5A), suggesting that ColR binds to the *eptA*$_{Pa}$ promoter. It was then tested whether overexpression of *colR* could induce *eptA*$_{Pa}$ transcription by semi-quantitative RT-PCR of cDNA. Response regulators *pmrA* and *phoP*, which directly or indirectly regulate *eptA* transcription in *S. enterica*, were also tested. Only overexpression of *colR* resulted in detectable transcription of *eptA*$_{Pa}$ (Fig 5B). Lipid A was modified with pEtN upon overexpression of *colR*, as demonstrated by both TLC separation of ^{32}Plabeled lipid A (Fig 5C, lane 4) and MALDI-TOF MS analysis of PA14 + p*colR* (Fig 5D).

To determine whether ColR induction of *eptA*$_{Pa}$ transcription is dependent on Zn^{2+}, a PA14 *colR* deletion mutant was generated and assessed for transcription of *eptA*$_{Pa}$ in the presence or absence of Zn^{2+} by both quantitative and semi-quantitative RT-PCR analysis. Although a Zn^{2+} concentration of 2mM had been used in the initial Zn^{2+} assay experiments, the PA14Δ*colR* mutant was sensitive to 2mM Zn^{2+}. Instead, 1mM Zn^{2+} was used, which was sufficient to visualize pEtN modification in PA14 (Fig. 6A, lane 2). Minimal *eptA*$_{Pa}$ transcription was detected in response to Zn^{2+} upon deletion of *colR*; complementation of this mutant restored Zn^{2+}-dependent *eptA*$_{Pa}$ transcription by >4-fold (Fig. 6B, Fig. S5). While 1mM Zn^{2+} induced pEtN modification of PA14 lipid A (Fig. 6A, lane 2), lipid A from the PA14Δ*colR* was not modified with pEtN in response to Zn^{2+}, as determined by both TLC separation of ^{32}P-labeled lipid A and MALDI-TOF MS analysis (Fig. 6A, lane 3 and Fig 6C). pEtN addition was restored upon complementation of the *colR* mutant with p*colR*$_{nprom}$ (Fig. 6A, lane 4 and Fig 6D). A PA14 *colS* mutant and complemented mutant were also tested for pEtN addition to lipid A in response to Zn^{2+} by TLC separation of ^{32}P-labeled lipid A. As for the *colR* mutant, lipid A modification with pEtN was not detected in

the *colS* mutant grown in the presence of 1mM Zn^{2+}, but was restored upon complementation of *colS* with p$colS_{nprom}$ (Fig. S6, lanes 3 and 4). These results demonstrate that the ColRS system induces pEtN addition to lipid A upon sensing Zn^{2+}.

We also investigated whether the downregulation of *arnT* transcription in the presence of Zn^{2+} was dependent on the ColR response regulator. Gene expression of *arnT* was analyzed by quantitative RT-PCR in the PA14 *colR* mutant and complemented strains in the presence of 1mM Zn^{2+}. Transcription of *arnT* was reduced by approximately 10-fold upon complementation of *colR* (Fig. 6B). This result indicates that ColR activates transcription of $eptA_{Pa}$ in response to Zn^{2+} while downregulating *arnT* transcription.

Discussion

Changes in the environment require bacterial outer membrane remodeling, including LPS structural changes, to promote membrane stability (Whitfield and Trent, 2014). L-Ara4N addition to lipid A phosphate groups contributes to cationic antimicrobial peptide resistance in *P. aeruginosa*, *E. coli* and *S. enterica* (Lee *et al.*, 2004; Herrera *et al.*, 2010; Fernández *et al.*, 2013). The addition of the amine-containing residue pEtN can also result in increased peptide resistance (Tran *et al.*, 2006; Herrera *et al.*, 2010), and in some organisms is a crucial factor for host infection (Cullen *et al.*, 2013; Hobbs *et al.*, 2013). Lipid A modifications in *P. aeruginosa* have been well-studied, yet despite the existence of *eptA* orthologs, pEtN addition has never been observed. Due to the importance of pEtN lipid A modification in other organisms, we investigated the functionality and regulation of *P. aeruginosa eptA* orthologs. In this report, we identify and characterize a functional *P. aeruginosa* lipid A pEtN transferase and determine that Zn^{2+} induces transcription of $eptA_{Pa}$ via the *Pseudomonas*-specific ColRS system (Fig. 7).

Overexpression of three *P. aeruginosa eptA* orthologs in *E. coli* revealed that PA14_39020 ($eptA_{Pa}$) was able to modify lipid A with pEtN (Fig. S1A and C). It is likely that the other two orthologs add pEtN to other targets in the cell. Based on its homology to *S. enterica* CptA, PA14_58610 may be the enzyme responsible for adding pEtN to the core of *P. aeruginosa* LPS (Kooistra *et al.*, 2003; Tamayo *et al.*, 2005). While a very minor amount of lipid A modification is detected by TLC separation of lipid A upon expression of PA14_21210 in PA14, lipid A is probably not the primary target of this enzyme. It is possible that this enzyme modifies an as yet unidentified target, as pEtN transferase enzymes in other organisms have been shown to modify structural proteins of the flagellum and pilus (Hegge *et al.*, 2004; Cullen and Trent, 2010), and in doing so has some very minor, non-specific activity toward lipid A. This activity toward lipid A, however, is so minor that is cannot be detected by mass spectrometry analysis (data not shown).

We characterized the site-specificity of pEtN addition to lipid A due to the potential for competition with other modification groups. Whereas pEtN addition occurs specifically or preferentially at the 1-phosphate group of lipid A in *H. pylori* (Tran *et al.*, 2004) and *S. enterica* (Herrera *et al.*, 2010), respectively, analysis of $EptA_{Pa}$ activity in *E. coli* revealed that this enzyme acts solely at the 4' position (Figs. 3, S2). $EptA_{Pa}$ activity thus differs from

ArnT and LpxT enzymes in *P. aeruginosa* that can modify either lipid A phosphate group (Bhat *et al.*, 1990; Nowicki *et al.*, 2014).

Investigation of conditions that induce pEtN modification revealed that excess Zn^{2+} acts as the activating signal for *eptA*$_{Pa}$ transcription. *Pseudomonas* species are readily found in the soil and aqueous environments which can be contaminated with metals due to waste runoff from mines, smelting, and other industrial facilities (Teitzel and Parsek, 2003; Raja *et al.*, 2006; Mathiyazhagan and Natarajan, 2011). In such environments, *Pseudomonas* can be exposed to high levels of metal pollutants and has thus evolved the ability to alter gene expression to promote metal tolerance (Perron *et al.*, 2004; Ha *et al.*, 2004; Hu and Zhao, 2007; Caille *et al.*, 2007). Excess Zn^{2+} may also be relevant in healthcare settings as concentrations up to 1mM can leach out from latex catheters and gloves (Perron *et al.*, 2004; Ballesta *et al.*, 2006). Deletion of *eptA*$_{Pa}$, however, does not result in increased sensitivity to Zn^{2+} or to Cd^{2+}, Ga^{3+}, Fe^{3+}, Co^{2+}, Cu^{2+}, Mn^{2+} or Ni^{2+}. Under laboratory settings Zn^{2+}-induced pEtN addition to lipid A has no effect on polymyxin resistance, biocide tolerance, or biofilm formation (data not shown). As the ColRS system was previously implicated to play a role in polymyxin resistance, unidentified genes other than *eptA*$_{Pa}$ within the ColRS regulon are likely involved in this resistance. The fact that *P. aeruginosa* has evolved regulatory mechanisms to control pEtN addition to lipid A, however, suggests the importance of this modification for conditions we have not yet identified.

Extracellular metals are sensed by one of three two-component systems in *Pseudomonas* species: CzcRS (Perron *et al.*, 2004), CopRS (Caille *et al.*, 2007) and ColRS (Hu and Zhao, 2007; Ainsaar *et al.*, 2014). Both CzcRS (activated by Zn^{2+}) and CopRS (activated by Cu^{2+}) induce expression of the heavy metal efflux pump CzcCBA while downregulating the OprD porin, leading to decreased carbapenem and imipenem uptake (Perron *et al.*, 2004; Caille *et al.*, 2007). In *P. putida*, the ColRS system senses Zn^{2+}, Fe^{3+}, Mn^{2+} and Cd^{2+}, and mutants of *colR* and *colS* display lower tolerance to these metals (Ainsaar *et al.*, 2014). We have now determined Zn^{2+} to be an activating signal for ColRS in *P. aeruginosa*. Additionally, the PA14 *colR* and *colS* mutants are more sensitive to Zn^{2+} than wild-type, suggesting a role for the ColRS system in Zn^{2+} tolerance in *P. aeruginosa*. While the ColRS system is important for metal tolerance in *P. putida* and deletion of multiple genes in the ColRS regulon results in increased metal sensitivity, no individual ColRS-regulated gene has a major contribution to metal tolerance (Ainsaar *et al.*, 2014). It is likely that multiple genes in the *P. aeruginosa* ColRS regulon are involved in Zn^{2+} tolerance, which could explain why the *eptA*$_{Pa}$ isogenic mutant does not have any growth defect in media with Zn^{2+}.

Our demonstration of pEtN-modified lipid A via the ColRS system in response to Zn^{2+} (Fig. 7) reveals that lipid A remodeling in *P. aeruginosa* is more complex than previously realized. Extracellular Zn^{2+} specifically induces transcription of *eptA*$_{Pa}$ and not *arnT* through the ColR response regulator, demonstrating coordinated control over lipid A modifications. In addition to selectively inducing expression of *eptA*$_{Pa}$ Zn^{2+} downregulates *arnT* transcription by over 4-fold (Fig. 4B). This is interesting given that ArnT-mediated L-Ara4N modification typically plays a more significant role in virulence and antimicrobial peptide resistance than *eptA* in organisms possessing both modification enzymes (Tamayo *et al.*, 2005; Herrera *et al.*, 2010). While *eptA*$_{Pa}$ does not seem to be involved in metal or

polymyxin resistance, there is likely an evolutionary reason for this targeted induction of *eptA*$_{Pa}$ transcription. Our findings demonstrate the tight control of *P. aeruginosa* lipid A modification systems, and suggest the need for further studies to better elucidate the mechanisms involved in outer membrane remodeling and its contribution to bacterial persistence and versatility.

Experimental Procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* strains were cultured in LB broth or agar (Difco) at 37°C. *P. aeruginosa* strains were grown on LB agar plates, and initial liquid cultures were grown overnight in LB broth at 37°C. The next day, *P. aeruginosa* cultures were diluted to an OD_{600} of ~0.05 in either LB broth or in morpholinepropanesulfonic acid (MOPS)-buffered minimal medium (50mM MOPS, 93mM NH_4Cl, 43mM NaCl, 2mM KH_2PO_4) supplemented with 3.5μM $FeSO_4 \cdot 7H_2O$, 20mM sodium succinate, and 1mM $MgSO_4$. Chloramphenicol was used at a concentration of 30μg/mL for *E. coli*. Ampicillin or carbenicillin was used at a concentration of 100μg/mL or 300μg/mL for *E. coli* or *P. aeruginosa*, respectively. For growth of *P. aeruginosa* in medium with added metals, LB was used to prevent metals from crashing out of solution. For the initial screen of lipid A modifications, metal salts were added in the following concentrations: 2mM $ZnSO_4$, 0.2mM $CdSO_4$, 0.1mM $Ga(III)NO_3$, 0.2mM $FeSO_4$, 0.1mM $CoCl_2$, 2mM $CuSO_4$, 2mM $MnSO_4$, and 2mM $NiSO_4$. The highest concentration of metal that did not significantly reduce growth (defined as a >50% reduction) in liquid medium was used, with the exception of Fe^{3+}, for which a lower, more physiologically relevant concentration was used based on concentrations known to induce lipid A modification in other organisms (Herrera *et al.*, 2010).

DNA and RNA preparation

Before preparing *P. aeruginosa* genomic DNA from an overnight culture in LB broth, two washes with 0.1M NaCl were performed. Genomic DNA was prepared using the Easy-DNA Kit (Invitrogen). Total RNA was extracted from cells grown to an OD_{600} of ~0.6 using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. To eliminate residual DNA contamination, total RNA was treated with DNase from the RNase-Free DNase Set (Qiagen). cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Recombinant DNA methods

Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). Chromosomal DNA for insertion into plasmid constructs was amplified using either the DNA polymerase *PfuTurbo*^{R} (Stratagene) or Takara *Ex Taq* (Takara). PCR products were separated on an agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). All primers were purchased from Integrated DNA Technologies (Table S2). Restriction endonucleases, T4 DNA ligase, and Antarctic Phosphatase used in this study for generation of plasmid constructs were purchased from New England Biolabs and used according to the manufacturer's instructions.

Generation of chromosomal gene deletion mutants

In-frame, markerless gene deletions were generated in *P. aeruginosa* by homologous recombination using the suicide plasmid pEX18Gm. ~1Kb DNA fragments flanking the target gene up or downstream were amplified using primers listed in Table S2. An assembly PCR was then carried out to stitch together these flanking regions. Assembly PCR fragments were digested with restriction endonucleases EcoRI and HindIII or BamHI, and ligated into pEX18Gm. The suicide plasmid constructs, pEX18-*eptA*del, pEX18-*colR*del or pEX18-*colS*del were introduced into *P. aeruginosa* via conjugation with *E. coli* strain SM10. Deletion mutants were then screened for as described previously (Hoang *et al.*, 1998). Deletions were confirmed by PCR.

Plasmid constructs

To construct pPA14_39020 (pAC*eptA*$_{Ec}$), pPA14_58610 and pPA14_21210, each gene was amplified along with the native RBS and cloned into the medium copy vector pACYC184 using EcoRV and SalI restriction endonucleases. For generation of p*eptA*$_{Pa}$, p*colR*, p*pmrA* and p*phoP*, each gene and its native RBS were amplified and digested with EcoRI and HindIII to clone into pEX1.8. All constructs were confirmed by sequencing. The *eptA*$_{Pa}$ and *colR* genes were amplified along with their native promoters and cloned into the medium copy vector pEX1.8 (Pearson *et al*., 1997) by digestion with SalI or SalI and HindIII, respectively, generating p*eptA*$_{nprom}$ and p *colR*$_{nprom}$. For generation of p*colS*$_{nprom}$, the *colRS* promoter was first amplified and cloned into pEX1.8 by digestion with BamHI and EcoRI. The *colS* coding sequence was then amplified and cloned into pEX1.8 (containing the *colRS* promoter) with EcoRI and HindIII.

Isolation and analysis of labeled lipid A

Overnight cultures were diluted to an OD_{600} of ~0.05 in 5mL of fresh medium (as indicated within each figure legend) and labeled with 2.5μCi/mL $^{32}P_i$ (Perkin-Elmer). Cells were harvested at an OD_{600} of ~1.0, and lipid A was isolated by mild acid hydrolysis as described previously (Zhou *et al*., 1999; Tran *et al*., 2004). $^{32}P_i$-labeled lipid A species were spotted on a TLC plate at ~5,000 cpm per lane (10,000 cpm for *E. coli*), and run in a solvent prepared in a 50:50:16:5 (v/v) ratio of chloroform, pyridine, 88% formic acid, and water, respectively. TLC plates were dried, set on a phosphor screen overnight and imaged using a phosphor-imager (BioRad PMI).

Large scale lipid A isolation and MALDI-TOF mass spectrometry

For large scale lipid A analysis, 250mL cultures were grown at 37°C to an OD_{600} of ~1.0 in the medium indicated. Lipid A was prepared by mild acid hydrolysis, washed and resuspended in chloroform/methanol/water (2:3:1, v/v), as described previously (Hankins *et al*., 2013). The sample was run through a DEAE cellulose column, washed with chloroform/methanol/water (2:3:1, v/v), and eluted in individual fractions with chloroform/methanol/increasing concentrations of ammonium acetate, as described previously (Odegaard *et al*., 1997; Hankins *et al*., 2013). Typically, hydrophilic or monophosphorylated species fractionate at lower ammonium acetate concentrations (flow-through, wash, 60 or 120mM elution fractions), while more hydrophobic, unmodified or phosphate-modified species

fractionate at the highest concentration, 480mM ammonium acetate. MALDI-TOF mass spectrometry was performed as described using a MALDI-TOF/TOF mass spectrometer (ABI 4700 Proteomics Analyser) (Hankins *et al*., 2013).

ESI and UVPD mass spectrometry

Lipid A was isolated and prepared as described above for MALDI-TOF analysis. All mass spectrometry experiments were executed on a Thermo Scientific Orbitrap Elite mass spectrometer (Bremen, Germany) modified to perform ultraviolet photodissociation (UVPD). The mass spectrometer was equipped with a 193-nm Coherent ExciStar XS excimer laser (Santa Clara, CA) and operated in the negative ion mode using a previously described set-up (Shaw *et al*., 2013). Briefly, solutions containing 1–5 μM lipid A in 50:50 methanol/ chloroform were directly infused using an electrospray ionization (ESI) source at a flow rate of 3 μl/min. The ESI voltage was set to 4 kV. UVPD mass spectra were collected using 10 laser pulses per spectrum (at 4–5 mJ/pulse) and were interpreted as described previously (Madsen *et al*., 2011).

Quantitative PCR methods

Primers for semi-quantitative and quantitative PCR (qPCR) were designed using the Primer-BLAST tool (NCBI) and are listed in Table S2. Semi-quantitative PCR was performed by amplifying cDNA obtained from samples cultured in the conditions or with the p*pmrA*, p*phoP* and p*colR* expression constructs as indicated, using primers specific for $eptA_{Pa}$ or *clpX* as a reference gene (Palmer *et al*., 2005). qPCR was performed in a OneStep thermocycler (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions, as described previously (Pfaffl, 2001; Nowicki *et al*., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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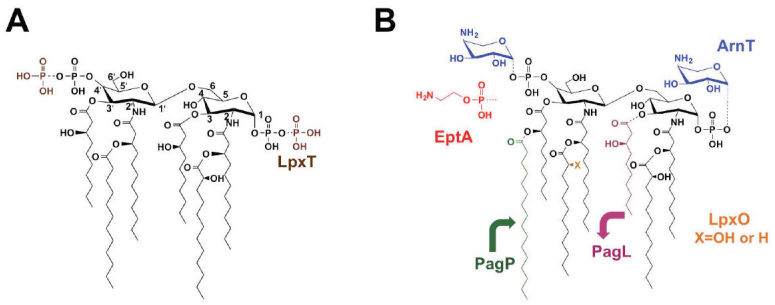

Fig 1.

Lipid A structure of *P. aeruginosa* with and without inducible modifications. A) Canonical, hexa-acylated, *bis*-phosphorylated lipid A structure is shown in black. Phosphorylation of the lipid A phosphate groups by LpxT, which occurs in standard growth media, is indicated in brown. B) Inducible modifications to *P. aeruginosa* lipid A are indicated in color, including addition of a palmitate chain by PagP (green), removal of the 3-hydroxydecanoate acyl chain by PagL (pink), hydroxylation of the C12 secondary acyl chain(s) by LpxO (orange), addition of L-Ara4N at the lipid A phosphate groups by ArnT (blue), and pEtN addition by EptA (red).

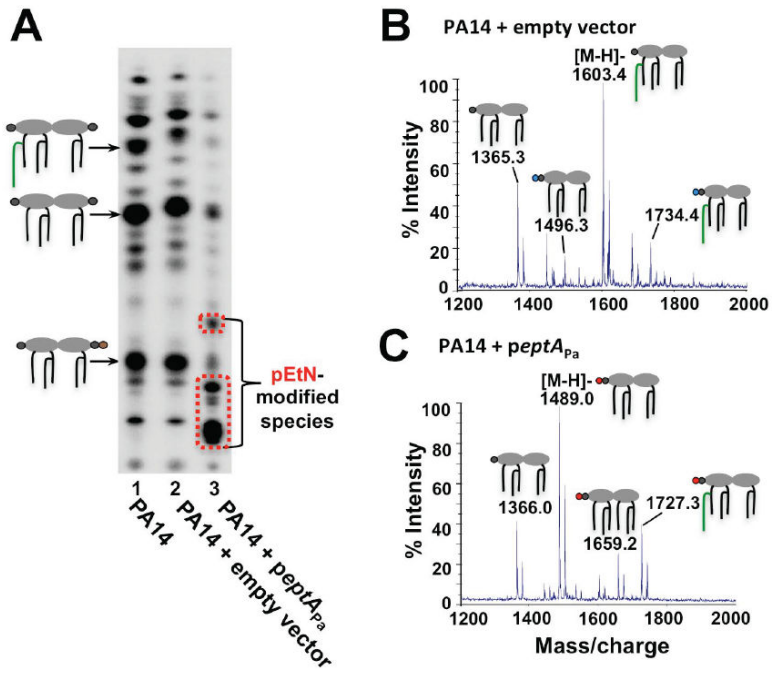

Fig 2.

Heterologous expression of a *P. aeruginosa eptA* ortholog results in pEtN addition to the lipid A. A) Cells were grown in MOPS minimal medium. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Expression of PA14_39020 (*eptA*$_{Pa}$) in *P. aeruginosa* results in modified lipid A species. B) MALDI-TOF MS analysis of PA14 + empty vector grown in MOPS minimal medium reveals no pEtN addition to the molecule, while C) analysis of PA14 + p*eptA*$_{Pa}$ shows pEtN modification of the lipid A. The fractions most representative of pEtN modification are shown.

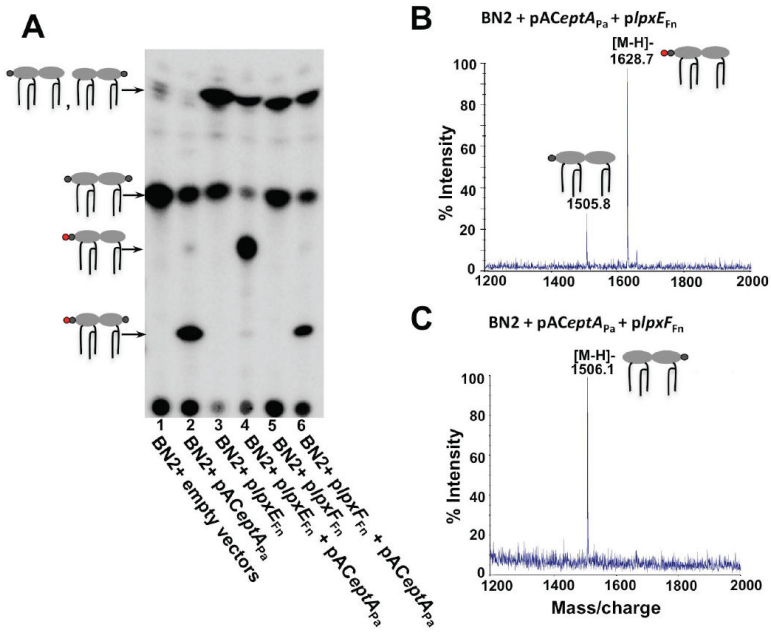

Fig 3.

EptA$_{Pa}$ adds pEtN exclusively to the lipid A 4' phosphate group. A) Cells were grown in LB broth. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Heterologous expression of *eptA*$_{Pa}$ in BN2 results in a pEtN-modified species, while expression of either the *lpxE*$_{Fn}$ or *lpxF*$_{Fn}$ phosphatase results in an increased of monophosphorylated species. Co-expression of *lpxE*$_{Fn}$ and *eptA*$_{Pa}$ results in pEtN addition to the 1-dephosphorylated lipid A molecule, while no pEtN addition of 4' –dephosphorylated species is detected. B) MALDI-TOF analysis of lipid A isolated from BN2 coexpressing *lpxE*$_{Fn}$ and *eptA*$_{Pa}$ corroborates the presence of a monophosphorylated, pEtN-modified species. C) MALDI-TOF analysis of lipid A isolated from BN2 coexpressing *lpxF*$_{Fn}$ and *eptA*$_{Pa}$ reveals that when the 4' phosphate group is removed, pEtN addition does not occur.

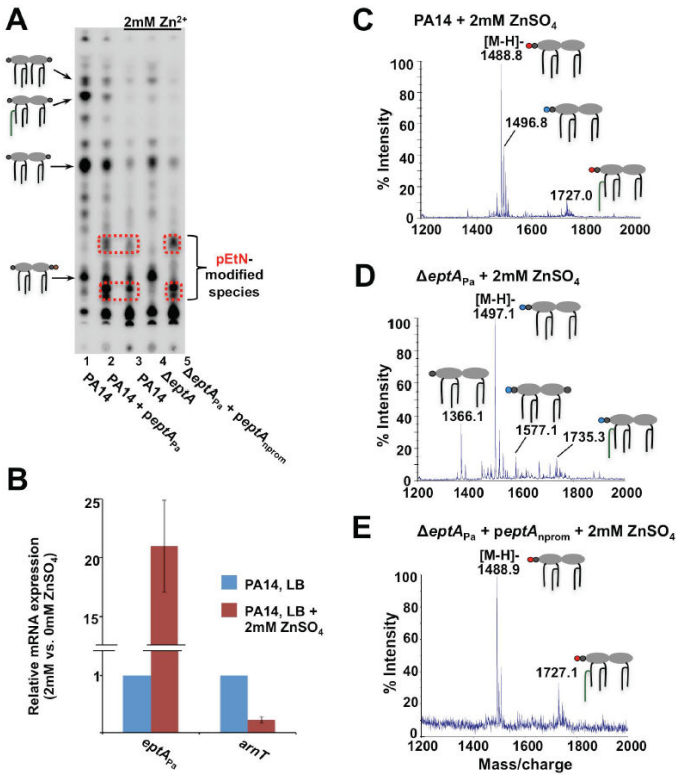

Fig 4.

Zn^{2+} induces transcription of *eptA*$_{Pa}$. A) Cells were grown in LB broth. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Both heterologous expression of *eptA*$_{Pa}$ as well as addition of 2mM $ZnSO_4$ to the media results in pEtN addition to lipid A. This modification is not detectable in the *eptA*$_{Pa}$ mutant, but restored upon complementation with p*eptA*$_{nprom}$. B) Relative gene expression of *eptA*$_{Pa}$ and *arnT* in response to Zn^{2+}. Transcription of *eptA*$_{Pa}$ is induced by 2mM $ZnSO_4$ approximately 21-fold. Zn^{2+} downregulates *arnT* transcription by >4-fold. Ratios were standardized relative to expression of the housekeeping control gene, *clpX*. C), D) and E). MALDI-TOF MS analysis of lipid A prepared from cells grown in LB broth. C) Analysis of PA14 + 2mM $ZnSO_4$ reveals pEtN addition to lipid A, while D) Δ*eptA*$_{Pa}$ + 2mM $ZnSO_4$ shows no pEtN modification, but instead L-Ara4N addition. E) Complementation of Δ*eptA*$_{Pa}$ with p*eptA*$_{nprom}$ restores pEtN addition to the lipid A in response to Zn^{2+}. The fractions most representative of pEtN modification are shown.

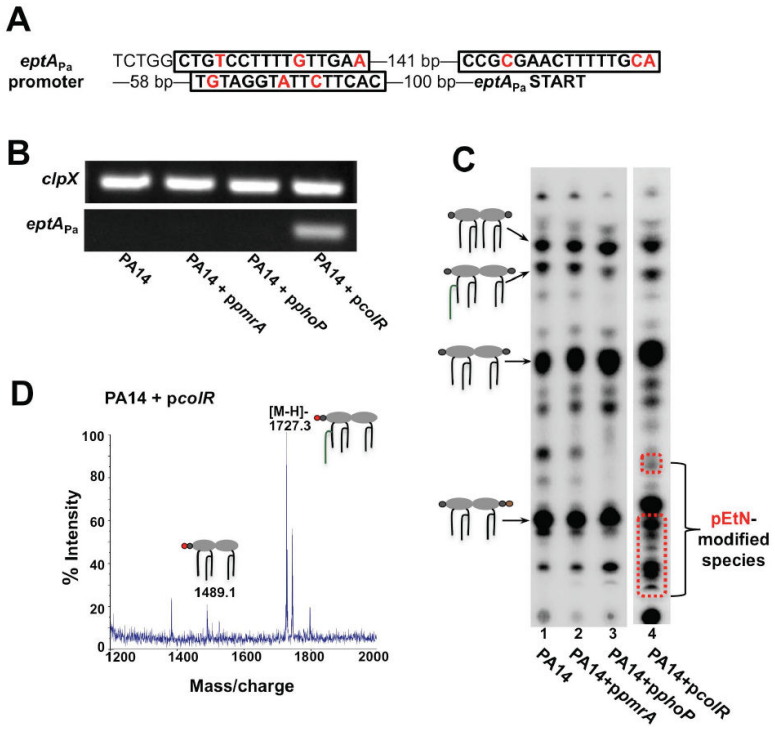

Fig 5.

The two-component system response regulator ColR activates *eptA*$_{Pa}$ transcription. A) Putative *eptA*$_{Pa}$ promoter ColR binding sites are in bold and boxed; nucleotides that deviate from the conserved recognition sequence in *P. putida* ((T/C)(T/C)NA(C/G)NN(T/C)TTTTT(C/G)AC) are indicated in red. The number of base pairs between ColR sites or upstream of the start codon is indicated. B) Semi-quantitative RT-PCR of cDNA prepared from cells grown in MOPS minimal medium. While *eptA*$_{Pa}$ is not transcribed in PA14, expression of *colR in trans* results in *eptA*$_{Pa}$ transcription. C) Lipid A was isolated from ^{32}P-labeled cells grown in MOPS minimal medium and separated by TLC. Only expression of the *colR* response regulator, and not *pmrA* or *phoP*, results in pEtN modification of lipid A. D) MALDI-TOF MS analysis of PA14 + p*colR* grown in MOPS minimal medium reveals pEtN-modified lipid A. The fraction most representative of pEtN modification is shown.

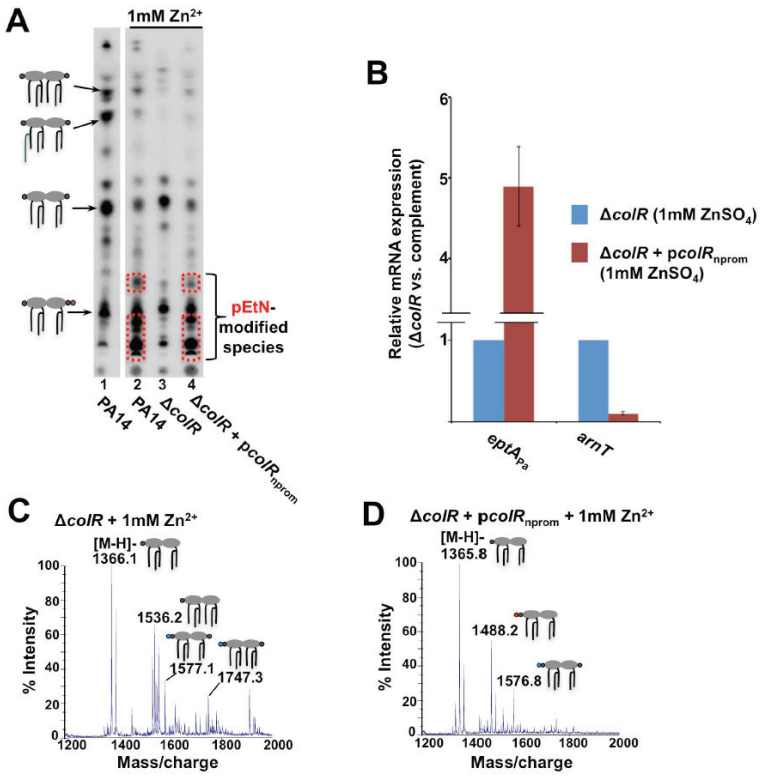

Fig 6.

Deletion of *colR* results in loss of Zn^{2+}-induced pEtN modification of *P. aeruginosa* lipid A. A) Lipid A was isolated from ^{32}P-labeled cells grown in LB broth and separated by TLC. While pEtN modification of lipid A is detectable for PA14 + 1mM $ZnSO_4$, no such modification occurs in PA14Δ*colR* in response to Zn^{2+}. Modification is restored in the complemented mutant. B) Relative gene expression of $eptA_{Pa}$ and *arnT* in response to Zn^{2+} in the Δ*colR* mutant or complemented mutant. Transcription of $eptA_{Pa}$ in the presence of 1mM $ZnSO_4$ is induced >4-fold in a ColR-dependent manner. An approximately 10-fold decrease in *arnT* transcription in the presence of 1mM $ZnSO_4$ is also dependent on ColR. Ratios were standardized relative to expression of the housekeeping control gene, *clpX*. C) and D). MALDI-TOF MS analysis of lipid A prepared from cells grown in LB broth. C) No pEtN modification is detected in the PA14Δ*colR* mutant grown in LB + 1mM $ZnSO_4$. D) Complementation of PA14Δ*colR* with p$colR_{nprom}$ restores the Zn^{2+}-dependent pEtN addition to the lipid A. The fractions most representative of pEtN modification are shown.

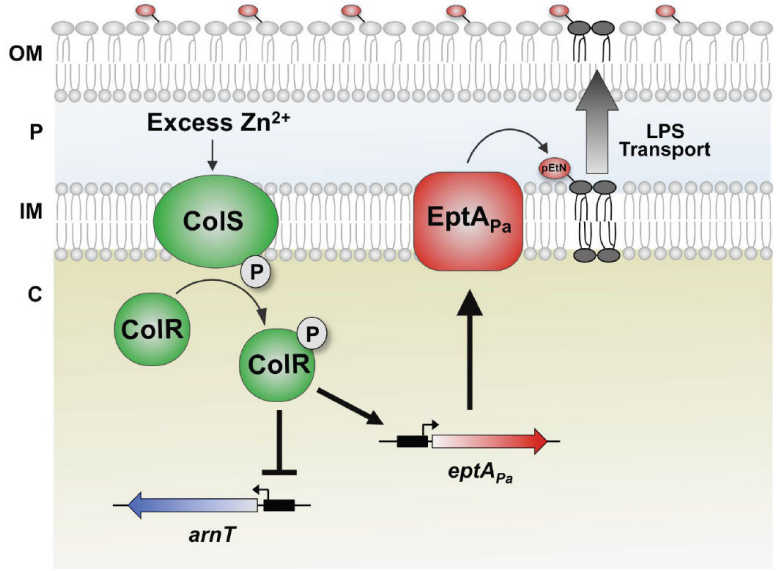

Fig 7.

Proposed model of pEtN addition to *P. aeruginosa* lipid A. Upon sensing excess Zn^{2+}, the ColS sensor kinase (green) autophosphorylates and transfers a phosphate group to the response regulator ColR (green). ColR then acts as a transcription factor, inducing transcription of *eptA*$_{Pa}$ (red) while inhibiting that of *arnT* (blue). EptA$_{Pa}$ protein is synthesized and transfers pEtN to the 4′-phosphate group of lipid A in the inner membrane. Lipid A is then transported to the bacterial cell surface. Following transport to the outer membrane, the 3-hydroxydecanoate acyl chain is removed by PagL (indicated in the model). In some instances, PagP can modify the lipid A (not shown). Cellular components are labelled as follows: OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm).