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Bordetella pertussis Lipid A Glucosamine Modification Confers Resistance to Cationic Antimicrobial Peptides and Increases Resistance to Outer Membrane Perturbation

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***Bordetella pertussis*, the causative agent of whooping cough, has many strategies for evading the human immune system. Lipopolysaccharide (LPS) is an important Gram-negative bacterial surface structure that activates the immune system via Toll-like receptor 4 and enables susceptibility to cationic antimicrobial peptides (CAMPs). We show modification of the lipid A region of LPS with glucosamine increased resistance to numerous CAMPs, including LL-37. Furthermore, we demonstrate that this glucosamine modification increased resistance to outer membrane perturbation.**

Cationic antimicrobial peptides (CAMPs) comprise an important human innate immune defense against pathogens (1). *Bordetella pertussis*, the causative agent of whooping cough, colonizes the human respiratory tract and has to contend with numerous bacterial clearance mechanisms, including several CAMPs (1, 2). Modification of lipid A, the membrane-proximal region of lipopolysaccharide (LPS) that makes up the outer layer of the outer membrane (OM), is one strategy used by select Gram-negative bacteria to confer resistance to CAMPs.

We have previously shown that LgmA, LgmB, and LgmC are required to modify the lipid A of *B. pertussis* with glucosamine (GlcN) moieties at the phosphate groups and that this modification increases human Toll-like receptor 4 (TLR4) activation by LPS (3, 4). Here, we investigate the effect of the GlcN modification on resistance to various CAMPs (Table 1) and resistance to OM perturbation.

Briefly, *B. pertussis* strains BP338 and the $GlcN^-$ isogenic strain BP338lgmABCDKO (hereby referred to as the GlcN mutant), were grown in Stainer-Scholte (SS) broth for 24 to 30 h, as previously described (3). Bacteria were diluted in SS salts (10.72 g glutamic acid, 0.24 g proline, 2.50 g NaCl, 0.50 g KH_2PO_4, 0.20 g KCl, 0.10 g $MgCl_2 \cdot 6H_2O$, 0.02 g $CaCl_2$, 3.175 g Tris-HCl, 0.59 g Tris base dissolved in 1 liter of distilled deionized water [pH 7.6]) to an optical density at 600 nm (OD_{600}) of 0.002, mixed in equal volume with the killing agent in polypropylene 96-well plates, and incubated at 37°C for 2 h. Subsequently, bacteria were plated onto Bordet-Gengou (BG) agar, grown at 37°C for 72 h, and enumerated to determine percent survival (5).

Compared to the GlcN mutant, a greater percentage of *B. pertussis* BP338 survived exposure to the different CAMPs (Fig. 1A), including LL-37, a CAMP found in human neutrophil granules and airway surface liquid (1). GlcN-modified lipid A provided greater resistance to some CAMPs than to others (Fig. 1A), though no pattern was observed based on the charge of the CAMPs at physiological pH. However, we noted greater resistance to the polymyxins, which are cyclic, lipidated CAMPs, and this is likely due to different interactions between lipid A and peptides of different sequences and structures. Complementation of the GlcN mutant restored GlcN modification of lipid A (3) and also restored wild-type levels of polymyxin B resistance (Fig. 1B). We observed no difference in susceptibility between these two *B. pertussis* strains to the positively charged aminoglycoside antibiotic gentamicin (Fig. 1A). This highlights the specificity of the GlcN modification resistance mechanism to CAMPs. We previously suggested GlcN-modified lipid A did not affect polymyxin B resistance in *B. pertussis* (4). However, in that study, the bacteria were grown on BG agar, which results in decreased transcription of *lgmA* and *lgmB* (BP0399 and BP0398, respectively) (6). Thus, the lipid A of these bacteria was likely not optimally modified with GlcN.

B. pertussis shows a range of susceptibility to numerous

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TABLE 1 CAMPs and antibiotics used in this study

CAMP or antibiotic (source)	Sequence	Net charge[a]	Structure	Class
Polymyxin B (Sigma-Aldrich)		+5	Cyclic, lipidated	Polymyxin
Polymyxin E (Sigma-Aldrich)		+5	Cyclic, lipidated	Polymyxin
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6	α helical	Cathelicidin
Indolicidin	ILPWKWPWWPWRR-NH_2	+3	Extended	Cathelicidin
HHC-10	KRWWKWIRW-NH_2	+3	α helical	Synthetic
CP28	KWKLFKKIGIGAVLKVLTTGLPALKLTK-NH_2	+7	α helical	Insect hybrid
Gentamicin (Sigma-Aldrich)		+5	Trisaccharide	Aminoglycoside

[a] The estimated charges are at pH 7.0 and expressed to the nearest integer value.

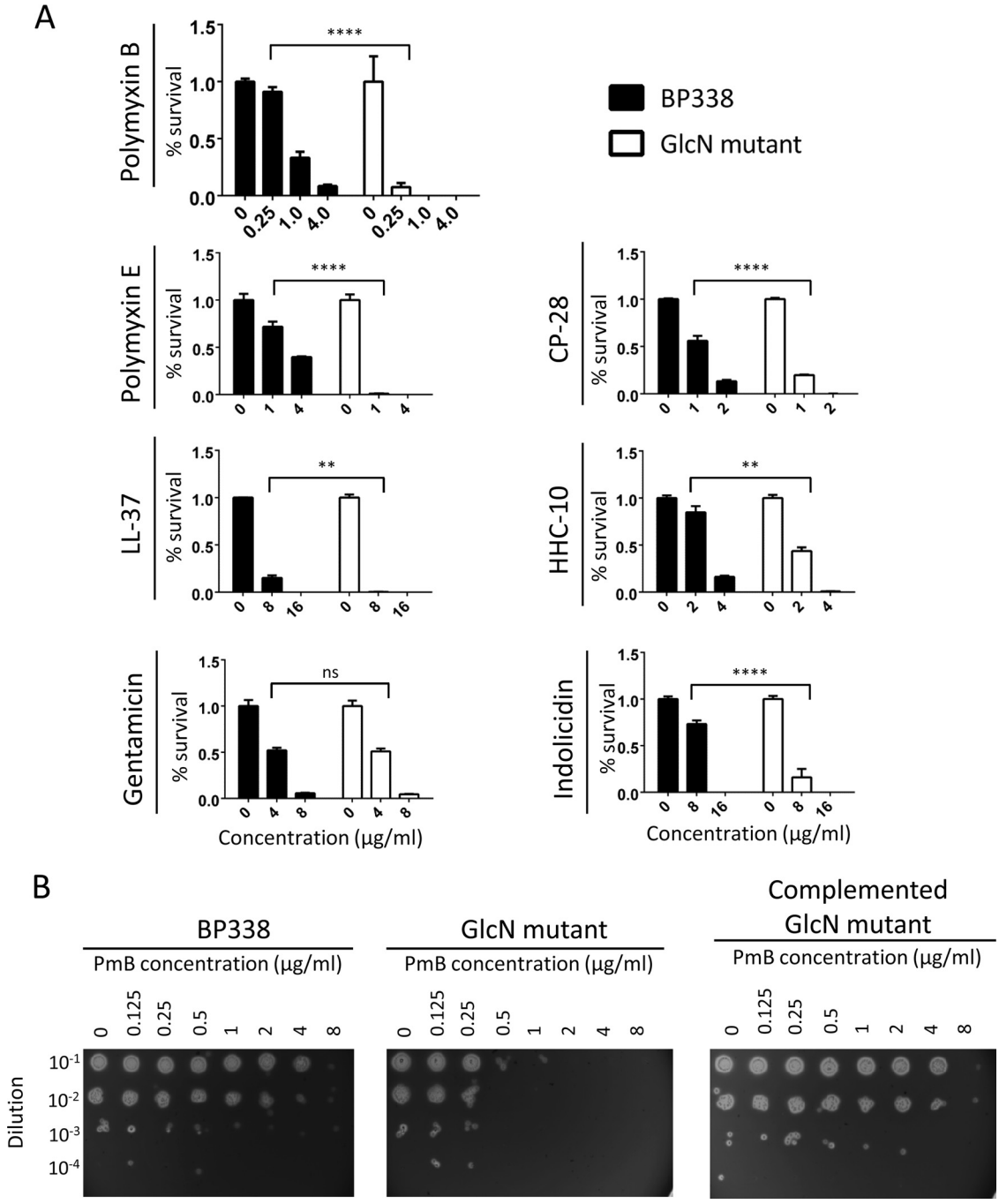

FIG 1 Influence of the *B. pertussis* lipid A GlcN modification on CAMP susceptibility. (A) *B. pertussis* lipid A GlcN modification increased resistance to CAMPs. *B. pertussis* BP338 and GlcN mutant strains were incubated with a range of CAMPs and antibiotics, including bacterial polymyxin B, bacterial polymyxin E (colistin), human LL-37, insect CP28, synthetic HHC-10, bovine indolicidin, and gentamicin. The minimum bactericidal concentrations (in micrograms per milliliter as determined by the assay depicted in panel B) for bacterial polymyxin B, bacterial polymyxin E (colistin), human LL-37, insect CP28, synthetic HHC-10, bovine indolicidin, and gentamicin are as follows (value for BP338 and then the value for the GlcN mutant): 8.0 and 2.0, >16 and 4.0, 32.0 and 16.0, 8.0 and 8.0, 8.0 and 8.0, 32.0 and 32.0, and 16.0 and 16.0, respectively. Gentamicin was used as a control. $n = 3$ for each sample. The results of one representative experiment of three experiments are shown. Data were analyzed with GraphPad Prism 6. Statistical significance was determined by analysis of variance (ANOVA), with a Bonferroni posttest to compare the values for the groups. Values that were significantly different are indicated by asterisks as follows: ****, $P < 0.0001$; **, $P < 0.01$. Values that were not significantly different (ns) are indicated. (B) Complementation of the *B. pertussis* GlcN mutant rescues resistance to polymyxin B. *B. pertussis* BP338, the GlcN mutant, and the GlcN mutant complemented with *lgmABCD* (BP338*lgmABCD*KO plus pPtacLgmABCD) (3) were incubated with a range of polymyxin B (PmB) concentrations, followed by 1/10 dilution, and 2-μl portions of these dilutions were spotted onto BG agar and grown at 37°C for 72 h to observe bacterial survival. The results of one representative experiment of three experiments are shown. Images were converted to gray scale from color images with PowerPoint (Microsoft).

CAMPs (7), and porcine beta-defensin 1 (pBD1) protects against *B. pertussis* infection in a newborn piglet model, demonstrating the protective capability of CAMPs against this pathogen (8). Recently, an LPS-independent mechanism in *B. pertussis* was also shown to increase CAMP resistance (9). *B. pertussis* has thus evolved at least two separate mechanisms for CAMP resistance, suggesting that this strategy plays an important role in the survival of this human-restricted pathogen.

Specific lipid A modifications in different species do not always have the same effect (10). In this context, the recent findings that the *arnT* (*Bordetella bronchiseptica lgmB* [$lgmB_{Br}$]) gene in *Bordetella bronchiseptica* controls resistance to polymyxin B and pBD1 (11), may not have applied *a priori* to *B. pertussis*, especially considering the differences in LPS between these two species. *B. pertussis* has penta-acyl LPS with no O antigen, whereas *B. bronchiseptica* LPS is hexa-acylated and has a long O antigen, which has been shown to confer resistance to CAMPs (6, 11, 12). Despite this variation, GlcN modification still confers resistance to polymyxin B in both these species. When comparing the effect on TLR4 activation in the natural hosts—humans versus small mammals, the $LgmB_{Br}$-mediated modification in *B. bronchiseptica* does not affect activation of mouse TLR4, whereas the GlcN modification in *B. pertussis* increases activation of human TLR4 (4, 11). This adds a layer of complexity in the *B. pertussis* and human system that is not present in *B. bronchiseptica* and mice.

The OM provides a barrier to many antimicrobial factors in the airway, such as lysozyme, which needs to permeate the OM to access its substrate peptidoglycan (1). CAMPs interact with the OM at sites where adjacent LPS molecules are bridged by divalent cations, causing perturbation of the OM and consequent self-promoted uptake of the CAMP (13). We tested the effect of lipid A GlcN modification on protection against OM perturbation by incubating *B. pertussis* BP338 and the GlcN mutant with EDTA, lysozyme, or both. EDTA destabilizes the OM by chelating cations that bridge negatively charged phosphate groups of lipid A (13). We found that strain BP338 was more resistant to killing by EDTA alone than the GlcN mutant was (Fig. 2). This shows that the GlcN modification increased resistance to OM perturbation. There was no significant difference in survival when incubated with lysozyme alone. However, membrane perturbation by EDTA rendered both bacterial strains more susceptible to killing by lysozyme, although the GlcN mutant had a larger decrease in survival than BP338 did.

We hypothesize that in wild-type *B. pertussis*, which modifies the phosphates of a proportion of its lipid A with GlcN, these positively charged GlcN groups coordinate negatively charged phosphate groups on other unmodified lipid A molecules, thereby stabilizing the OM, even in the absence of cations. Stabilization of the OM is critical for protection against infection-clearing agents in the airway, such as lysozyme (1, 13). Agents in the respiratory tract, such as lactoferrin, destabilize the OM, thus allowing lysozyme greater access to peptidoglycan (1). This type of synergy has been found between many antimicrobial factors in the airway (1). Therefore, the OM stabilization afforded by lipid A GlcN modification may also protect *B. pertussis* against lysozyme and other infection-clearing mechanisms.

In conclusion, we found that the *B. pertussis* lipid A GlcN modification protects against numerous CAMPs and OM perturbation. Recently, the GlcN modification in *B. bronchiseptica* was linked to successful transmission between mice and colonization at lower infectious doses (11). It remains to be seen whether this also holds true for *B. pertussis* infections in humans.

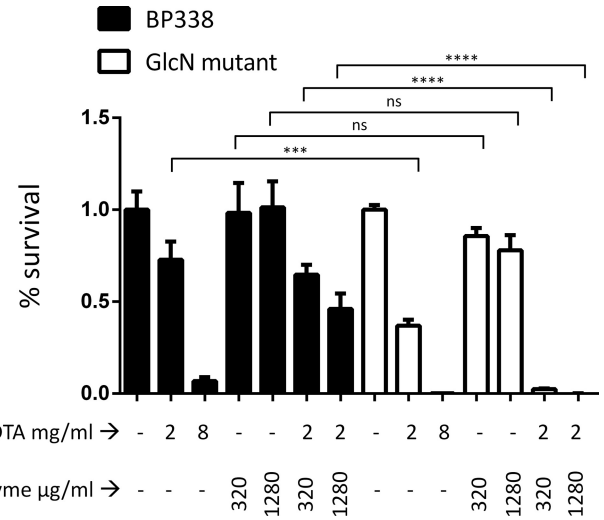

FIG 2 Influence of the *B. pertussis* lipid A GlcN modification on resistance to OM perturbation. *B. pertussis* lipid A GlcN modification increased resistance to OM perturbation by comparing EDTA, lysozyme, and EDTA plus lysozyme. *B. pertussis* BP338 and GlcN mutant strains were incubated with a range of EDTA, lysozyme, and EDTA plus lysozyme. $n = 3$ for each sample. The results from one representative experiment of three experiments are shown. Data were analyzed with GraphPad Prism 6. Statistical significance was determined by ANOVA, with a Bonferroni posttest to compare the values for the groups. Values that were significantly different are indicated by asterisks as follows: ****, $P < 0.0001$; ***, $P < 0.001$. Values that were not significantly different (ns) are indicated.

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