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## Removal of the outer Kdo from *Helicobacter pylori* lipopolysaccharide and its impact on the bacterial surface

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### Abstract

*Helicobacter pylori* produces a unique surface lipopolysaccharide (LPS) characterized by strikingly low endotoxicity that is thought to aid the organism in evading the host immune response. This reduction in endotoxicity is predicted to arise from the modification of the Kdo-lipid A domain of *Helicobacter* LPS by a series of membrane bound enzymes including a Kdo (3-deoxy-D-manno-octulosonic acid) hydrolase responsible for the modification of the core-oligosaccharide. Here we report that Kdo hydrolase activity is dependent upon a putative two-protein complex composed of proteins Hp0579 and Hp0580. Inactivation of Kdo hydrolase activity produced two phenotypes associated with cationic antimicrobial peptide (CAMP) resistance and O-antigen expression. Kdo hydrolase mutants were highly sensitive to polymyxin B, which could be attributed to a defect in downstream modifications to the lipid A 4'-phosphate group. Production of a fully extended O-antigen was also diminished in a Kdo hydrolase mutant, with a consequent increase in core-lipid A. Finally, expression of O-antigen Lewis X and Y epitopes, known to mimic glycoconjugates found on human tissues, was also affected. Taken together, we have demonstrated that loss of Kdo hydrolase activity affects all three domains of *H. pylori* LPS, thus highlighting its role in the maintenance of the bacterial surface.

### Introduction

*Helicobacter pylori* is a Gram-negative bacterium with only one well defined niche, the human stomach. After colonizing the stomach *H. pylori* can persist for several years without manifesting any symptoms, although over time serious sequelae can appear, including peptic ulcer disease and gastric cancer (Blaser, 1996, Parsonnet *et al.*, 1991). Like the majority of Gram-negative bacteria the outer surface of *H. pylori* is composed primarily of lipopolysaccharide (LPS), consisting of three domains known as lipid A, core and O-antigen.

The core and O-antigen represent the polysaccharide component of LPS. The core sugars are generally well conserved within a bacterial species; however, the O-antigen can show great diversity. *H. pylori* O-antigen expression is highly variable and generally mimics human blood group antigens (Appelmelk *et al.*, 1998, Wang *et al.*, 2000). This molecular mimicry

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is thought to contribute to the chronic nature of *H. pylori* infections by making the bacterium appear like its host and preventing detection by the immune system (Wirth *et al.*, 1997, Appelmelk *et al.*, 2000). *H. pylori* O-antigen has also been implicated in other aspects of pathogenesis, including adhesion to gastric epithelial cells and immune system modulation, mediated by interaction with the C-type lectin DC-SIGN (Bergman *et al.*, 2004).

The lipid A domain of LPS acts as a hydrophobic anchor holding the LPS molecule in the outer membrane. Lipid A is also responsible for the endotoxic properties associated with LPS. Biosynthesis of lipid A is well conserved throughout Gram-negative bacteria and proceeds via a nine-step enzymatic pathway (Raetz & Whitfield, 2002). Despite its conserved synthesis, a great deal of variation is seen when comparing the lipid A structures of various Gram-negative bacterial species (Raetz *et al.*, 2007). This variation is generated primarily by the action of lipid A modification enzymes and, with human pathogens, may well be a consequence of evolutionary pressure applied by the human innate immune system. For example, the modification of phosphate groups present on the lipid A disaccharide backbone provides resistance to cationic anti-microbial peptides (CAMPs). CAMPs are small positively charged peptides that bind to negatively charged structural motifs (e.g. lipid A) present on the surface of bacteria leading to eventual cell lysis and death (Diamond *et al.*, 2009). Gram-negative bacteria resist the action of CAMPs by adding positively charged substituents, such as phosphoethanolamine or L-4-aminoarabinose, to the negatively charged phosphate groups of lipid A or by removing the phosphate groups of lipid A (Tran *et al.*, 2006, Gunn *et al.*, 1998).

In a number of organisms lipid A modifications are regulated and only occur after specific environmental cues are detected; however, it appears that in the case of *H. pylori*, lipid A modifications are constitutive. *H. pylori* lipid A modification is a complex process that occurs via a five step enzymatic pathway (Fig. 1) and in the laboratory a single distinct lipid A species is produced. A lack of regulation could be explained by the fact that *H. pylori* has only one known reservoir, the human stomach, thus rendering adaptation unnecessary. However, it is not possible to rule out whether or not the organism is capable of changing its lipid A domain within its human host.

Several of the *H. pylori* lipid A modification enzymes have already been characterized by previous studies from our laboratory, including the identification of a novel 3-deoxy-D-manno-octulosonic acid-hydrolase (Kdo-hydrolase) enzymatic activity present in *H. pylori* membranes (Stead *et al.*, 2005). The Kdo-hydrolase removes the outer Kdo sugar from the core region, with optimal activity occurring after the prior removal of the 1-phosphate group by a dedicated inner membrane phosphatase encoded by *hp0021*. The presence of a single Kdo sugar had previously been attributed to a mono-functional Kdo transferase (KdtA). Investigations into *H. pylori* KdtA functionality proved it to be bi-functional, indicating that it was no longer possible to predict how many Kdo sugars a particular KdtA may transfer based upon characterization of the Kdo-lipid A species alone. A similar situation was thought to exist in *Francisella novicida*, which has also been reported to produce a Kdo-hydrolase (Wang *et al.*, 2004).

Although the Kdo-hydrolase enzymatic activity is well characterized, the gene responsible for encoding the enzyme has yet to be determined. Here we report the identification of the Kdo-hydrolase of *Helicobacter pylori* and show that Kdo-hydrolase activity is, in fact, dependent upon two gene products. We also demonstrate that Kdo-hydrolase activity is necessary for subsequent lipid A modifications (Fig. 1), resistance to CAMPs, as well as efficient O-antigen expression, indicating a key role in *H. pylori* pathogenesis.

## Results

### The *H. pylori* Kdo-hydrolase is encoded by hp0579 & hp0580

Given that membranes isolated from both *Helicobacter pylori* and *Francisella novicida* had been shown to catalyze the removal of the outer Kdo sugar from Kdo<sub>2</sub>-lipid A substrates (Stead et al., 2005, Wang et al., 2004), a genomic comparison of the two organisms was performed to identify a possible gene encoding for the Kdo hydrolase (see accompanying manuscript by Raetz and Zhao). The genomic comparison yielded a candidate gene annotated as *FTN\_0495* in *Francisella* and *hp0580* in *Helicobacter* strain 26695 (Fig. S1). PSI-BLAST (Altschul et al., 1997) analysis shows that the soluble C-terminal domain of both Hp0580 (Fig. S2) and its counterpart in *Francisella* show distant homology to the sialidase NanI (Newstead et al., 2008) produced by *Clostridium perfringens* (see Fig. S3 of the accompanying manuscript, Zhao and Raetz). Sialidases function to bind and hydrolyze a terminal monosaccharide from glycoconjugates, lending support to the prediction of Hp0580 functioning in the cleavage of the outer Kdo sugar. *Hp0580* was cloned into pET21a, heterologously expressed in *E. coli* HMS174 and membranes were isolated for use in an *in vitro* assay. The *in vitro* assay system makes use of radiolabeled lipid A substrates, which are incubated with an enzyme source followed by separation of the reaction products via thin layer chromatography (TLC). The solvent used for TLC separates species according to hydrophobicity, such that more hydrophobic products migrate faster. In this case we utilized Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate and membranes isolated from HMS174/pHp0580 as the enzyme source. After analysis of the reactant products by TLC, we were surprised to see no removal of a Kdo sugar from the starting substrate (Fig. 2, lane 5). Membranes from *H. pylori* showed Kdo hydrolase activity (lane 2) and served as the positive control.

This anomalous result prompted closer inspection of the *FTN\_0495* and *hp0580* genomic regions. In the case of *Francisella* it appeared that *FTN\_0495* could be transcribed as part of an operon, which contained only one other gene, annotated as *FTN\_0494*, with no predicted function. The genome of *H. pylori* showed a similar organization, with the gene encoding the putative sialidase (*hp0580*) possibly organized in an operon with *hp0579* (Fig. S1). Unlike *hp0580*, *hp0579* is not homologous to its *Francisella* counterpart *FTN\_0494*. However, hydropathy analysis of *hp0579* and *FTN\_0494* indicated that both proteins contain multiple transmembrane domains (6 or 7) absent of additional notable structural features (Fig. S2). Therefore, we speculated that *hp0579* might also be required for Kdo hydrolase activity in *Helicobacter*. Both *hp0579* and *hp0580* were cloned into pET21a and overexpressed for use in an *in vitro* assay. When using Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate and membranes isolated from HMS174/pHp0579-80 as the enzyme source, we observed a robust Kdo hydrolase activity (Fig. 2, lane 6), suggesting that both Hp0579 and Hp0580 were required for Kdo hydrolase activity. Confirmation that the product spot was in fact generated by a Kdo hydrolase was provided after performing mass spectrometry on the products from a large-scale Kdo hydrolase reaction (Fig S3). To demonstrate that Hp0579 alone wasn't responsible for the Kdo hydrolase activity, *hp0579* was cloned into pET21a and assayed using Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate and, as expected, no Kdo hydrolase activity was apparent (Fig. 2, lane 4). Kdo hydrolase activity could also be generated by mixing membranes isolated from HMS174/pHp0579 with membranes isolated from HMS174/pHp0580 in the presence of Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A substrate (Fig. 2, lane 7), further supporting evidence for a two-protein enzymatic complex.

The results obtained with heterologous expression of *hp0579* and *hp0580* prompted us to generate mutations in *H. pylori*. Two *H. pylori* strains were chosen for this study: strain G27 for its ability to be readily transformed and the gerbil adapted strain 7.13 for its potential in animal studies (Censini et al., 1996, Franco et al., 2005). Furthermore, it is important to confirm results in a second strain due to the high degree of phase variation found with *H.*

*pylori*, which can lead to strain specific phenotypes (Tran et al., 2006). Mutations were made in the catalytic subunit (*hp0580*) along with a double mutation, which removed both *hp0579* and *hp0580*, as described in experimental procedures. Each of these mutations were also complemented chromosomally by insertion of the relevant gene(s) into the *rdxA* locus. Although G27 is now sequenced, this was not the case when these mutants were constructed and consequently complemented, which necessitated the use of strain 26695 genome sequence (Baltrus *et al.*, 2009). The G27 homolog of Hp0580 shows 93% identity (E-value of 0.0 with 96% coverage) and the Hp0579 homolog shows 96% identity (E-value of  $1 \times 10^{-82}$  with 97% coverage) to those of *H. pylori* 26695. Membranes were isolated from each of the mutants and complemented mutants for use in an *in vitro* assay, using 1-dephosphorylated-Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate. The assay clearly showed complete loss of Kdo hydrolase function in both strains G27 and 7.13 for the *hp0580* single mutant (Fig. 3, lanes 3 & 8) and the *hp0579-hp0580* double mutant (Fig. 3, lanes 5 & 10). Kdo hydrolase activity was fully restored in each of the complemented mutants for both strains (Fig. 3, lanes 4, 6, 9 & 11), ruling out any polar effects. This data, along with the heterologous expression data, demonstrates that Hp0579 and Hp0580 are responsible for removal of the Kdo sugar and that both proteins are required for activity.

### Demonstration of Kdo hydrolase activity *in vivo*

Analysis of Kdo hydrolase activity *in vivo* is complicated by the fact that standard lipid A analyses, such as radiolabeling or mass spectrometry, rely upon removal of the polysaccharide portion of LPS for lipid A isolation. The polysaccharides are removed by mild acid hydrolysis at 100°C, which cleaves the bond between the Kdo sugar and the lipid A backbone, preventing any further analysis of the Kdo sugars. To circumvent this problem, we made use of a previously well characterized *H. pylori* heptosyl transferase mutant (26695 *hp1191::kan*), which produces a severely truncated LPS with only a Kdo and heptose remaining in the core region (Fig. 4A) (Chandan *et al.*, 2007). This truncation allows accurate analysis of LPS via SDS-PAGE, using a 16% tricine gel, to show differences in mass corresponding to one sugar (Lesse *et al.*, 1990). We proceeded to make a mutation in the *hp0579* and *hp0580* genes of strain 26695 *hp1193::kan* to produce the double mutant. One would expect to see a slower migrating LPS species in the heptosyl transferase/Kdo hydrolase double mutant as compared to the heptosyl transferase mutant, because the number of sugar moieties present in the core region should increase from 2 to 3 (Fig. 4A). SDS-PAGE analysis of the LPS from the two mutants displays the expected phenotype (Fig. 4B), confirming the function of Hp0579 and Hp0580, using an *in vivo* system.

### Hp0580 has a periplasmic active site

Our previous studies characterizing the Kdo hydrolase activity present in *H. pylori* membranes indicated that the enzyme utilized a 1-dephosphorylated lipid A substrate when working in the linear range of activity (Stead et al., 2005). The enzyme responsible for removing the 1-phosphate group of *H. pylori* lipid A, Hp0021 (LpxE) (Fig. 1), has been shown to have a periplasmic active site (Tran *et al.*, 2004). This would suggest that Hp0580 also has a periplasmic active site, given its dependence on the prior removal of the 1-phosphate group. Furthermore, one would predict that it would be energetically unfavorable to remove the outer Kdo sugar in the cytoplasm given that both Kdo sugars are assembled into the core oligosaccharide in the same cytoplasmic compartment by the Kdo transferase, WaaA (Brozek *et al.*, 1989). Therefore, we sought to confirm the location of the Hp0580 active site by expressing C-terminal PhoA (alkaline phosphatase) and GFP (green fluorescent protein) fusion proteins (Fig. 5A).

PhoA is only active if present in the periplasm (Hoffman & Wright, 1985) and therefore a C-terminal Hp0580-PhoA fusion protein would display phosphatase activity only if the

Hp0580 soluble sialidase domain was periplasmic. Phosphatase activity can be determined by growing the bacteria on solid agar medium containing the colorimetric substrate X-phosphate, which produces a blue colony phenotype after cleavage by a phosphatase. *E. coli* PhoA mutants expressing either the Hp0580-PhoA fusion or PhoA minus its signal peptide, and the vector control were grown in the presence of X-phosphate. Only the strain expressing the Hp0580-PhoA fusion protein produced blue colonies, indicating that the Hp0580 active site is in fact periplasmic (Fig. 5B). This finding was corroborated with an Hp0580-GFP fusion protein. The inverse scenario to PhoA is true with GFP, in that GFP will fluoresce only if it is folded in the cytoplasm, meaning that the Hp0580-GFP fusion protein will not fluoresce if the Hp0580 active site is periplasmic (Feilmeier *et al.*, 2000). Fluorescence microscopy of *E. coli* expressing the Hp0580-GFP fusion protein revealed no fluorescent bacteria, while the positive control strain expressing cytoplasmic GFP only, produced fluorescent bacteria (Fig. 5C), confirming the periplasmic orientation of the Hp0580 active site.

### Inactivation of the Kdo hydrolase leads to an increase in polymyxin B sensitivity

CAMPs primary mode of action is to bind to the negative charges present on the LPS molecule, prior to exerting their bactericidal activity (Diamond *et al.*, 2009). Kdo sugars contain a carboxylic acid moiety, which may be negatively charged at neutral pH. Therefore, the Kdo hydrolase mutant could increase the net negative charge present at the bacterial surface, thereby increasing sensitivity to CAMPs. We tested this hypothesis using polymyxin B, a common experimental substitute for CAMPs, which has a similar mechanism of action (Gutsmann *et al.*, 2005). Minimum inhibitory concentrations (MICs) were determined for the *hp0579-hp0580* mutants and *hp0579-hp0580* complemented mutants in both strains G27 and 7.13, using Etest<sup>®</sup> strips, as described in experimental procedures. The G27 *hp0579-hp0580* mutant showed over a 250-fold decrease in its polymyxin B MIC (Table 1) and the 7.13 *hp0579-hp0580* mutant showed over a 180-fold decrease in its polymyxin B MIC (Table 1) as compared to wild type levels. The complemented Hp0579-Hp0580 mutants of each strain regained wild type levels of polymyxin B resistance, eliminating the possibility of any downstream polar effects.

This initial experiment utilized the Kdo hydrolase double mutant, which is deficient in both Hp0579 and Hp0580. Hp0580 contains homology to a sialidase and, therefore, is likely responsible for the actual cleavage of the Kdo-Kdo linkage within the LPS core oligosaccharide. Hp0579, however, has no predicted function and, therefore, could also be involved in other distinct processes. To confirm that the increase in polymyxin B sensitivity is a result of the action of the Kdo hydrolase and not a secondary function attributable to Hp0579 we repeated the experiment using the Hp0580 single mutant and Hp0580 complemented mutant. We saw identical results as compared to the Hp0579-Hp0580 mutant and Hp0579-Hp0580 complemented mutant in both strains, indicating that the increase in sensitivity is attributable to the action of the Kdo hydrolase (Table 1).

The dramatic increase in polymyxin B sensitivity of the Kdo hydrolase mutants is comparable to that of the increase in polymyxin B sensitivity observed when lipid A phosphate modifications are disrupted (Tran *et al.*, 2006). This was a surprising result given that a phosphate group carries more negative charges as compared to a carboxylic acid. To explain this phenomenon we hypothesized that loss of Kdo hydrolase function could affect other downstream lipid A modifications, such as removal of the 4'-phosphate group (Fig. 1). The requirement of *H. pylori* lipid A modification enzymes for a specific substrate has been shown previously by our group, lending credence to our hypothesis (Tran *et al.*, 2004, Stead *et al.*, 2005). We isolated lipid A from G27 wild type, the G27 Hp0579-Hp0580 mutant and the G27 Hp0579-Hp0580 complemented mutant and subjected each lipid A species to analysis by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass

spectrometry in the negative ion mode. The wild type spectrum showed a predominant peak at  $m/z$  1547.9 consistent with the  $[M-H]^-$  ion of the wild type structure of *H. pylori* lipid A (predicted  $[M-H]^-$  at  $m/z$  1548.1), which is tetra-acylated without a phosphate group at the 4'-position and a phosphoethanolamine residue at the 1-position (Fig. 6). The Hp0579-Hp0580 mutant spectrum showed two predominant peaks at  $m/z$  1547.7 and  $m/z$  2091.0 (Fig. 6). The peak at  $m/z$  1547.7 is consistent with the  $[M-H]^-$  ion of the wild type structure; however, the peak at  $m/z$  2091.0 is consistent with the  $[M-H]^-$  of a lipid A species that is hexa-acylated with a phosphate group at the 4'-position and a phosphoethanolamine at the 1-position (predicted  $[M-H]^-$  at  $m/z$  2092.5). This indicates that the 4'-phosphatase and 3'-O-deacylase are no longer 100% efficient, suggesting an ordered lipid A modification pathway, determined by enzyme substrate specificity. The Hp0579-Hp0580 complemented mutant displayed a predominant peak at  $m/z$  1547.4, consistent with a wild type lipid A species. These same analyses were completed in strain 7.13 harboring the *hp0579-hp0580* mutation (Fig. S4) and in strains harboring the single *hp0580* mutation (data not shown). In all cases the results were identical to those shown in Fig. 6.

The presence of a lipid A sub-population still possessing a 4'-phosphate group may explain the increase in polymyxin B sensitivity seen with the Kdo hydrolase mutants, although it does not rule out a possible direct contribution to polymyxin B sensitivity brought about by the presence of a second Kdo sugar and its associated carboxylic acid moiety. *Francisella* mutants lacking a 4'-phosphatase also show an increase in polymyxin B sensitivity, which correlates to an attenuation of virulence (Wang *et al.*, 2007), highlighting the importance of such modifications for bacterial survival.

### Kdo hydrolase activity modulates O-antigen expression

Addition of core sugars to the Kdo-lipid A domain occurs at the cytoplasmic membrane, prior to transport of core-lipid A across the inner membrane by MsbA (Raetz & Whitfield, 2002). O-antigen is synthesized, transported across the inner membrane and ligated to the core-lipid A, by the ligase WaaL, to produce the finished LPS molecule (Raetz & Whitfield, 2002). To determine if the presence of a second Kdo sugar, present in a Kdo hydrolase mutant, had any effect on the LPS synthesis pathway we analyzed the LPS profile of each Kdo hydrolase mutant by SDS-PAGE. The resultant gel shows that in both strains G27 and 7.13 the Hp0580 mutant (Fig. 7A, lanes 2 & 7) and Hp0579-Hp0580 mutant (Fig. 7A, lanes 4 & 9) have a reduced amount of fully extended LPS and, inversely, the amount of core-lipid A increases as compared to wild type bacteria (Fig. 7A, lanes 1 & 6). Complementation of the Hp0580 and Hp0579-Hp0580 mutants fully restores the wild type phenotype (Fig. 7A, lanes 3, 5, 8 & 10), ruling out any polar effects.

*H. pylori* O-antigen usually mimics human blood group antigens, although the specific epitope seen can be highly variable (Appelmelk *et al.*, 1998, Wang *et al.*, 2000). Despite the possibility for variation, Lewis X and Lewis Y blood group antigens are the most prevalent epitopes found on the bacterial cell surface (Simoons-Smit *et al.*, 1996). The Lewis X motif is composed of a galactose and N-acetylglucosamine backbone decorated with fucose on the N-acetylglucosamine sugar by the  $\alpha(1,3)$ -fucosyl transferase FutA (Moran, 2008). Lewis Y is a terminal motif with a galactose and N-acetylglucosamine backbone decorated with a fucose on both sugars by the  $\alpha(1,3)$ -fucosyl transferase FutA and the  $\alpha(1,2)$ -fucosyl transferase FutC (Moran, 2008). The presence of poly-C tracts in the fucosyl transferase genes allows the proteins to effectively be 'on' or 'off', providing the mechanism of variation in O-antigen expression (Appelmelk *et al.*, 1999, Wang *et al.*, 1999b).

After observing a reduction in fully extended O-antigen with the Kdo hydrolase mutants, it was logical to see if this reduction also corresponded with a change in blood group antigen expression. To achieve this aim LPS SDS-PAGE gels were blotted to a nitrocellulose

membrane and the O-antigen profile was determined using specific antibodies, as described in the experimental procedures. Wild type G27 expressed both Lewis X & Y antigens (Fig. 7B). Interestingly this was not the case for the Kdo hydrolase mutants. Both the Hp0580 and Hp0579-Hp0580 mutants no longer expressed the Lewis X antigen; however, expression of the Lewis Y antigen was unaffected (Fig. 7B). The wild type phenotype was fully restored in the Hp0580 and Hp0579-Hp0580 complemented mutants (Fig. 7B). To prove that this phenotype was not a consequence of variation in the levels of Lewis X and Y undecaprenyl-linked precursors, each sample was subjected to mild acid hydrolysis followed by SDS-PAGE and immunoblot analysis. Mild acid hydrolysis cleaves undecaprenyl-linked sugars while leaving LPS intact. Figure S5 shows no changes in the Kdo hydrolase mutants after mild acid hydrolysis, indicating that a variation in Lewis antigen expression is not responsible for the observed phenotype. Each strain was also probed with anti-H type 1 (Fig. 7B), anti-Lewis A (data not shown) and anti-Lewis B (data not shown) antibodies and shown not to express any of those epitopes. Not only does the Kdo hydrolase have an effect on the amount of fully extended O-antigen produced, it is also capable of modulating which blood group antigen is presented at the bacterial cell surface.

Strain 7.13 did not have the same phenotype as strain G27 because it did not express the Lewis X or Lewis Y antigens (Fig. 7B). Blots probing with anti-Lewis A and anti-Lewis B antibodies also produced negative results (data not shown). However, we were able to show that wild type strain 7.13 expressed the H type 1 epitope (Fig. 7B). Although the production of an H type 1 epitope did not disappear in the 7.13 Hp0580 and Hp0579-Hp0580 mutants, it was reduced (Fig. 7B), mirroring the results seen in the LPS gel (Fig. 7A).

## Discussion

Modification of the Kdo-Lipid A domain of LPS is a common theme amongst Gram-negative pathogens, which invariably provide a direct benefit to the invading microbe. *H. pylori* is no exception to this rule and produces a highly modified Kdo-lipid A, which provides resistance to the human innate immune system in an *in vitro* setting (Tran et al., 2006). One step of the *H. pylori* Kdo-lipid A modification pathway involves the removal of a Kdo sugar, a trait known to be shared with only one other bacterium – *Francisella tularensis* (Stead et al., 2005, Wang et al., 2004). Although other bacteria can produce lipid A species with only one Kdo sugar, this arrangement is generated by a mono-functional Kdo transferase and the Kdo sugar is consequently modified by the addition of a phosphate containing group (Hankins & Trent, 2009, Isobe *et al.*, 1999, White *et al.*, 1997).

After previously characterizing the *H. pylori* Kdo hydrolase we have now shown that the Kdo hydrolase enzymatic activity is dependent on the presence of two proteins, Hp0579 and Hp0580, *in vitro*. Hp0580 has homology to a sialidase, a family of sugar cleaving enzymes, indicating that Hp0580 is likely responsible for the actual cleavage of the Kdo-lipid A linkage. However, Hp0580 cannot function without the co-expression of Hp0579, suggesting that both proteins work in concert to remove the outer Kdo sugar. This raises questions as to the exact role played by Hp0579 during the cleavage process. Hp0579 has numerous alpha-helical trans-membrane domains (Fig. S2), a feature that is often found in inner membrane transport proteins. Perhaps Hp0580 actually transfers the Kdo sugar to Hp0579, which then shuttles the Kdo sugar across the inner membrane, back to the cytoplasm, for recycling. Another intriguing possibility is that Hp0579 acts as a 'docking station', presenting the Kdo<sub>2</sub>-lipid A substrate to Hp0580 in a specific conformation, making it amenable for cleavage. Although easy to speculate, determining the exact function of Hp0579 will undoubtedly be a challenging project for future research.

What makes the Kdo hydrolase stand out from other *H. pylori* Kdo<sub>2</sub>-lipid A modification enzymes is the downstream effect it has on *H. pylori* CAMP resistance and O-antigen expression. In an *H. pylori* Kdo hydrolase mutant two lipid A species are present, one representing a lipid A species typically seen in wild type *H. pylori* the other a hexa-acylated lipid A species, with an intact 4'-phosphate group. The appearance of a 4'-phosphorylated lipid A sub-population in the Kdo hydrolase mutant goes a long way towards explaining the increase in polymyxin B sensitivity. It is well documented that removal or 'masking' of lipid A phosphate groups is the primary mechanism involved in CAMP resistance (Trent *et al.*, 2006). The reason for inefficient removal of the 4'-phosphate group could be explained by substrate specificity, a mechanism previously shown to be important for Kdo hydrolase function and the *Helicobacter* lipid A 1-phosphatase, Hp0021 (LpxE) (Stead *et al.*, 2005, Tran *et al.*, 2004). Thus, it appears that the enzymatic machinery responsible for these modifications has evolved into a highly ordered pathway producing a lipid A that is perhaps more suited for the unique lifestyle of *H. pylori*.

It has been documented that in *Salmonella* species, ligation of the O-antigen to core-lipid A is specific for a particular core structure (Kaniuk *et al.*, 2004). This specificity was shown not to be imparted by the WaaL ligase itself, but rather by an unknown accessory molecule. *H. pylori* Kdo hydrolase mutants also showed differences in O-antigen expression after alteration to the core region, in the form of an extra Kdo sugar. In both strains G27 and 7.13, production of a fully extended LPS was diminished with the Kdo hydrolase mutants, suggesting that WaaL could no longer function at optimal efficiency. The reduction in fully extended LPS correlated to an increase in core-lipid A, as would be expected. Given that *H. pylori* O-antigen has been well characterized as a virulence factor, it will be interesting to see if merely a reduction in surface exposed O-antigen, as seen with the Kdo hydrolase mutant, has an any impact on pathogenesis, or if a complete truncation is required to see attenuation. However, these results could be complicated by the increased sensitivity of the Kdo hydrolase mutant to polymyxin B.

The G27 Kdo hydrolase mutant also possessed a unique O-antigen phenotype, related to the expression of specific blood group antigens. Wild type G27 expresses both Lewis X and Lewis Y blood group antigens, as shown in Fig. 7B. However, after the introduction of a Kdo hydrolase mutation, only Lewis X expression was affected. Because FutA activity is required for the generation of both epitopes and the fucosyl transferases do not have overlapping activity (Wang *et al.*, 1999a, Ge *et al.*, 1997), this phenomenon cannot be explained by a FutA transcriptional effect. Perhaps strain G27 encodes two WaaL accessory molecules, one specific for Lewis X and the other for Lewis Y, and only the Lewis X accessory molecule is sensitive to the presence of a second Kdo sugar. Recent studies directed towards *H. pylori* WaaL showed that the enzyme was capable of ligating diverse substrates in an *in vitro* assay (Hug *et al.*, 2010); however, because the ligation assay used purified WaaL it is possible that the WaaL accessory molecules were not present in the reaction mixture.

Variation in *H. pylori* O-antigen expression is a well studied process, which has been shown to occur at the level of fucosyl transferase transcription (Appelmek *et al.*, 1999, Wang *et al.*, 1999b). The fucosyl transferases decorate the O-antigen backbone with fucose sugars to produce the blood group antigen epitopes. Each fucosyl transferase gene contains a poly-C tract, which makes transcription prone to mistakes, leading to production of non-functional proteins. Inactivation of a fucosyl transferase produces a distinct clonal population of bacteria, which under favorable conditions can outgrow the progenitor strain and become predominant. Although this system can generate changes in O-antigen expression, it is not capable of producing a rapid response to sudden environmental changes. It is tempting to



speculate that the Kdo hydrolase gene is actually regulated and could turn off unnecessary modifications in an entire bacterial population.

A definitive role for the Kdo hydrolase enzyme has yet to be determined in relationship to *H. pylori* pathogenesis, although we have clearly demonstrated its importance for CAMP resistance and O-antigen expression, two well characterized virulence determinants. Thus, the periplasmic modification of the Kdo domain is critical for maintenance of the *H. pylori* outer surface. We have also demonstrated previously a Kdo hydrolase activity in several *H. pylori* clinical strains (Stead et al., 2005), supporting the importance for a functional Kdo hydrolase enzyme in the *H. pylori* life cycle.

## Experimental procedures

### Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are summarized in Table 2. Primary plate cultures of *H. pylori* were grown from methyl cellulose stocks on blood agar medium at 37 °C for 24–48 h in a microaerobic atmosphere (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>). The resultant colonies were inoculated into Brucella broth supplemented with 7% fetal bovine serum (Hyclone) and vancomycin (10 µg/ml). Cells were grown to an A<sub>600</sub> of ~ 1.0 at 37 °C under microaerobic conditions for 24–48 h. Prior to every experiment, confirmation of *H. pylori* was performed by both Gram stain and urease test (Jerris, 1995). *E. coli* were typically grown at 37 °C in LB broth (Miller, 1972). When required for selection of plasmids, cells were grown in the presence of ampicillin (100 µg/ml). Antibiotics ampicillin (100 µg/ml), kanamycin (8 µg/ml), chloramphenicol (8 µg/ml) and metronidazole (12 µg/ml) were used where appropriate.

### Recombinant DNA techniques

Plasmids were isolated using the QIAprep Spin Minirep Kit (Qiagen). Custom primers were obtained from Integrated DNA Technologies (Table S1). PCR reagents were purchased from Stratagene and PCR products were isolated using Qiaquick PCR Purification Kit (Qiagen). DNA fragments were isolated from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen). All other modifying enzymes were purchased from New England Biolabs and were used according to the manufacturers' instructions.

### Overexpression of the Kdo hydrolase proteins behind a T7lac promoter

*Hp0579* and *hp0580* were both separately and jointly subcloned into pET21a behind the *T7lac* promoter, using the following primer sets: *hp0579* – Fhp0579 & Rhp0579, *hp0580* – Fhp0580 & Rhp0580, *hp0579-hp0580* – Fhp0580 & Rhp0579 (Table S1). The generated plasmids pHp0579, pHp0580, and pHp0579-80 were transformed into HMS174 (DE3) for over-expression of the protein.

### Construction of *H. pylori* Kdo hydrolase defective non-polar mutants

*Hp0580* and *hp0579-hp0580* knock out vectors were constructed as described previously (Chalker *et al.*, 2001). Briefly an upstream (primers Hp0580P1 and Hp0580P2 for both *hp0580* and *hp0579-hp0580*) and downstream (primers Hp0580P3 and Hp0580P4 for *hp0580* and primers Hp0579P3 and Hp0579P4 for *hp0579-hp0580*) region of each gene was PCR amplified from *H. pylori* strain 26695 genomic DNA. Each P2 and P3 primer also incorporated an overhang complementary to the chloramphenicol resistance (Cam) cassette from the plasmid pHel2. The Cam cassette was PCR amplified from pHel2 (primers Cam<sup>F</sup> and Cam<sup>R</sup>) using a reverse primer, which incorporated a ribosomal binding sequence and start codon to enable generation of a non-polar mutant. The upstream and downstream amplicons for each individual gene were then combined with the Cam amplicon, plus the

corresponding P1 and P4 primers, in a PCR reaction to generate the knockout inserts. The inserts were then cloned into pBluescript SK II + to yield the completed knockout vectors. The knockout vectors were transformed into *H. pylori* by natural transformation and resistant colonies selected on blood agar plates containing 8 µg/ml of chloramphenicol. Resistant colonies were re-purified on chloramphenicol containing plates and the successful insertion of the resistance cassette was verified by PCR of genomic DNA.

### Chromosomal complementation of Kdo hydrolase mutants

*RdxA* (*hp0954*) was chosen as the site for chromosomal complementation. *RdxA* is a nitroreductase, which is capable of converting metronidazole from an inactive pro-drug, into its active form (Gerrits *et al.*, 2004). The disruption and consequent inactivation of *rdxA*, by insertion of *hp0580* or *hp0579-hp0580*, will render the bacteria resistant to metronidazole. The *hp0580* and *hp0579-hp0580* complementation vectors were constructed using the previously made vector pET634comp (Stead *et al.*, 2008). Plasmid pET634comp was digested with BamHI and EcoRI to remove the *jhp0634* insert and gel purified, leaving the *rdxA* flanking regions intact. *Hp0580* (primers F580comp and R580comp) and *hp0579-hp0580* (primers F580comp and R579comp) plus approximately 100 base pairs of upstream sequence were PCR amplified from *H. pylori* strain 26695 using primers, which incorporated BamHI and EcoRI restriction sites. Each amplicon was then ligated between the *rdxA* flanking regions of the gel-isolated vector to generate the complementation vectors. The complementation vectors were transformed into *H. pylori* by natural transformation and resistant colonies selected on blood agar plates containing 12 µg/ml of metronidazole. Resistant colonies were re-purified on metronidazole containing plates and the successful insertion of the complementation cassette was verified by PCR of genomic DNA.

### Preparation of cell-free extracts, double-spun cytosol, and washed membrane

Typically, 200 ml of culture was grown to an  $A_{600}$  of ~ 1.0 at 37 °C and harvested by centrifugation at 10,000 × g for 10 min. All samples were prepared at 4 °C. Cell-free extract, membrane-free cytosol, and washed membranes were prepared as previously described (Tran *et al.*, 2004) and were stored in aliquots at -20 °C. Protein concentration was determined by the bicinchoninic acid method, using bovine serum albumin as the standard.

### Preparation of radiolabeled substrates

Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A and 1-dephosphorylated Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A were prepared as previously described (Stead *et al.*, 2005).

### Assay of Kdo hydrolase activity

Kdo hydrolase activity was assayed under optimized conditions in a 10-µl reaction mixture containing 50 mM HEPES, pH 8, 0.1% Triton X-100 and 2.5 µM lipid A substrate (Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A or 1-dephosphorylated Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A at ~5000 cpm/nmol). Washed membranes at 0.5 mg/ml for *E. coli* over expressed samples and 1 mg/ml for *H. pylori* samples were employed as the enzyme source, as indicated. Enzymatic reactions were incubated at 30 °C for the indicated times and terminated by spotting 4.5 µl portions of the mixtures onto silica gel 60 TLC plates. The reaction products were separated using the following solvent system: chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v). TLC plates were exposed overnight to a PhosphorImager Screen and product formation detected and analyzed using a Bio-Rad Molecular Imager PhosphorImager equipped with Quantity One Software.

## Large-scale isolation of lipid A and mass spectrometry analysis

Typically, 25 ml cultures of each strain were grown at 37 °C until each culture reached an  $A_{600}$  of <1.0. Lipid A was released from cells and purified as described previously (Tran *et al.*, 2006). The lipid A species were analyzed in the UT-Austin Analytical Instrumentation Facility Core using a MALDI-TOF (ABI Voyager-DE PRO) mass spectrometer equipped with a N<sub>2</sub> laser (337 nm) using a 20 Hz firing rate. The spectra were acquired in negative ion reflectron mode. The matrix used was a saturated solution of 6-aza-2-thiothymine in 50% acetonitrile and 5% tribasic ammonium citrate (20:1, v/v). 0.6µl of matrix solution was deposited on the sample plate, followed by 0.4µl of sample dissolved in chloroform-methanol (4:1, v/v).

## Construction of hp0580-phoA fusion vectors

*Hp0580* minus the stop codon was PCR amplified from *H. pylori* strain 26695 genomic DNA using primers F580phoA and R580phoA, which introduced XbaI and PstI restriction sites, respectively. R580phoA also incorporated an AvrII restriction site to facilitate insertion of *phoA*. *Hp0580* was inserted between the XbaI and PstI restriction sites of pBAD24 to create the vector pBAD580. *PhoA* was PCR amplified from *E. coli* W3110 genomic DNA using primers FphoAfus and RphoAfus, which introduced AvrII and HindIII restriction sites, respectively. FphoAfus was designed to remove the first 13 codons from *phoA*, thereby disrupting the signal peptide (Hoffman & Wright, 1985). The *phoA* amplicon was inserted into pBAD580 between the AvrII and HindIII restriction sites to produce the vector pBAD580phoA. The completed *hp0580-phoA* fusion insert was PCR amplified from pBAD580phoA using the primers F580phoApet and RphoAfus, which introduced NdeI and HindIII restriction sites, respectively. The *hp0580-phoA* amplicon was inserted between the NdeI and HindIII restriction sites of pET21a to produce the vector pHp0580-PhoA. *PhoA* minus the first 13 codons was cloned into pET21a using the primer set FphoA and RphoAfus, to act as a negative control. The FphoA primer incorporated a start codon to allow transcription of the gene. Both pET580phoA and pHp0580-PhoA were transformed into W3110 (DE3) *phoA::kan* for use in consequent assays.

## Construction of hp0580-gfp fusion vectors

*Hp0580* minus the stop codon was PCR amplified from *H. pylori* strain 26695 genomic DNA using primers F580gfp and R580gfp, which introduced HindIII and XbaI restriction sites, respectively. F580gfp also incorporated an NheI restriction site to facilitate insertion of *hp0580-gfp* into pET21a. *Hp0580* was inserted between the HindIII and XbaI restriction sites of pGFPuv to create the vector pGFPuv580. *Hp0580-gfp* was excised from pGFPuv using NheI and EcoRI restriction enzymes, followed by insertion into pET21a, to produce the vector pHp0580-GFP. *Gfp* was PCR amplified from pGFPuv using primers Fgfp and Rgfp, then cloned into pET21a, to act as a positive control. Both pHp0580-GFP and pGFP were transformed into W3110 (DE3) for use in consequent assays.

## Construction of E. coli W3110 (DE3) phoA defective mutant

P1 *vir* phage transduction was used to move the selectable *phoA::kan* mutation from *E. coli* strain JW0374 (Keio collection) to strain W3110 (DE3) as previously described (Sambrook & Russell, 2001).

## Alkaline phosphatase assay

The alkaline phosphatase activity of the Hp0580-PhoA fusion protein was determined qualitatively by growth of W3110 (DE3) *phoA::kan*/pHp0580-PhoA on LB agar plates containing 100µg/ml ampicillin, 0.05mM IPTG and 40µg/ml 5-bromo-4-chloro-3-indoyl phosphate (X-phosphate, Sigma). X-phosphate is a colorimetric substrate, which turns blue

in the presence of a phosphatase, leading to the production of blue colonies on the agar plate.

### Fluorescence Microscopy

Cultures were grown to an  $A_{600}$  of  $\sim 0.7$ , then induced with 1mM IPTG and allowed to grow for a further 2 hours. 20  $\mu$ l of a 1:1 culture dilution was added to a poly-L-lysine coated slide (Electron Microscopy Sciences) and a coverslip added. Bacteria were viewed at a magnification of 1000 $\times$  with a Nikon Eclipse 80i microscope equipped with a 100 $\times$  1.4NA PLAN APO lens, a GFP band pass emission filter set with a  $480 \pm 15$  nm excitation range and a  $535 \pm 20$  nm emission range and a Photometrics Cool SNAP HQ<sup>2</sup> camera. NIS-Elements AR 3.0 software was used to capture the images.

### Determination of polymyxin B minimum inhibitory concentrations (MIC)

MICs were determined using Polymyxin B Etest<sup>®</sup> strips (Biomérieux). 200 $\mu$ l of culture at an  $A_{600}$  of 0.1 was spread evenly onto a blood agar plate and allowed to dry completely, followed by addition of the Etest<sup>®</sup> strip to the center of the plate. The plates were incubated at 37 $^{\circ}$ C in a microaerobic atmosphere (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>) for 24 hours before reading. Each experiment was repeated in triplicate, the results averaged and a standard deviation calculated.

### H. pylori LPS analyses

Cultures were grown to an  $A_{600}$  of  $\sim 1$ , then the equivalent to 1 ml of culture at an  $A_{600}$  of 1 was harvested at 16,000  $\times$  g in a microcentrifuge and washed once with 1 $\times$  PBS. Cell pellets were resuspended in 100 $\mu$ l of 1 $\times$  LDS sample buffer (Invitrogen) + 4% BME. Cell suspensions were boiled for 10 minutes to lyse the cells and allowed to cool. Proteinase K (New England Biolabs) was added to a concentration of 125 ng/ $\mu$ l and the mixture was incubated at 55 $^{\circ}$ C for 16 hours. The proteinase K was heat inactivated at 100 $^{\circ}$ C for 5 minutes. The proteinase K treated whole cell lysates were separated by SDS polyacrylamide gel electrophoresis using a 4–12% bis-tris gradient gel (Invitrogen). The gels were either stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes) or blotted to a nitrocellulose membrane (Invitrogen) for immunodetection. The blots were probed with the following primary mouse monoclonal antibodies (Covance): anti-BG-4 (H type 1 chain) clone 17–206, anti-BG-5 (Lewis A) clone T174, anti-BG-6 (Lewis B) clone T218, anti-BG-7 (Lewis X) clone P12, anti-BG-8 (Lewis Y) clone F3. The secondary antibody was goat anti-mouse conjugated to peroxidase (MP Biomedicals, Cappel). Bands were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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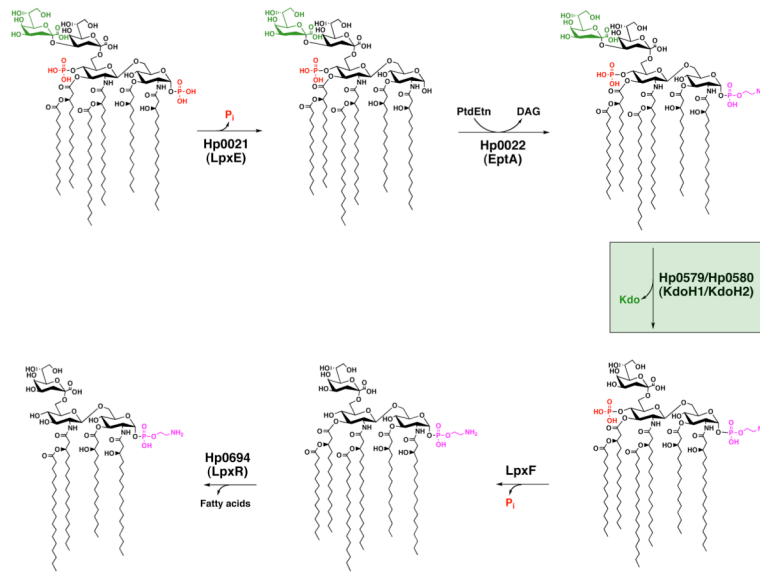
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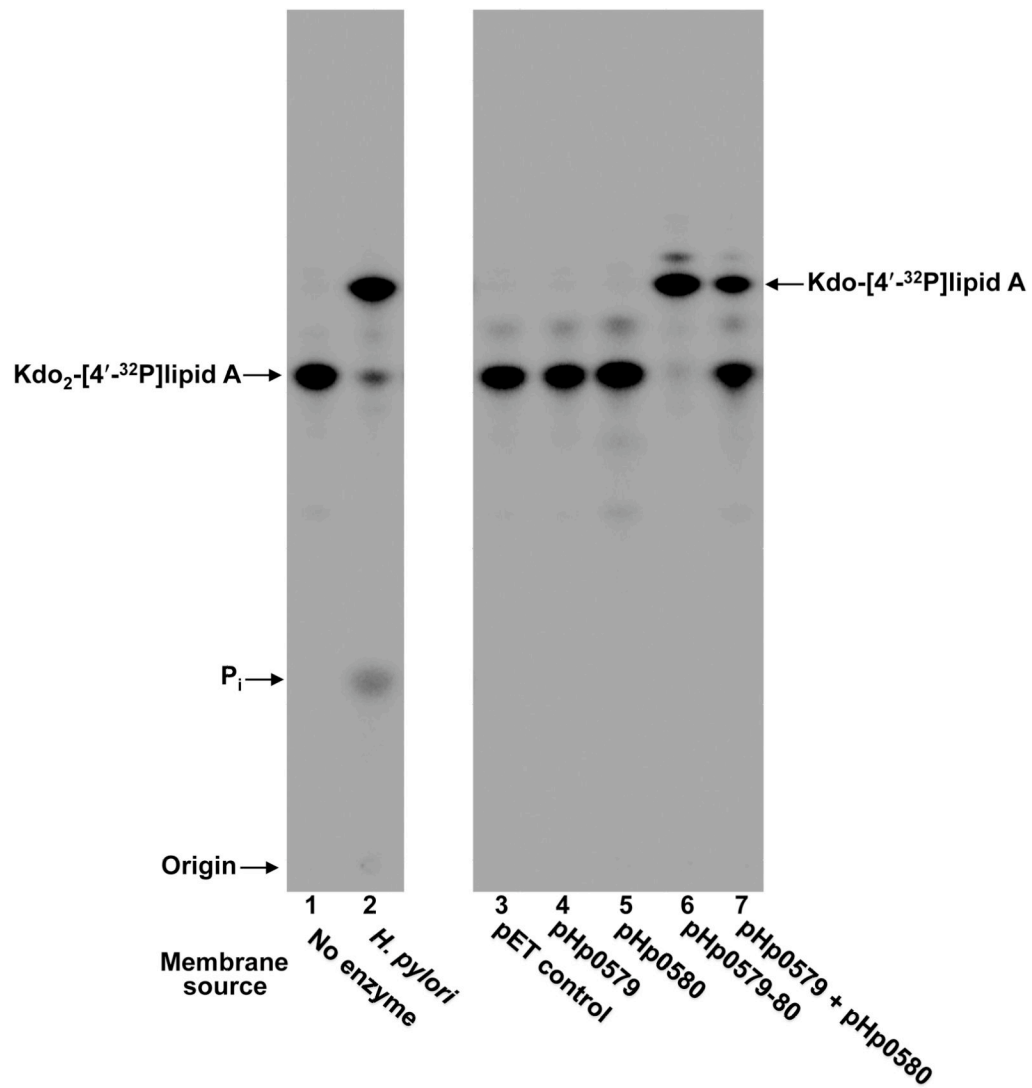
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**Fig. 1. Modification pathway of *H. pylori* Kdo-lipid A**

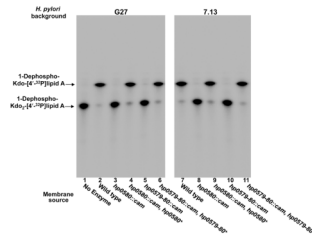
*H. pylori* produces a highly modified lipid A species via a five step enzymatic pathway. The 1-phosphate is first cleaved by Hp0021 (LpxE), leaving a free hydroxyl group, followed by addition of a phosphoethanolamine by Hp0022 (EptA). Presumably, phosphatidylethanolamine (PtdEtN) serves as the donor for the latter. Next Hp0579 (KdoH1) and Hp0580 (KdoH2) work in concert to remove the terminal Kdo sugar. The 4'-phosphate group is removed by an as yet unidentified phosphatase; however, the remaining hydroxyl group is not further modified as is the case at the 1 position. The final step involves the removal of the 3'-O-linked acyl chains by Hp0694 (LpxR), resulting in a tetra-acylated lipid A.





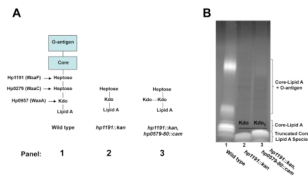
**Fig. 2. *In vitro* assay of Kdo hydrolase candidate proteins, Hp0579 and Hp0580, heterologously expressed in *E. coli***

Hp0579, Hp0580 and Hp0579-Hp0580 were overexpressed in *E. coli* HMS174 and membranes were isolated for use as the enzyme source in an *in vitro* Kdo-lipid A modification assay, using Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate. *H. pylori* membranes isolated from strain Hp7-91 harboring a mutation in *hp0021* (1-phosphatase) acted as a positive control and the empty pET21a vector acted as a negative control, demonstrating that *E. coli* has no endogenous Kdo hydrolase activity. When assayed individually, Hp0579 and Hp0580 were devoid of Kdo hydrolase activity; however, after co-expression a robust Kdo hydrolase activity could be detected by the appearance of a faster migrating product, which co-migrated with the positive control. Mixing membranes from individually expressed Hp0579 and Hp0580 could also generate Kdo hydrolase activity, reinforcing the necessity for a two protein complex. The minor product spots present above both the starting substrate Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A and the major product Kdo-[4'-<sup>32</sup>P]lipid A can be attributed to endogenous *E. coli* PagP activity, an outer membrane lipid A acyl transferase (Bishop *et al.*, 2000).



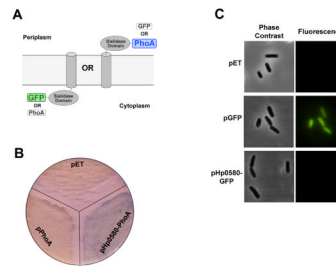
**Fig. 3. *In vitro* assay of *H. pylori* Kdo hydrolase mutants**

Membranes from both Hp0580 and Hp0579-Hp0580 mutants in strains G27 and 7.13 were assayed using 1-dephosphorylated-Kdo<sub>2</sub>-[4'-<sup>32</sup>P]lipid A as the substrate. Each of the mutants showed a complete loss of Kdo hydrolase activity, which was fully restored in complemented strains.



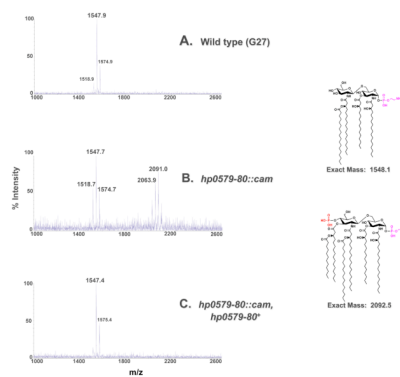
**Fig. 4. SDS-PAGE analysis of *H. pylori* 26695 *hp1191::kan* and *hp1191::kan*, *hp0579-80::cam* LPS profiles**

**A.** Hp1191 is a heptosyl transferase that is responsible for transferring the second heptose sugar found in the core region of *H. pylori* 26695 LPS (panel 1). An Hp1191 mutant produces a severely truncated LPS with only two sugars, heptose and Kdo, extending past the lipid A domain (panel 2). The *in vitro* assays depicted in Figs. 2 and 3 would predict that an *hp1191*, *hp0579-hp0580* mutant would produce an LPS species with 3 sugars extended past the lipid A domain (panel 3). **B.** The predicted difference in LPS composition, between the *hp1191* and *hp1191*, *hp0579-hp0580* mutants was evaluated by SDS-PAGE. As expected the *hp1191*, *hp0579-hp0580* mutant produced a higher mass LPS (lane 3) as compared to the *hp1191* mutant (lane 2), demonstrating an *in vivo* activity for the Kdo hydrolase.



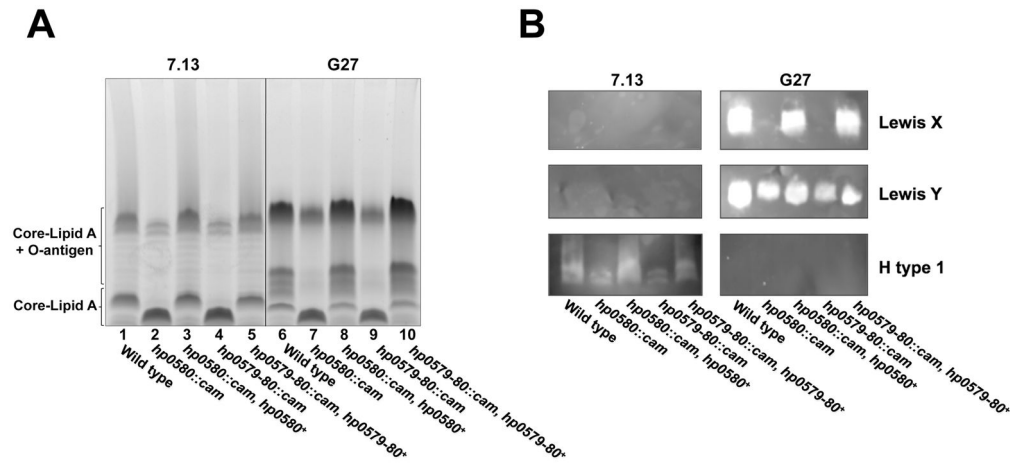
**Fig. 5. Hp0580 topology studies**

**A.** Hp0580 topology was investigated using PhoA and GFP C-terminal fusion proteins. PhoA is only active when located in the periplasm, whilst GFP will only fluoresce when folded in the cytoplasm (see text for details). **B.** W3110 (DE3) PhoA<sup>-</sup> expressing *hp0580-phoA* was grown on LB agar in the presence of X-phosphate substrate. The appearance of blue colonies indicates a periplasmic orientation for the Hp0580 soluble domain. W3110 (DE3) PhoA<sup>-</sup> plus empty vector and W3110 (DE3) PhoA<sup>-</sup> expressing *phoA* minus a signal peptide acted as negative controls. **C.** W3110 (DE3) expressing *hp0580-GFP* or GFP alone was visualized under a fluorescent microscope. Only the strain expressing GFP alone fluoresced, indicating a periplasmic orientation for the Hp0580 soluble domain.



**Fig. 6. Mass spectrometry of the G27 Kdo hydrolase mutant**

Lipid A was isolated from G27, G27 *hp0579-80::cam* and G27 *hp0579-80::cam*, *hp0579-80*<sup>+</sup> and analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. G27 produced a peak at 1547.9 m/z corresponding to the published wild type *H. pylori* lipid A mass (A). In addition to the wild type peak at m/z 1547.7 G27 *hp0579-80::cam* also displayed a peak at m/z 2091.0 (B). 2091.0 corresponds to a hexa-acylated lipid A species, with the 4'-phosphate still present. G27 *hp0579-80::cam*, *hp0579-80*<sup>+</sup> showed complete reversion to the wild type phenotype, with a single peak present at a m/z of 1547.4 (C).



**Fig. 7. SDS-PAGE analysis of Kdo hydrolase mutants and complements**

Proteinase K treated whole cell lysates from Hp0580, Hp0579-Hp0580 mutants and Hp0580, Hp0579-Hp0580 complemented mutants, in strains G27 and 7.13, were separated by SDS-PAGE and stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (**A**) or blotted to a nitrocellulose membrane and probed with antibodies raised against various human blood group antigens (**B**). The Kdo hydrolase mutants in both strains displayed a reduced amount of fully extended O-antigen and an increase in core-lipid A, as compared to wild type. A wild type phenotype was fully restored in each of the complemented Kdo hydrolase mutants. Strain 7.13 only expressed the H type 1 blood group antigen, which was reduced in the Kdo hydrolase mutants. Strain G27 expressed both Lewis X and Lewis Y blood group antigens. Lewis Y expression was unaffected in the Kdo hydrolase mutants; however, Lewis X expression was absent in each of the Kdo hydrolase mutants.

Table 1

Polymyxin minimal inhibitory concentration (MIC) of *H. pylori* strains

Strain	Kdo Hydrolase Activity	Polymyxin B MIC ( $\mu\text{g/ml}$ )
7.13	+	256.0 $\pm$ 0.0
7.13/hp0580::cam	-	1.0 $\pm$ 0.0
7.13/hp0580::cam, hp0580 <sup>+</sup>	+	234.7 $\pm$ 37.0
7.13/hp0579-80::cam	-	1.0 $\pm$ 0.0
7.13/hp0579-80::cam, hp0579-80 <sup>+</sup>	+	256.0 $\pm$ 0.0
G27	+	128.0 $\pm$ 0.0
G27/hp0580::cam	-	0.7 $\pm$ 0.1
G27/hp0580::cam, hp0580 <sup>+</sup>	+	117.3 $\pm$ 18.5
G27/hp0579-80::cam	-	0.5 $\pm$ 0.0
G27/hp0579-80::cam, hp0579-80 <sup>+</sup>	+	128.0 $\pm$ 0.0

Table 2

Bacterial strains and plasmids used in this study

Strain or Plasmid	Genotype or Description	Source or Reference
Strains		
<i>Helicobacter pylori</i>		
26695	Wild type laboratory strain	ATCC 700392
7.13	Gerbil adapted B128	Boneca, I.G.
G27	Wild type laboratory strain	Salama, N.R.
J99	Wild type laboratory strain	ATCC 700824
Hp7-91	Hp7-91 with chloramphenicol resistance cassette in <i>hp0021</i> ( <i>lpxE</i> )	(Tran et al., 2006)
26695/ <i>hp1191::kan</i>	26695 with kanamycin resistance cassette in <i>hp1191</i> ( <i>waaf</i> )	(Chandan et al., 2007)
26695/ <i>hp1191::kan/hp0579-80::cam</i>	26695 with kanamycin resistance cassette in <i>hp1191</i> ( <i>waaf</i> ) & chloramphenicol resistance cassette in <i>hp0579</i> & <i>hp0580</i>	This work
7.13/ <i>hp0580::cam</i>	7.13 with chloramphenicol resistance cassette in <i>hp0580</i>	This work
7.13/ <i>hp0580::cam, hp0580<sup>+</sup></i>	7.13/ <i>hp0580::cam, rdxA::hp0580</i>	This work
7.13/ <i>hp0579-80::cam</i>	7.13 with chloramphenicol resistance cassette in <i>hp0579</i> & <i>hp0580</i>	This work
7.13/ <i>hp0579-80::cam, hp0579-80<sup>+</sup></i>	7.13/ <i>hp0579-80::cam, rdxA::hp0579-80</i>	This work
G27/ <i>hp0580::cam</i>	G27 with chloramphenicol resistance cassette in <i>hp0580</i>	This work
G27/ <i>hp0580::cam, hp0580<sup>+</sup></i>	G27/ <i>hp0580::cam, rdxA::hp0580</i>	This work
G27/ <i>hp0579-80::cam</i>	G27 with chloramphenicol resistance cassette in <i>hp0579</i> & <i>hp0580</i>	This work
G27/ <i>hp0579-80::cam, hp0579-80<sup>+</sup></i>	G27/ <i>hp0579-80::cam, rdxA::hp0579-80</i>	This work
<i>Escherichia coli</i>		
XL-1 Blue	<i>recA endA1 gyrA96/hi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacZ</i> ΔM15::Tn10 (Tet <sup>R</sup> )]	Stratagene
HMS174 (DE3)	F <sup>-</sup> <i>recA1 hsdR</i> (r <sub>K12</sub> <sup>-</sup> m <sub>K12</sub> <sup>+</sup> ) (DE3) Rf <sup>R</sup>	Novagen
W3110 (DE3)	W3110, (DE3- novagen), Tet <sup>R</sup>	(Cullen & Trent, 2010)
W3110 (DE3) <i>phoA</i> <sup>-</sup>	W3110, (DE3- novagen), <i>phoA::kan</i> , Tet <sup>R</sup>	This work
JW0374	BW25113 <i>phoA::kan</i> (Keio Collection)	(Baba et al., 2006)
Plasmids		
pBluescript II SK (+)	Cloning vector; Amp <sup>R</sup> ; lac promoter ( <i>lacZ</i> ), fl, ColE1	Stratagene
pET21a	Vector containing a T7 promoter, Amp <sup>r</sup>	Novagen
pGFPuv	Vector containing the "cycle 3" variant of GFP, Amp <sup>r</sup>	Clontech
pBAD24	Bacterial expression vector, Amp <sup>r</sup>	(Guzman et al., 1995)



Strain or Plasmid	Genotype or Description	Source or Reference
pHp0579	pET21a containing <i>hp0579</i>	This work
pHp0580	pET21a containing <i>hp0580</i>	This work
pHp0579-80	pET21a containing <i>hp0579-80</i>	This work
pBS580KO	pBluescript II SK (+) containing <i>hp0580</i> flanking regions with a chloramphenicol resistance cassette insert	This work
pBS579-80KO	pBluescript II SK (+) containing <i>hp0579-80</i> flanking regions with a chloramphenicol resistance cassette insert	This work
pET1634comp	<i>jlhp0634</i> complement plasmid	(Stead et al., 2008)
pET580comp	pEThp0954 with <i>hp0580</i> inserted in <i>hp0954</i>	This work
pET579-80comp	pEThp0954 with <i>hp0579-80</i> inserted in <i>hp0954</i>	This work
pPhoA	pET21a containing <i>phoA</i>	This work
pBAD580	pBAD24 containing <i>hp0580</i>	This work
pBAD580phoA	pBAD580 containing <i>phoA</i>	This work
pHp0580-PhoA	pET21a containing <i>hp0580</i> with an C-terminal <i>phoA</i> fusion	This work
pGFP	pET21a containing <i>gfp</i>	This work
pGFPuv580	pGFPuv containing <i>hp0580</i>	This work
pHp0580-GFP	pET21a containing <i>hp0580</i> with an C-terminal <i>gfp</i> fusion	This work