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A Two-component Kdo Hydrolase in the Inner Membrane of *Francisella novicida*

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Abstract

Lipid A coats the outer surface of the outer membrane of Gram-negative bacteria. In *Francisella tularensis* subspecies *novicida* lipid A is present either as the covalently attached anchor of lipopolysaccharide (LPS) or as free lipid A. The lipid A moiety of *Francisella* LPS is linked to the core domain by a single 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) residue. *F. novicida* KdtA is bifunctional, but *F. novicida* contains a membrane-bound Kdo hydrolase that removes the outer Kdo unit. The hydrolase consists of two proteins (KdoH1 and KdoH2), which are expressed from adjacent, co-transcribed genes. KdoH1 (related to sialidases) has a single predicted *N*-terminal transmembrane segment. KdoH2 contains 7 putative transmembrane sequences. Neither protein alone catalyzes Kdo cleavage when expressed in *E. coli*. Activity requires simultaneous expression of both proteins or mixing of membranes from strains expressing the individual proteins under in vitro assay conditions in the presence of non-ionic detergent. In *E. coli* expressing KdoH1 and KdoH2, hydrolase activity is localized in the inner membrane. WBB06, a heptose-deficient *E. coli* mutant that makes Kdo_2-lipid A as its sole LPS, accumulates Kdo-lipid A when expressing the both hydrolase components, and 1-dephospho-Kdo-lipid A when expressing both the hydrolase and the *Francisella* lipid A 1-phosphatase (LpxE).

Introduction

Francisella tularensis, a highly infectious Gram-negative pathogen, is the causative agent of tularemia in mammals (Ellis *et al.*, 2002, McLendon *et al.*, 2006). As few as 10 bacteria of *F. tularensis* subsp. *tularensis* can cause fatal disease in humans, and *F. tularensis* is therefore considered a potential bio-weapon. The environmental isolate, *F. tularensis* subsp. *novicida* (*F. novicida*), is a mouse pathogen and is not infectious to humans (Rohmer *et al.*, 2007), making it an excellent model system with which to study *F. tularensis* biochemistry. *F. novicida*, like other species of *F. tularensis*, is an intracellular pathogen that can survive and replicate within the mouse macrophage cytoplasm after its escape from phagolysosomes (McLendon et al., 2006, Checroun *et al.*, 2006).

Lipid A, the hydrophobic moiety of lipopolysaccharide (LPS), is a major component of the outer leaflet of outer membranes of Gram-negative bacteria (Fig. 1A) (Raetz & Whitfield, 2002,Raetz *et al.*, 2007). Although *F. novicida* and other strains of *Francisella* contain LPS (Vinogradov *et al.*, 2002), over 70% of their lipid A is present in a “free” form (Fig. 1B), which is not covalently attached to the usual core and O-antigen sugars (Wang *et al.*, 2006b). The two main species of free lipid A in *F. novicida* are designated A1 (Fig. 1B) and A2 (not shown) (Wang et al., 2006b). The minor component A2 differs from A1 in that it is further glycosylated at 6′-position with a glucose residue (Wang et al., 2006b). Both A1 and

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A2 lack the 4′-phosphate moiety and the 3′-hydroxyacyl chain present in *E. coli* lipid A (Wang et al., 2006b), and both are modified with a galactosamine residue on the 1-phosphate group (Wang et al., 2006b,Wang *et al.*, 2009,Song *et al.*, 2009) (Fig. 1B). Both A1 and A2 contain longer acyl chains when compared to *E. coli* lipid A (Figs. 1A and 1B), and *Francisella* lipid A displays much greater micro-heterogeneity in the lengths of its acyl chains (Shaffer *et al.*, 2007).

Despite these differences in lipid A structure, the *F. novicida* genome encodes orthologs of the key *E. coli* enzymes involved in Kdo_2-lipid A biosynthesis, including the 4′-kinase LpxK and the Kdo transferase KdtA (Rohmer et al., 2007). The lack of a 4′-phosphate group is explained by the presence of a specific phosphatase in *F. novicida*, termed LpxF, which removes the 4′-phosphate moiety of both free lipid A and LPS on the outer surface of the inner membrane (Fig. 2) (Wang *et al.*, 2006a). Mutants lacking LpxF (Wang *et al.*, 2007) synthesize lipid A molecules that retain their 4′-phosphate group as well as their 3′-hydroxyacyl chain, suggesting an obligatory order of extra-cellular processing (Fig. 2). LpxF mutants of *F. novicida* are hypersensitive to cationic anti-microbial peptides and are highly attenuated in a mouse infection model (Wang et al., 2007).

The lipid A moiety of *Francisella* LPS shares many structural features with the free lipid A of *Francisella*, such as the absence of the 4′-phosphate group and the 3′-hydroxyacyl chain (Figs. 1B and C) (Vinogradov et al., 2002). Unlike the free lipid A (Fig. 1B) (Wang et al., 2006b), however, the lipid A component of *Francisella* LPS also lacks the 1-phosphate group and its attached GalN residue (Fig. 1C) (Vinogradov et al., 2002). The 1-phosphate group is removed on the outer surface of the inner membrane by a selective, Kdo-dependent phosphatase, designated LpxE (Fig. 2) (Wang *et al.*, 2004). Furthermore, the LPS that is made by *F. tularensis* contains only one Kdo moiety in its core domain (Fig. 1C) (Vinogradov et al., 2002). The presence of one Kdo unit in the LPS core could be explained by a mono-functional Kdo-transferase KdtA, as seen in *Haemophilus influenza* (White *et al.*, 1997, Chung & Raetz, 2010). However, we now demonstrate that *F. novicida* encodes a bifunctional Kdo-transferase. In addition, we have characterized a Kdo trimming activity in *F. novicida* membranes that removes the outer Kdo moiety from model substrates, such as *E. coli* Kdo_2-lipid A (Fig. 1A) or its tetra-acylated precursor Kdo_2-lipid IV_A (Wang et al., 2004). We have identified two adjacent genes that encode the *Francisella* Kdo hydrolase, which consists of two inner membrane proteins designated KdoH1 and KdoH2. KdoH1 is the putative catalytic component, because it is distantly related to bacterial sialidases like NanI (Newstead *et al.*, 2008), with which it appears to share several key active site residues. Extracts of *F. novicida* cells harboring mutations in either or both genes encoding the Kdo hydrolase lack Kdo trimming activity, but these deletions have no effect to the viability of *F. novicida*. Both proteins are likewise required for activity *in vitro* and *in vivo* when expressed in *E. coli*. A similar Kdo trimming activity is present in *Helicobacter pylori* (Stead *et al.*, 2005). As shown in the accompanying manuscript (Stead *et al.*, 2010), the *H. pylori* Kdo hydrolase also consists of two protein components. The availability of the genes encoding these Kdo hydrolases will be useful for re-engineering lipid A and LPS structures in diverse Gram-negative bacteria with possible applications in vaccine development.

Results

A bi-functional Kdo transferase in *F. Novicida.*

Kdo transferases (KdtAs) of Gram-negative bacteria can add one or more Kdo residues derived from the donor substrate CMP-Kdo to lipid IV_A, a tetra-acylated precursor of lipid A (Supporting Fig. 1) (Raetz & Whitfield, 2002). KdtA of *E. coli* is a bi-functional enzyme (Belunis & Raetz, 1992) in that it incorporates both the inner and outer Kdo residues to generate the intermediate Kdo_2-lipid IV_A (Supporting Fig. 1). To determine whether *F.*

novicida kdtA encodes a mono- or bi-functional Kdo transferase, the *FnkdtA* gene was cloned into pBAD33, generating pBAD-FnKdtA. Both the vector and pBAD-FnKdtA were then transformed into CMR300 (Reynolds & Raetz, 2009), an *E. coli* mutant with a deletion in its chromosomal *kdtA* gene. CMR300 harbors the covering plasmid pWMsbA that over-expresses MsbA (Reynolds & Raetz, 2009), an ABC transporter for core-lipid A (Zhou *et al.*, 1998, Doerrler *et al.*, 2001), enabling growth on rich medium at 37 °C with lipid IV_A as the only remaining LPS substructure. Membranes were prepared from both constructs, as well as wild-type *E. coli*, and assayed for Kdo transferase activity (Fig. 3). The reactions were stopped after two hours at 30° C by spotting portions of the assay mixtures onto a TLC plate, which was developed with the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v). The membranes of the vector control CMR300/pBAD33 did not convert any [4′-^{32}P]lipid IV_A to more slowly migrating glycosylated substances. However, membranes of CMR300/pBAD-FnKdtA converted [4′-^{32}P]lipid IV_A to two products. The predominant slowly-migrating product corresponds to Kdo_2- [4′-^{32}P]lipid IV_A, as shown by assaying membranes of wild-type *E. coli* in parallel (Fig. 3). The second product seen with the CMR300/pBAD-FnKdtA membranes corresponds to the Kdo-lipid IV_A intermediate (Belunis & Raetz, 1992, White et al., 1997). The results show that FnKdtA is a bifunctional enzyme in vitro (Fig. 3).

Mutants of *F. novicida* lacking Kdo hydrolase activity

The Kdo hydrolase likely accounts for the presence of the single Kdo residue seen in the inner core of *F. novicida* LPS (Vinogradov et al., 2002, Wang et al., 2004). Although not present in *E. coli*, Kdo hydrolase activity has also been reported in *Helicobacter pylori* (Stead et al., 2005). The *Helicobacter* activity exhibits a strong dependency on the prior removal of the 1- phosphate group of Kdo_2-lipid A (Stead et al., 2005). We postulated that the Kdo hydrolase sequences of *F. novicida* and *H. pylori* should be homologous to each other, but not to any proteins of *E. coli*. To identify potential Kdo hydrolase gene(s), we took advantage of the phylogenetic profiler service of the Integrated Microbial Genomes System (Markowitz *et al.*, 2008). To include as many potential candidates as possible, the minimal percentage of identity at the protein level was set at 20%. In this manner, we identified 45 structural genes present in both *F. novicida* and *H. pylori* J99 that were absent in *E. coli*. The ones predicted to produce cytosolic proteins were ignored, because the Kdo hydrolase is membrane associated. Of the remaining 38 candidate genes, 35 were available with at least one transposon insertion mutation (Gallagher *et al.*, 2007), which were obtained through the service available at www.francisella.org. Membranes were prepared from all these mutants and assayed for Kdo hydrolase activity. Under the conditions employed, the 4′-phosphatase activity of *F. novicida*, encoded by *lpxF* (Wang et al., 2006a), competes for the substrate (Supporting Fig. 1), generating $^{32}P_i$. In order to minimize the loss of label caused by LpxF, short reaction times (1 and 5 minutes) were used in these screening assays. Membranes of a mutant with a transposon insertion in the gene FTN_0495 (renamed *kdoH1*) (Fig. 4) did not show any Kdo trimming activity under standard assay conditions. To confirm this result, we constructed an in-frame deletion mutant, designated JZ-H1, in which the *kdoH1* gene replaced by kanamycin resistant cassette, as described in the methods section. Again, no Kdo hydrolase activity was detected in membranes of this strain, either in the presence (Fig. 5, lanes 5/6) or absence (not shown) of the shuttle vector pMP529 (Maier *et al.*, 2004).

A second gene (locus tag FTN_0494), transcribed in the same direction, is located next to *kdoH1* on the *F. novicida* chromosome with 8 overlapping base pairs (Fig. 4). An in-frame deletion mutation in this downstream gene was constructed as for *kdoH1*, which likewise eliminated Kdo trimming activity when membranes were assayed in vitro (Fig. 5, lanes 9/10). This gene was therefore designated as *kdoH2* (Fig. 4), and the correspondingly

mutated strain as JZ-H2. Because of the overlap of these two genes, the kanamycin cassette that was used to replace *kdoH2* was preceded by a ribosome binding sequence, located immediately upstream of *kdoH2* and downstream of *kdoH1*. The mutant, JZ-H1H2, harboring an in-frame deletion of both *kdoH1* and *kdoH2* with replacement by a kanamycin resistance cassette, likewise lost its Kdo hydrolase activity (Fig. 5, lanes 13/14).

Phenotypes of mutants and hydropathy analysis of KdoH1 and KdoH2

The doubling time of the *kdtA* deletion mutant of *F. novicida* was 56 minutes when grown at 37° C, while that of wild-type cells was 40 minutes. LPS was absent in these mutants (Supporting Fig. 2A), but the levels of free lipid A were slightly higher than wild-type (Supporting Fig. 2B). Deletion mutants in either or both protein components of the Kdo hydrolase displayed normal growth rates under laboratory conditions. Their LPS profiles were normal, as judged by gel electrophoresis (Supporting Fig. 2A), and the amount of free lipid A was unchanged (Supporting Fig. 2B). The Kdo hydrolase mutants were not hypersensitive to polymyxin.

Based on a PSI-BLAST analysis (Altschul *et al.*, 1997), the *C*-terminal portion of the protein expressed from the *kdoH1* gene shows distant homology to the soluble sialidase NanI of Clostridium perfringens (Supporting Fig. 3A), which is secreted into the growth medium (Newstead et al., 2008). However, KdoH1 is predicted to contain one *N*-terminal transmembrane helix, while KdoH2 is predicted to have 7 transmembrane segments without any large connecting loops or other unusual structural features, as judged by the TMHMM algorithm (Krogh *et al.*, 2001) (Fig. 4). A significant homolog of KdoH1 is present in *H. pylori* (see accompanying manuscript and Supporting Fig. 3B) (Stead et al., 2010). No obvious homologues of KdoH2 were detected in any genomes other than related strains of *Francisella*. However, as in *F. novicida*, a second gene encoding a protein with 6 predicted transmembrane helices follows the *kdoH1* homolog of *H. pylori* with 3 base pairs of overlap (Stead et al., 2010). This downstream *H. pylori* gene product likewise displays no homology to any known protein sequences, but its appears to have a similar function to *F. novicida kdoH2* (see accompanying manuscript) (Stead et al., 2010).

Complementation of *F. novicida* mutants defective in the Kdo hydrolase

A shuttle vector for *E. coli* and *Francisella*, pMP529, kindly provided by Dr. T. Zahrt of The Medical College of Wisconsin (Maier et al., 2004), carries a hygromycin resistance cassette under the control of the *F. novicida* groESL promoter. A unique MluI restriction enzyme site was used for inserting *kdoH1* and/or *kdoH2*. Three plasmids were constructed: pMP-H1, pMP-H2, and pMP-H1H2, which express KdoH1, KdoH2 or both. In each case, the 300-base pair sequence immediately upstream of *kdoH1* in *F. novicida* was used as the promoter and ribosomal binding site. These plasmids and the control vector were transformed into our three *F. novicida* mutants, JZ-H1, JZ-H2 and JZ-H1H2 (Fig. 5). Membranes derived from the three Kdo hydrolase mutants carrying the pMP529 vector displayed no Kdo trimming activity in vitro (Fig. 5, lanes 5/6, 9/10, and 13/14). The Kdo hydrolase activity of mutants JZ-H1 and JZ-H2 could be reconstituted by plasmids pMP-H1 and pMP-H2, respectively (Fig. 5, lanes 7/8 and 11/12), but they could not rescue the enzyme defect in the double deletion mutant JZ-H1H2 (Fig. 5, lanes 15/16 and 17/18). Only plasmid pMP-H1H2 expressing both proteins restored Kdo hydrolase activity to membranes of JZ-H1H2 (Fig. 5, lanes 19/20).

Reconstitution of hydrolase activity by mixing of membranes or co-expression in *E. coli*

Membranes from the *F. novicida* strains JZ-H1 and JZ-H2, harboring the single protein mutations, showed no Kdo hydrolase activity under standard assay conditions in the presence of 0.1 % Triton X-100 (Fig. 5). To demonstrate that hydrolase activity could be

reconstituted, the membranes from the two mutant strains were solubilized by diluting them into 1% Triton X-100 in phosphate buffered saline (PBS), pH 7.4, containing 250mM NaCl, at a final protein concentration of 1 mg/mL. After 30 minutes at 4° C and removal of insoluble materials by centrifugation at 200,000 × g for 1 h, the supernatants were added to a Kdo hydrolase assay mixture at a final concentration of 0.1 mg/mL each. The reactions were incubated at 30° C for 25 hours. This resulted in the cleavage of about 30 % of the substrate when the mixed membranes were used, but not with the individual membranes (Supporting Fig. 4).

The *kdoH1* and *kdoH2* genes, or both, were cloned into the vector pWSK29 (Wang & Kushner, 1991) with a ribosome binding site derived from pET21b. The resulting constructs were designated pWSK-H1, pWSK-H2 and pWSK-H1H2. These plasmids and the vector control pWSK29 were transformed into wild-type *E. coli* W3110 (Table 1). Membranes were prepared and used in the standard Kdo hydrolase in vitro assay at 0.1 mg/mL in the presence of 0.1 % Triton X-100. The reactions were stopped after 30 minutes at 30° C by spotting onto a TLC plate that was developed in chloroform, methanol, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v). Membranes from cells carrying the vector pWSK29, or the plasmids pWSK-H1 or pWSK-H2 expressing the single proteins, showed no Kdo trimming activity (Fig. 6A, lanes 1–3). However, membranes from W3110/pWSK-H1H2 displayed robust activity (Fig. 6A, lane 4). Alternatively, hydrolase activity could be reconstituted by mixing solubilized membranes from W3110 cells expressing the individual proteins derived from pWSK-H1 or pWSK-H2 (Fig. 6A, lane 5).

These results were confirmed with a quantitative time course assay shown in Fig. 6B, which was carried out at 0.005 mg/mL of each membrane protein preparation. Membranes expressing the individual proteins had no detectable hydrolase activity (Fig. 6B). Taken together, these results strongly support the idea that the *kdoH1* and *kdoH2* are structural genes encoding a novel two-component Kdo trimming enzyme.

Substrate selectivity of the Kdo hydrolase

As demonstrated previously, membranes of wild-type *F. novicida* catalyze the cleavage of the outer Kdo unit from Kdo_2-lipid IV_A or Kdo_2-lipid A (Wang et al., 2004). Prior removal of the phosphate group from the 1-position of lipid A by LpxE greatly accelerates Kdo trimming activity (Wang et al., 2004). Although tetra-acylated 1-dephospho-Kdo_2-lipid IV_A was used as the substrate in our initial experiments (Figs. 5 - 8), the hydrolase was ~5 fold more active with hexa-acylated 1-dephospho-Kdo_2-lipid A as the substrate under our standard assay conditions. With membranes from *E. coli* cells expressing both KdoH1 and KdoH2, the specific activity of Kdo hydrolysis was 0.022 nmol/min/mg with 5 μM Kdo_2-lipid IV_A, 6.6 nmol/min/mg with 5 μM 1-dephospho,Kdo_2-lipid IV_A, and 33.8 nmol/min/mg with 5 μM 1-dephospho-Kdo_2-lipid A.

Dependence of the recombinant Kdo hydrolase on pH and detergent

Membranes of *E. coli* cells expressing both Kdo hydrolase components were assayed over the pH range of 4.0 to 9.0, using a triple buffer system consisting of 100 mM sodium acetate, 50 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)hexane, and 50 mM Tris (McClerren *et al.*, 2005). The optimal pH for Kdo hydrolase activity was 5.0 under these conditions, and product formation was proportional to time (Fig. 7A). In a phosphate buffered assay system, the pH optimum was around 6.0 (not shown). Although the pH optima were slightly different in the two systems, the Kdo hydrolase activity was always higher under mildly acidic conditions.

To study the detergent dependence of the hydrolase, both the non-radioactive carrier and the radiolabeled 1-dephospho-Kdo_2-lipid IV_A substrates (Supporting Fig. 1) were initially suspended in 25 mM Tris-HCl, pH 7.8, containing 1 mM EDTA and EGTA, in the absence of Triton X-100. The assay concentrations of Triton X-100 could then be varied from 0% to 2% in w/v in the assay system by dilution from a 10% stock (Fig. 7B). Again, 0.005 mg/mL of total membrane protein from cells expressing both KdoH1 and KdoH2 was used as the enzyme source. The reactions were stopped after 10 min and 30 min at 30 ° C, and the maximal conversion of substrate to product was always less than 30%. Over the range of Triton X-100 concentrations tested, Kdo hydrolase activity was highest at 0.02%, but was strongly inhibited by 2% (Fig. 7B). The activity was not completely abolished at very low detergent concentrations (Fig. 7B) or in the absence of Triton X-100 (data not shown).

Membrane localization of the *F. novicida* Kdo hydrolase expressed in *E. coli*

Kdo hydrolase activity is associated with membranes whether expressed in *F. novicida* or *E. coli*. The cytosolic fraction contained little or no activity. To determine whether the hydrolase is localized in outer or inner membranes when expressed in *E. coli*, wild-type cells of strain W3110, harboring the plasmid pWSK-H1H2 (Table 1), were grown in LB medium and induced with 1 mM IPTG. The inner and outer membranes were separated by a minor modification of the sucrose gradient centrifugation method described previously (Osborn & Munson, 1974,Trent *et al.*, 2001). Twenty five fractions were collected. Analysis of the protein concentration revealed two major peaks (Fig. 8), which were confirmed to be inner and outer membranes by assaying for the marker enzymes NADH oxidase and phospholipase A, respectively (Zhou et al., 1998,Trent et al., 2001) (Fig. 8). Kdo hydrolase assays were performed using equal volumes of each fraction. The highest protein concentration in the assay was 0.1 mg/mL for fraction 12. The reactions were stopped after 90 seconds at 30° C, and Kdo hydrolysis was analyzed by TLC (Fig. 8, lower panel). The Kdo hydrolase activity coincided with the inner membrane marker, strongly suggesting that the KdoH1 and KdoH2 are localized to the inner membrane when expressed in *E. coli*, consistent with their hydropathy profiles (Fig. 4).

Identification of the in vitro reaction products of the recombinant Kdo hydrolase

LC-ESI/MS in the negative ion mode (Fig. 9) was used to confirm the proposed structures of the reaction products generated in vitro by the recombinant Kdo hydrolase. In this experiment 100 μM 1-dephospho-Kdo_2-lipid A was used as the substrate (Fig. 9A), which was isolated from *E. coli* WBB06 (Brabetz *et al.*, 1997) expressing LpxE (Wang et al., 2004). Analysis of the chloroform-soluble lipid product in the acidic Bligh-Dyer lower phase (Bligh & Dyer, 1959,Nishijima & Raetz, 1979) of a complete reaction mixture containing 0.5 mg/ml of recombinant KdoH1 and KdoH2 showed a strong peak at *m/z* 967.642 (Fig. 9B), interpreted as the $[M\text{-}2H]^{2-}$ ion of 1-dephospho-Kdo-lipid A (predicted $[M\text{-}2H]^{2-}$ at *m/z* 967.648). The compound migrated as a discrete peak between minutes 21 and 22, as judged by analysis of the extracted ion current (EIC) (Fig. 9C), which is diagnostic for the presence of 1-dephospho-Kdo-lipid A in the column elution profile. The no enzyme control reaction (Fig. 9A) contained very little 1-dephospho-Kdo-lipid A, but showed the appropriate substrate peak at *m/z* 1077.678, as expected for the $[M\text{-}2H]^{2-}$ ion of 1-dephospho-Kdo_2-lipid A (predicted $[M\text{-}2H]^{2-}$ at *m/z* 1077.677).

Analysis of the water-soluble product(s) (Figs. 9D, 9E and 9F) revealed a strong peak at *m/z* 237.060 in the Kdo hydrolase sample (Fig. 9E) but not in the no enzyme control (Fig. 9D), consistent with $[M\text{-}H]^-$ ion of the released Kdo (predicted $[M\text{-}H]^-$ at *m/z* 237.061). The Kdo product migrated as a broad peak between minutes 26 and 29, as judged by analysis of the EIC (Fig. 9F).

Modification of Kdo_2-lipid A in *E. coli* by the combined action of the lipid A 1-phosphatase and the Kdo hydrolase

Expression of the *F. novicida* phosphatase LpxE in the heptose deficient *E. coli* mutant WBB06, which makes Kdo_2-lipid A without any other core sugars (Brabetz et al., 1997, Kanipes *et al.*, 2001), removes the phosphate group from the 1-position of Kdo_2-lipid A, resulting in the accumulation of 1-dephospho-Kdo_2-lipid A in living cells (Wang et al., 2004). When both LpxE and KdoH1/KdoH2 were co-expressed in WBB06, they modified Kdo_2-lipid A synergistically. In this case, a version of WBB06 was constructed that carries both the pBAD-FnLpxE and the pWSK-H1H2 plasmids (Fig. 10A). In general, 0.2% arabinose and 1mM IPTG were added as protein expression inducers when cell cultures reached density of A_{600} ~0.5. Addition of the inducers to WBB06 expressing both LpxE and KdoH1/KdoH2 at the time of inoculation was toxic (data not shown).

The lipids were extracted by the acidic Bligh-Dyer method (Nishijima & Raetz, 1979) and subjected to TLC analysis in chloroform, methanol, acetic acid, water (25:15:4:4, v/v/v/v) (Fig. 10A). In WBB06 cells containing only the vectors pWSK29 and pBAD33, Kdo_2-lipid A was found as the major lipid A species (Fig. 10A, lane 1). WBB06/pWSK29/pBAD-FnLpxE, which expresses LpxE, produces a new LPS species, previously identified as 1-dephospho-Kdo_2- lipid A (Fig. 10A, lane 2). The strain WBB06/pWSK-H1H2/pBAD33, which expresses KdoH1/KdoH2, produces some Kdo-lipid A, which migrates differently than the 1-dephospho-Kdo_2-lipid A (Fig. 10A, lane 3). When both modification enzymes were expressed simultaneously in WBB06/pWSK-H1H2/pBAD-FnLpxE, a different lipid A species was produced, which migrated faster than others, as expected for 1-dephospho-Kdo-lipid A (Fig. 10A, lane 4).

The compositions of the predicted lipid A species were confirmed by ESI/MS analysis (Figs. 10B to 10E). In the strain containing the vectors only, the $[M-2H]^{2-}$ ion at *m/z* 1117.661, which arises from *E. coli* Kdo_2-lipid A and was used to calibrate the spectrum, was predominant, followed by its sodium adduct ion $[M+Na-3H]^{2-}$ (predicted *m/z* 1128.651) at *m/z* 1128.649 (Fig. 10B). When LpxE was expressed from the plasmid pBAD-FnLpxE in WBB06, a major peak consistent with the $[M-2H]^{2-}$ ion of 1-dephospho-Kdo_2-lipid A (predicted *m/z* 1077.677) was observed at *m/z* 1077.684 (Fig. 10C). Similarly, the $[M-2H]^{2-}$ ion for Kdo-lipid A (predicted *m/z* 1007.631) was observed at *m/z* 1007.658 in WBB06 expressing KdoH1/KdoH2 (Fig. 10D). Lastly, a large peak at *m/z* 967.656, consistent with the $[M-2H]^{2-}$ of 1-dephospho-Kdo-lipid A (predicted *m/z* 967.648), was observed in WBB06 expressing both KdoH1/KdoH2 and LpxE (Fig. 10E). In each case, lower levels of sodium adduct ions were also detected.

In summary, when KdoH1 and KdoH2 are expressed in an *E. coli* mutant lacking heptose, significant amounts of Kdo-lipid A accumulate in living cells (Fig. 10D). When KdoH1 and KdoH2 are expressed together with the 1-phosphatase LpxE, the cells produce even larger amounts of 1-dephospho-Kdo-lipid A (Fig. 10E). These findings, together with the in vitro assays described above, provide definitive evidence that *kdoH1* and *kdoH2* are the structural genes encoding the Kdo hydrolase.

Discussion

Most Gram-negative bacteria synthesize LPS molecules containing two or three Kdo residues within their inner core domains (Raetz & Whitfield, 2002, Belunis *et al.*, 1992), but strains of *Haemophilus influenzae* (Zamze *et al.*, 1987), *Bordetella pertussis* (Isobe *et al.*, 1999), *Helicobacter pylori* (Stead et al., 2005), and *Francisella tularensis* (Vinogradov et al., 2002), among others, synthesize LPS with only a single Kdo unit attached to their lipid A. In *Haemophilus* and *Bordetella*, the Kdo transferase (KdtA) is monofunctional (White et

al., 1997, Isobe et al., 1999), whereas in *E. coli* and most other organisms KdtA is bifunctional (Belunis & Raetz, 1992, Raetz & Whitfield, 2002). Bacteria with a monofunctional Kdo transferase usually contain a Kdo kinase (White et al., 1997, White *et al.*, 1999), which adds a phosphate group to the Kdo 4-position that is usually occupied by the outer Kdo residue. There is no homolog of Kdo kinase in either *F. novicida* or *H. pylori*. However, the *F. novicida* and *H. pylori* Kdo transferases are bifunctional. The single Kdo unit found in their LPS arises as a consequence of a novel Kdo trimming reaction, catalyzed by an unusual hetero-dimeric Kdo hydrolase. This enzyme is associated with the inner bacterial membrane. It consists of a putative catalytic protein that is oriented towards the periplasm (see accompanying manuscript) (Stead et al., 2010) and a polytopic inner membrane protein that probably serves as part of the membrane anchor or is needed to deliver the nascent LPS substrate to the catalytic domain. Both proteins are absolutely required for function (Figs. 5 and 6), as judged by genetic analysis and reconstitution of enzymatic activity in *E. coli*.

The genes encoding the enzymes that catalyze Kdo cleavage have not been reported previously. The *Francisella* enzyme that we have investigated removes only the outer Kdo residue. It would be interesting to explore the possibility that it could remove more than one Kdo moiety from LPS cores of organisms like *Chlamydia* that contain three or four Kdo units (Brade, 1999). The putative catalytic component of KdoH1 is distantly related to secreted Gram-positive bacterial sialidases, some of which have been studied by x-ray crystallography (Newstead et al., 2008). Interestingly, the two essential arginine residues that interact with the carboxylic acid moiety of sialic acid substrate in the sialidase are also conserved in KdoH1 (Newstead et al., 2008) (Supporting Fig. 3A). Bacterial sialidases cleave terminal sialic acid residues from the oligosaccharides of glycoproteins and glycolipids on eukaryotic cells (Corfield, 1992). The physiological significance of the sialidase activity has been linked with the pathogenicity and nutrition of some bacterial strains (Corfield, 1992).

The function of the Kdo hydrolase is not yet known. The pH dependence profile of KdoH1/KdoH2 shows that a slight acidic condition is preferred (Fig. 7). Other *F. novicida* lipid A modification enzymes, such as LpxE, LpxF and FlmK, also prefer a pH of around 6.0 (Wang et al., 2004,Wang et al., 2006a,Wang et al., 2009). This observation may reflect the low pH environment within macrophages in which *F. novicida* cells replicate (McLendon et al., 2006). *Francisella* mutants lacking the hydrolase continue to make LPS (Supporting Fig. 2A), albeit with two Kdo moieties (J. Zhao and C. R. H. Raetz, in preparation), and they grow normally under laboratory conditions. Their virulence in animal models remains to be examined. As shown in the accompanying manuscript (Stead et al., 2010), *H. pylori* mutants lacking the hydrolase are hypersensitive to cationic anti-microbial peptides because their LpxF phosphatase cannot efficiently remove the 4′-phosphate group of their lipid A, implying an obligatory order of lipid A processing by the various extracellular modification enzymes of that organism.

A unique feature of the *Francisella* system is the relative abundance of free lipid A (Wang et al., 2006b), which is apparently exported to the outer membrane without prior attachment of Kdo (Fig. 2). Free lipid A does not arise by cleavage of the inner Kdo moiety, which is not a substrate for KdoH1/KdoH2. Deletion of *kdtA* in *F. novicida* results in the complete loss of LPS (Supporting Fig. 2A) with a modest (~25%) increase in free lipid A (Supporting Fig. 2B) and a reduction in growth rate. In wild-type *Francisella*, free lipid A is more abundant than LPS-bound lipid A (Wang et al., 2006b), which makes up less than 30% of the total.

The core region of *Francisella* lacks heptose, which is replaced by mannose (Vinogradov et al., 2002), a structurally similar sugar that is also found in *Rhizobium etli* (Kadrmas *et al.*, 1998, Forsberg & Carlson, 1998). The *Rhizobium* mannosyl transferase, encoded by the *lpcC* gene (Kadrmas et al., 1998), requires a Kdo disaccharide for activity (Kanipes *et al.*, 2003), and it is an intracellular enzyme. LpcC is inactive with substrates containing a single Kdo unit (Kanipes et al., 2003). If *Francisella* incorporated only a single Kdo moiety into its nascent LPS, it would presumably be unable to assemble its core and O-antigen domains. To test this idea one could replace the bifunctional Kdo transferase of *Francisella* with the monofunctional Kdo transferase of *H. influenzae* and analyze the resulting LPS and free lipid A structures.

A unique feature of the *F. novicida* and *H. pylori* Kdo hydrolases is the requirement for two distinct proteins. So far, we have been unable to complement our *Francisella* mutants with the *Helicobacter* genes encoding KdoH1 and KdoH2 (Stead et al., 2010). Given that reconstitution by mixing of membranes expressing the individual protein components is quite efficient (Fig. 6), it should be possible to examine the kinetics and affinity of these two components for each other in a purified system. So far, it has been difficult to over-express both proteins at high levels in *E. coli*. Use of codon optimized genes or expression in *Francisella* itself, which contains some unusual membrane phospholipids (Wang et al., 2004), may be necessary to achieve high levels of protein expression for structural studies.

As shown in previous work, mutation of lipid A or LPS modification enzymes can have profound effects on pathogenesis, often resulting in attenuation (Gunn, 2001, Wang et al., 2007, Raetz et al., 2007). Characterization of the pathogenesis phenotypes of the Kdo hydrolase mutants therefore requires further investigation. Expression of modification enzymes in foreign hosts, as shown for KdoH1/KdoH2 in the case of *E. coli* (Fig. 10), permits unusual structural modifications of LPS that likewise may have profound effects on outer membrane assembly and pathogenesis. KdoH1/KdoH2 can modify lipid A in *E. coli* synergistically with LpxE, the lipid A 1-phosphatase. The product of both KdoH1/KdoH2 and LpxE, 1-dephospho-Kdo-lipid A, accumulates to high levels in the *E. coli* strains expressing both enzymes (Fig. 10). The loss of the 1-phosphate group and the outer Kdo residue greatly reduces the charge of the LPS molecules and likely alters the electrostatic interactions between them. The stability of the outer membrane may be compromised when 1-dephospho-Kdo-lipid A is the major component of outer leaflet of outer membrane. Combining heterologous modification enzymes in diverse pathogens that normally do not contain them could lead to the development of novel attenuated strains with utility as vaccines.

Experimental procedures

Materials and reagents

Trypticase soy broth, Mueller-Hinton broth, yeast extract, tryptone and Bacto agar were purchased from Difco, Detroit, MI. Glass-backed silica gel 60 thin layer chromatography (TLC) plates were from E. Merck, Darmstadt, Germany. Chloroform, pyridine, methanol, acetic acid and 88% formic acid were from EMD Science, Gibbstown, NJ. [γ -^{32}P]-ATP was purchased from PerkinElmer, Waltham, MA.

Bacterial growth and membrane preparation

E. coli strains were usually grown in LB broth (1% tryptone, 0.5% yeast extract and 1% NaCl) at 37° C. *F. novicida* U112 (Rohmer et al., 2007) and derived strains were grown in 3% trypticase soy broth supplemented with 0.1% cysteine (w/v). When necessary, ampicillin, chloramphenicol, kanamycin, hygromycin or tetracycline was added at 100, 25,

30, 200 or 12.5 μg/mL, respectively for *E. coli* strains, while kanamycin, hygromycin or tetracycline was added at 15, 200 or 12.5 μg/mL respectively for *F. novicida* strains.

To prepare membranes, the bacteria were typically grown in 100 mL of medium at 37° C and were harvested when A_{600} reached 1.0. The inducers, IPTG or L-arabinose, were added when necessary as indicated. Cells were harvested by low-speed centrifugation and washed twice with 50 mL PBS, pH7.4. The cells were then re-suspended in 2 mL PBS and passed through French pressure cell once at 16,000 psi. The unbroken cells and large debris were removed by centrifugation at 10,000 × g for 15 minutes at 4 ° C. The supernatant was subjected to ultracentrifugation at 200,000 × g for 1 hour at 4° C. The membrane-containing pellets were resuspended in 0.4 mL PBS, pH7.4, and stored at −80° C in aliquots. The protein concentration of the membrane preparations was determined with the bicinchoninic acid assay (Pierce, St. Louis, MO) (Smith *et al.*, 1985).

Expression cloning of the *F. novicida kdtA*, *lpxE* and Kdo hydrolase genes

The *F. novicida kdtA* (*FnkdtA*) was amplified by PCR and cloned into the pBAD33 vector at the SacI and PstI restriction sites (Table 1). The forward primer nKdtA_for and the reverse primer nKdtA_rev used for this purpose are shown in Supporting Table 1. The PCR was performed with Pfu turbo polymerase (Invitrogen, Carlsbad, CA) and *F. novicida* genomic DNA as template, prepared with the Easy-DNA kit (Invitrogen). The 50-μL reaction mixture contained 100 ng template, 200 ng of each primers, and 2.5 units of Pfu polymerase. The reaction was started at 95° C for 1 min, followed by 25 cycles of denaturation (1 min at 95° C), annealing (1 min at 55° C) and extension (3 min at 72° C). A 10-min extension at 72° C was used at the end. The DNA product was located on 0.7% Agarose gel, and the desired band was excised and purified with Gel Extraction Kit (Qiagen, Valencia, CA). The PCR product was digested with SacI and PstI, and ligated into similarly digested and Antarctic Phosphatase treated pBAD33 vector. The ligation mixture was transformed into *E. coli* DH5α and a portion of the cells were spread onto a LB agar plate, containing chloramphenicol. The insertion of *FnkdtA* into pBAD33 was confirmed by DNA sequencing, and the desired plasmid was designated as pBAD-FnKdtA. All restriction enzymes, T4 DNA ligase and phosphatase were purchased from New England BioLabs (Ipswich, MA). The plasmid pBAD-FnLpxE was constructed similarly by cloning the *F. novicida lpxE* gene into the pBAD33 vector, and was kindly provided by Dr. B. Ma (Ma et al., 2008).

The individual protein components of the *F. novicida* Kdo hydrolase, or both, were cloned into the *E. coli* vector pWSK29 (Wang & Kushner, 1991) or into pMP529 (Maier et al., 2004), a shuttle vector for *E. coli* and *F. novicida*. To clone *kdoH1* or both *kdoH1* and *kdoH2* into pMP529, the same forward primer nMpH1_for was used for PCR (Supporting Table 1). For cloning *kdoH1* alone, the reverse primer nMpH1_for was employed (Supporting Table 1). The reverse primer nMpH2_rev (Supporting Table 1) was used for cloning both *kdoH1* and *kdoH2*. The PCR products were digested with MluI, purified and ligated to pMP529 at the MluI restriction site, using similar procedure previously described for pBAD-FnKdtA. The resulting plasmids carrying *kdoH1* and *kdoH1-kdoH2* are designated as pMP-H1 and pMP-H1H2, respectively. The shuttle vector and its derivatives were transformed to *F. novicida* by electroporation (LoVullo *et al.*, 2006).

The *kdoH2* gene was cloned into pMP529 preceded by the promoter region upstream of *kdoH1* to allow expression *in trans*. A two-step PCR protocol was applied for plasmid construction. First, the promoter and the *kdoH2* coding region were amplified separately. The region about 300bp immediately upstream of *kdoH1* contains the promoter and ribosomal binding site. To amplify the promoter region, an additional sequence matching the beginning of *kdoH2* was incorporated into the reverse primer. This region was required for

the fusion PCR in the second step to anneal to the *kdoH2* coding sequence and generate the final DNA product containing the promoter, ribosomal binding site and *kdoH2*. The promoter region was amplified by PCR using the forward primer nMpH1_for (as above) about 300bp upstream of *kdoH1* and a reverse primer nPromH2_rev (Supporting Table 1). The *kdoH2* coding region was amplified with the forward primer nMpH2_for (Supporting Table 1); the reverse primer nMpH2_rev (Supporting Table 1) was the same as used for cloning *kdoH1-kdoH2*. Both above PCR reactions used *F. novicida* genomic DNA as template. In the second PCR step, 50 ng each of the purified PCR products of promoter region and the *kdoH2* coding sequence were used as templates. About 200 ng of the common forward primer for the promoter region and the reverse primer for *kdoH2* (Supporting Table 1) were used in a reaction that included 1 unit of KOD Hot Start DNA polymerase (Novagen). The reaction started with 2 min incubation at 95° C, followed by 30 cycles of 30 seconds denaturation at 95° C, 30 seconds annealing at 50° C and 1 min extension at 70 ° C. A 10-min extension at 70° C was added in the end. The PCR product was purified, digested with appropriate restriction enzyme, and ligated into pMP529 at the MluI site. The resulting plasmid carrying *kdoH2* is designated as pMP-H2.

To clone *kdoH1* into pWSK29, the gene was amplified with the forward primer nWskH1_for and the reverse primer nWskH1_rev (Supporting Table 1). The *kdoH2* gene was amplified with forward primer nWskH2_for and the reverse primer nWskH2_rev (Supporting Table 1). To clone both *kdoH1* and *kdoH2*, the forward primer for *kdoH1* and the reverse primer for *kdoH2* were used in the PCR reaction (Supporting Table 1). The PCR products were purified, digested with XbaI and BamHI, and ligated into pWSK29. The plasmid constructs harboring *kdoH1*, *kdoH2* or *kdoH1* and *kdoH2* were designated pWSK-H1, pWSK-H2 or pWSK-H1H2, respectively.

Construction of *F. novicida* mutations with deletions in *kdoH*1 and *kdoH2*

The kanamycin cassette was amplified from the pET28b vector with the forward primer nKan_for and the reverse primer nKan_rev (Supporting Table 1).

To construct *F. novicida* mutants with deletions in *kdoH1*, *kdoH2*, or both, the following primers were used. To amplify the 2kb region upstream of *kdoH1*, forward primer nHmut_for was used together with reverse primer nH1Kan_rev (Supporting Table 1). To amplify the 2kb region downstream of *kdoH1*, reverse primer nHmut_rev was used in conjunction with forward primer nH1Kan_for (Supporting Table 1). Both PCR reactions used *F. novicida* genomic DNA as the template.

The two PCR products and the kanamycin cassette were then used as templates in a final PCR that was done with forward primer nHmut_for and reverse primer nHmut_rev (Supporting Table 1). The desired DNA species was purified and transformed to wild type *F. novicida* that had been rendered competent by chemical treatment (Anthony *et al.*, 1991). The mutants were selected on 3% trypticase soy broth agar plates supplemented with kanamycin and 0.1% cysteine.

To construct the *kdoH2* deletion mutant, the 2kb upstream sequence of *kdoH1* was amplified with forward primer nHmut_for and reverse primer nH2Kan_rev (Supporting Table 1). The downstream 2kb sequence of *kdoH2* was amplified with reverse nHmut_rev and forward primer nH2Kan_for (Supporting Table 1). Finally, a fusion PCR reaction was set up as described above for *kdoH1*, and the PCR product was transformed into wild type *F. novicida* followed by selection on kanamycin plates.

The DNA for constructing the *kdoH1-kdoH2* deletion mutant was prepared in a fusion PCR reaction using the 2kb upstream sequence for *kdoH1*, the 2kb downstream sequence for

kdoH2 and the kanamycin cassette as templates with forward primer nHmut_for and reverse primer nHmut_rev. The linear DNA product was transformed into wild type *F. novicida*, and the *kdoH1-kdoH2* mutant was selected as above. The deletions in *kdoH1*, *kdoH2*, or *kdoH1-kdoH2* were confirmed by DNA sequencing, and the mutated strains were designated JZ-H1, JZ-H2 and JZ-H1H2, respectively.

Preparation of 1-dephospho-Kdo$_2$-[4′-^{32}P]lipid IV$_A$

The tetraacyl-disaccharide 1-phosphate precursor of lipid A was converted to [4′-^{32}P]lipid IV$_A$ using [γ -^{32}P]ATP and BLR(DE3)/pLysS/pJK2 membranes containing the overexpressed 4′-kinase LpxK (Garrett *et al.*, 1997). Purified *E. coli* Kdo transferase (KdtA) (Basu *et al.*, 1999), was used to convert [4′-^{32}P]lipid IV$_A$ to Kdo$_2$-[4′-^{32}P]lipid IV$_A$ (Supporting Fig. 1). The 1- dephospho-Kdo$_2$-[4′-^{32}P]lipid IV$_A$ was generated from Kdo$_2$-[4′-^{32}P]lipid IV$_A$ with *E. coli* membranes containing recombinant *F. novicida* 1-phosphatase LpxE (Supporting Fig. 1) (Wang et al., 2004). The 1-dephospho-Kdo$_2$-[4′-^{32}P]lipid IV$_A$ was purified by preparative TLC (Basu et al., 1999).

In vitro assay for the *F. novicida* Kdo hydrolase

Kdo hydrolase assays were usually done in a 15 μL reaction mixture at 30° C, containing 50 mM potassium phosphate buffer, pH 6.0, 0.1% Trition X-100 and 5 μM 1-dephospho-Kdo$_2$-[4′-^{32}P]lipid IV$_A$ (100,000 cpm/nmol). The reactions were terminated by spotting 4 μL samples onto a TLC plate. The reaction times were varied as indicated. The plate was dried under a cool air stream and developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v). The plate was dried with hot air stream and exposed to a PhosphorImager screen. Reactants and products were quantified using a Molecular Dynamics PhosphorImager equipped with ImageQuant software. Specific activity was calculated from the amount of product in nmol generated at a given time in the assay volume, divided by the incubation time in minutes and the total amount of protein in the given assay volume in mg.

Lipid preparation

Unless otherwise indicated, lipids were extracted using the Bligh-Dyer system (Bligh & Dyer, 1959). Typically, 100 mL cultures were grown to A_{600} of 1.0. The cells were harvested by centrifugation and washed twice with 50 mL PBS, pH7.4. The cells were resuspended in 8 mL PBS (for the neutral condition) or 0.1N HCl (for the acidic condition), and then 10 mL chloroform and 20 mL methanol were added to form a single phase mixture (chloroform, methanol and aqueous ratio of 1:2:0.8, v/v/v). After incubation at room temperature for 60 min, the insoluble debris was removed by centrifugation at 3000 × g for 30 min. The supernatant was transferred to clean solvent-resistant bottles, and then 10 mL chloroform and 10 mL PBS (for the neutral extraction) or 0.1N HCl (for the acidic extraction) were added to generate a two-phase system. After thorough mixing, the samples were centrifuged at 3000 × g for 15 min, and the lower phase was collected; for the acidic extraction, several drops of pyridine were added. The sample was dried under a stream of nitrogen.

ESI/MS analysis

Lipid samples were dissolved in chloroform and methanol (2:1, v/v) containing 1% piperidine and immediately subjected to ESI/MS analysis in negative ion mode (Raetz *et al.*, 2006, Wang et al., 2007) via direct infusion, as previously described. All mass spectra were collected on a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex) equipped with an ESI source. Data acquisition and analysis were performed with Analyst QS software supplied by manufacturer.

LC-ESI/MS analysis

Normal phase LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system coupled to a QSTAR XL quadrupole time-of-flighttandem mass spectrometer (as above). An Ascentis^R Si HPLC column (5 μm, 25 cm × 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/ aqueous ammonium hydroxide (600:340:50:5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 μl/min. The post-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer. Nitrogen was used as the collision gas for MS/MS experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EIC	extracted ion current
ESI-MS	electrospray ionization mass spectrometry
IPTG	isopropyl-β-D-thiogalactoside
Kdo	2-keto-3-deoxy-D-*manno*-octulosonic acid
LC	liquid chromatography
LPS	lipopolysaccharide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

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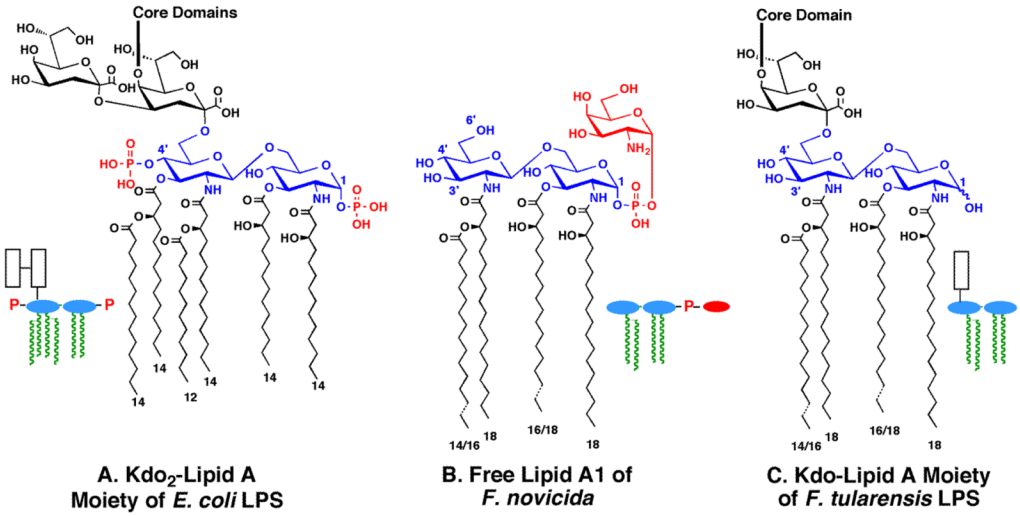

Fig. 1. Structures of lipid A species found in *E. coli* and *F. novicida*

Panel A. The Kdo_2-lipid A moiety of *E. coli* LPS accumulates in heptose deficient mutants like WBB06 (Brabetz et al., 1997, Raetz et al., 2006). The lengths of the acyl chains are indicated by the numbers at the bottom. The 1 and 4′ positions are the sites of phosphate group attachment on the glucosamine disaccharide backbone. The core domain of *E. coli* LPS is attached to the inner Kdo residue, as indicated (Raetz & Whitfield, 2002). No free lipid A is present in wild-type *E. coli*. **Panel B**. Free lipid A makes up over 70% of the total lipid A of *F. novicida*. The A1 component of free lipid A is the predominant species (Wang et al., 2006b). The less abundant A2 component (not shown) contains an additional α-linked glucose residue at position 6′ (Wang et al., 2006b). **Panel C**. The Kdo-lipid A moiety of *F. novicida* LPS has a different structure than free lipid A (Wang et al., 2006b, Vinogradov et al., 2002). The LPS-bound lipid A of *Francisella* (less than 30% of the total lipid A) lacks the 1-phosphate group (Vinogradov et al., 2002), consistent with the presence of the LpxE 1-phosphatase (Wang et al., 2004) in *Francisella*. The rest of the core and O-antigen regions are not shown. Colors: *black*, Kdo; *blue*, glucosamine residues of lipid A; *red*, phosphate groups and galactosamine. The corresponding schematic structures are used throughout the paper: *white boxes*, Kdo; *blue*, glucosamine; *red*, phosphate groups or galactosamine; *green*, acyl chains.

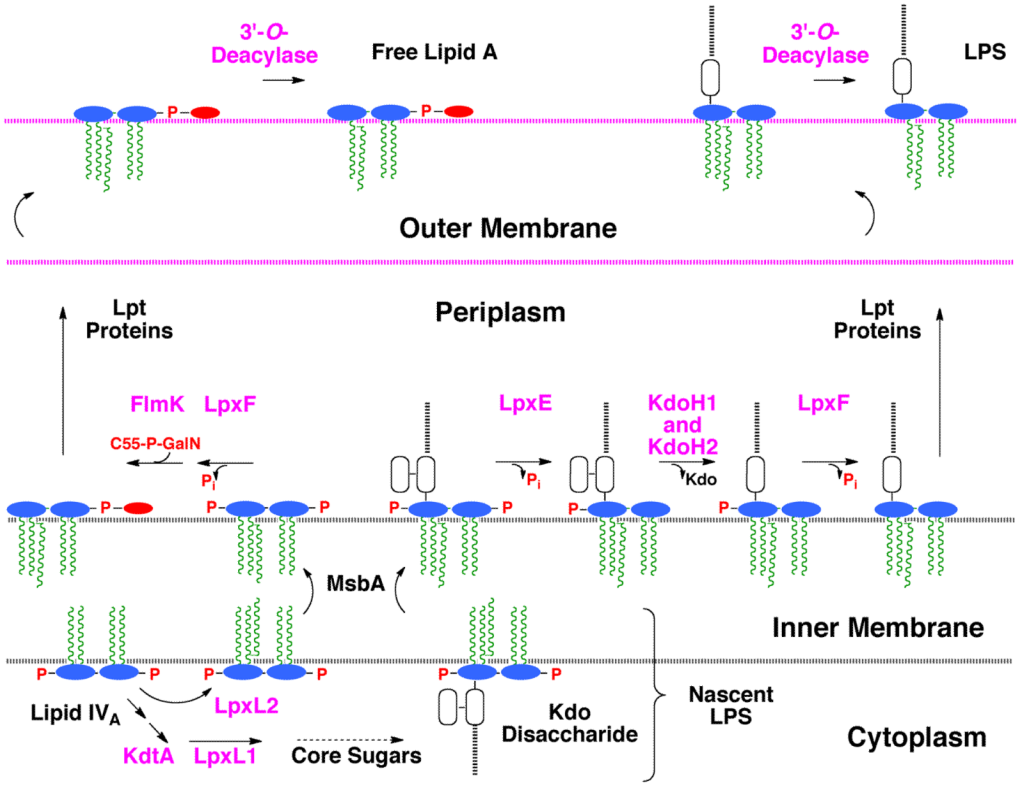

Fig. 2. Topography of lipid A modification enzymes in the *F. novicida* cell envelope

The differences and similarities in the processing of free lipid A and LPS-bound lipid A are compared to highlight the role of the Kdo hydrolase. The pathway diverges at the lipid IV_A stage, which is acylated by LpxL2 and exported by MsbA in the case of free lipid A (Raetz *et al.*, 2009), or is glycosylated by KdtA on the way to generating LPS. LpxE does not dephosphorylate free lipid A because it requires the Kdo moiety for activity (Wang et al., 2004). LpxE and LpxF are MsbA-dependent (Wang et al., 2004, Wang et al., 2006a), when expressed in *E. coli*, and therefore have active sites that face the periplasm. The order of LpxF action (if any) relative to FlmK (Wang et al., 2009) or KdoH1/H2 is not yet known. The gene encoding the *F. novicida* 3′-O-deacylase has not yet been identified. The enzyme probably resides in the outer membrane, based on studies with *Salmonella* LpxR (Rutten *et al.*, 2009), which catalyzes a similar deacylation reaction. The schematic lipid A structures and the color schemes are the same as in Fig. 1.

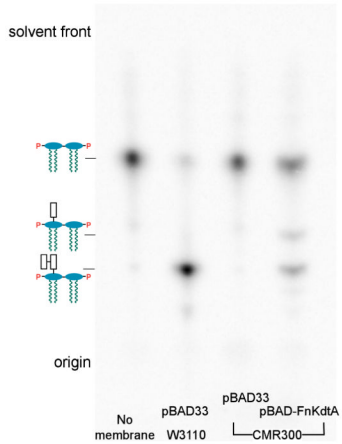

Fig. 3. Kdo transferase assays of *E. coli* membranes and recombinant *F. novicida* KdtA

Membranes (1 mg/mL) of *E. coli* W3110/pBAD33 or CMR300/pBAD-FnKdtA were assayed with 5 μM [4′-^{32}P]lipid IV_A (10,000 cpm/nmol) as the substrate. The first lane contains no membrane in the reaction mixture. The reactions were stopped after 2 hours at 30° C by spotting 4 μL onto a TLC plate, which was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v).

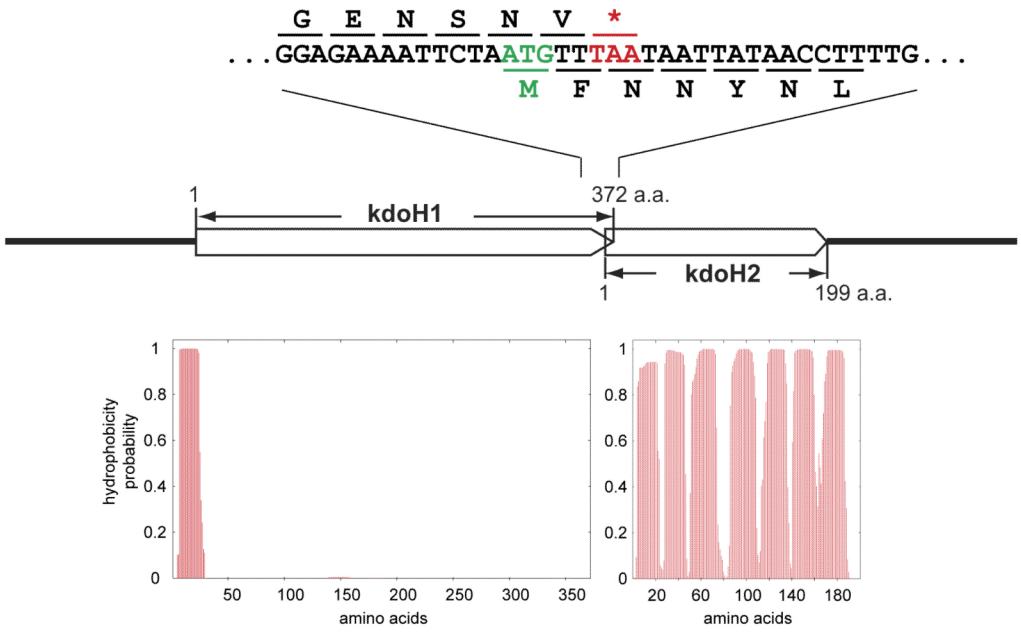

Fig. 4. Overlapping genes encoding the *F. novicida* Kdo hydrolase components and hydropathy analysis of the predicted proteins

Panel A. The organization of the *F. novicida* genes encoding the two protein components of the Kdo hydrolase (Rohmer et al., 2007). Panel B. The putative transmembrane segments of each Kdo hydrolase protein, as predicted by the TMHMM algorithm (Krogh et al., 2001).

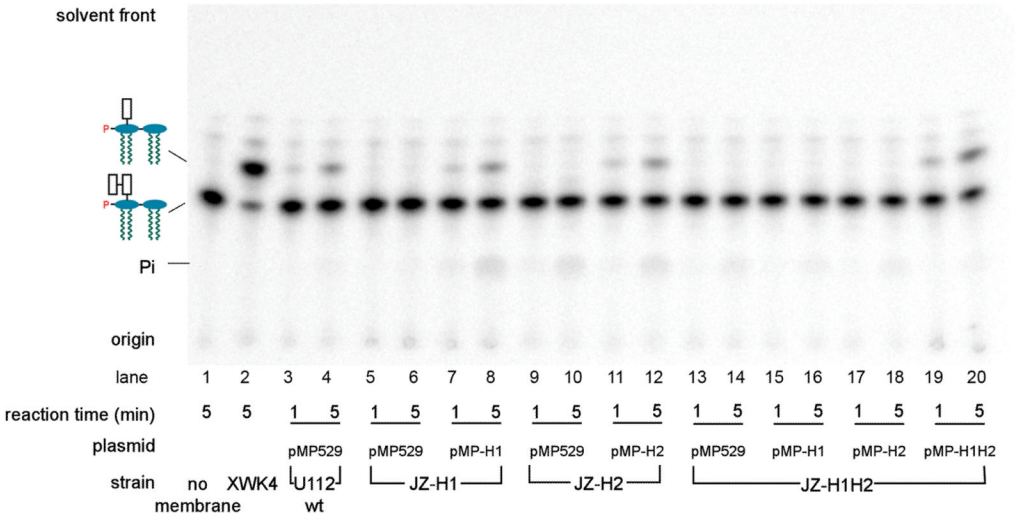

Fig. 5. Absence of Kdo hydrolase activity in membranes of *KdoH1* and *KdoH2* mutants of *F. novicida* and its restoration by complementation

The in vitro assays were carried out with 1-dephospho-Kdo-[4′-^{32}P]lipid IV_A as the substrate (Supporting Fig. 1). The membranes were from the *F. novicida* U112 wild-type, or from the mutants JZ-H1, JZ-H2 or JZ-H1H2, each harboring either the vector pMP529 (Maier et al., 2004) or the plasmids pMP-H1, pMP-H2, or pMP-H1H2 (Table 1), as indicated. The reactions were stopped at the indicated times at 30° C by spotting a portion of each reaction mixture onto a TLC plate. The products were separated using the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v). A negative control without added membranes and a positive control using membranes of the *F. novicida* 4′-phosphatase mutant XWK4 (Wang et al., 2007) are shown in the left two lanes.

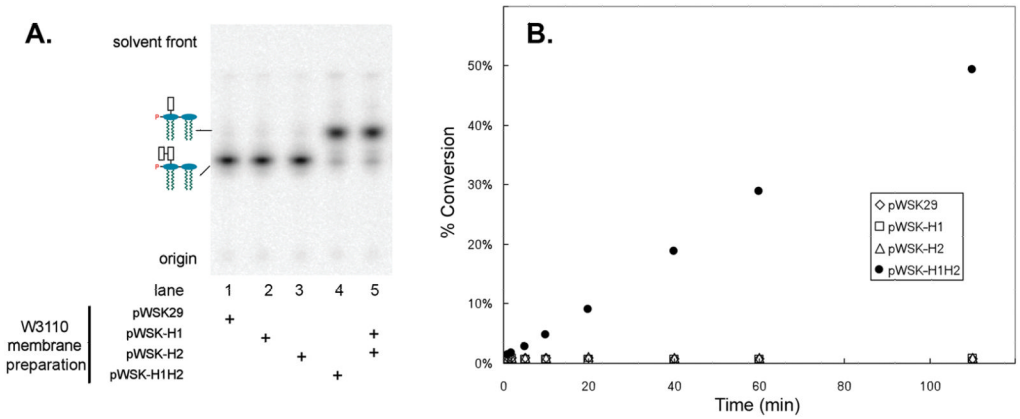

Fig. 6. Hydrolase activity in membranes of *E. coli* cells expressing both KdoH1 and KdoH2 or by mixing membranes containing the individual protein components

Panel A. Membranes, prepared from *E. coli* W3110 harboring pWSK29, pWSK-H1, pWSK-H2 or pWSK-H1H2, were used in the Kdo hydrolase assays at 0.1 mg/mL each with 1-dephospho-Kdo-[4′-^{32}P]lipid IV_A as the substrate (Supporting Fig. 1). The reactions were stopped after 30 min at 30° C, and the products were separated as in Fig. 5. **Panel B**. Time course of product formation at membrane concentrations of 0.005 mg/mL. The reactions at 30° C were stopped at the indicated times by spotting a portion of each reaction mixture onto a TLC plate, as above. Product formation was quantified by PhosphorImager analysis.

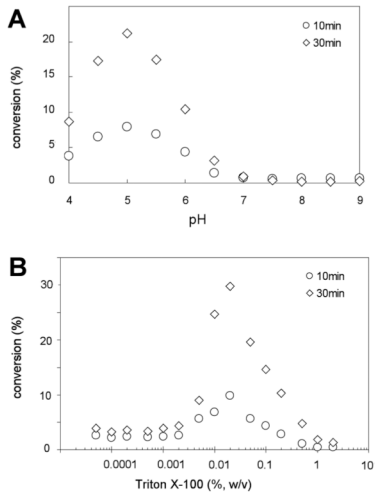

Fig. 7. pH and Triton X-100 dependence of the recombinant Kdo hydrolase

Panel A. The pH dependence of the Kdo hydrolase activity was determined in a triple buffer system (McClerren et al., 2005). **Panel B.** The Triton X-100 dependency was analyzed in the range from 0.00005% to 2% w/v. The residual activity was the same in the absence of Triton X-100 as at 0.00005%.

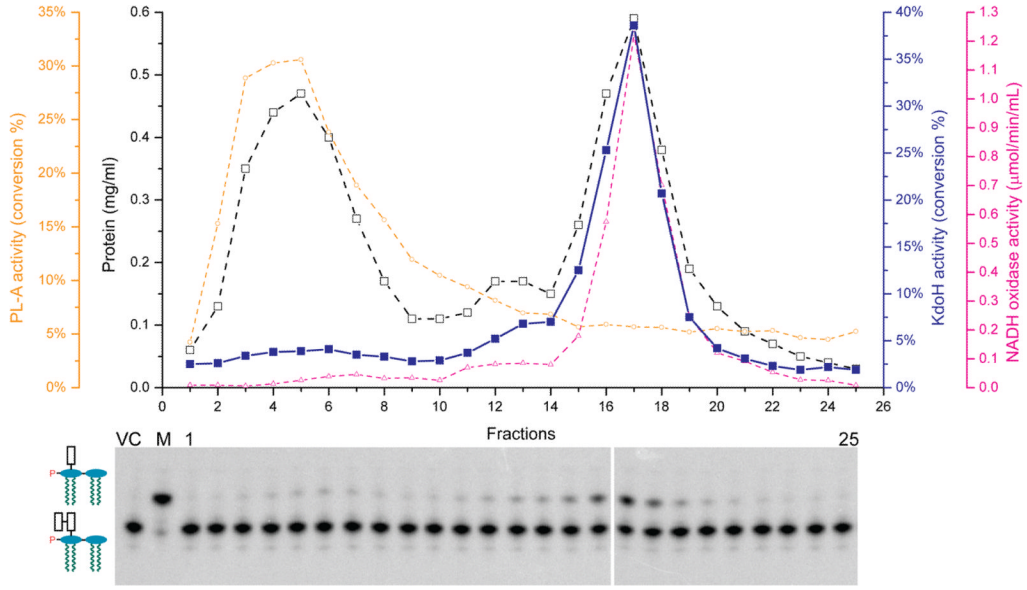

Fig. 8. Inner membrane localization of the recombinant Kdo hydrolase in *E. coli*

Kdo hydrolase was expressed from the plasmid pWSK-H1H2 in *E. coli* W3110. The inner and outer membranes were separated by sucrose gradient centrifugation (Osborn & Munson, 1974). Each fraction from was assayed for protein concentration using the bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL) (Smith et al., 1985) (*black* open squares), the outer membrane marker phospholipase A (*yellow* open circles), the inner membrane marker NADH oxidase (*magenta* open triangles) (Zhou et al., 1998), and the Kdo hydrolase (*blue* solid squares and lower panel). VC, vector control; M, total membranes; 1 and 25, the first and last fractions of the gradient.

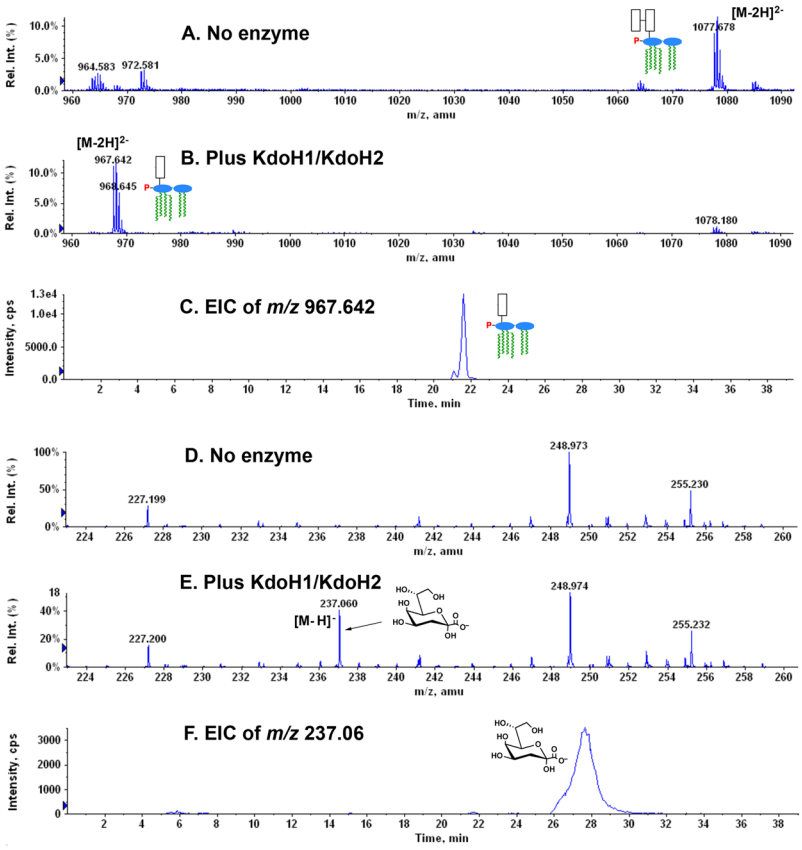

Fig. 9. Identification of the in vitro reaction products generated by the recombinant Kdo hydrolase by normal phase LC/ESI/MS

For this purpose, 100 μM hexa-acylated 1-dephospho-Kdo_2-lipid A was used as the substrate together with 0.5 mg/mL of membranes from *E. coli* expressing both proteins. **Panels A, B and C**. Analysis of the chloroform-soluble (lower phase) substrate and product before or after incubation with KdoH1/KdoH2. **Panels D, E and F.** Analysis of the water-soluble (upper phase) product. The reaction mixtures were extracted with a two phase Bligh-Dyer system (Bligh & Dyer, 1959), and the compounds in the lower and upper phases were subjected to normal phase LC/ESI/MS analysis in the negative ion mode. EIC, extracted ion current.

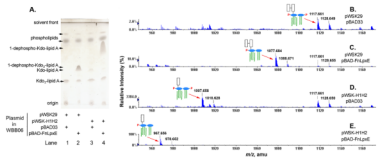

Fig. 10. Tandem modification of Kdo_2-lipid A by LpxE and KdoH1/KdoH2 in *E. coli*

Panel A. The heptose deficient strain, WBB06 (Brabetz et al., 1997), was used as the host to express LpxE, KdoH1/KdoH2, or both. When expressed individually, a new lipid A species appears in each strain, but migrating with differently. When both LpxE and KdoH1/KdoH2 are expressed together, a rapidly migrating species accumulates, indicating the combined effect of both enzymes on Kdo_2-lipid A. **Panel B**. The ESI/MS analysis in the negative ion mode of the total lipids confirms that some Kdo_2-lipid A (Raetz et al., 2006) is still present in all the strains, which was used to calibrate the spectra. Expression of LpxE causes accumulation of 1-dephospho- Kdo_2-lipid A, whereas Kdo hydrolase expressed from pWSK-H1H2 removes one Kdo residue from Kdo_2-lipid A. When both KdoH1/KdoH2 and LpxE are expressed, the combination generates the novel species 1-dephospho-Kdo-lipid A.

Table 1

Bacterial strains and plasmids.

Strain or plasmids	Description	Source
Strains		
W3110	Wild-type *E. coli*, F^-, λ^-	*E. coli* Gentic Stock Center, Yale University
WBB06	W3110 (*waaC-waaF::tet6*)	(Brabetz et al., 1997, Raetz et al., 2006)
CMR300	W3110 (*kdtA::kan*) pWMsbA	(Reynolds & Raetz, 2009)
U112	Wild-type *F. novicida*	Dr. F. Nano, University of Victoria, Canada
XWK4	U112 (*lpxF::kan*)	(Wang et al., 2007)
JZ-H1	U112 (*kdoH1::kan*)	this work
JZ-H2	U112 (*kdoH2::kan*)	this work
JZ-H1H2	U112 (*kdoH1-kdoH2::kan*)	this work
Plasmids		
pACYC184	Medium copy vector, Tet^R, Cam^R	New England Biolabs
pWSK29	Low copy vector, *lac* promoter, Amp^R	(Wang & Kushner, 1991)
pWMsbA	pWSK29 harboring *E. coli msbA*	(Reynolds & Raetz, 2009)
pWSK-H1	pWSK29 harboring *F. novicida kdoH1*	this work
pWSK-H2	pWSK29 harboring *F. novicida kdoH2*	this work
pWSK-H1H2	pWSK29 harboring *F. novicida kdoH1* and *kdoH2*	this work
pBAD33	Medium copy vector, Cam^R	(Guzman *et al.*, 1995)
pBAD-FnLpxE	pBAD33 harboring *F. novicida lpxE* (Previously designated pBAD33-lpxE)	(Ma et al., 2008)
pBAD-FnKdtA	pBAD33 harboring *F. novicida kdtA*	this work
pMP529	Shuttle vector for *F. novicida* and *E. coli*	(Maier et al., 2004)
pMP-H1	pMP529 harboring *F. novicida kdoH1*	this work
pMP-H2	pMP529 harboring *F. novicida kdoH2*	this work
pMP-H1H2	pMP529 harboring *F. novicida kdoH1* and *kdoH2*	this work