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Kdo hydroxylase is an inner core assembly enzyme in the Ko-containing lipopolysaccharide biosynthesis

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ABSTRACT

The lipopolysaccharide (LPS) isolated from certain important Gram-negative pathogens including a human pathogen *Yersinia pestis* and opportunistic pathogens *Burkholderia mallei* and *Burkholderia pseudomallei* contains D-glycero-D-*talo*-oct-2-ulosonic acid (Ko), an isosteric analog of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Kdo 3-hydroxylase (KdoO), a Fe^{2+}/α-KG/O_2 dependent dioxygenase from *Burkholderia ambifaria* and *Yersinia pestis* is responsible for Ko formation with Kdo_2-lipid A as a substrate, but in which stage KdoO functions during the LPS biosynthesis has not been established. Here we purify KdoO from *B. ambifaria* (BaKdoO) to homogeneity for the first time and characterize its substrates. BaKdoO utilizes Kdo_2-lipid IV_A or Kdo_2-lipid A as a substrate, but not Kdo-lipid IV_A *in vivo* as well as *in vitro* and Kdo-(Hep)kdo-lipid A *in vitro*. These data suggest that KdoO is an inner core assembly enzyme that functions after the Kdo-transferase KdtA but before the heptosyl-transferase WaaC enzyme during the Ko-containing LPS biosynthesis.

1. Introduction

The outer membrane of Gram-negative bacteria is an asymmetric lipid bilayer that consists of phospholipids in its inner leaflet and lipopolysaccharide (LPS) in its outer leaflet and functions as a permeability barrier against hydrophobic compounds, such as detergents, bile salts, and antibiotics [1]. LPS is composed of three major parts: the hydrophobic lipid A (endotoxin), a non-repeating core oligosaccharide including two 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, and an O-antigen polymer [2,3].

The *Burkholderia cepacia* complex (BCC) comprises 17 closely related species that are isolated from diverse ecological niches, such as soils, rhizospheres, water, plants, fungi, animals, and infected humans [4]. Some members of the BCC have been used for efficient plant growth promotion [5] and bioremediation [6]. On the other hand, other members of the BCC are opportunistic human pathogens that can cause severe necrotizing pneumonia and septicemia in cystic fibrosis patients and in immuno-compromised individuals [7]. These Gram-negative bacteria synthesize an unusual isosteric analog of Kdo, known as D-glycero-D-*talo*-oct-2-ulosonic acid (Ko) [8–12], in which the axial hydrogen atom at the 3-position is replaced with a hydroxyl group [8] (Fig. 1A). We previously reported that heterologous expression of Kdo 3-hydroxylase (KdoO) from *Burkholderia ambifaria* (*B. ambifaria*) and *Yersinia pestis* (*Y. pestis*) in heptosyl transferase-deficient *Escherichia coli* WBB06 elicits hydroxylation of the 3-deoxy carbon in the outer Kdo unit of Kdo_2-lipid A, resulting in Ko formation. KdoO exhibits

Abbreviations: α-KG, alpha-ketoglutarate; Ara4N, 4-amino-4-deoxy-L-arabinose; BaKdoO, *Burkholderia ambifaria* KdoO; BCA, bicinchoninic acid; BCC, *Burkholderia cepacia* complex; BSA, bovine serum albumin; DEAE-cellulose, Diethylaminoethyl cellulose; DTT, dithiothreitol; EcKdtA, *Escherichia coli* KdtA; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FPLC, fast protein liquid chromatography; Hep, heptose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HiKdtA, *Haemophilus influenza* KdtA; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; KdtA, Kdo transferase; KdoO, Kdo hydroxylase; Ko, D-glycero-D-*talo*-oct-2-ulosonic acid; LPS, lipopolysaccharide; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TLC, thin layer chromatography; WaaC, heptosyl transferase to Kdo_2-lipid A; WaaF, heptosyl tansferase to Kdo-(Hep)Kdo-lipid A; YpKdoO, *Yersinia pestis* KdoO.

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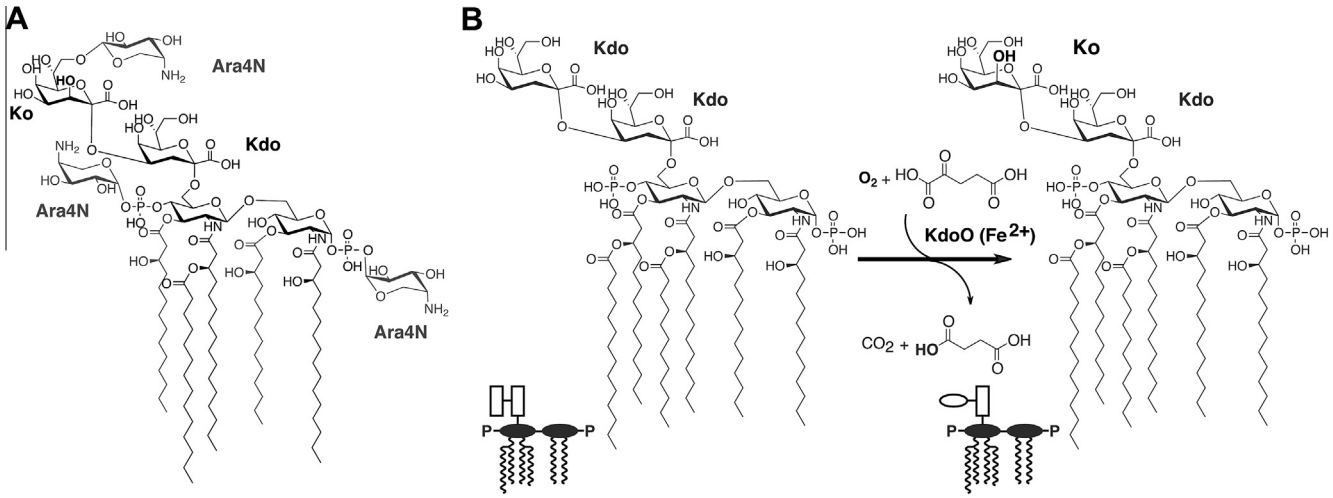

Fig. 1. (A) Ko-Kdo-lipid A structure of BCC. Gray colored residues are present in non-stoichiometric amounts. (B) Conversion of Kdo to Ko by KdoO. Kdo, *black boxes*; Ko, *empty oval*; glucosamine, *filled gray ovals*; acyl chains, *gray squiggles*: Phosphate group, *P*.

hydroxylase activity in an O_2, Fe^{2+} and α-ketoglutarate (α-KG) dependent manner [13] (Fig. 1B). KdoO belongs to the Domain of Unknown Function 2843 (DUF2843) family (Accession Number: PF11004, http://pfam.sanger.ac.uk/family/PF11004.3) that was annotated as the bacterial protein family with unknown function. KdoO homologues are found in human pathogens such as *Burkholderia mallei, Burkholderia pseudomallei, Klebsiella pneumoniae*, and *Coxiella burnetii*, in plant pathogens such as *Ralstonia solanacearum*, and in some environmental organisms such as *Methylobacterium extorquens*. [13]. It has been speculated that the extra OH group in Ko-containing LPS may provide an advantage under stressful environmental conditions by facilitating hydrogen bonding between adjacent LPS molecules in the outer membrane, and enhance or modulate the binging of LPS to TLR4/MD2 [13,14]. In addition, Ko-Kdo-lipid A was shown to be more resistant to mild acid hydrolysis than Kdo_2-lipid A *in vitro*, demonstrating the enhanced chemical stability of the Ko-Kdo and (Ko-Kdo)-lipid A linkages [13]. Therefore, understanding Ko formation in LPS assembly will provide insights into the biochemical and physiological role of outer membrane biogenesis. While KdoO belongs to the Fe^{2+}/α-KG/O_2 dependent dioxygenase family, it does not share high sequence similarity with any characterized Fe^{2+}/α-KG/O_2 dependent dioxygenase and has never been purified and characterized before.

Here, we report the purification of *B. ambifaria* KdoO (BaKdoO) to near homogeneity. After stabilizing its activity, we establish that KdoO is an inner core assembly enzyme and functions after the Kdo transferase but before the heptosyl transferase in the Ko-containing LPS biosynthesis.

2. Experimental: materials and methods

2.1. Materials

Chloroform, methanol, and silica gel 60 (0.25 mm) thin layer chromatograph (TLC) plates and high-performance analytical TLC (HPTLC) plates were from EMD Chemicals (Gibbstown, NJ). Tryptone, yeast extract, and agar were from BD Sciences (Franklin Lakes, NJ). Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was from Invitrogen (Carlsbad, CA). [γ-^{32}P]ATP (3 mCi/nmol) and γ-$^{32}P_i$ were from PerkinElmer (Waltham, MA). All other chemicals including α-KG, $Fe(NH_4)_2(SO_4)_2$, were of reagent grade. Purified Kdo_2-lipid A was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Bacterial strains

Bacterial strains used in this study are described in supporting Table SI.

2.3. Molecular biology techniques

Protocols for handling DNA and preparing *E. coli* cells for electroporation were those of Sambrook and Russell [15]. Chemical transformation-competent *E. coli* cells were prepared according to the method of Inoue et al. [16].

2.4. Plasmid constructions and transformations into E. coli C41(DE3), CMR300, JW3596, or JW3595

A C-terminally His_6-tagged pBAKdoO-His_6 was constructed using primers prHSC167 (5′-GGCGCAGCATATGAGCGAATCCCAGAT CATCGA-3′) and prHSC171 (5′-GCAGAAGCTTAACCAGCGCCCGGC-3′), and the pET21b vector. The resulting plasmid was confirmed with primers T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′) and transformed into C41(DE3) [17]. pBaKdoO-HiKdtA was constructed with the extended PCR method described in reference [18] using primer pairs prHSC167/prHSC229 (5′-GCAAGCTGGTATAAAAAAAA CGCCACATTG GTATAT CTCCTTCTTATCAAACCAGCGCCCGG-3′, and prHSC228 (5′-CCGG GCGC TGGTTTGATAAGAAGGAGATATACCAATGTGGCGTTTTTTTTAT ACCAGCTTGC-3′)/prHSC7 (5′-GCAGAAGCTTTCATACATTGCGCTCCA AATAAGG TTTT-3′), with pBaKdoO.1 and pHiKdtA (Table S1) as PCR templates, respectively. The resulting PCR products from both reactions were mixed in a 1:1 ratio and used as a template for the second PCR, which was performed using primers prHSC167 and prHSC7. The resulting PCR products were ligated into the pBAD33.1. The sequence of the resulting plasmid was confirmed with primers 33F (5′-CTGTTTCTCCATACCCGTT-3′), 33R (5′-AATTCT GTTTTATCAGACCGCTT-3), and prHSC33 (5′-TGAGATCATATTTAATA TTGCCCGTGATATTCA-3′). These plasmids, pHiKdtA containing *Haemophilus influenza kdtA* [18] and pBAKdo-HiKdtA, were transformed into CMR300 [19], JW3596, and JW3595 [20].

2.5. Purification of BaKdoO-His_6

The overall purification scheme for BaKdoO-His_6 is shown in Fig. S2. C41(DE3)/pBaKdoO-His_6 was grown, induced at 18 °C for 20 h with 1 mM IPTG. The cells were then harvested and washed

with phosphate-buffered saline (PBS) [21]. They were suspended with buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, supplemented with 100 mM NaCl. Cells were lysed by passage through a French Pressure cell at 17,000 psi, and the lysates were centrifuged at 8000×*g* to remove cell debris. A portion of the supernatant was retained as the "cell-free lysate". The cell-free lysate from *E. coli* over-expressing BaKdoO-His$_6$ was centrifuged at 40,000 rpm (~140,000×*g*) in a Beckman 50.2 rotor for 1 h at 4 °C. The supernatant was the "membrane-free lysate". The membrane pellet was re-suspended, homogenized, and then immediately washed with 50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA. The solution was centrifuged immediately at 40,000 rpm (~140,000×*g*) in a Beckman 50.2 rotor for 1 h at 4 °C. The supernatant was the "EDTA wash" fraction. The membrane proteins were solubilized with 50 mM HEPES, pH 7.5, 300 mM NaCl, 20% glycerol (buffer A) containing 1.5% Triton X-100. The solubilized BaKdoO-His$_6$ was purified by Ni–NTA affinity purification. BaKdoO-His$_6$ was eluted with 300 mM imidazole in buffer A and immediately supplemented with 1 M dithiothreitol (DTT) and 0.5 M EDTA, pH 7.5 to yield final concentrations of 2 mM DTT and 1 mM EDTA, and kept at 4 °C. The concentrated sample was purified in a 320 mL calibrated size-exclusion column (HiLoad 26/600 Superdex 200 prep grade; GE Healthcare, Waukesha, WI), with buffer A containing 2 mM DTT and 1 mM EDTA at 4 °C using an AKTA FPLC system equipped with the UNICORN program (GE Healthcare, Waukesha, WI) at 4 °C. Fractions containing BaKdoO-His$_6$, as judged by A_{280} and SDS–PAGE, were pooled and concentrated to 7.3 mg/mL. Protein was diluted into 1–5 mg/mL and stored at −80 °C. Protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL) or Bradford assay (Biorad, Hercules, CA) with bovine serum albumin (BSA) as a standard [22] and by measuring absorbance at 280 nm(A_{280}) using theoretical molar extinction coefficient, 34,950 M^{-1} cm^{-1} (http://web.expasy.org/protparam/).

2.6. Extraction of total lipids from CMR300/pHiKdtA, CMR300/pBaKdoO-HiKdtA, JW3596/pHiKdtA, JW3596/pBaKdoO-HiKdtA, JW3595/pHiKdtA, and JW3595/pBaKdoO-HiKdtA

Cells harboring suitable plasmids were grown from overnight cultures and diluted 1:100 into 100 mL LB broth [23], containing 20 μg/mL chloramphenicol and/or 50 μg/mL ampicillin and 0.2% l-Ara. Cells were grown at 30 °C with shaking at 220 rpm. When the A_{600} reached 0.8–1.0, cells were harvested by centrifugation at 4000×*g* for 20 min, washed once with 30 mL of PBS [21]. Total lipids were extracted by Bligh–Dyer (B/D) system [24] and analyzed by thin layer chromatograph (TLC) as described in reference [13].

2.7. Preparation of Kdo-lipid IV_A, Kdo_2-lipid IVA, Kdo_2-lipid A, Kdo-(Hep)Kdo-lipid A

Kdo-lipid IV_A and Kdo-(Hep)Kdo-lipid A were extracted by the similar method described above from 2 L of CMR300/pHiKdtA and JW3595, respectively. Dried lipids were purified with DEAE-cellulose column chromatography as described by Kanjilal-Kolar and Raetz [25]. Kdo_2-lipid IV_A was obtained as described [26]. Kdo_2-lipid A was purchased from Avanti Polar Lipids. Inc (Alabaster, USA).

2.8. Preparation of radiolabeled substrates

^{32}P-labeled Kdo_2-lipid A substrate was prepared according to published procedures [27]. ^{32}P-labeled lipid IV_A [18] was converted to ^{32}P-labeled Kdo-lipid IV_A and Kdo_2-lipid IV_A by *in vitro* conversion in the presence of 50 mM Hepes (pH 7.5), 1 mM Kdo, 0.1% Triton X-100, 20 μM [4′-^{32}P]lipid IV_A (~2 mCi/nmol), 2.5 mM CTP, 10 mM $MgCl_2$, 1.8 milliunits of partially purified CMP-Kdo synthase and CMR300/pHiKdtA membrane. Products were mixture (2:3 ratio) of Kdo-lipid IV_A and Kdo_2-lipid IV_A. These two products were purified by preparative TLC method [13] developed in 30:70:16:10 (Chroloform: pyridine: 88% formic acid: water) tank. ^{32}P-labeled Kdo-(Hep)Kdo-lipid A was extracted by two-phase B/D method from JW3595 with whole cell ^{32}P-labeling method similar to ^{32}P-labeled lipid X preparation [28]. ^{32}P-labeled Kdo-(Hep)Kdo-lipid A was purified by preparative TLC method.

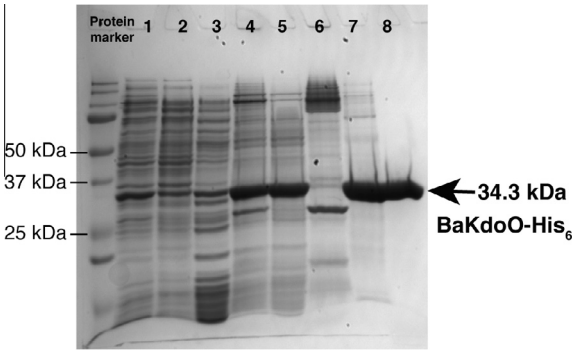

Fig. 2. Purification of C-terminally His$_6$-tagged BaKdoO (BaKdoO-His$_6$). SDS–PAGE analysis of ~15 μg of protein samples from each step of the BaKdoO-His$_6$ purification. Numbers denote fractions obtained from purification steps in methods. 1: cell free, 2: membrane free, 3: EDTA wash, 4: membrane, 5: solubilized membrane, 6: insoluble membrane, 7: Ni–NTA purification, and 8: size exclusion purification.

2.9. In vitro assays of BaKdoO-His$_6$

In vitro assays for purified BaKdoO-His$_6$ were carried out at 30 °C, quenched, and quantified with method described in reference [13] with modifications. The reaction mixture (typically in a final volume of 20 μL) contained 50 mM HEPES, pH 7.5, 1 mM α-KG, 2 mM ascorbate, 15 μM $Fe(NH_4)_2(SO_4)_2$, 0.1% Triton X-100, 4 mM DTT, 0.5 mg/mL BSA, and 5 μM Kdo_2-[4′-^{32}P]lipid A (~300,000 cpm/nmol), 5 μM Kdo_2-[4′-^{32}P]lipid IV_A (~300,000 cpm/nmol), 5 μM Kdo-(Hep)Kdo-[4′-^{32}P]lipid A (~300,000 cpm/nmol) or 5 μM Kdo-[4′-^{32}P]lipid IV_A (~300,000 cpm/nmol). Just prior to assaying, BaKdoO-His$_6$ from stock solutions (1–5 mg/mL) were diluted with buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, and 0.5 mg/mL BSA.

3. Results

3.1. Purification and activity stabilization of BaKdoO-His$_6$

C41(DE3)/pBaKdoO-His$_6$ showed significant overexpression of BaKdoO-His$_6$ (Fig. 2, lane 1) and most of the expressed protein was solubilized from membranes by 1.5% w/v Triton X-100 (Fig. 2, lane 5 vs 6). From a 1.4 L (A_{600} ~3.9) cell culture, ~15 mg of BaKdoO-His$_6$ was purified to greater than 95% homogeneity using Ni–NTA affinity and size-exclusion chromatography (Fig. 2). Despite minor loss of membrane-associated BaKdoO-His$_6$ when membranes were washed with 1 mM EDTA, 50 mM HEPES (pH 7.5), and 100 mM NaCl, this wash step significantly improved the purity of the protein (Fig. 2, lane 3 vs 4). BaKdoO-His$_6$ with a calculated MW of 34,327 Da was eluted at 229.6 mL from a calibrated size exclusion column, where it chromatographed as a symmetric peak, implying that BaKdoO-His$_6$ behaved as a monomer in solution. Initially, we attempted to purify and store BaKdoO-His$_6$ without DTT and EDTA, but the activity of BaKdoO-His$_6$ had a

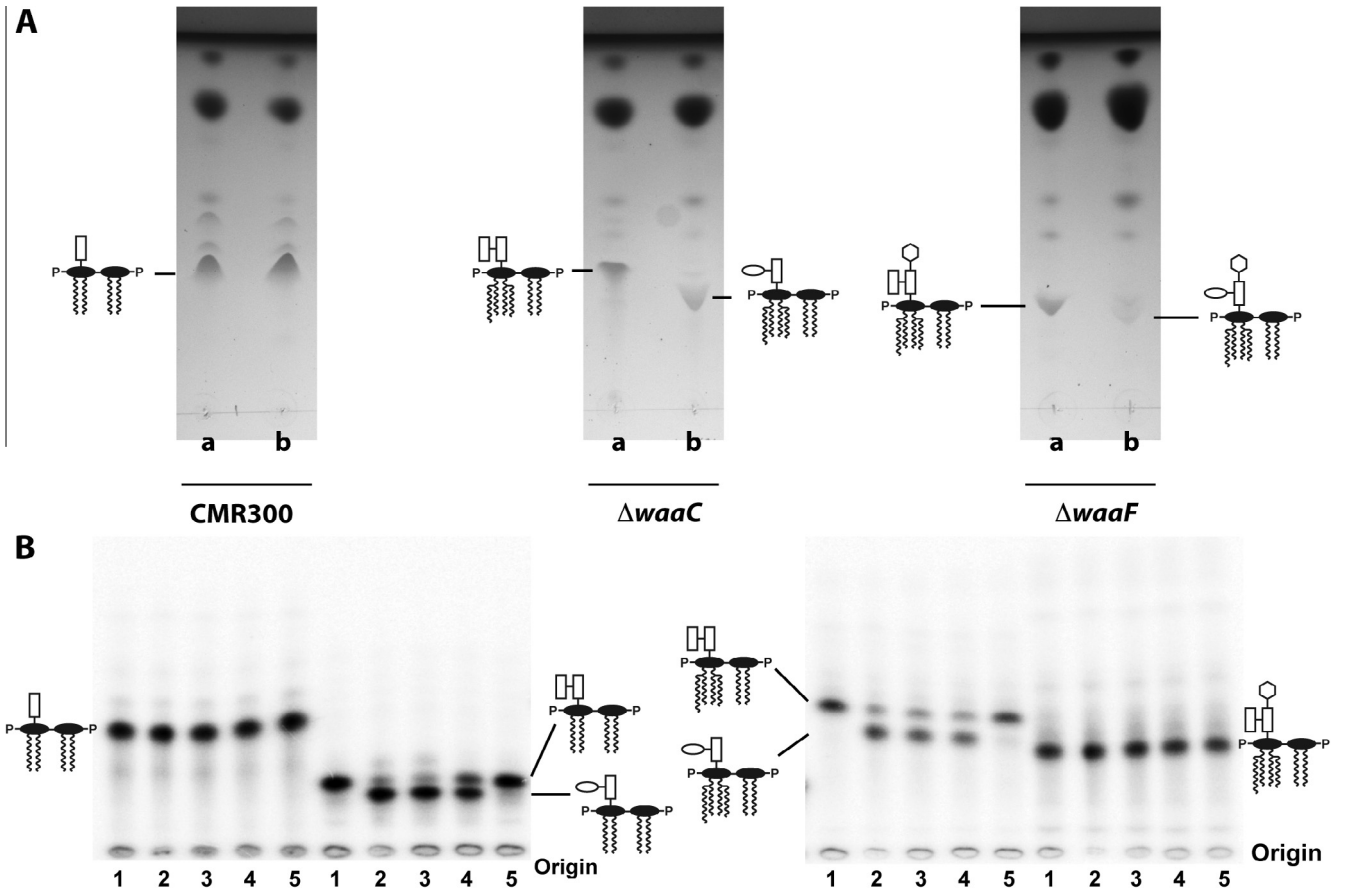

Fig. 3. *In vivo* and *in vitro* substrate selectivity of BaKdoO. (A) TLC plates of Lipid A species extracted from CMR300, JW3596 (Δ*waaC*), and JW3595 (Δ*waaF*) carrying pHiKdtA (a) or pBaKdoO-HiKdtA (b). The TLC plates were developed in chloroform:methanol:acetic acid:H_2O (25:15:3.5:4 v/v) and visualized with a charring method. (B) *In vitro* KdoO assays with different substrates and enzyme concentrations- Kdo-[4′-^{32}P]lipid IV_A, Kdo_2-[4′-^{32}P]lipid IV_A, Kdo_2-[4′-^{32}P]lipid A, and Kdo-(Hep)Kdo-[4′-^{32}P]lipid A at 5 μM and 1: no enzyme, 2: 200 μg/mL, 3: 20 μg/mL, 4: 2 μg/mL, or 5: 0.2 μg/mL of purified BaKdoO-His_6. The TLC plates were developed in chloroform:methanol:acetic acid:H_2O (25:15:3.5:4 v/v) and visualized with a PhosphorImager screen. Kdo, *black boxes*; Ko, *empty oval*; heptose, *hexagon*; glucosamine, *filled gray ovals*; acyl chains, *gray squiggles*; Phosphate group, *P*.

half-life of approximately one week under these storage conditions. The activity of BaKdoO-His_6 was fully maintained for more than 3 months when DTT and EDTA were included in the final purification step. The optimized purification scheme for BaKdoO-His_6 is shown in Fig. S1. The specific activity of the protein increased ~10-fold after purification (Table S2), consistent with the enrichment of BaKdoO-His_6 observed by SDS–PAGE (Fig. 2). The total activity of the purified protein was ~18% of the cell-free lysate (Table S2). Purity and homogeneity of the purified BaKdoO-His6 is shown by SDS–PAGE and size exclusion chromatogram (column: GEhealthcare, Superdex 200 10/300 GL) in Figs. 2 and S2.

3.2. Substrate selectivity and the biosynthetic pathway of Ko-containing LPS

3.2.1. Substrate selectivity of BaKdoO in vivo

To better place KdoO into the overall biosynthetic pathway for organisms that produce Ko-containing LPS, we heterologously expressed BaKdoO in suitable *E. coli* mutant strains: strain CMR300 [19] producing lipid IV_A, strain JW3596 (Δ*waaC*) [20] producing Kdo_2-lipid A, or strain JW3595 (Δ*waaF*) [20] producing Kdo-(Hep)Kdo-lipid A as their primary LPS species. An expression plasmid containing the monofunctional KdtA from *Haemophilus influenza* (pHiKdtA), that can only add one Kdo residue to lipid-IV_A, or pBaKdoO-HiKdtA, an expression plasmid encoding both BaKdoO and HiKdtA, in CMR300 resulted in identical lipid A profiles based on TLC (Fig. 3A, left panel) analysis. Electrospray ionization mass spectrometry (ESI-MS) confirmed that the major LPS species is Kdo-lipid IV_A (data not shown). This result indicated that at least 2 Kdo sugars must be present for optimal KdoO activity. Consistent with this hypothesis overexpression of pBaKdoO-HiKdtA in JW3596 expressing endogenous *E. coli* KdtA (EcKdtA) from its chromosome resulted in the production of Ko-Kdo-lipid A as the major LPS species (Fig. 3A, middle panel). Together, these results indicated that Kdo-lipid IV_A is unlikely a substrate of BaKdoO, while Kdo_2-lipid A serves as a good substrate *in vivo*. Overexpression of pHiKdtA or pBaKdoO-HiKdtA in JW3595 resulted in Kdo-(Hep)Kdo-lipid A or a mixture of Kdo-(Hep)Kdo-lipid A/Ko-(Hep)Kdo-lipid A respectively (Fig. 3A right panel). One possible explanation for the incomplete conversion of Kdo to Ko in JW3595 is that Kdo-(Hep)Kdo-lipid A might be a less preferred substrate of BaKdoO than Kdo_2-lipid A, resulting in a situation where BaKdoO and the LPS export system (including the flippase MsbA [29,30]) could compete for the same substrate. Another possible hypothesis is that Kdo-(Hep)Kdo-lipid A is not a substrate of BaKdoO at all, where WaaC competes with BaKdoO for Kdo_2-lipid A.

3.2.2. Substrate selectivity of the purified BaKdoO-His_6 in vitro

To clarify the substrate specificity of BaKdoO, we performed *in vitro* assays using different potential substrates: including Kdo-lipid IV_A, Kdo_2-lipid IV_A, Kdo_2-lipid A, and Kdo-(Hep)Kdo-lipid A and tested their conversion to corresponding Ko containing products in the presence of BaKdoO-His_6 (Fig. 3B). Kdo-lipid IV_A was not

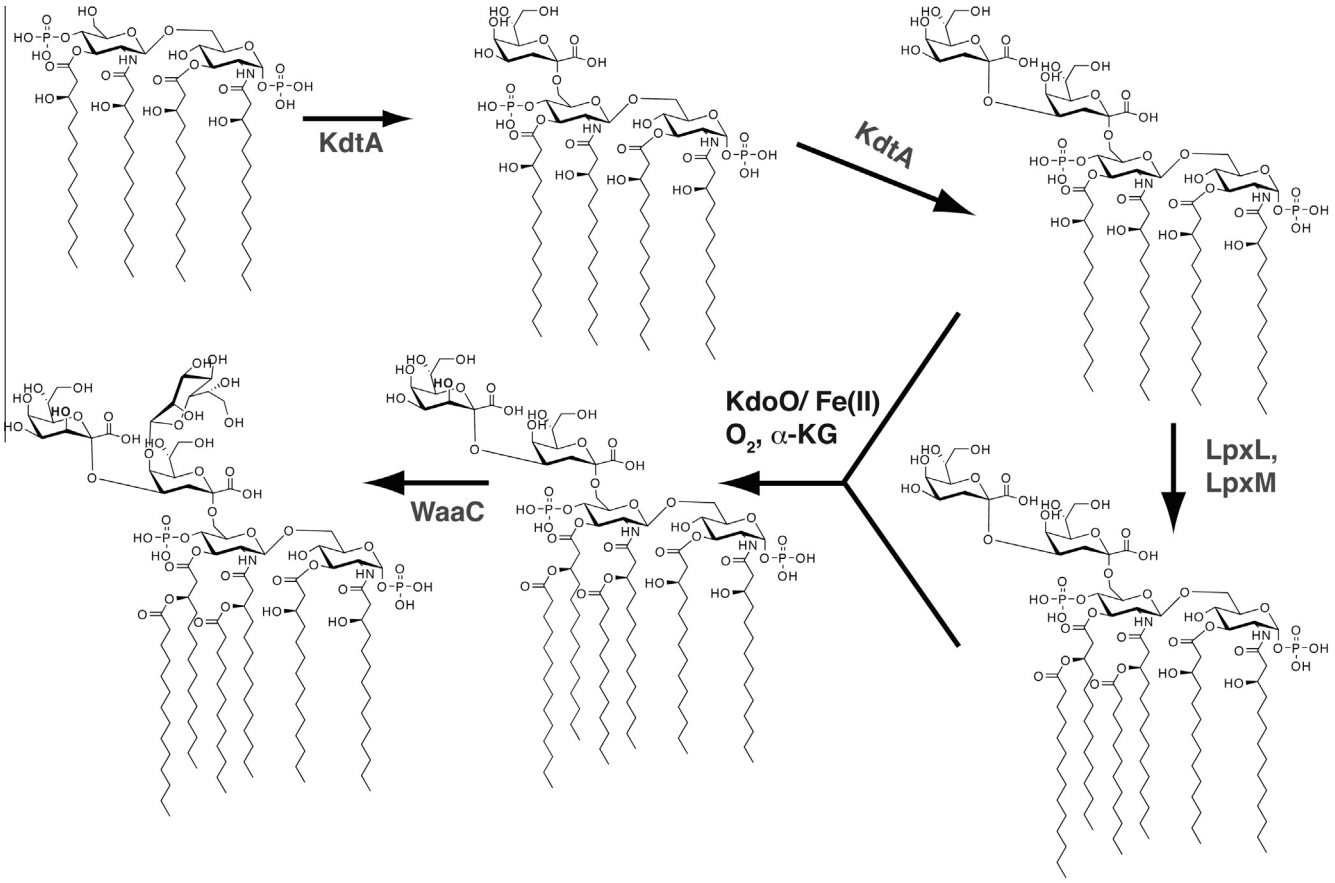

Fig. 4. Proposed biosynthetic pathway of Ko-Kdo containing lipid A species in respect to LPS assembly. KdoO functions after KdtA but before WaaC in the LPS biosynthesis.

converted to a Ko containing species, as no migration shift was observed by TLC analysis (Fig. 3B). However, Ko-Kdo-lipid IV_A or Ko-Kdo-lipid A was formed in the presence of 0.2–2 μg/mL of BaKdoO-His_6, when Kdo_2-lipid IV_A or Kdo_2-lipid A was supplied as a substrate, respectively. This result implied that the presence of secondary acyl chains on the lipid A domain does not affect KdoO activity, and further supported the hypothesis that 2 Kdo sugars are required. A Ko containing product was not observed when Kdo-(Hep)Kdo-lipid A was provided as a substrate, even in the presence of 200 μg/mL of BaKdoO-His_6 (Fig. 3B), in agreement with the strict requirement of BaKdoO for the presence of 2, and only 2, Kdo sugars.

4. Discussion

While quantitative amounts of Ko [31,32] are found in BCC LPS, a Ko-derivatized lipid A moiety is observed as a minor component of LPS isolated from *Y. pestis* grown at 6–7 °C [33]. This difference might result from temperature dependent gene expression in Yersinia or the endogenous character of each KdoO (52% sequence identity and 64% sequence similarity between BaKdoO and YpKdoO) but it might also reflect the relative efficiency of WaaC and KdoO in each species, as these enzymes compete for common substrates.

BCC exhibits extraordinary intrinsic resistance to antimicrobial peptides such as polymyxin. In most other Gram-negative bacteria 4-amino-4-deoxy-l-arabinose (Ara4N) is added only to lipid A, and Ara4N in BCC is added to the lipid A as well as in the C8 position of the Ko residue (Fig. 1A) and which is essential for BCC survival and polymyxin resistance [34,35]. At this point, it is not clear if C3 hydroxylation of Kdo (i.e. Ko formation) is required for Ara4N addition in BCC and contributes to polymyxin resistance. However, we have clearly demonstrated that BaKdoO functions after KdtA, but before WaaC in the LPS inner core assembly (Fig. 4) based on *in vivo* and *in vitro* substrate selectivity. Since Ara4N modification to lipid A usually occurs after LPS inner core assembly in the periplasmic site of inner membrane [36], Ko-containing LPS must be a substrate for Ara4N modification enzyme in a majority of BCC. It is noteworthy that *kdoO* is located in a gene cluster consisting of *waaC* and *kdtA*, constitutive inner core assembly enzymes involved in the LPS biosynthesis [37] in chromosome 1, and accordingly *kdoO* is very likely co-translated with *kdtA* and *waaC* in a majority of BCC. In addition, BaKdoO's substrate specificity for lipid A precursors, its requirement for Fe^{2+}, and its membrane-associated characteristics suggest that BaKdoO is a peripheral inner membrane protein facing the cytoplasm and a part of the inner core biosynthetic enzymes rather than a modification enzyme of Ko-containing LPS in a majority of BCC (Fig. 4).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.153.

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