The biosynthetic pathway for Kdo8N in S. oneidensis

The origin of 8-amino-3,8-dideoxy-D-manno-octulosonic acid (Kdo8N) in the lipopolysaccharide of *Shewanella oneidensis*.

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**Background:** The lipopolysaccharide of *Shewanella oneidensis* contains a unique sugar, Kdo8N.

**Results:** The Kdo8N biosynthetic genes were identified and characterized.

**Conclusion:** Kdo8N is synthesized from Kdo via a two-step mechanism utilizing O₂ and glutamate.

**Significance:** These results expand the known lipid A biosynthetic enzymes, which is important for various human health applications, and present evidence for a potentially novel class of alcohol oxidases.

**SUMMARY**

Lipopolysaccharide (LPS, endotoxin) is an essential component of the outer monolayer of nearly all Gram-negative bacteria. LPS is composed of a hydrophobic anchor, known as lipid A, an inner core oligosaccharide, and a repeating O-antigen polysaccharide. The first sugar bridging the hydrophobic lipid A and the polysaccharide domain is 3-deoxy-D-manno-octulosonic acid (Kdo) in nearly all species, and thus is critically important for LPS biosynthesis. Modifications to lipid A have been shown to be important for resistance to antimicrobial peptides as well as modulating recognition by the mammalian innate immune system. Therefore, lipid A derivatives have been used for development of vaccine strains and vaccine adjuvants. One derivative that has yet to be studied is 8-amino-3,8-dideoxy-D-manno-octulosonic acid (Kdo8N), which is found exclusively in marine bacteria of the genus *Shewanella*. Using bioinformatics, a candidate gene cluster for Kdo8N biosynthesis was identified in *Shewanella oneidensis*. Expression of these genes recombinantly in *E. coli* resulted in lipid A containing Kdo8N, and in vitro assays confirmed their proposed enzymatic function. Both the in vivo and in vitro data were consistent with direct conversion of Kdo to Kdo8N prior to its incorporation into the Kdo8N-lipid A domain of LPS by a metal-dependent oxidase, followed by a glutamate-dependent aminotransferase. To our knowledge, this oxidase is the first enzyme shown to oxidize an alcohol using a metal and molecular oxygen, not NAD(P)⁺. Creation of an *S. oneidensis* in-frame deletion strain showed increased sensitivity to the cationic antimicrobial peptide.
polymyxin, as well as bile salts, suggesting a role in outer-membrane integrity.

INTRODUCTION

Bacteria of the genus Shewanella are ubiquitous marine organisms known for their remarkable metabolic capabilities (1). They have attracted substantial interest for industrial applications, such as bioremediation of heavy metal contamination, generation of electrical power from biomass, and synthesis of omega-3 polyunsaturated fatty acids (1,2). More recently, Shewanella species have also been identified as opportunistic pathogens, typically from exposure of broken skin to the marine environment (3-5).

Like nearly all Gram-negative bacteria, Shewanella possess both an inner and an outer membrane, in which the outer-most layer of the outer membrane is primarily composed of lipopolysaccharide (LPS). LPS contains a hydrophobic lipid anchor known as lipid A, an inner-core oligosaccharide, and a repeating O-antigen domain that is highly variable between species (6,7). The sugar 3-deoxy-D-manno-octulosonic acid (Kdo) is the first sugar added to lipid A, and laboratory strains of E. coli cannot survive without synthesizing the minimal LPS substructure Kdo2-lipid A (Figure 1), unless compensatory mutations are introduced. The biosynthesis of lipid A is largely conserved across Gram-negative organisms. Many species, such as E. coli, add two Kdo sugars with a single Kdo transferase, while others, including Shewanella, add a single Kdo which is then phosphorylated by a separate enzyme (Figure 2A). In strains of Shewanella, including the model organism S. oneidensis, Kdo is further modified, in which the C8 hydroxyl group is converted to a primary amine (Kdo8N, Figure 2A) (8-10). Here, we report the identification of a three-gene cluster in S. oneidensis that is responsible for biosynthesis of Kdo8N. When heterologously expressed in E. coli, >75% of the extracted LPS contains Kdo8N. Expression of all three genes is required for incorporation of Kdo8N into E. coli lipid A, allowing for a biosynthetic pathway to be proposed in which Kdo is directly converted to Kdo8N, followed by incorporation into lipid A. Purification of the enzymes and generation of an in vitro assay was also consistent with the proposed biosynthetic pathway. Furthermore, chromosomal deletion of the Kdo8N biosynthetic genes in S. oneidensis results in increased sensitivity to compounds known to perturb the outer membrane, such as polymyxin B and bile salts, suggesting the presence of Kdo8N may increase the integrity of the Shewanella outer-membrane.

EXPERIMENTAL PROCEDURES

General methods. Unless otherwise stated, all reagents were obtained from Sigma and were reagent grade or better. Primers were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). All primers, plasmids, and strains are listed in Table 1. Bacterial cultures were maintained in LB containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, supplemented with ampicillin (100 µg/mL), gentamicin (15 µg/mL), or dianimopimelate (DAP, 100 µg/mL), as needed.

Genomic database searching. The genome of Shewanella oneidensis was searched using NCBI/BLAST with the known aminosugar biosynthetic enzyme GnmB (11) from A. ferrooxidans as a query sequence. The neighboring genes on the chromosome were examined using the gene entry on the NCBI website.

Cloning and molecular biology. The SO_2476, SO_2477, and SO_2478 genes were amplified from S. oneidensis genomic DNA (ATCC). The genes of unknown function were renamed kdnA (SO_2476) and kdnB (SO_2477), while SO_2478 was presumed to be a CMP-Kdo synthase (KdsB, Figure 1) on the basis of homology (45% identical/58% similar to E. coli KdsB). PCR was performed using the KOD polymerase (EMD Biosciences) and the primers listed in Table 1. DNA fragments were generated for all three genes (Primers 1 and 6), each individual gene (primer combinations of 1 and 2, 3 and 4, 5 and 6), and for two gene constructs (primer combinations of 1 and 4, 3 and 6). Each DNA fragment contained a 5'-XbaI site and a 3'-XhoI site, as well as a ribosome binding site derived from pET21b. DNA fragments were digested with the restriction enzymes XbaI...
and XhoI (NEB), purified by a DNA binding spin-column (Qiagen), and ligated into pWSK29 (12) that had been similarly digested and purified using T4 DNA ligase (NEB). Plasmids, including a pWSK29 vector control, were transformed in E. coli strain WBB06 using the TSS method (13) and grown in LB supplemented with ampicillin and IPTG. The resulting plasmids and strains are listed in Table 1.

**Chromosomal deletion of kdnA/kdnB in S. oneidensis.** An in-frame deletion of SO_2476 and SO_2477 was created as previously described (14). Briefly, approximately 500 bp of sequence directly upstream and downstream of the target genes (SO_2476/kdnA and SO_2477/kdnB) was amplified by PCR using the *S. oneidensis* genomic DNA and primers 5O and 5I, 3O and 3I, respectively (Table 1). The primers were designed with a complementary sequence to the plasmid pDS3.1 (15), which had been similarly digested. This plasmid contains a gentamicin resistance marker, a SacB sucrose sensitivity marker, and utilizes an R6K origin of replication which can only replicate in strains harboring pWSK29 vector control (16) and XhoI (NEB). The fragment was digested by NotI (NEB) and purified as described above, and ligated into the plasmid pWSK29 vector control (16) in the presence of 100 µg/mL gentamicin were screened for the marker, and utilized an R6K origin of replication which can only replicate in strains harboring pWSK29 vector control (16).

**Lipid biosynthesis pathway for Kdo8N in S. oneidensis**. The biosynthetic pathway for Kdo8N in *S. oneidensis* was confirmed by PCR using primers 5O and 3O and primers 1 and 4.

**Lipid extraction.** Strains of *E. coli* WBB06 (18) harboring pWSK29-derived plasmids are listed in Table 1. Each strain was grown to saturation overnight in 5 mL LB supplemented with ampicillin. The cultures were then diluted 1:100 into 100 mL LB supplemented with ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were grown at 37°C to an OD<sub>600</sub> of 1.0, then harvested by centrifugation. Cells were washed once with 5 mL phosphate buffered saline (PBS), then frozen at -80°C. For lipid extraction, cells were thawed and resuspended in 5 mL PBS. The aqueous mixture was then converted to a one-phase Bligh-Dyer (19) by addition of 12.5 mL methanol and 6.25 mL chloroform. The cells were incubated at room temperature for 1 hour, then centrifuged at 2,000 x g for 30 min. The supernatant was then converted to an acidic two-phase Bligh-Dyer by addition of 6.25 mL each 0.1 N HCl and chloroform. The lower (chloroform) phase was removed, and the upper phase was washed once with 12.5 mL chloroform followed by removal of the lower phase. The combined chloroform fractions were neutralized with pyridine (~1 drop per 10 mL), dried under a nitrogen stream, and stored at -20°C.

**Lipid analysis by TLC and mass spectrometry.** Dried samples were dissolved in 1 mL 4:1 CHCl₃:MeOH and dispersed by sonication in a water bath. For thin-layer-chromatography (TLC), samples were spotted on a glass-backed 10 cm x 10 cm silica TLC plate, and developed in 25:15:3.5:4 CHCl₃:MeOH:CH₃COOH:H₂O. Plates were sprayed with 10% sulfuric acid in ethanol and charred on a hot plate. Separately, samples for mass spectrometry were diluted ~20 fold in 4:1 CHCl₃:MeOH supplemented with 1% piperidine. Samples were immediately infused at 5 µL/min and analyzed in the negative ion mode. Spectra were obtained on a QStar XL quadrupole time-of-flight mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) in direct injection mode equipped with an ESI source and analyzed using Analyst QS v.1.1.
Purification and activity assays of the kdnA and kdnB gene products. His-tagged expression constructs were made as follows: each gene was amplified from genomic DNA to incorporate a 5’ XbaI site and a 3’ XhoI site (KdnA) or a 5’ NdeI site and a 3’ BamHI site (KdnB). The PCR products were digested with the appropriate enzymes, purified, and ligated with T4 DNA ligase into pET21b (KdnA) or pET16b (KdnB) vectors, which had been similarly digested. Plasmids were confirmed by DNA sequencing (Eton biosciences) and transformed into chemically competent E. coli strain C41 (20). For KdnA, 2L of LB were inoculated at 37˚C and grown to an OD600 = 0.6, then induced with 1 mM IPTG and grown overnight. KdnB was expressed similarly, but the temperature was reduced to 25˚C at induction. After harvesting the cell pellet by centrifugation, cells were resuspended in 0.1 M Tris pH 8.0, 150 mM NaCl, 2 mM tris(carboxyethyl)phosphine (TCEP). Cells were lysed by 2 passages through a French Press, then the cell debris was removed by centrifugation at 20,000 x g for 1 hour. The cell free extract was loaded onto a Ni-NTA column equilibrated in the same buffer, washed with buffer plus 10 mM imidazole, and eluted with buffer plus 250 mM imidazole. Pure fractions of either KdnA or KdnB were pooled, concentrated, and dialyzed to remove imidazole. Pure KdnA was diluted to 50 µM in 0.1 M Tris pH 8.0, 150 mM NaCl, 2 mM TCEP, and 200 µM pyridoxal-5’-phosphate (PLP), and incubated on ice for 1 hour to allow for incorporation of the cofactor. Excess PLP was removed by buffer exchange, and the enzyme stored in aliquots at -80˚C. A sample of the purified KdnB enzyme was subjected to inductively coupled plasma mass spectrometry (ICP-MS) analysis (Keck Elemental Geochemistry Laboratory, University of Michigan). Pure Apo-KdnB was prepared by dilution to approximately 50 µM in 10 mM Tris, pH 8.0, 100 mM dipicolinic acid, 100 µM EDTA on ice for one hour. The enzyme was then desalted over a PD-10 column equilibrated with 10 mM Tris, pH 8.0, and stored in aliquots at -80˚C.

Assays were conducted using 14C-labeled Kdo, which was synthesized from 14C-labeled pyruvate (American Radiolabeled Chemicals Inc, St. Louis, MO) as previously described (21). A typical assay mixture contained 0.1 M Tris pH 8.0, 1 mM DTT, ~5,700 cpm 14C-Kdo, 50 µM Kdo (unlabeled), 100 mM L-glutamic acid, and an appropriate amount of enzyme. KdnB was supplemented with MnSO4 (one equivalent) or 1 mM MnSO4 was added to the assay, as indicated in the appropriate figure legends. Reactions were quenched by spotting on glass-backed silica TLC plates, and run in a tank containing 6:2:1 isopropanol:aqueous ammonia:water. TLC plates were exposed to a storage phosphor screen (GE Healthcare) for ~24 hours and then scanned by a phosphorimager and quantified using ImageQuant 5.0. Where appropriate, the above assays without 14C-Kdo were analyzed for hydrogen peroxide formation using the Amplex Red Hydrogen Peroxide/Peroxidase Activity Kit (Invitrogen) according to the manufacturer instructions.

Minimum inhibitory concentration (MIC) assays of wild-type and ΔkdnAΔkdnB S. oneidensis. MIC assays of S. oneidensis were conducted in 96-well plates as previously described (22). Briefly, 2-fold serial dilutions of compound (50 – 0.024 µg/mL polymyxin; 10 – 0.005 mg/mL bile salts) were inoculated with wild-type or ΔkdnAΔkdnB S. oneidensis to an OD600 = 0.05 in a total of 200 µL. Cells were grown overnight at 30˚C, and then stained with 50 µL 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours. Living cells metabolize MTT to a purple formazan, which was quantified by visual inspection. Assays were performed in quadruplicate in 96-well plates and repeated in 3 independent experiments.

RESULTS
Identification of the putative Kdo8N biosynthetic genes. BLAST searches of the S. oneidensis genome revealed a gene with 34% identity to the known aminosugar biosynthetic enzyme GnnB (11). This gene (SO_2476) was annotated as an aspartate aminotransferase. Adjacent to this gene was a putative alcohol dehydrogenase (SO_2477),
followed directly downstream by the *kdsB* gene (SO_2478, Figure 2B), which encodes the enzyme responsible for activating Kdo to the sugar-nucleotide donor CMP-Kdo (Figure 1). The annotated enzymatic functions and location of SO_2476 and SO_2477 adjacent to *kdsB* strongly suggested these genes could be responsible for Kdo8N biosynthesis. We renamed these genes *kdnA* (SO_2476) and *kdnB* (SO_2477).

**Expression of kdnA, kdnB, and kdsB results in Kdo8N biosynthesis.** To assess the function of the putative biosynthetic cluster, the lipid A products were analyzed in the background of WBB06 (18), an *E. coli* strain that synthesizes Kdo2-lipid A as its sole LPS species (Table 1). Strain SG1, which contains all three putative biosynthetic genes, was grown and the lipids extracted as described in Experimental Procedures. While the vector control strain produced the expected single band of Kdo2-lipid A (Figure 3, lane A), TLC analysis of the lipids from the strain expressing all three genes showed a mixture of 3 lipid A-like species (Figure 3, lane B). These bands consisted of Kdo2-lipid A and two more polar (slower migrating) species, which were presumed to indicate incorporation of one or two 8-amino-Kdo (Kdo8N) moieties in place of Kdo.

Kdo2-lipid A has an exact mass of 2235.32 amu, which appears by ESI-MS as a doubly charged species with an *m/z* = 1117.66. Replacement of Kdo with Kdo8N results in a 0.985 amu decrease; therefore (Kdo8N)2-lipid A has an expected mass of 2233.35 and will appear as a doubly-charged species ([M-2H]+2) with *m/z* = 1116.68, while a mixed Kdo/Kdo8N species would be 0.49 amu larger. The lipid A from the strain expressing the entire gene cluster shows peaks at 1117.477 and 1116.978, consistent with incorporation of 1 or 2 Kdo8N moieties in place of Kdo, respectively (Figure 4A). Furthermore, ESI-MS/MS fragmentation revealed no mass difference in the lipid A fragment, indicating there is no modification to the lipid A anchor (Figure 4, B and C). In contrast, there is a 0.434 amu difference in the [M-2H]+2 *m/z* for the fragment ion corresponding to loss of one Kdo (1007.907 vs. 1007.473), indicating the modification is in the Kdo moiety (Figure 4, B and C). This is further confirmed by a 0.986 amu decrease in the Kdo fragment, corresponding to a single amine substituent, and a 1.969 amu difference in the Kdo2-disaccharide moiety, corresponding to two amines (Figure 4, B and C). The cumulative evidence indicates the genes identified are sufficient for biosynthesis of Kdo8N.

The entire gene cluster is required for Kdo8N biosynthesis. Strains were constructed containing each of the individual genes from the Kdo8N biosynthetic cluster in a pWSK29 plasmid in WBB06 (Table 1). When these strains were grown and the lipids extracted as described above, no Kdo8N was observed by TLC (Figure 5) or ESI-MS analysis (data not shown). Furthermore, when the putative alcohol dehydrogenase and amino transferase genes were expressed with the CMP-Kdo synthetase gene *kdsB* omitted, still no Kdo8N was observed (Figure 5). Because *E. coli* possesses a *kdsB* gene, this result suggests the *S. oneidensis* *kdsB* gene product has a functional difference from its *E. coli* homologue that allows for Kdo8N biosynthesis.

Recombinant purified KdnA and KdnB can synthesize Kdo8N in vitro. Bioinformatic predictions suggested NAD⁺ or NADP⁺ and an amino acid such as aspartate or glutamate as co-substrates for KdnB and KdnA, respectively. Preliminary experiments using extracts suggested L-glutamate yielded the highest activity (supplemental figure S1). Interestingly, product formation occurred in extracts in the absence of added pyridine nucleotide and was not stimulated by additional NAD⁺, suggesting the classification of KdnB as an NAD-dependent alcohol dehydrogenase was incorrect. Both KdnA and KdnB were purified to homogeneity (Figure 6) and assayed in vitro as described in Experimental Procedures. Incubation of both purified enzymes with Kdo and L-Glu resulted in Kdo8N formation, confirming that Kdo is a direct substrate for the reaction (Figure 7, lane 8). KdnB alone, which is predicted to be the first step in the reaction, does not lead to formation of a detectable intermediate (Figure
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7, lane 4), suggesting two possibilities: (a) the reaction catalyzed by KdnB is thermodynamically unfavorable, and requires the second reaction catalyzed by KdnA to drive product formation, or (b) the two enzymes are active only in a complex. Since many alcohol dehydrogenases favor reduction of the aldehyde, we believe the first possibility is most likely. ICP-MS analysis showed KdnB contained 0.51 ± 0.03 moles of Fe per mole of enzyme, as well as 0.24 ± 0.01 equivalents of Zn. Metal activation experiments showed highest activity with Mn$^{2+}$ and Fe$^{2+}$ (supplemental figure S2), and inhibition by EDTA (Figure 7, lanes 3 and 7). Because Fe$^{2+}$ is unstable in the presence of oxygen, we chose to complete our initial studies with apo-KdnB supplemented with MnSO$_4$. As purified, KdnA did not contain PLP, and had minimal activity in the *in vitro* assay (not shown), but did exhibit robust activity after incubation with PLP (Figure 7), suggesting that KdnA requires PLP for activity. When both enzymes are present, no product formation is observed if L-Glu is withheld or EDTA is included (Figure 7, lanes 6 and 7), consistent with the proposal that KdnB is a metal-dependent enzyme, KdnA is a PLP-dependent aminotransferase that utilizes L-glutamate, and both enzymes are required for product formation.

Perhaps the most surprising result was that NAD(P) was not required for Kdo8N formation. We hypothesized that the most reasonable electron acceptor was molecular oxygen. Indeed, we observed production of H$_2$O$_2$ when Mn-KdnB and PLP-KdnA were incubated with Kdo and L-Glu, while no H$_2$O$_2$ was observed when the reaction was performed with apo-KdnB or when EDTA was included (Figure 7B). Furthermore, inclusion of NAD$^+$ only slightly inhibited H$_2$O$_2$ formation, suggesting that O$_2$ is the preferred oxidant. In addition, no product inhibition was observed for NADH up to 30 mM, further suggesting it is not a product of the reaction (supplemental figure S3). Therefore, KdnB appears to be an alcohol oxidase as opposed to an alcohol dehydrogenase. To our knowledge, no metal-dependent alcohol oxidases have previously been reported, suggesting this enzyme could represent a novel class of alcohol oxidases. Further mechanistic studies are required to fully address this question.

**Loss of Kdo8N increases susceptibility to polymyxin and bile salts.** The MIC values for wild-type and Δ*kdnAkdnB* *S. oneidensis* are shown in Table 2. The knockout strain showed increased sensitivity to polymyxin B (~3-fold) and bile salts (~2-fold). Both of these compounds affect outer membrane permeability, suggesting that Kdo8N contributes modestly to outer-membrane integrity.

**DISCUSSION**

*Identification of the Kdo8N biosynthetic genes.* Kdo8N was first observed by direct analysis of the LPS from *S. oneidensis* (10). This modification to Kdo had not previously been observed, and the genes responsible for its formation were completely unknown. The chemical conversion of Kdo to Kdo8N consists of replacing the C8 hydroxyl of Kdo with a primary amine. This chemistry has been observed frequently in deoxy sugar modification pathways (23). One such pathway is the conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) (Figure 1) to the 3-amino derivative UDP-GlcNAc3N by the enzymes GnnA/GnnB (11), which is the origin of lipid A species containing only N-linked acyl chains in certain Gram-negative bacteria (24). This reaction is accomplished by oxidation of the 3-OH of UDP-GlcNAc to a ketone by an NAD-dependent alcohol dehydrogenase, followed by transamination by a PLP-dependent glutamate-aminotransferase. We reasoned that a similar pathway might be responsible for Kdo8N biosynthesis, and searched the *S. oneidensis* genome for homologues of GnnA and GnnB. While no significant homologues for GnnA were found, a homologue to GnnB with 34% identity was found in a gene cluster adjacent to a putative alcohol dehydrogenase and a homologue of the *kdsB* gene (SO_2478), the last step in CMP-Kdo biosynthesis (Figure 1). The putative alcohol dehydrogenase showed very little similarity to GnnA, suggesting a distinct function from the GnnA/GnnB reaction. The
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two enzymes upstream of *kdsB* were renamed *kdnA* (SO_2476) and *kdnB* (SO_2477).

Expression of all three genes (*kdnA*, *kdnB*, and *kdsB*) was carried out in *E. coli* model strain WBB06, which harbors deletions in WaaC and WaaF, the glycosyl transferases necessary for initial extension of the core sugars in LPS (Table 1). As a result, the final lipid-A species obtained from this strain is Kdo2-lipid A, which partitions to the chloroform phase of a Bligh-Dyer extraction. WBB06 expressing the entire cluster was analyzed by TLC and mass spectrometry, which were consistent with Kdo8N formation (Figures 3 and 4). Taken together, this evidence strongly indicates that *kdnA*, *kdnB*, and *kdsB* are sufficient for Kdo8N biosynthesis in *Shewanella oneidensis*.

**Proposed pathway for Kdo8N.** In Gram-negative bacteria, Kdo is synthesized from phosphoenol pyruvate and arabinose-5-phosphate, dephosphorylated, and then activated by conversion to a sugar nucleotide, CMP-Kdo (Figure 1). This activated species is then transferred to a tetra-acylated lipid A precursor, known as lipid IVₐ, and processed to Kdo2-lipid A by two acyl transferases, LpxL and LpxM (6). In many species, including *E. coli*, a single enzyme transfers two Kdo moieties to lipid IVₐ (referred to as a bifunctional KdtA), while in others, including *S. oneidensis* and *Haemophilus influenzae*, only a single Kdo is transferred (monofunctional KdtA) followed by phosphorylation at C4 by an additional Kdo kinase enzyme (Figure 2A). Because Kdo exists in several forms in *E. coli* (free sugar, sugar nucleotide, Kdo2-lipid IVₐ, Kdo2-lipid A), several possible pathways exist for producing Kdo8N₂-lipid A. One such pathway is direct modification of Kdo2-lipid A to Kdo8N₂-lipid A (or the Kdo2-lipid IVₐ precursor). Given the differences in activities between the *E. coli* and *S. oneidensis* KdtA enzymes (monofunctional vs. bifunctional), this pathway, which bypasses the Kdo transferase step, might have been expected. However, when any of the genes is expressed individually, no modification is observed, suggesting direct modification of Kdo2-lipid A does not occur. If the lipid A was directly modified, but the oxidized intermediate was unstable, co-expression of *kdnA* and *kdnB* should be sufficient to produce Kdo8N₂-lipid A. Still, expression of *kdnA* and *kdnB* in the absence of the *S. oneidensis kdsB* gene resulted in no observed modification. Therefore it is most likely that the amine-modified species is formed prior to glycosylation of lipid IVₐ, and implies the *E. coli* Kdo transferase can use CMP-Kdo8N as a substrate.

Formation of the sugar nucleotide CMP-Kdo is catalyzed by the enzyme CMP-Kdo synthase (CKS, gene product of *kdsB*). All of the WBB06-derived strains expressing *S. oneidensis* proteins contained a chromosomal copy of the *E. coli kdsB*. Therefore, if *S. oneidensis* CKS (SoCKS) was functionally identical to *E. coli* CKS, there should be no difference between the strain expressing all three genes and the strain expressing *kdnA* and *kdnB* but omitting *S. oneidensis* *kdsB*. Since all three genes are required to observe Kdo8N in the extracted lipid A, this suggests that SoCKS has a function distinct from the *E. coli* enzyme. This can be explained by the pathway proposed in Figure 8, wherein Kdo is converted to Kdo8N by KdnA and KdnB, presumably through an aldehyde intermediate, followed by activation to CMP-Kdo8N by a CKS with expanded substrate specificity recognizing Kdo8N. This proposed biosynthetic pathway explains the failure to observe intermediates when expressing any of the single genes, and explains the requirement of SoCKS to observe Kdo8N in LPS. This pathway is also consistent with the results of *in vitro* assays of purified KdnA and KdnB, where we observe Kdo8N formation directly from Kdo without an enzyme to make CMP-Kdo. Indeed, when CTP and Mg²⁺ were included in the presence of SoCKS to allow formation of CMP-Kdo, product formation was inhibited, suggesting CMP-Kdo is a poor substrate (supplemental figure S4). Furthermore, the observed activity in the absence of NAD⁺ suggests that molecular oxygen could function as the electron acceptor in the reaction, which was confirmed by detection of H₂O₂ production. Interestingly, NAD⁺ did not inhibit H₂O₂...
production, suggesting that it is not a better substrate than O₂. This, in turn, suggests either (a) molecular oxygen is the physiological substrate for KdnB, or (b) another redox cofactor is used in *S. oneidensis*. Preliminary experiments showed no rate enhancement with *E. coli* cytochrome C or ubiquinone (not shown), but it is still possible that a specific *S. oneidensis* redox cofactor is used *in vivo*.

In addition to *S. oneidensis*, Kdo8N has been observed in all species of *Shewanella* studied to date, and significant homologues of *kdnA* and *kdnB* exist in all those with sequenced genomes. No other species to date has been found to contain Kdo8N, but several organisms that have not been subjected to detailed analysis of their LPS contain a homologous cluster with at least 50% identity, including *Thiomicrospira crunogena* XCL-2, *Thioalkalimicrobium cyclicum* cyclicum ALM1, and *Chitinophaga pinensis* DSM 2588. *T. crunogena* and *T. cyclicum* are obligate sulfur-oxidizing bacteria (25,26), while *C. pinensis* is notable for its ability to degrade the polysaccharide chitin (27). All three are marine organisms, and the incorporation of Kdo8N may be helpful in some aspect of these organisms’ ecological niches. Lipid A modifications that incorporate an amino-substituent have previously been shown to confer resistance to cationic antimicrobial peptides (28), presumably due to unfavorable interactions with a positively charged amine. Since deletion of *kdnA/kdnB* in *S. oneidensis* led to ~3-fold increased sensitivity to polymyxin and ~2-fold increased sensitivity to bile salts (Table 2), we speculate that Kdo8N may contribute modestly to outer membrane integrity.

Kdo is an 8-carbon aldulosonic acid. C9 aldulosonic acids also exist, including the neuraminic acids, also known as sialic acids. Naturally occurring neuraminic acids contain one or more acetamido-groups, which are derived biosynthetically from UDP-GlcNAc. Like Kdo, sialic acids must be activated as CMP-derivatives prior to glycosylation. In *Leptospira interrogans*, we observed a gene cluster similar to the *kdnA/kdnB* genes (34% and 48% identity, respectively), but adjacent to a CMP-neuraminic acid synthase instead of a CMP-Kdo synthase. This raises the possibility that additional amine modifications could be present in LPS or glycosylated proteins at residues other than Kdo, perhaps initiated by a specific class of alcohol oxidases that recognize aldulosonic acids. The detailed carbohydrate analysis of *Leptospira* LPS required to answer this question has yet to be performed.

The physiological role of Kdo8N is not clear. Besides the role in stabilizing the membrane, it is also possible that Kdo8N may alter recognition of lipid A by the MD2/TLR4 receptor complex, which senses lipid A as part of the mammalian innate immune system. Previously characterized lipid A modifications, such as dephosphorylation of the 1-phosphate in *Francisella novicida*, have been shown to have attenuated virulence (29). Altered TLR4 activation by modified lipid A species has previously been shown to be useful for the development of vaccine strains as well as vaccine adjuvants.

Interestingly, the function of KdnB diverges from its predicted activity. Oxidation of an alcohol to an aldehyde can occur by a variety of mechanisms, all of which involve transfer of electrons from the alcohol to an acceptor (see reference (30) for a review of alcohol dehydrogenase/oxidase classifications). The metal-dependent alcohol dehydrogenases typically use NAD(P)+ as the electron acceptor. Metal-independent alcohol dehydrogenases use pyrroloquinoline quinone (PQQ) as a cofactor to transfer electrons to another biological electron acceptor, such as ubiquinone. Probing the sequence of KdnB in the Conserved Domain Database (CDD, (31-33)) suggest that KdnB is an iron-containing alcohol dehydrogenase class 3 (Fe-ADH3), within the dehydroquinase synthase-like and iron-containing alcohol dehydrogenase (DHQ_Fe-ADH) superfamily. Proteins of this specific family have not been well characterized, but proteins within the DHQ_Fe-ADH superfamily typically use NAD or NADP. KdnB is clearly metal-dependent, but does not require any redox cofactor or pyridine nucleotide, and instead utilizes molecular oxygen. An enzyme that can oxidize an alcohol directly with O₂ is an
alcohol oxidase, but all known examples contain a flavin cofactor (FAD or FMN). KdnB shows no similarity to these flavin-dependent oxidases. This hybrid activity, wherein we observe metal-dependent alcohol oxidase activity, has never been observed to our knowledge, and more detailed mechanistic studies are warranted to probe a potentially novel enzyme sub-family. Since members of the Fe-ADH3 family have not been well characterized, it is difficult to say whether KdnB should be placed in a new family, or if the Fe-ADH3 family contains a mixture of dehydrogenases and oxidases. This biosynthetic pathway is also unusual because it involves the direct modification of a free sugar rather than the sugar-nucleotide, which is generally observed (23). By uncovering the biosynthetic pathway for Kdo8N, we have set the stage for illuminating the physiological role and potential applications of this unstudied lipid A modification and present evidence for a novel class of alcohol oxidases.

SUPPLEMENTAL DATA
Supplemental figures S1 – S4 are available via the internet.

ACKNOWLEDGMENTS
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FIGURE LEGENDS

Figure 1: Truncated pathway for Kdo2-lipid A biosynthesis in E. coli. Both inner-core Kdo sugars are added to lipid IV\textsubscript{A} by a single Kdo-transferase enzyme (KdtA) using the sugar-nucleotide donor CMP-Kdo. Schematic illustrations represent acyl chains (black lines), glucosamine (black ovals), and Kdo (blue ovals).

Figure 2: 8-amino-Kdo (Kdo8N) and its proposed biosynthetic cluster. (A) Chemical structure of the Kdo-moiety in LPS from E. coli (Kdo\textsubscript{2}) and S. oneidensis (Kdo8N-4-phosphate), where R represents the lipid A anchor. (B) S. oneidensis gene cluster and potential functions in Kdo8N biosynthesis.

Figure 3: TLC analysis of lipid A from E. coli expressing potential Kdo8N biosynthetic genes. Lane A, WBB06 expressing empty pWSK29 control; lane B, WBB06 expressing kdnA, kdnB, and kdsB. Lipid A samples were extracted and run on silica TLC plates in 25:15:3.5:4 CHCl\textsubscript{3}:MeOH:CH\textsubscript{3}COOH:H\textsubscript{2}O, and visualized by charring on a hot plate, as described in Experimental Procedures. Proposed products are illustrated as acyl chains (black lines), glucosamine (black ovals), Kdo (blue ovals), and Kdo8N (red ovals).

Figure 4: ESI-MS of lipid A species from WBB06 expressing Kdo8N biosynthetic enzymes. Isolated lipid A samples were prepared and injected into a QStar XL mass spectrometer as described in Experimental Procedures. (A) Overlay of spectra obtained for extracted lipids from vector control strain (blue) and all three genes kdnA, kdnB, and kdsB, expanded to show Kdo\textsubscript{2}-lipid A species. (B) MS/MS of Kdo\textsubscript{2}-lipid A from vector control strain. (C) MS/MS of Kdo8N-2-lipid A from strain expressing kdnA, kdnB, and kdsB. Proposed structures are shown adjacent to relevant peaks.

Figure 5: TLC analysis of lipid A from E. coli expressing individual Kdo8N biosynthetic genes. Lipid A species were extracted and run on silica TLC plates in 25:15:3.5:4 CHCl\textsubscript{3}:MeOH:CH\textsubscript{3}COOH:H\textsubscript{2}O, and visualized by charring on a hot plate, as described in Experimental Procedures. Samples were obtained from WBB06 strains expressing an empty pWSK29 vector control (lane A), kdnA (lane B), kdnB (lane C), kdsB (lane D), kdnA and kdnB (lane E), and kdnA, kdnB, and kdsB (lane F). Proposed products are illustrated as acyl chains (black lines), glucosamine (black ovals), Kdo (blue ovals), and Kdo8N (red ovals).

Figure 6: SDS-PAGE of KdnA and KdnB purifications. Lane L, protein molecular weight marker. Approximately 1.5 µg of KdnA and KdnB as cell free extract (CFE) or purified protein was loaded, as indicated, and ran on a 4-20% Tris-HCl gel.

Figure 7: In vitro activity of KdnA and KdnB. (A) TLC based assay of cell extracts and purified KdnB and KdnA. Assays were conducted using 0.1 M Tris pH 8.0, 1 mM DTT, 50 µM Kdo, 5,700 cpm \textsuperscript{14}C-Kdo, 1 mM MnSO\textsubscript{4}, and ± the following components as indicated: 15 µg cell extract, 30 µM apo-KdnB, 30 µM PLP-KdnA, 0.1 M L-glutamate, 2 mM EDTA in 10 µL. After 60 min, 2.5 µL of reaction mixture was spotted and run on a TLC plate in 6:2:1 iProH:NH\textsubscript{4}OH:H\textsubscript{2}O as described in Experimental Procedures. (B) Hydrogen peroxide formation catalyzed by KdnA/KdnB. H\textsubscript{2}O\textsubscript{2} formation was monitored using the Amplex Red Hydrogen Peroxide kit (Invitrogen). Assays were performed in 100 µL with 0.1 M Tris, pH 8.0, 50 µM Kdo, 50 µM Amplex Red, 0.1 U/mL horse radish peroxidase, 0.1 M L-glutamate, and one of the following: No enzyme control (open circles), no enzyme/5 mM NAD (open squares), 10 µM apo-KdnB/PLP-KdnA (open diamonds), 10 µM Mn-KdnB/PLP-KdnA (closed triangles), 10 µM Mn-
The biosynthetic pathway for Kdo8N in *S. oneidensis*

KdnB/PLP-KdnA + 5 mM NAD (closed squares), 10 μM Mn-KdnB/PLP-KdnA + 2 mM EDTA (open triangles).

**Figure 8:** Proposed pathway for Kdo8N biosynthesis and incorporation into lipid A.
TABLES

### TABLE 1

**Primers**

| Primer | Sequence |
|--------|----------|
| 1      | GCGCAGTCTAGAGAAGGAGATATACATATGCCCGGTGTTTGAATTTTGCTCA |
| 2      | GCAGCTCGAGTTTATGGGCAAATGCTTTCTAATGTTTCAATACCGC |
| 3      | GCGCAGTCTAGAGAAGGAGATATACATATGAGTTTTAAAAATTTTAAGTGTGAAAAATGATCTTCGGTC |
| 4      | GCAGCTCGAGTTAATGGGAAGGAGATATACATACCTGTTAATCCGGCCG |
| 5      | GCAGCTCGAGTTAATGGGAAGGAGATATACATAGTTTTAAAAATTTTAAGTGTGAAAAATGATCTTCGGTC |
| 6      | GCAGCTCGAGTTAATGGGAAGGAGATATACATACCTGTTAATCCGGCCG |

**Plasmids**

| Plasmid | Description | Source/Reference |
|---------|-------------|-----------------|
| pWSK29  | Low copy expression vector, Amp<sup>R</sup> | Ref. 12 |
| pDS3.1  | R6K origin, SacB, Gm<sup>R</sup> | Ref. 15 |
| pΔ7677  | pDS3.1 containing SO<sub>2476</sub> and SO<sub>2477</sub> flanking sequences | This work |
| pSG1    | pWSK29 containing <i>kdnA</i>, <i>kdnB</i>, and <i>kdsB</i> from <i>S. oneidensis</i>. | This work |
| pSG2    | pWSK29 containing <i>kdnA</i> from <i>S. oneidensis</i>. | This work |
| pSG3    | pWSK29 containing <i>kdnB</i> from <i>S. oneidensis</i>. | This work |
| pSG4    | pWSK29 containing <i>kdsB</i> from <i>S. oneidensis</i>. | This work |
| pSG5    | pWSK29 containing <i>kdnA</i> and <i>kdnB</i> from <i>S. oneidensis</i>. | This work |

**Strains**

| Strain | Genotype/Description | Source/Reference |
|--------|----------------------|-----------------|
| S. oneidensis MR-1 | Wild type <i>S. oneidensis</i> MR-1, isolated from Lake Oneida, NY | ATCC |
| WBB06  | W3110 mtl, Δ(lacC-<i>lacF</i>)-tet6 | Ref. 18 |
| EC100D | Host strain for R6K<sub>γ</sub> replicons | Epicentre |
| p2155  | RP4-2-<i>γ</i>:<i>C</i>:<i>Δ</i>(erm-<i>piv</i>) thrB1004, pro, thi, strA, hsdS, lacZ ΔM15, (F<sup>+</sup> lac<sup>+</sup> ΔM15 lacIq, traD<sup>+</sup>, proA<sup>+</sup>, proB<sup>+</sup>) [Km<sup>R</sup> Sm<sup>R</sup> Em<sup>R</sup>]<sup>+</sup> | Ref. 16 |
| SGVC   | WBB06 containing pWSK29 | This work |
| SG1    | WBB06 containing pSG1 | This work |
| SG2    | WBB06 containing pSG2 | This work |
| SG3    | WBB06 containing pSG3 | This work |
| SG4    | WBB06 containing pSG4 | This work |
| SG5    | WBB06 containing pSG5 | This work |
Table 2:

| S. oneidensis strain | Minimum inhibitory concentration (MIC)* | Polymyxin (µg/mL) | Bile salts (mg/mL) |
|----------------------|----------------------------------------|--------------------|--------------------|
| Wild-type (MR-1)     |                                        | 0.16 ± 0.05        | 1.25 ± 0           |
| ΔkdnAΔkdnB           |                                        | 0.05 ± 0.01        | 0.63 ± 0           |

* MIC values were determined in quadruplicate and repeated for 3 independent experiments. Values are expressed as the mean and standard deviation of the minimum concentration that inhibited growth as described in Experimental Procedures.
The biosynthetic pathway for Kdo8N in S. oneidensis

FIGURES

Figure 1:
The biosynthetic pathway for Kdo8N in S. oneidensis

Figure 2:

A

E. coli  S. oneidensis

B

kdnA  kdnB  kdsB
SO_2476  SO_2477  SO_2478
Putative aminotransferase  Putative alcohol dehydrogenase  CMP-Kdo synthase (CKS)
Figure 3:
The biosynthetic pathway for Kdo8N in S. oneidensis

Figure 4:
Figure 5:
The biosynthetic pathway for Kdo8N in S. oneidensis

Figure 6:
Figure 7:

A

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------|---|---|---|---|---|---|---|---|
| Vector (extracts) | + | - | - | - | - | - | - | - |
| SG1 (extracts) | - | + | + | - | - | - | - | - |
| KdnB      | - | - | - | + | - | + | + | + |
| KdnA      | - | - | - | - | + | + | + | + |
| L-Glu     | + | + | + | + | + | - | + | + |
| EDTA      | - | - | + | - | - | - | + | - |

B

![Graph showing the biosynthetic pathway for Kdo8N in S. oneidensis](image)

The biosynthetic pathway for Kdo8N in S. oneidensis
The biosynthetic pathway for Kdo8N in S. oneidensis

Figure 8:

- **Kdo**
  - KdoB: Fe^{2+}/Mn^{2+} → O_2 / H_2O_2
  - KdoA: PLP → L-Glu / α-ketoglutarate → Kdo8N

- **Kdo8N-lipid IV_A**
  - KdtA: Lipid IV_A → Lipid IV_A
  - CMP-Kdo8N
The origin of 8-amino-3,8-dideoxy-D-manno-octulosonic acid (Kdo8N) in the lipopolysaccharide of Shewanella oneidensis
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