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Interchangeable Domains in the Kdo Transferases of *Escherichia coli* and *Haemophilus influenzae*

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Abstract

Kdo₂-lipid A, a conserved substructure of lipopolysaccharide, plays critical roles in Gram-negative bacterial survival and interaction with host organisms. Inhibition of Kdo biosynthesis in *Escherichia coli* results in cell death and accumulation of the tetra-acylated precursor lipid IV_A. *E. coli* KdtA (EcKdtA) is a bi-functional enzyme that transfers two Kdo units from two CMP-Kdo molecules to lipid IV_A. In contrast, *H. influenzae* KdtA (HiKdtA) transfers only one Kdo unit. *E. coli* CMR300, which lacks Kdo-transferase because of a deletion in *kdtA*, can be rescued to grow in broth at 37 °C if multiple copies of *msbA* are provided in *trans*. MsbA, the inner membrane transporter for nascent lipopolysaccharide, prefers hexa-acylated to tetra-acylated lipid A, but with the excess MsbA present in CMR300, lipid IV_A is efficiently exported to the outer membrane. CMR300 is hypersensitive to hydrophobic antibiotics and bile salts, and does not grow at 42 °C. Expressing HiKdtA in CMR300 results in the accumulation of Kdo-lipid IV_A in place of lipid IV_A without suppression of its growth phenotypes at 30 °C. EcKdtA restores intact lipopolysaccharide, together with normal antibiotic resistance, detergent resistance and growth at 42 °C. To determine which residues are important for the mono- or bi-functional character of KdtA, protein chimeras were constructed using EcKdtA and HiKdtA. These chimeras, which are catalytically active, were characterized by *in vitro* assays and *in vivo* complementation. The N-terminal half of KdtA, especially the first 30 amino acid residues, specifies whether one or two Kdo units are transferred to lipid IV_A.

The outer membrane (OM) of Gram-negative bacteria is an asymmetric lipid bilayer in which the inner leaflet is comprised of phospholipids and the outer leaflet of lipopolysaccharide (LPS) (1–3). The OM serves as a permeability barrier that protects cells from the entry of hydrophobic compounds, such as detergents and certain antibiotics, and it allows enteric Gram-negative bacteria to survive in the intestine in the presence of high concentrations of bile salts (1).

LPS is composed of three covalently linked regions: 1) the lipid A moiety (also known as endotoxin), which is the OM anchor; 2) a non-repeating core oligosaccharide; and 3) the O-antigen polymer, which is absent in laboratory strains of *Escherichia coli* K-12 (4,5). The Kdo₂-lipid A substructure of *E. coli* LPS (Fig. 1) is relatively conserved in most Gram-negative bacteria and is assembled on the cytoplasmic surface of the inner membrane (5). When lipid A or Kdo biosynthesis is inhibited, the integrity of the OM is compromised, and the cells are rendered non-viable (5). Mutations in the O-antigen or in other core sugars are compatible with growth under laboratory conditions but usually reduce virulence in animal models of infection (4). The lipid A moiety of LPS activates the TLR4/MD2 receptor of the mammalian innate

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Supporting information available

The primers used to generate the various KdtA chimeras or to construct relevant strains are shown in Supporting Tables 1 and 2. Supporting Figure 1 provides the general scheme for the PCR generation of *kdtA* gene chimeras. Supporting Figure 2 shows typical levels of expression seen for EcKdtA, HiKdtA and two chimeras, as well as the results of assays of the corresponding membrane preparations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

immune system (6), stimulating inflammation and production of cytokines, such as TNF- α and IL-1 β (7,8). During severe sepsis, excessive inflammation can damage small blood vessels, contributing to Gram-negative septic shock (9). Therefore, the enzymes of lipid A biosynthesis are attractive targets not only for the design of new antibiotics but also for the development of strategies to reduce the risks associated with septic shock (10,11).

Most Gram-negative bacteria make LPS that contains a Kdo disaccharide attached to the 6'-position of lipid A, as seen in *E. coli* (Fig. 1) (5,12). The *E. coli* Kdo transferase (EcKdtA) is a bi-functional glycosyltransferase that is responsible for the incorporation of both Kdo units (13,14). Very little of the intermediate Kdo-lipid IV_A is detected either *in vitro* or *in vivo*. However, in some other organisms, including *Haemophilus influenzae*, *Bordetella pertussis*, *Aquifex aeolicus*, and *Vibrio cholerae* only a single Kdo moiety is present in the LPS core. The Kdo transferases of these bacteria incorporate just one Kdo unit (Fig. 1) (15,16). The outer Kdo residue is replaced with a phosphate group (17), which is added by a special kinase, termed KdkA (Fig. 1) (18). This kinase is not present in *E. coli*.

EcKdtA and HiKdtA consist of single polypeptide chains that display 48% identity and 64% similarity over their entire lengths (Fig. 2). Both EcKdtA and HiKdtA are membrane proteins (14,15), and their active sites are presumed to face the cytoplasm. Despite their important roles in bacterial survival and virulence, nothing is known about their active sites, mechanisms, or structures. In order to determine which regions are important for the mono- or bi-functionality of these two enzymes, we generated KdtA chimeras by swapping domains of EcKdtA and HiKdtA (Fig. 3). These constructs are catalytically active, and were characterized by *in vitro* assays and *in vivo* complementation studies in an *E. coli* strain lacking its own chromosomal *kdtA* gene (19). The mono- or bi-functionality of KdtA is determined by the N-terminal half of the enzyme, in which residues 20 to 28 appear to play an especially critical role. Our findings are consistent with the idea that the N-terminal domain of KdtA may bind the acceptor lipid substrate, whereas the C-terminal domain binds the donor nucleotide, CMP-Kdo.

MATERIALS AND METHODS

Materials

Chloroform, methanol, 0.25 mm silica gel 60 thin layer chromatography (TLC) plates, and high-performance analytical thin layer chromatography (HPTLC) plates were from EMD Chemicals Inc. (Gibbstown, NJ). Tryptone, yeast extract, and agar were from Becton, Dickinson and Co. (Franklin Lakes, NJ). MacConkey-agar contains neutral red, which is responsible for the red color of the plates. [γ -³²P]ATP (3 mCi/nmol) was from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). All other chemicals including L-arabinose were reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or Mallinckrodt-Baker, Inc. (Phillipsburg, NJ), and used without a further purification.

Bacterial strains

Bacterial strains used in this study are described in Table 1. Typically, bacteria were grown in LB medium, which contains 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter (20). In addition, all strains were tested for viability on MacConkey-agar plates (20). When required for the selection of plasmids, cells were grown in the presence of 50 μ g/mL ampicillin, 25 μ g/mL chloramphenicol, and/or 20 μ g/mL kanamycin.

Molecular biology applications

Protocols for handling of DNA samples and preparing *E. coli* for electroporation were those of Sambrook and Russell (21). Transformation-competent cells of *E. coli* were prepared by the method of Inoue *et al.* (22). Plasmids were isolated from cell cultures using the QIAprep

Miniprep kit or extracted from agarose gel with the QIAquick gel extraction kit (Qiagen, Valencia, CA). Genomic DNA was isolated using the protocol for bacterial cultures in the Easy-DNA kit (Invitrogen, Carlsbad, CA). T4 DNA ligase (Invitrogen, Carlsbad, CA or NEB, Ipswich, MA), restriction endonucleases (NEB, Ipswich, MA), and calf intestinal alkaline phosphatase (NEB, Ipswich, MA) were used according to the manufacturers' instructions. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from MWG-Biotech (Huntsville, AL) or IDT, Inc. (Coralville, IA).

Plasmids construction and transformation in CMR300

The plasmid pBAD33 does not contain a ribosomal binding sites (23). To make the constructs discussed below, the nucleotides between the XbaI and HindIII sites in pBAD33 were replaced with those of pET21b (EMD Chemicals, Gibbstown, NJ). The multi-cloning site in pET21b was amplified by PCR using T7F and T7R primers (Table S1). The resulting PCR products and pBAD33 vector were digested by XbaI and HindIII. Digested pBAD33 was treated with calf intestinal alkaline phosphatase, and digested PCR products were ligated into the digested and treated pBAD33, using T4 ligase. The sequence of the resulting plasmid was confirmed with primers 33F and 33R (Table S1), and was named pBAD33.1, as it now contained a ribosomal binding site with an extended multi-cloning site. All the constructs used in this study were derived from pBAD33.1 using the NdeI and HindIII restriction sites, unless otherwise stated. The plasmid pBAD33.1 contains the P_{BAD} promoter, which can be induced with L-arabinose and can be suppressed with D-glucose and D-fucose. Therefore, the level of transcription of the gene that is located downstream of the P_{BAD} promoter can be controlled depending on the type and amount of sugar.

The genes encoding EckdtA and HikdtA were amplified from *E. coli* W3110 genomic DNA or *H. influenzae* genomic DNA (ATCC51907D). Most of the inserts encoding the various protein chimeras (Table 1) were amplified by the extended PCR method (Fig. S1, Table S1 and S2), using the primers and templates listed in Table S2. Inserts for pHE-5 (Table 1), encoding KdtA chimera HE-5, were amplified with the prHSC61 and prHSC3 primers from pEcKdtA (Table S1). Plasmids pHEH-1 through pHEH-4 (Table 1) were generated by the Quikchange PCR protocol provided by Stratagene, using pHiKdtA as the template in conjunction with the suitable primers (Table S1). The resulting plasmids were all confirmed by sequencing using the primers 33F and 33R at the Duke University DNA Analysis Facility.

All plasmids were electroporated into electrocompetent CMR300 cells prepared by the procedure described previously (19). pEcKdtA and pHEH-1 were also transferred into chemically competent DY330 cells (24) prepared by the method of Inoue *et al.* (22).

In vivo complementation of CMR300 with chimeric KdtAs

Plasmids, pEcKdtA, pHiKdtA, pEH-1 to pEH-4, pHE-1 to pHE-6, and pHEH-1 to pHEH-4 (Table 1) were electroporated into CMR300 and grown at 30 °C in LB medium for 1 h. Transformed cells were plated on LB-agar plate containing 50 µg/mL ampicillin, 25 µg/mL chloramphenicol, and 20 µg/mL kanamycin, and grown at 30 °C overnight. The resulting colonies were purified twice by re-streaking from single colonies on plates supplemented with suitable antibiotics. A purified single colony of each strain was then streaked in parallel on LB-agar or MacConkey-agar plates, containing 0%, 0.02%, or 0.2% L-arabinose to test the toxicity of over-expression or the requirement of over-expression of each construct for complementation, and grown overnight at 30 °C or 42 °C, as indicated.

Preparation of cell-free extracts and membrane fractions

All constructs were grown in 50 mL LB medium containing 50 µg/mL ampicillin and 25 µg/mL chloramphenicol with either 0%, 0.02 %, or 0.2 % L-arabinose at 30 °C, as noted. Cells were harvested at $A_{600} \sim 1.0$, and cell pellets were washed with phosphate-buffered saline (PBS) (25). The cell pellets were re-suspended in 8 mL of 50 mM PBS, pH 7.5, supplemented with 300 mM sodium chloride. Cells were lysed using a French Pressure cell at 17000 psi and centrifuged at $5000 \times g$ to remove cell debris. The supernatant was retained as the cell free lysate. To prepare membranes, the cell-free lysate was centrifuged at 45,000 rpm ($\sim 140,000 \times g$) in a Beckman type 70.1 Ti rotor for 1 hour at 4 °C. The pellet was suspended and homogenized in 8 mL of 50 mM PBS, pH 7.5, supplemented with 300 mM sodium chloride, and centrifuged again at 45,000 rpm for 1 hour at 4 °C. The resulting pellet was suspended and homogenized with 1.5 mL of the same buffer. Protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL) with BSA as the standard (26).

Enzymatic Synthesis and Purification of KdtA Substrates

The [$4'$ - ^{32}P]lipid IV_A was prepared enzymatically using the overexpressed 4-kinase present in membranes of *E. coli* BLR(DE3)/pLysS/pJK2 (27), with tetraacyl disaccharide 1-phosphate as the acceptor substrate, according to published procedures (28). Unlabeled lipid IV_A was extracted and purified from CMR300 (19,29). All lipid substrates were dispersed in aqueous buffers by sonic irradiation in bath apparatus for approximately 1 minute prior to use. Recombinant *E. coli* CMP-Kdo synthetase was partially purified as described by Brozek *et al.* (30) from JM103/pTJB201.2 (31).

In vitro KdtA activity assay

The transfer of Kdo to its acceptor substrate, [γ - ^{32}P]lipid IV_A, was assayed by the method of Brozek *et al.* (30) with minor modifications. Reaction mixtures (10–20 µl) contained 50 mM Hepes (pH 7.5), 1 mM Kdo, 0.1% Triton X-100, 20 µM [γ - ^{32}P]lipid IV_A (3,000–6,000 cpm/nmol), 2.5 mM CTP, 10 mM MgCl₂, and 1.8 milliunits of partially purified CMP-Kdo synthase. Standard assays were initiated by addition of cell free lysate or membranes, as indicated, and incubated at 30 °C for 60 minutes. The reactions were terminated by spotting 3–5 µl of the reaction mixtures onto a TLC plate. The plate was dried with a cold air stream and developed in chloroform/pyridine/88% formic acid/H₂O (30:70:16:10, v/v). The solvent was evaporated under a hot air stream, and the plate was exposed to a PhosphorImager screen for 12–16 h. The extent of conversion of [γ - ^{32}P]lipid IV_A to the products of interest was determined with a Molecular Dynamics PhosphorImager, equipped with ImageQuant software.

Construction of kdtA deletion strains in E. coli DY330 harboring pEcKdtA or pHEH-1

These deletions were constructed following a reported procedure (19) with minor modifications. PCR was used to construct a linear piece of DNA containing the kanamycin resistance cassette (*kan*), flanked on the 5' end by a ribosome binding site plus 39 bp of chromosomal DNA upstream of *kdtA*, and flanked on the 3' end by 40 bp of chromosomal DNA located downstream of *kdtA*. The sequences of the forward (KdtAKOFOR) and reverse (KdtAKOREV) primers used to construct this PCR product are shown in Table S1. The kanamycin resistance gene (Tn903) from plasmid pWSK130 (32) served as the template. The PCR reaction was carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The PCR product was resolved on a 1.0% Agarose gel and purified with the QIAquick gel kit. DY330/pEcKdtA and DY330/pHEH-1 (Table 1) had been grown at 30 °C in 50 mL LB medium in the presence of 25 µg/mL chloramphenicol (DY330/pEcKdtA) or 25 µg/mL chloramphenicol and 0.2% L-arabinose (for DY330/pHEH-1) until A_{600} reached ≈ 0.4 , and then each culture was shifted to 42 °C for 15 min to activate the λ -RED

genes (24). Next, the cells were washed twice with 30 mL ice cold sterilized water. Cell pellets were re-suspended in 500 μ L of ice cold sterilized water. The cells (100 μ L) were then electroporated with 100–250 ng of the PCR product. The resulting cells were allowed to recover for 2 h at 30 °C in 1 mL LB broth containing 25 μ g/mL chloramphenicol (for DY330/pEcKdtA) or 25 μ g/mL chloramphenicol and 0.2% L-arabinose (for DY330/pHEH-1). The cells were then plated onto LB-agar, containing 20 μ g/mL kanamycin, 25 μ g/mL chloramphenicol (for DY330/pEcKdtA) or 20 μ g/mL kanamycin, 25 μ g/mL chloramphenicol and 0.2% L-arabinose (for DY330/pHEH-1). The plates were incubated at 30 °C overnight. The resulting colonies were re-purified on plates at 30 °C. The *kdtA::kan* replacement on the chromosome of DY330 harboring the covering plasmids was verified by PCR using external primers KdtACFor and KdtACRev (Table S1). The PCR products were resolved on a 1% Agarose gel, purified with the QIAquick gel extraction kit, and sequenced to confirm the replacement. The *kdtA::kan* derivatives DY330/pEcKdtA and DY330/pHEH-1 were designated as HSC1/pEcKdtA and HSC1/pHEH-1, respectively. The presence of 0.2% L-arabinose was toxic to HSC1/pEcKdtA, but it was required for the growth of HSC1/pHEH-1. Therefore, HSC1/pEcKdtA was grown in the presence 25 μ g/mL chloramphenicol and 20 μ g/mL kanamycin, and HSC1/pHEH-1 was grown with 25 μ g/mL chloramphenicol, 20 μ g/mL kanamycin, and 0.2% L-arabinose in all subsequent experiments.

Isolation of lipids of CMR300/pBAD33, CMR300/pEcKdtA, CMR300/pHiKdtA, CMR300/pHEH-1, DY330, HSC1/pEcKdtA and HSC1/pHEH-1

The various CMR300 strains harboring different plasmids (Table 1) were grown from overnight cultures and diluted 1:100 into 100 mL LB broth containing 25 μ g/mL chloramphenicol, 50 μ g/mL ampicillin, 20 μ g/mL Kanamycin and 0.2% L-arabinose, and grown at 30 °C with shaking at 220 rpm. DY330, HSC1/pEcKdtA and HSC1/pHEH-1 were grown from overnight cultures and diluted 1:100 into 100 mL LB broth containing the supplements antibiotics as appropriate at 30 °C. When the A_{600} of the cultures reached 1.0, they were harvested by centrifugation at $4000 \times g$ for 20 min. Cells were washed once with 30 mL of PBS (25) and then re-suspended in 16 mL of PBS and transferred to glass tubes, or to solvent resistant bottles. Chloroform (20 mL) and methanol (40 mL) were added to make a one-phase Bligh-Dyer system (33). The mixture was incubated at room temperature for 1 h with occasional shaking and then centrifuged at $2500 \times g$ for 30 min. The supernatant contained phospholipids, fatty acid and free lipid A, and the pellet contained the intact LPS and other insoluble debris. The supernatant was converted to a two-phase Bligh-Dyer system (33) by the addition of chloroform (20 mL) and 0.1 N hydrochloric acid (20 mL), followed by thorough mixing and centrifugation at $2500 \times g$ for 20 min. The organic phase was removed, and the aqueous phase was extracted a second time by the addition of pre-equilibrated organic phase. The organic phases were pooled, neutralized with a few drops of pyridine, and dried by rotary evaporation. Lipids were dissolved in 5 mL chloroform/methanol (4:1, v/v) and subjected to sonic irradiation in a bath apparatus. The lipids were transferred to fresh glass tubes, dried under N_2 gas at room temperature, and stored at -80 °C.

Release of lipid A from the cell residue by mild acid hydrolysis

The pellet containing the LPS and cell debris was washed twice with 25 mL of a single-phase Bligh/Dyer mixture (33) and collected by low speed centrifugation. The washed pellet was suspended in 8 mL of 50 mM sodium acetate, pH 4.5, and was dispersed using a Branson probe sonicator. The suspension was boiled for 30 min in a water bath and then cooled to room temperature. The mixture was adjusted to pH ~1.5 by the addition of aqueous HCl and converted to a two-phase Bligh and Dyer mixture (33) by the addition of chloroform (20 mL), methanol (20 mL) and 0.1 N hydrochloric acid (10 mL). Lipids were recovered and dried using the same procedure described above.

For TLC analysis, one-third of the total phospholipids fraction and all of lipid A from a 100 mL culture were dissolved in 200 μ L chloroform/methanol (4:1, v/v). Next, 5–10 μ L portions were spotted onto 10 $\times$ 10 cm HPTLC plates, which were subjected to chromatography in chloroform/pyridine/88 % formic acid/H₂O (30:70:16:10 v/v), followed by charring with 10 % sulfuric acid in ethanol.

RESULTS

Evidence for domains within EcKdtA and HiKdtA

The mono-functional HiKdtA and the bi-functional EcKdtA enzymes (Fig. 1) show 48% identity and 64% similarity over their entire lengths (Fig. 2). Both proteins appear to be anchored to the inner surface of the inner membrane by short *N*-terminal transmembrane segments (residues 1–19 in EcKdtA and residues 4–26 in HiKdtA). Bioinformatic analyses further suggest that both EcKdtA and HiKdtA contain glycosyltransferase N (GTase N) pfam motifs (34) within their *N*-terminal halves and glycosyltransferase 1 (GTase 1) motifs within their *C*-terminal halves (Fig. 3). A putative linker connects these two domains, the location of which between residues 196 and 224 is suggested by the relatively low levels of sequence similarity of that region (Fig. 2).

Enzymatic activity of EcKdtA/HiKdtA chimeras

Subtle variations in a few active site residues or sequence motifs could be sufficient to explain for the difference in the functionality of these two enzymes. However, random mutagenesis of the *H. influenzae kdtA* gene on a plasmid did not produce point mutants that rescued the ability of the *kdtA* deletion mutant CMR300 (Table 1) to grow at 42 °C in the presence of bile salts (data not shown). We therefore explored a broader domain swapping approach. The plasmids pEH-1, and pHE-1 were generated by exchanging the *C*- and *N*- terminal halves of EcKdtA and HiKdtA (Fig. 3), and were transformed into CMR300 (Table 1) (19). Cell-free extracts were then assayed for their ability to transfer one or two Kdo residues to lipid IV_A (Fig. 4A). Extracts of the vector control CMR300/pBAD33 did not show any KdtA activity (Fig. 4A, lane 2), consistent with the deletion of *kdtA* in CMR300. Cell-free extracts of CMR300/pEcKdtA were bi-functional, as expected for wild-type EcKdtA, converting lipid IV_A to Kdo₂-lipid IV_A without much accumulation of the Kdo-lipid IV_A intermediate (Fig. 4A, lane 3). Extracts of CMR300/pHiKdtA were mono-functional, generating only Kdo-lipid IV_A (Fig. 4A, lane 4). The small amount of Kdo₂-lipid IV_A-like material (Fig. 4A, lane 4) may indeed reflect the formation of some Kdo₂-lipid IV_A when HiKdtA was greatly over-expressed, or it may be some other product, such as the 1-pyrophosphate derivative of lipid A that is generated by the incorporation of an additional phosphate unit by LpxT (35). Extracts of CMR300/pEH-1 transferred two Kdo units to lipid IV_A with little accumulation of Kdo-lipid IV_A, showing that the bi-functional character of EcKdtA was retained despite the replacement of the *C*-terminal half of EcKdtA with HiKdtA (Fig. 4A, lane 5). Conversely, cell-free extracts of CMR300/pHE-1 (Fig. 4A, lane 6) transferred only one Kdo residue to lipid IV_A under these assay conditions.

The relative levels of over-expression of the Kdo transferase proteins were estimated by gel electrophoresis of membrane fractions (Fig. S2, Panel A), followed by densitometry. Over-expression of HE-1 was about 20% of that of EcKdtA, as estimated from the protein gel. The Kdo transferase specific activities of the membranes, not corrected for over-expression, were 353.6 nmol/min/mg (CMR300/pEcKdtA), 161.2 nmol/min/mg (CMR300/pHiKdtA), 66.9 nmol/min/mg (CMR300/pEH-1), and 1.3 nmol/min/mg (CMR300/pHE-1) under our standard assay conditions (Fig. S2, Panel B). When corrected for the expression level of CMR300/pHE-1 relative to CMR300/EcKdtA, the specific activity of CMR300/pHE-1 membranes was 5.2 ~ 7.8 nmol/min/mg.

In summary, the assays (Fig. 4A) revealed that the *N*-terminal halves of HiKdtA and EcKdtA were responsible for determining whether these enzymes are mono- or bi-functional Kdo transferases, respectively.

Complementation of a *kdtA* deletion mutant with the EcKdtA/HiKdtA chimeras

The hybrid plasmids were tested for their ability to restore growth of CMR300 at 42°C on MacConkey-agar plates, which contain bile salts (Fig. 4B). The OM of wild type *E. coli* prevents bile salts from penetrating into cells. Because CMR300/pBAD33 does not contain KdtA activity (Fig. 4A, lane 2), its OM is compromised, and the cells are therefore sensitive to bile salts (Fig. 4B). The vector control pBAD33 did not support growth of cells in CMR300 on MacConkey-agar plates at 42 °C. However, the two plasmids encoding bi-functional enzymes, pEcKdtA and pEH-1 (Fig. 4A), supported normal growth of CMR300 under these conditions, whereas the mono-functional constructs, pHiKdtA and pHE-1, did not (Fig. 4B). The results of the *in vivo* complementation studies and the *in vitro* assays are therefore consistent with each other, supporting the idea the *N*-terminal domain of KdtA plays a key role in determining the number of Kdo residues that are transferred to lipid IV_A.

Converting a bi-functional KdtA into a mono-functional KdtA

The bi-functionality of KdtA may require the proper placement of several motifs or residues around the active site. To find the determinants of bi-functionality, we followed the loss of bi-functionality from EcKdtA and its acquisition by HiKdtA. In order to determine the subset of residues participating in determination of the bi-functionality of EcKdtA, we constructed a series of active KdtA chimeras (encoded by pEH-1 through pEH-4) that contain progressively fewer *N*-terminal residues of EcKdtA and more residues of HiKdtA (Fig. 5A). All the constructs were transformed into CMR300 and analyzed as described above. Whereas cell-free extracts of CMR300/pEcKdtA, CMR300/pEH-2, and CMR300/pEH-3 transfer two Kdo units from CMP-Kdo to [4'-³²P]lipid IV_A (Fig. 5A, lanes 3, 5 and 6), cell-free extracts of CMR300/pEH-4 transferred only a single Kdo unit (Fig. 5A, lane 7). These results imply that amino acid residues between positions 92 and 165 are important for conferring bi-functionality to EcKdtA. If we exclude identical amino acids (Fig. 2), one or more of the 27 amino acids that differ in this region could be required for the bi-functionality.

We next tested the ability of pEH-3 and pEH-4 to complement the inability of CMR300 to grow on MacConkey-agar plates (Fig. 5B). The plasmid pEH-3, encoding a bi-functional enzyme, supported the growth of CMR300 on MacConkey-agar plates at 42 °C, whereas pEH-4, encoding a mono-functional enzyme, did not. The plasmid pEH-2, encoding a bi-functional enzyme *in vitro*, also supported the growth of CMR300 on MacConkey-agar plates at 42 °C (data not shown)

Converting a mono-functional KdtA to a bi-functional KdtA

Residues between positions 92–165 in EcKdtA may not be the only ones required for bi-functionality. We next constructed a series of KdtA chimeras (encoded by pHE-1 through pHE-6), containing progressively fewer *N*-terminal residues of HiKdtA and more residues of EcKdtA (Fig. 6A). Whereas cell-free extracts of CMR300/pHE-2 through CMR300/pHE-5 were mono-functional (Fig. 6A, lane 5, 6, 7, and 8), cell-free extracts of CMR300/pHE-6 were bi-functional, as judged by the *in vitro* assay (Fig. 6A, lane 9). The chimeras HE-5 and HE-6 differ by only 11 amino acids (residues 20 to 30). The mono-functional chimera HE-5 consists entirely of EcKdtA except for the first 30 amino acid residues, which are derived from HiKdtA (Fig. 6A), whereas the bi-functional chimera HE-6 is essentially the same as HE-5, except that it contains only the first 19 residues of HiKdtA. Thus, the segment from residues 20 to 30 of the *N*-terminal region is crucial for conferring bi-functionality to the remaining 395 C-terminal residues of EcKdtA (Fig. 6A, lanes 8 versus 9). Introduction of pHE-6 into CMR300 rescued

cell growth on MacConkey-agar plates at 42 °C; however, over-expression of pHE-6 in the presence of 0.2 % L-arabinose was toxic (Fig. 6B), demonstrating the need to maintain an optimal level of expression when working with these chimeras. The combined data suggest that residues between positions 20 and 30 (Fig. 6A), as well as between positions 92 and 165 (Fig. 5A), contribute to the bi-functionality of EcKdtA.

Replacement of nine residues of HiKdtA with their EcKdtA counterparts confers partial bi-functionality

We next asked whether a single short sequence motif from EcKdtA, taken from segments between residues 20–165, could be grafted into HiKdtA and confer bi-functionality in the context of a protein that otherwise consists entirely of HiKdtA. Accordingly, we generated plasmids pHEH-1 through pHEH-4, which encode chimeras consisting mostly of HiKdtA with short embedded EcKdtA sequences, as shown for EcKdtA residues 20–28 in the case of pHEH-1 (Fig. 7). Introduction of pHEH-1 into CMR300 restored growth on MacConkey-agar plates at 42 °C, but only when induced with 0.2 % L-arabinose (Fig. 7A). Cell-free extracts of CMR300/pHEH-1 grown with 0.2 % L-arabinose at 30 °C catalyzed the formation of a mixture of Kdo-lipid IV_A and Kdo₂-lipid IV_A in about 4:3 molar ratio (Fig. 7B, lane 5). Even though pHEH-1 encoded a chimera that was not fully bi-functional *in vitro* (Fig. 7B, lane 5), it apparently converted enough of lipid IV_A to Kdo₂-lipid IV_A *in vivo* to restore OM integrity, as judged by growth on MacConkey-agar plates (Fig. 7A). None of the other constructs of this kind (Fig. 7A) restored OM integrity.

Even though residues between positions 20 and 30, as well as between 91 and 165, are important for the bi-functionality of EcKdtA (Figs. 5 and 6), replacement of residues 20 to 28 of HiKdtA with the corresponding EcKdtA sequence was sufficient to confer partial bi-functionality to the HiKdtA protein (Fig. 7A). The explanation for this apparent anomaly will require structural studies, which should explain how different portions of EcKdtA and HiKdtA fit together and interact with each other during catalysis.

Lipid composition of CMR300 cells expressing KdtA chimeras

As previously reported (19), CMR300/pBAD33 accumulates lipid IV_A as its only remaining LPS substructure (Fig. 8, lane 1). CMR300/pHiKdtA accumulates Kdo-lipid IV_A to similar levels (Fig. 8, lane 3). Very little mature lipid A is generated by this construct (Fig. 8, lane 7), even when HiKdtA is induced with 0.2 % L-arabinose, because *E. coli* LpxL (29) and WaaC (36) require the presence of the Kdo disaccharide to be optimally active. In contrast, mature lipid A species were generated when pHEH-1 was induced with 0.2 % L-arabinose (Fig. 8, lane 8), demonstrating that the enzyme chimera encoded by pHEH-1 was sufficiently bi-functional to convert most of the lipid IV_A to Kdo₂-lipid IV_A in cells, and to restore full length LPS *in vivo*, as seen with pEcKdtA (Fig. 8, lane 6). Since CMR300 also harbors pWMSbA (encoding *msbA*), which results in significant expression of MsbA even in the absence of induction, excess MsbA is always present in these constructs, resulting in the export of tetra- and penta-acylated species, as well as hexa-acylated LPS, when either pEcKdtA or pHEH-1 are induced (Fig. 8, lanes 6 and 8).

Complementation of a *kdtA::kan* deletion by pEcKdtA or pHEH-1 in DY330

All of the above studies were done in strain CMR300, harboring pWMSbA (encoding *msbA*), which functions as a multi-copy suppressor of the *kdtA* deletion present in this background. To determine whether pHEH-1 (Fig. 7) can also complement a *kdtA* deletion in the absence of *msbA* over-expression, we transformed pEcKdtA or pHEH-1 into DY330, and then replaced the chromosomal *kdtA* gene with kanamycin resistant cassette by homologous recombination at 30 °C (Fig. 9B). Whereas induction of pEcKdtA at 30 °C was toxic to cells in this genetic setting (Fig. 9A), induction of pHEH-1 was required to support cell growth at 30 °C on LB

agar plates (Fig. 9A), indicating that pHEH-1 can complement the chromosomal *kdtA* deletion in DY330, if expressed at an appropriate level.

The lipids present in HSC1/pHEH-1 were the same as those recovered from DY330 or HSC1/pEcKdtA (Fig. 9C), confirming that the chimera encoded by pHEH-1 is indeed bi-functional *in vivo* at 30 °C in DY330. With TLC analysis (Fig. 9C), we detected only mature hexa-acylated lipid A, which was released from LPS by mild acid hydrolysis. No tetra- or penta-acylated lipid A species were present because MsbA was not over-expressed in this construct.

DISCUSSION

KdtA belongs to Carbohydrate Active enZyme (CAZy) family #30, which is a member of the glycosyl transferase-B super-family (37). Family 30 consists exclusively of putative KdtA orthologs from different Gram-negative bacteria. KdtA orthologs are conserved inner membrane proteins, typically consisting of a single polypeptide chain of 350 to 450 amino acids, that transfer one or more Kdo units from CMP-Kdo to the precursor lipid IV_A (Fig. 1) (5). EcKdtA transfers two Kdo residues to lipid IV_A, resulting in an α ,2–6 linkage between the distal glucosamine residue of lipid IV_A and the first Kdo moiety, and an α ,2–4 linkage between two Kdo units (13,30). In contrast, KdtA of *H. influenzae* (15), *Aquifex aeolicus* (38) and *Bordetella pertussis* (16) transfer only a single Kdo unit to lipid IV_A (Fig. 1). Organisms containing mono-functional Kdo transferases often encode a special Kdo kinase (18), which adds a phosphate group to the same site occupied by the outer Kdo unit in *E. coli* LPS (Fig. 1). The mono-functional enzyme encoded by *H. influenzae kdtA* cannot complement an *E. coli kdtA* deletion at elevated growth temperatures (30 °C or above) (39), because *E. coli* LpxL functions poorly with substrates containing one or no Kdo residue (29,44). Reduced LpxL activity results in accumulation of tetra-acylated lipid A, which is exported relatively slowly (40). Likewise, WaaC does not efficiently incorporate heptose units into LPS precursors containing only one Kdo residue (36), and therefore a complete LPS core is not generated.

While EcKdtA is bi-functional and HiKdtA is mono-functional, they nevertheless share 48 % identity and 64% similarity in their amino acid sequences and are of similar lengths (Fig. 2). Comparison of the amino acid sequences of HiKdtA and EcKdtA does not readily explain their respective mono- or bi-functionality. KdtA orthologs of *Chlamydia* can transfer three or even four Kdo units to lipid IV_A (41,42), giving rise to the so-called genus-specific epitope characteristic of these organisms (43).

Subtle differences in specific amino acid residues or sequence motifs must account for KdtA functionality, but we were unable to convert HiKdtA to a bi-functional enzyme by chemical mutagenesis of a *kdtA* bearing plasmid (data not shown). Bioinformatic considerations suggested that KdtA might consist of two distinct domains (Fig. 3). The relatively low levels of sequence similarity of the amino acid residues connecting these domains (Fig. 2) suggested that it might function as a linker. Therefore, we initially constructed chimeras in which the N- and C-terminal halves of the proteins were switched, demonstrating conclusively that the N-terminal half of KdtA was responsible for conferring mono- or bi-functionality (Fig. 4). Next, we found that many other segments of EcKdtA and HiKdtA could be interchanged without complete loss of Kdo transferase activity. The least active chimeras retained about 1% of the wild-type specific activity. By replacing portions of EcKdtA with HiKdtA we found that residues 20–30 and 92–165 are important for the addition of the second Kdo unit (Figs. 5 and 6). When we replaced residues 20–28 of HiKdtA with the corresponding segment of EcKdtA, enzyme activity was partially shifted from mono-functionality to bi-functionality (Fig. 7), and the ability of CMR300 to grow on MacConkey-agar plates was restored (Fig. 7). The complementation analysis of CMR300 by all the chimeras shown in Figs. 4 through 7

confirmed that bi-functional Kdo transferase activity was absolutely necessary for restoration of growth on MacConkey-agar plates and LPS biosynthesis in *E. coli* (Fig. 8).

Residues 20–26 of HiKdtA are much more hydrophobic than their EcKdtA counterparts (Fig. 2) and may comprise part of a transmembrane α -helix. In EcKdtA three arginine residues are present in this same segment, replacing the hydrophobic amino acids of HiKdtA. This hydrophilic tether might allow the EcKdtA active site to move towards the cytosol and farther away from the inner membrane surface. This feature might account for the fact that EcKdtA can access the 4-position of the Kdo unit of Kdo-lipid IV_A, whereas HiKdtA cannot. Deletion of the transmembrane segment of EcKdtA (residues 1 to 19) eliminates enzyme activity (data not shown). When the corresponding transmembrane segment of HiKdtA (residues 1–26) is used to replace EcKdtA residues 1–19, but with retention of *E. coli* residues 20–425 (Fig. 2), the resulting construct complemented the growth of CMR300 on MacConkey-agar plates at 42 °C, when grown with either 0 % and 0.02% L-arabinose. However when it was grown with 0.2% L-arabinose, this construct was toxic. In cell extracts this protein chimera produced a mixture of products, consisting of Kdo-lipid IV_A and Kdo₂-lipid IV_A (data not shown), in a 2:1 molar ratio, similar to the results for HEH-1 (Fig. 7) (data not shown). These observations show that a transmembrane segment is required not only for the localization of EcKdtA in the inner membrane where lipid IV_A is but also for the function of EcKdtA, and that the basic segment from residues 20–28 that follows the transmembrane segment in EcKdtA (Fig. 2) is especially important for conferring bi-functionality.

Recently, Mamat and coauthors demonstrated that purified KdtA of *Aquifex aeolicus* (AaKdtA) is also mono-functional (38). AaKdtA is one of the smallest KdtA orthologues and is not predicted to have an *N*-terminal transmembrane segment. In fact, AaKdtA displays very low sequence similarity to EcKdtA throughout its first 48 amino acid residues. The residues required for the bi-functionality of EcKdtA are missing in the *N*-terminus of AaKdtA.

Only a few dozen structures have been reported for the members of the GTase-B superfamily (37), and no structural characterization is available for any of the KdtA orthologs, despite their important role in outer membrane assembly and virulence. Consequently, very little is known about the active sites and the mechanisms of KdtA orthologs. Our findings set the stage for the systematic study of the kinetics, mechanism and structural biology of the Kdo transferases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations are

EcKdtA	<i>E. coli</i> Kdo transferase
HiKdtA	<i>H. influenzae</i> Kdo transferase
Kdo	2-keto-3-deoxy-D-manno-octulosonic acid
LPS	lipopolysaccharide

OM	outer membrane
PBS	phosphate-buffered saline

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Figure 1.

Reactions catalyzed by EcKdtA and HiKdtA in the late steps of lipid A biosynthesis. EcKdtA is bi-functional (14), whereas HiKdtA is strictly mono-functional (15). In *H. influenzae*, Kdo kinase phosphorylates the inner Kdo residue at the 4-position (18), the same site at which the outer Kdo residue is attached by EcKdtA. *E. coli* does not encode the kinase KdkA, but its heterologous expression together with HiKdtA in *E. coli* results in the production of phospho-Kdo-lipid A, which can substitute for Kdo₂-lipid A in supporting *E. coli* growth at 37 °C or lower (39).

EcKdtA	MLELLYTAALLYLIQPLIWIRLWVRGRKAPAYRKRWGERYC	40
HiKdtA	MWRFFYSLLILICQPLILCFIGLLSVSPRYQQLAERYC	40
EcKdtA	FYRHP--LKFPGSIMLHSVSVGETLAAIPLVRAIRHRYFDE	78
HiKdtA	FYGNASCPPFQGIIFIHAA SVGEVIAATPLVROQQDYPHL	80
EcKdtA	PITVTMTPTGSESVQSAFGKDQHVLPYDLFDALNRRL	118
HiKdtA	SITFTTFTPTGSESVKATFGDSVFHYLPLDLFFSIHRFI	120
EcKdtA	NKVDPKLVLMETELWPNLIAALHKIKIPLVIANARLSAR	158
HiKdtA	NFVQPKLCIVMETELWPNLIHQFLFNIPFVIANARLSAR	160
EcKdtA	SAAGYANLGKFVRRLRRITLIAAQNEEDGARFVALGAKN	198
HiKdtA	SAHRVGIKAHLQTMWSQISLIAQDNISGKRYATLGYPK	200
EcKdtA	NQVTVTQSLKFDISVTPQLAAKAVTLERQWAPHRPVWIA	238
HiKdtA	EKLNIQNIKYDLNTNDELRRKIDSLETLWKQDRPIWIA	240
EcKdtA	STHEGESVVIAAHQALLQQFPNLLLIIVPRHPERFPDAI	278
HiKdtA	STHNGEDEIILKSHRALLAKYPNLLLIIVPRHPERFNVVA	280
EcKdtA	NIVRQAGLSYITRSSEVSTSTQVVVSDTMGELMLLYGI	318
HiKdtA	DILKKEKFQFIRRSSTNELNENTQVILSDSMGELMLMYGI	320
EcKdtA	ADLAFVGGSLVERGGHNPLEAAAHAIPLVMGPHTFNFKDI	358
HiKdtA	SDIAFVGGSLVKHGGHNPLEPLAFKMPVITCKHTFNFPET	360
EcKdtA	CARLEQASGLITVT-DATTLAKEVSSLLTDADYSFYGRH	397
HiKdtA	FRMLVEVQGVLEVNSTADALERAVEALLNSKESERLENA	400
EcKdtA	AVEVLYQNQALQRLQLLEPYLPPKTH	425
HiKdtA	GYEVLMEVRGALQRLLDLLKPYLERNV-	427

Figure 2.

Sequence alignment of EcKdtA and HiKdtA. These proteins show 48% identity and 64% amino acid similarity over their full lengths. The central region of *E. coli* KdtA from residues 196 to 224 is much less similar to its HiKdtA counterpart, suggesting that it might function as the linker between the *N*- and *C*-terminal domains. Residues 20 to 26 of EcKdtA contain three arginine residues in contrast to the same part of HiKdtA, which appears to be part of a longer *N*-terminal transmembrane segment.

A. Wild-type KdtA **B. Chimeric KdtA** **Figure 3.**

Schematic diagram of the predicted EcKdtA and HiKdtA domains. These domain predictions are based on the pfam algorithm available at <http://pfam.sanger.ac.uk/protein?acc=P0AC75> for EcKdtA and <http://pfam.sanger.ac.uk/protein?acc=P44806> for HiKdtA (34).

Figure 4.

The *N*-terminal half of KdtA determines mono- versus bi-functionality. Panel A. Conversion of [4'-³²P]-lipid IV_A (IV_A) to [4'-³²P]-Kdo-lipid IV_A (KDO-IV_A) and/or [4'-³²P]-Kdo₂-lipid IV_A (KDO₂-IV_A) by cell-free extracts derived from the indicated constructs induced by 0.2 % L-arabinose. Final protein concentrations in the assays were 0.25 mg/mL for CMR300/pEcKdtA, CMR300/pHiKdtA, and CMR300/pEH-1, and 0.50 mg/mL for CMR300/pBAD33, CMR300/pHE-1. The reactions were allowed to proceed for 60 minutes at 30 °C. Panel B. Growth of the *kdtA* deletion mutant CMR300 harboring the indicated *kdtA* constructs on a LB-agar plate at 30 °C, and the complementation of CMR300 by the same plasmids on MacConkey-agar plates at 42 °C in the presence or absence of 0.2 % L-arabinose.

Figure 5. Construction of EcKdtA-HiKdtA chimeras that lose their bi-functionality. Panel A. TLC analysis showing the conversion of [4'-³²P]-lipid IV_A (IV_A) to [4'-³²P]-Kdo-lipid IV_A (KDO-IV_A) and/or [4'-³²P]-Kdo₂-lipid IV_A (KDO₂-IV_A) by cell-free extracts derived from the indicated constructs. A final concentration of 0.25 mg/mL for CMR300/pEcKdtA, CMR300/pHiKdtA, CMR300/pEH-2, and CMR300/pEH-3, and 1.0 mg/mL for CMR300/pBAD33, CMR300/pEH-4 were used in the assay at 30 °C for 60 minutes. All constructs were induced with 0.2 % L-arabinose. Panel B. Growth of the *kdtA* deletion mutant CMR300 harboring the indicated *kdtA* chimeras on a LB-agar plate at 30 °C and complementation on MacConkey-agar plates at 42 °C in the presence or absence of 0.2 % L-arabinose. The plasmids pEH-2 and pEH-3, encoding bi-functional KdtA chimeras, complemented the CMR300 phenotype on MacConkey-agar plates, but pEH-4, which encodes a mono-functional chimera, did not.

Figure 6. Construction of HiKdtA-EcKdtA chimeras that acquire bi-functionality. Panel A. TLC analysis showing conversion of [4'-³²P]-lipid IV_A (IV_A) to [4'-³²P]-Kdo-lipid IV_A (KDO-IV_A) and/or [4'-³²P]-Kdo₂-lipid IV_A (KDO₂-IV_A) by the indicated constructs. A final concentration of 0.25 mg/mL of the cell-free extracts from each construct was used in the assay (30 °C for 60 minutes). pEcKdtA, pHiKdtA, and pHE-2 through pHE-5 were induced with 0.2 % L-arabinose. pHE-6 was toxic to cells in the presence of 0.02 % or 0.2 % L-arabinose, and was therefore not induced. Panel B. The plasmid pHE-6, encoding a bi-functional KdtA chimera, complemented the CMR300 phenotype on MacConkey-agar plates, but pHE2-pHE5, which encode mono-functional chimeras, did not.

Figure 7. Induction of pHEH-1 complements growth of CMR300 and encodes a partially bi-functional enzyme. Panel A. Diagram of constructs pHEH-1, pHEH-2, pHEH-3, and pHEH-4, encoding KdtA chimeras that consist mostly of HiKdtA with selected EcKdtA inserts. Only the plasmid pHEH-1, when induced, complemented the CMR300 phenotype on MacConkey-agar plates at 42 °C. Panel B. Assays were performed as in Figs. 6 and 7, demonstrating the partial bi-functional character of the KdtA chimera HEH-1. A final concentration of 0.25 mg/mL of cell-free extract from each construct (induced with 0.2 % L-arabinose) was used in the assay at 30 °C for 60 minutes. Abbreviations: IV_A, [4'-³²P]-lipid IV_A; KDO-IV_A, [4'-³²P]-KDO-lipid IV_A; KDO₂-IV_A, [4'-³²P]-Kdo₂-lipid IV_A.

Figure 8.

Induction of pHEH-1 restores LPS production in *E. coli* CMR300. Induced cells of CMR300, containing the vector or the plasmids with the indicated inserts, were first extracted by the Bligh-Dyer method (33) to obtain the glycerophospholipid fraction (lanes 1, 2, 3, and 4), which also includes the free lipid IV_A and Kdo-IV_A, if present. Next, the extracted cell residues from the same strains (which contain the intact LPS) were subjected to mild acid hydrolysis to cleave the Kdo-lipid A linkage, and they were then subjected to a second Bligh-Dyer extraction to recover the released lipid A species (lanes 5, 6, 7, and 8). The lipids, separated by TLC in the solvent chloroform/pyridine/88 % formic acid/H₂O (30:70:16:10 v/v), were detected by charring with 10 % sulfuric acid in ethanol. The number of acyl chains present in each of the three major lipid A species are indicated as hexa, penta or tetra. Small amounts of the 1-dephosphorylated lipid A derivatives, which are formed as byproducts of the mild acid hydrolysis, are the two most rapidly migrating bands in lanes 5 through 8 that are not labeled. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

A.

B.

1: DY330
2: HSC1/pEcKdtA
3: HSC1/pHEH-1

C.

Lipid extracted from whole cell Lipid extracted after mild acid hydrolysis of LPS

Figure 9.

pHEH-1 complements the *kdtA::kan* deletion in DY330 at 30 °C. Panel A. Complementation of the *kdtA* deletion in *E. coli* DY330 by induced pHEH-1 or by uninduced pEcKdtA. Two colonies from each construct were tested on each plate. In *E. coli* DY330 the over-expression of pEcKdtA was toxic. Panel B. Confirmation by PCR of the *kdtA::kan* replacement in strains HSC1/pEcKdtA and HSC1/pHEH-1 strains. Panel C. Glycerophospholipid and lipid A profiles of DY330, HSC1/pEcKdtA and HSC1/pHEH-1. Lipids were prepared as in Fig. 8 and were detected by charring with 10 % sulfuric acid in ethanol.

Table 1

Bacterial strains and plasmids.

<i>E. coli</i> Strain	Relevant genotype	Source or References
W3110	Wild type, F ⁻ , λ ⁻	<i>E. coli</i> Genetic Stock center, Yale University
DY330	W3110 $\Delta lacU169 gal490 \lambda cI857 \Delta(cro-bioA)$	(24)
XL1-Blue	$\Delta mcrABC recA1 endA1 gyrA96 relA1 supE44 thi-1 lac$	Stratagene
CMR300	W3110 (<i>kdtA::kan</i>)/pWMsbA	(19)
HSC1/pEckdt A	DY330 (<i>kdtA::kan</i>)/pEcKdtA	This work
HSC1/pHEH-1	DY330 (<i>kdtA::kan</i>)/pHEH-1	This work
Plasmids		
pBAD33	Medium copy vector, Cam ^R	(23)
pBAD33.1	Medium copy vector, Cam ^R , replaced pBAD33 nucleotides between XbaI to HindIII with those of pET21b including ribosomal binding site.	This work
pEcKdtA	pBAD33.1 harboring <i>E. coli kdtA</i> 1–1278 [#]	This work
pHiKdtA	pBAD33.1 harboring <i>H. influenzae kdtA</i> 1–1284 [#]	This work
pEH-1	pBAD33.1 harboring <i>E. coli</i> 1–645 fused to <i>H. influenzae</i> 640–1284 [*]	This work
pEH-2	pBAD33.1 harboring <i>E. coli</i> 1–548 fused to <i>H. influenzae</i> 555–1284 [*]	This work
pEH-3	pBAD33.1 harboring <i>E. coli</i> 1–495 fused to <i>H. influenzae</i> 502–1284 [*]	This work
pEH-4	pBAD33.1 harboring <i>E. coli</i> 1–273 fused to <i>H. influenzae</i> 280–1284 [*]	This work
pHE-1	pBAD33.1 harboring <i>H. influenzae</i> 1–639 fused to <i>E. coli</i> 646–1278 [*]	This work
pHE-2	pBAD33.1 harboring <i>H. influenzae</i> 1–390 fused to <i>E. coli</i> 385–1278 [*]	This work
pHE-3	pBAD33.1 harboring <i>H. influenzae</i> 1–269 fused to <i>E. coli</i> 264–1278 [*]	This work
pHE-4	pBAD33.1 harboring <i>H. influenzae</i> 1–105 fused to <i>E. coli</i> 106–1278 [*]	This work
pHE-5	pBAD33.1 harboring <i>H. influenzae</i> 1–90 fused to <i>E. coli</i> 91–1278 [*]	This work
pHE-6	pBAD33.1 harboring <i>H. influenzae</i> 1–57 fused to <i>E. coli</i> 58–1278 [*]	This work
pHEH-1	pBAD33.1 harboring <i>H. influenzae</i> 1–57 fused to <i>E. coli</i> 58–84 and fused to <i>H. influenzae</i> 85–1284 [*]	This work
pHEH-2	pBAD33.1 harboring <i>H. influenzae</i> 1–213 fused to <i>E. coli</i> 208–240 and fused to <i>H. influenzae</i> 247–1284 [*]	This work
pHEH-3	pBAD33.1 harboring <i>H. influenzae</i> 1–420 fused to <i>E. coli</i> 415–444 and fused to <i>H. influenzae</i> 451–1284 [*]	This work
pHEH-4	pBAD33.1 harboring <i>H. influenzae</i> 1–126 fused to <i>E. coli</i> 127–147 and fused to <i>H. influenzae</i> 154–1284 [*]	This work

[#] Ec: Escherichia coli, Hi: Haemophilus influenzae

^{*} The numbers followed the original base pair numbering system in EckdtA or HikdtA.