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Dual Role of FtsH in Regulating Lipopolysaccharide Biosynthesis in *Escherichia coli*[▽]

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In *Escherichia coli*, FtsH (HflB) is a membrane-bound, ATP-dependent metalloendoprotease belonging to the AAA family (ATPases associated with diverse cellular activities). FtsH has a limited spectrum of known substrates, including the transcriptional activator σ^{32}. FtsH is the only known *E. coli* protease that is essential, as it regulates the concentration of LpxC, which carries out the first committed step in the synthesis of lipid A. Here we identify a new FtsH substrate—3-deoxy-D-*manno*-octulosonate (KDO) transferase—which carries out the attachment of two KDO residues to the lipid A precursor (lipid IV_A) to form the minimal essential structure of the lipopolysaccharide (LPS) (KDO_2-lipid A). Thus, FtsH regulates the concentration of the lipid moiety of LPS (lipid A) as well as the sugar moiety (KDO-based core oligosaccharides), ensuring a balanced synthesis of LPS.

FtsH (HflB) is an ATP-dependent, membrane-bound protease (17, 18) which belongs to the AAA family (ATPases associated with diverse cellular activities) (2, 26, 31, 36). FtsH degrades proteins tagged with the SsrA degradation peptide (12, 13) as well as YccA, SecY, and the ATPase F_0 when they are not complexed (1, 20). FtsH also has an important regulatory role in *Escherichia coli*, as it specifically degrades proteins such as the transcriptional activator σ^{32} and the viral regulatory proteins CII, CIII, and Xis (5, 14, 15, 21, 35, 37). However, although FtsH regulates the concentration of several key proteins, the spectrum of its specific substrates is quite limited.

FtsH is the only known essential ATP-dependent protease in *E. coli*. The lack of viability of Δ*ftsH* mutants is due to the accumulation of UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase (LpxC), which catalyzes the second reaction and the first committed step in the biosynthesis of lipid A (9, 30). The accumulation of LpxC results in unbalanced ratio of lipid A (in the biosynthesis of lipopolysaccharides [LPS]) to phospholipids, as both pathways use the same precursor (-3-hydroxymyristoyl-acyl carrier protein), which is present in limited amounts (30).

LPS is an essential component of the outer membrane of gram-negative bacteria and is present in high concentrations (about 10% of the total cell lipids). LPS is vital for bacterial pathogenesis, as virulence of mutants with altered LPS is substantially decreased or eliminated. Moreover, LPS—also referred to as endotoxin—is highly toxic and constitutes the major lethal factor in gram-negative bacterial sepsis (24, 27, 33, 34).

LPS is composed of lipid A and core oligosaccharides which start with 2 units of 3-deoxy-D-*manno*-octulosonate (KDO). The KDO residues are transferred in two sequential steps to lipid IV_A, the lipid A precursor, by the bifunctional enzyme KDO transferase (KdtA) (4). KDO_2-lipid A represents the minimal essential structure of the LPS and, therefore, KdtA is the only essential glycosyltransferase in the core oligosaccharide biosynthesis pathway. It should be noted that recent results indicate the abilities of several suppressor mutations to overcome the requirement for KDO (23).

Here we show that KdtA is subject to ATP-dependent proteolysis carried out by FtsH. This finding broadens the spectrum of known specific FtsH substrates. As FtsH is known to degrade LpxC, the finding that it also degrades KdtA reinforces its role as a regulator of LPS biosynthesis, as it effectively controls the concentrations of both the lipid A precursor and the precursor of sugar moiety (KDO).

MATERIALS AND METHODS

Bacteria and plasmids. All the experiments were carried out with *E. coli* K-12 MG1655 (wild type) and its derivatives. For a detailed description, see Table 1.

Media and growth conditions. Cells were grown on LB broth (Lennox; Difco) with aeration at 37°C unless otherwise stated. The medium was supplemented, when required, with 50 μg/ml kanamycin and 12.5 μg/ml tetracycline. The experiments were started when the cultures reached an optical density at 600 nm (OD_{600}) of 0.2 to 0.4.

Construction of the *kdtA*::FLAG strain. Chromosomal epitope tagging of *kdtA* was achieved using the system described by Uzzau et al. (38). Primers containing the *fabZ* promoter sequence upstream of the *kdtB* coding sequence were designed. A two-step PCR method was applied in which the pSUB11 plasmid served as a primary template, and the following primers were used: *kdtA* FLAG F, CGTCTGCTTCAACTGCTGGAACCTTACCTGCCACCGAAAACGCATGACTACAAAGACCATGACGG; and *kdtA* FLAG R1, ACAATAATAACACGGCCTGCCGCAATCGTAAGAATGAGACAGGCCGTAAAGTTTGGCGAACAAAAGATGGCATATGAATATCCTCCTTAG. The PCR product was used as a template for the second reaction using the same forward primer and the reverse primer *kdtA* FLAG R2, TGGGATCGAAAGTACCCGGATAAATCGCCCGTTTTTGCATGATACTCTTCCTGTCAAAATATAAGAAACGACAATAATAACACGGCCTG.

The kanamycin-resistant cassette achieved was then introduced by electroporation into competent cells containing the plasmid pKD46, and selection for kanamycin was applied. The colonies positive for kanamycin resistance were further verified by PCR and sequencing. The kanamycin-resistant cassette was then flipped out using the plasmid pCP20.

SDS-PAGE and immunoblotting. Gel electrophoresis was carried out according to published protocols using 10% sodium dodecyl sulfate-polyacrylamide gel

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TABLE 1. Bacterial strains

Strain	Description	Reference or source
MG1655 *kdtA*::FLAG	K-12 wild-type strain carrying the 3′ FLAG tag fusion for the chromosomal *kdtA* gene plus a *fabZ* promoter insertion upstream of *kdtB*	This study
Δ*lon kdtA*::FLAG	Km^r Δ*lon* carrying the *kdtA*::FLAG allele	This study
Δ*clp kdtA*::FLAG	Km^r Δ*clp* carrying the *kdtA*::FLAG allele	This study
KY2981 Triple mutant (Δ*lon* Δ*clpP* Δ*hslU)*	Δ(*clpP-lon*)*1196*::*cat* Δ*hslVU1172*::*tet* Δ*sulA2981*	19; kindly provided by Takashi Yura
Triple mutant *kdtA*::FLAG	*kdtA*::FLAG allele introduced into a *KY2981* triple mutant	This study
ftsH1	Temperature-sensitive *ftsH* allele	11; kindly provided by Christophe Herman and Philippe Bouloc
ftsH1 kdtA::FLAG	*kdtA*::FLAG allele introduced into a temperature-sensitive *ftsH* mutant (*ftsH1*)	This study

electrophoresis (SDS-PAGE). Mouse monoclonal anti-FLAG antibodies (Sigma, Ltd.) were used as the primary antibody, and horseradish peroxidase-conjugated anti-mouse immunoglobulin G was used as the secondary antibody. Detection was performed with the EZ-ECL chemiluminescence detection kit (Biological Industries, Israel). The films were scanned, and the bands were quantified by densitometry.

In vivo protein degradation assays. Cultures were grown exponentially. When turbidity reached an OD_{600} of 0.4, rifampin (200 μg/ml) and spectinomycin (300 μg/ml) were added to block transcription and translation, respectively. Samples (1 ml) were removed at intervals and centrifuged, and the pellets were frozen in acetone-dry ice and analyzed by SDS-PAGE. KdtA was detected and quantified by Western blotting with anti-FLAG antibodies. All the data presented here represent at least three independent experiments.

Protein purification. (i) FtsH purification. The sequence coding for the 549 C-terminal amino acid of FtsH was cloned downstream to a His-tagged maltose-binding protein (MBP) coding sequence on the inducible pMAL-C expression vector (NEB). Culture (800 ml) was grown to an OD_{600} of 0.8 when IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM. After 3 h, the cells were centrifuged and the pellet was resuspended in 30 ml buffer A (100 mM $NaPO_4$ [pH 7.5], 300 mM NaCl) and subjected to a French press. The lysate was centrifuged at 20,000 × *g* for 30 min to precipitate membrane debris. The clear supernatant was supplemented with imidazole to a final concentration of 20 mM and loaded on a gravity flow column containing 3 ml Ni-NTA agarose (Qiagen). The column was washed with 50 ml of buffer A supplemented with 50 mM imidazole and eluted with 5 ml of buffer A supplemented with 250 mM imidazole.

The eluted fraction was loaded onto a gravity flow column containing 5 ml of amylose resin (NEB), washed with 30 ml of buffer A, and eluted with 5 ml of buffer A supplemented with 10 mM maltose. The purified faction was further concentrated using Amicon Ultra (Millipore).

(ii) KdtA purification. The KdtA coding sequence was cloned into the His tag containing the pET-22b expression vector (Novagen) using NdeI and XhoI to remove the *pelB* leader sequence. Cultures were grown, induced, and harvested as above, and the pellet was resuspended in 30 ml of KdtA buffer (20 mM HEPES [pH 7.6], 5 mM β-mercaptoethanol, 0.2% Igepal) and centrifuged as described before. The supernatant was loaded onto a 16/10 SP FF cation exchange column (Amersham Biosciences) equilibrated with the same buffer and eluted with a 200-ml gradient of 0 to 1 M NaCl in the same buffer. Fractions containing KdtA (detected using SDS-PAGE chromatography) were collected, and imidazole was added to a final concentration of 3 mM. The sample was loaded on a gravity flow column containing 3 ml Ni-NTA agarose, washed with 30 ml of KdtA buffer supplemented with 0.5 M NaCl and 20 mM imidazole, and eluted with the same buffer containing 100 mM imidazole. The purified enzyme was further concentrated as described for FtsH.

In vitro degradation assay. The degradation assay was performed as described by Makinoa et al. (22), with minor modifications. The reaction was carried out in a final volume of 65 μl containing 50 mM Tris-acetate (pH 8.0), 5 mM magnesium acetate, 12.5 μM zinc acetate, 20 mM KCl, 100 mM NaCl, 2 mM β-mercaptoethanol, 15 μg MBP-FtsH, 3 μg His-KdtA. ATP (4 mM) or ADP (4 mM) was added as indicated. The reaction was carried out at 37°C, and samples (15 μl) were removed at the indicated times, mixed with sample buffer, and frozen.

Samples were subjected to SDS-PAGE on 10% polyacrylamide gels and stained with Coomassie brilliant blue.

RESULTS

KdtA is an unstable protein. A proteomic study to identify unstable proteins in *E. coli* provided data suggesting that KdtA may be an unstable protein with a short half-life ($t_{1/2}$). In order to study the concentration and stability of KdtA, we fused an oligonucleotide coding for the 15-amino acid FLAG tag to the 3′ end of the genomic *kdtA* (38) to enable detection and monitoring of the protein using anti-FLAG antibodies.

KdtA is coded for by the gene *kdtA* (*waaA*), an essential gene which is located in an operon upstream of the essential *kdtB* (*coaD*) gene. To prevent a lethal polar effect, we introduced an additional promoter downstream of the FLAG tag and upstream of the *kdtB* reading frame (Fig. 1, top). We chose the small *fabZ* promoter, which is regulated similarly to the *kdt* operon, as it participates in phospholipid biosynthesis. The *E.*

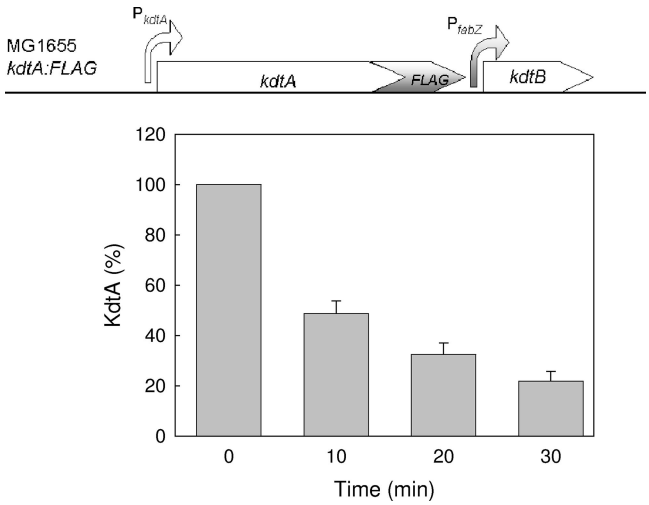

FIG. 1. Determination of KdtA stability. A chromosomal FLAG tag for the *kdtA* gene was introduced to an *E. coli* MG1655 strain as described by Uzzau et al. (38). The small *fabZ* promoter was added on the primers upstream of the *kdtB* coding sequence homology (top). Cultures of MG1655 *kdtA*::FLAG were grown to an OD_{600} of 0.4 at 37°C. Rifampin and spectinomycin were added to block transcription and translation, respectively, and samples were taken at intervals and subjected to Western blot analysis as described in Materials and Methods. The results represent an average of three independent experiments. A $t_{1/2}$ of <10 min was calculated in each individual experiment.

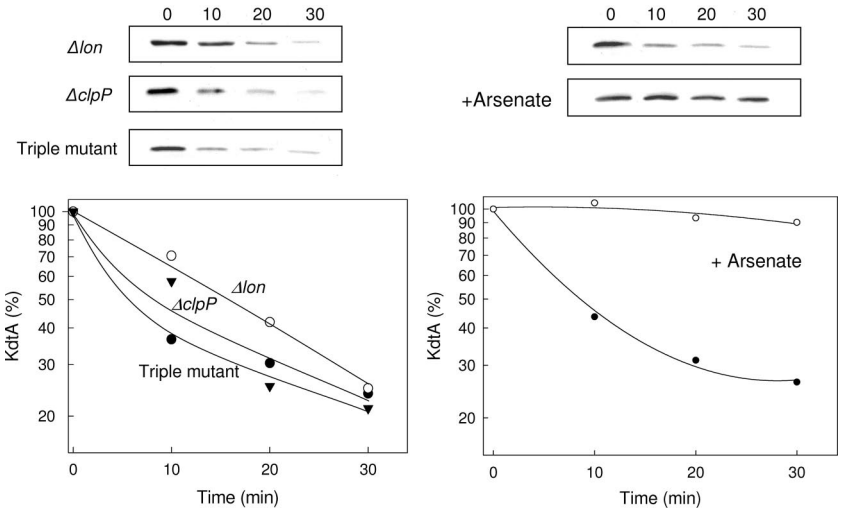

FIG. 2. Degradation of KdtA in several proteolysis-deficient mutants and in the presence of arsenate. Degradation assay of KdtA was performed in all the strains as described for Fig. 1. Left, degradation in proteolysis-deficient mutants; the FLAG-tagged *kdtA* allele was introduced into a *lon* mutant, a *clpP* mutant, and a triple mutant comprised of *lon*, *clpP*, and *hslVU*. Right, effect of 20 mM sodium arsenate.

coli strain containing the chromosomal FLAG-tagged KdtA grew as well as the wild-type bacteria, indicating that the function of these two essential genes (*kdtA* and *kdtB*) was not disturbed by the manipulations.

The *E. coli* strain containing the chromosomal FLAG-tagged KdtA was grown to an OD_{600} of 0.6, and stability of KdtA was determined as described in Materials and Methods by measuring the concentration of residual KdtA, following cessation of transcription and translation. The results indicate that KdtA is an unstable protein that is quickly degraded, with a $t_{1/2}$ shorter than 10 min (Fig. 1, bottom).

As we used a FLAG-tagged substrate, it is possible that the addition of the FLAG tag changes the specificity toward FtsH. This possibility appears unlikely in view of additional experiments indicating a similar degradation of His-tagged KdtA, both in vivo and in vitro.

KdtA degradation in mutants lacking ATP-dependent cytosolic proteases. Assuming that KdtA is proteolytically degraded, we examined the possible involvement of the four known cellular ATP-dependent proteases (10, 25). The FLAG-tagged *kdtA* allele was introduced into different mutant strains, and its stability was determined. The mutants used were *lon*, *clpP*, and the triple mutants *lon*, *clpP*, and *hslVU*, which lack the activities of Lon, ClpAP, ClpXP, and HslVU. KdtA degradation took place in all of the mutant strains examined, including the triple mutant lacking all four proteases (Fig. 2, left), demonstrating that proteolysis is independent of these proteases. It can be noted that proteolysis is even faster in the *lon* mutant. This result could imply that the active protease is upregulated in the absence of other proteases.

KdtA degradation is mediated by an energy-dependent protease. In view of the finding that KdtA was degraded even in mutants lacking all four cytosolic proteases, we examine whether metabolic energy is required for degradation. Arsenate was used to reduce the intracellular ATP content, as it is known to compete with phosphate in cellular phosphorylation processes (32). KdtA stability was determined in the presence of 20 mM sodium arsenate. The level of ATP during the treatment with arsenate decreased by more than 90% (6). At this concentration, the effect of arsenate is reversible, and ATP-dependent proteolysis is resumed after the arsenate is removed with no decrease in viability (3). The results presented in Fig. 2, right, show that KdtA was stabilized by the addition of arsenate, indicating that its degradation depends on a continuous supply of ATP.

KdtA is a substrate of FtsH (HflB). KdtA is degraded by an ATP-dependent protease, but not by any of the four cytosolic proteases Lon, ClpAP, ClpXP, and HslVU. An additional ATP-dependent protease that could play a role in KdtA degradation is the membrane-associated FtsH protease. However, in contrast to the cytosolic ATP-dependent proteases, FtsH is an essential gene and, therefore, it was not possible to use deletion mutants. Although there is an available deletion mutant with a suppressor in the *fabZ* gene (30), we used a temperature-sensitive mutant that loses FtsH activity above 42°C. A strain carrying a temperature-sensitive allele (*ftsH1*) of the *ftsH* gene (kindly provided by Christophe Herman and Philippe Bouloc) was used as a recipient for transduction of the gene coding for the FLAG-tagged KdtA. Cultures were grown exponentially at 30°C until turbidity reached an OD_{600} of 0.4 before they were transferred to 44°C. Rifampin and spectinomycin were added after 15 min at 44°C, and samples were removed as described earlier. At 37°C, KdtA was degraded at approximately the same rate in the *ftsh1* background as in the wild-type strain. At 44°C, when FtsH activity is lost, KdtA degradation was dramatically stabilized (Fig. 3). Furthermore, such stabilization is highly remarkable, since in the wild type the stability of KdtA decreases with an increase in temperature (see comparison of stability at 37°C and at 44°C [Fig. 3]). This result is in agreement with other findings, indicating that most proteases are more active at higher temperatures; moreover, for FtsH substrates it has been shown that they undergo partial unfolding at the higher temperatures, facilitating faster degradation (12).

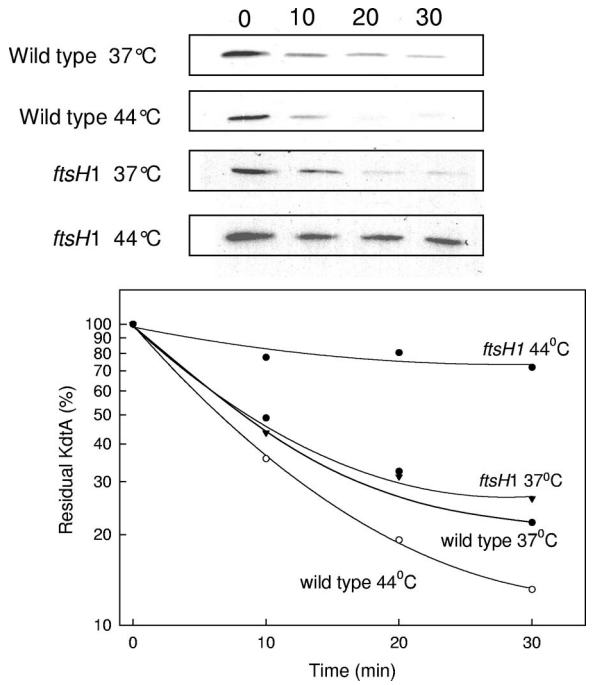

FIG. 3. Degradation of KdtA in a temperature-sensitive *ftsH* mutant (*ftsH1*). The FLAG-tagged *kdtA* allele was introduced into an *ftsH* temperature-sensitive strain (*ftsH1*). Cultures were grown at 37°C to an OD_{600} of 0.4 and divided into two, and one part was transferred to 44°C conditions. After 15 min, the degradation assay of KdtA was performed as described for Fig. 1.

In vitro degradation of KdtA by FtsH. As the number of FtsH substrates identified so far is very limited, it was important to demonstrate that the effect of FtsH on KdtA proteolysis is direct. These experiments required the purification of active FtsH and of KdtA, for which there was no available protocol. Here we present a protocol for KdtA purification as well as a novel quick protocol for tandem affinity purification of active FtsH.

In order to carry out an in vitro reaction, we fused the 549 C-terminal amino acids of FtsH, which include the active part and the first transmembrane domain, required for oligomerization, to MBP (22). Purified FtsH and KdtA were incubated as described in Material and Methods, and samples were removed at the times indicated. The results, presented in Fig. 4, indicate that the purified FtsH-MBP degraded purified KdtA in an ATP-dependent manner. Thus, no degradation was obtained if ATP was replaced by ADP (Fig. 4, right). These data indicate that KdtA is a specific substrate of FtsH.

DISCUSSION

Here we identified a new FtsH substrate, KdtA, a KDO transferase (7) which is essential and carries out the attachment of two KDO residues to the lipid A precursor (lipid IV_A) to form the minimal essential structure of the LPS (KDO_2-lipid A). Although FtsH has a limited spectrum of known specific substrates, one of these substrates is another protein involved in LPS synthesis, LpxC, which carries out the first committed step in the synthesis of lipid A.

The essential structure of LPS is composed of a lipid moiety (lipid A) and a sugar moiety (KDO). LpxC catalyzes the second step in lipid A biosynthesis, hydrolyzing UDP-3-*O*-(3-hydroxymyristoyl)-*N*-acetylglucosamine to acetate and UDP-3-*O*-(3-hydroxymyristoyl) glucosamine, which is the first committed step in lipid A biosynthesis. Therefore, by controlled proteolysis of LpxC, FtsH regulates the levels of lipid A. KdtA catalyzes the transfer of two KDO residues from the cytosolic donor molecule, CMP-KDO, to the acceptor membrane precursor lipid IV_A. After the transfer of KDO is complete, the KDO_2-lipid IV_A can further mature to the final, physiologically essential structure, KDO_2-lipid A. By controlled proteolysis of KdtA, FtsH also regulates the addition of the sugar moiety of the LPS and thus the maturation of the LPS precursor. This finding, that FtsH is also involved in controlling the transfer of the KDO molecules to the lipid A precursor, is compatible with the idea that FtsH-mediated proteolysis is essential for balancing the levels of the two components of the essential structure of LPS (Fig. 5).

Regulation of the concentration of KdtA may also be important as this enzyme has a rather low substrate specificity (7). Therefore, in the absence of a suitable substrate, this enzyme

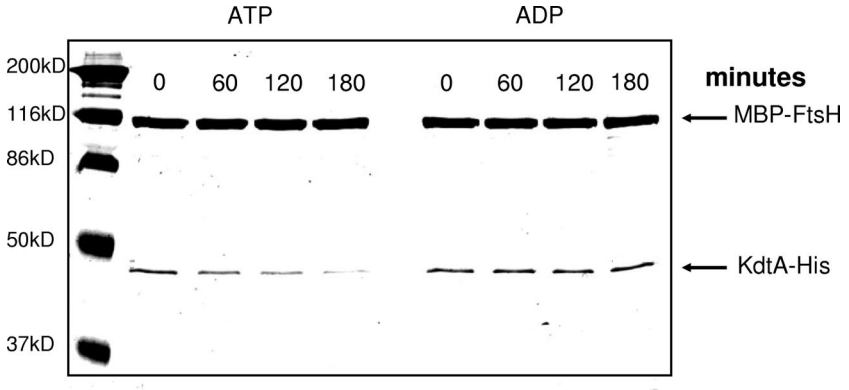

FIG. 4. In vitro proteolysis of KdtA by FtsH. In vitro degradation assay was performed as described in Materials and Methods with ATP (left) or ADP (right). Samples were collected at 60-min intervals and subjected to SDS-PAGE analysis and Coomassie blue staining. The reactions were carried out at 37°C.

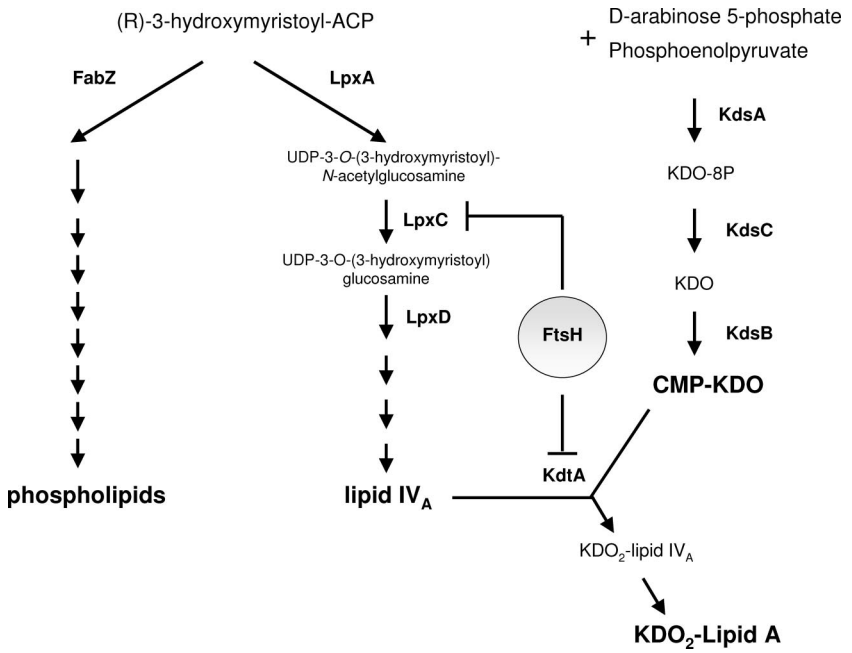

FIG. 5. Schematic representation of the involvement of FtsH in the LPS biosynthetic pathway. Phospholipids represent the major groups of phospholipids found in *E. coli*, including cardiolipin and phosphatidylethanolamine. T bars represent degradation by FtsH of LpxC and KdtA.

may be able to glycosylate alternative molecules, resulting in the accumulation of undesirable products.

Several recognition domains have been suggested for FtsH substrates. Thus, in the case of σ^{32}, the highly conserved region 2.1 appears to be essential for in vivo degradation (5, 16, 28, 29). However, this region is not sufficient for degradation. The other FtsH substrate—LpxC—is probably recognized by a hydrophobic region at the C terminus (8, 9). It has been suggested that a C-terminal sequence of UUXXXXXUUUU (U represents a hydrophobic amino acid and X represents any amino acid) is the degradation signal (F. Narberhaus, unpublished data). It is yet not known which domain of KdtA constitutes the FtsH recognition signal. The protein contains two hydrophobic stretches that are compatible with the suggested consensus recognition sequence. These sequences are PLEA AAHAIPVLM (UUXUUUXUUUUUU, starting at amino acid 336) and LLQLLEPYLPP (UUXUUXUXUUU, starting at amino acid 412). Support for the involvement of hydrophobic recognition between FtsH and KdtA was obtained from experiments indicating that proteolysis was not affected by high ionic strength but was inhibited by low concentrations of mild, nonionic, nondenaturing detergents, such as NP-40. However, it is possible that the inhibition by NP-40 results from disruption of the oligomeric state of FtsH. It has also been shown that FtsH degradation requires at least partial unfolding of the substrate (12), usually resulting from low intrinsic thermodynamic stability. KdtA appears to belong to this group of proteins, as purified KdtA tends to aggregate in a temperature- and concentration-dependent manner (C. Katz, unpublished data). At any rate, the identification and characterization of an additional FtsH substrate will advance our understanding of the specificity of its proteolytic activity.

The specific substrates of the membrane-bound protease FtsH include regulatory proteins, the transcriptional activator σ^{32}, and phage repressors. In addition, FtsH is essential for regulating the level of one essential protein involved in LPS synthesis, LpxC. The results presented here point out the involvement of FtsH in the proteolysis of an additional essential protein which also participates in the same pathway, KdtA. This proteolysis-based dual regulation may be essential for balancing the synthesis of the lipid component (lipid A) and the sugar component (KDO) of the LPS essential structure (Fig. 5). This dual regulation ensures the balanced synthesis of the two components of LPS, a compound crucial for growth and pathogenesis of gram-negative bacteria.

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