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Control of Lipopolysaccharide Biosynthesis by FtsH-Mediated Proteolysis of LpxC Is Conserved in Enterobacteria but Not in All Gram-Negative Bacteria▽†

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Despite the essential function of lipopolysaccharides (LPS) in Gram-negative bacteria, it is largely unknown how the exact amount of this molecule in the outer membrane is controlled. The first committed step in LPS biosynthesis is catalyzed by the LpxC enzyme. In *Escherichia coli*, the cellular concentration of LpxC is adjusted by the only essential protease in this organism, the membrane-anchored metalloprotease FtsH. Turnover of *E. coli* LpxC requires a length- and sequence-specific C-terminal degradation signal. LpxC proteins from *Salmonella*, *Yersinia*, and *Vibrio* species carry similar C-terminal ends and, like the *E. coli* enzyme, were degraded by FtsH. Although LpxC proteins are highly conserved in Gram-negative bacteria, there are striking differences in their C termini. The *Aquifex aeolicus* enzyme, which is devoid of the C-terminal extension, was stable in *E. coli*, whereas LpxC from the alphaproteobacteria *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* was degraded by the Lon protease. Proteolysis of the *A. tumefaciens* protein required the C-terminal end of LpxC. High stability of *Pseudomonas aeruginosa* LpxC in *E. coli* and *P. aeruginosa* suggested that *Pseudomonas* uses a proteolysis-independent strategy to control its LPS content. The differences in LpxC turnover along with previously reported differences in susceptibility against antimicrobial compounds have important implications for the potential of LpxC as a drug target.

Gram-negative cells are surrounded by an asymmetric outer membrane containing mostly lipopolysaccharides (LPS) in its outer leaflet. The LPS layer serves as a permeability barrier protecting the cell from harmful compounds, including antibiotics (46). Hence, a proper equilibrium between LPS and phospholipid molecules in the outer membrane is crucial for viability of most Gram-negative bacteria. LPS also plays an important role in symbiotic and pathogenic plant-microbe interactions as well as in mammalian infections (45, 49). The biosynthesis of LPS has gained a lot of attention (i) because lipid A, the hydrophobic anchor of LPS, is an endotoxin that causes severe sepsis upon Gram-negative infections and (ii) because LPS biosynthesis is both unique and essential for Gram-negative bacteria and therefore an attractive target for the design of novel antibiotics and vaccines (54, 55).

Both too much and too little LPS are detrimental in *Escherichia coli*. To avoid toxic accumulation of LPS, the membrane-bound and essential AAA protease (ATPases associated with various cellular activities) FtsH degrades two enzymes of the LPS biosynthesis pathway: LpxC and KdtA (35, 47). LpxC catalyzes the first committed step in biosynthesis of lipid A (64). Therefore, both accumulation and lack of LpxC are lethal for *E. coli* (15, 47, 59), making this enzyme the target of choice for drug design (8, 10, 36, 38, 48, 50). KdtA attaches the KDO sugar core moieties to lipid A, forming a minimal lipid A structure which is thought to allow growth of Gram-negative microorganisms (22).

The molecular mechanism of LpxC degradation is not yet fully understood (44). Degradation by the FtsH protease requires a length- and sequence-specific C-terminal degradation signal containing an LAXXXXXAVLA motif consisting of six nonpolar amino acids within the last 11 residues (15). Like the SsrA tag (9, 21, 28), this tail serves as a general degradation signal. Additional, not yet defined internal regions of LpxC are required to direct the enzyme exclusively to FtsH (16).

The exact composition of LPS is known to vary between different Gram-negative bacteria and can be modulated in response to changing environmental conditions, e.g., cold shock in *E. coli* (7, 54). Despite this variability, the first steps in lipid A biosynthesis are highly conserved and are referred to as the constitutive part of the LPS biosynthesis pathway. Interestingly, the C termini of LpxC proteins differ significantly between species (Fig. 1) although the overall sequence of LpxC enzymes is highly conserved (Table 1). Whether FtsH is essential in Gram-negative bacteria other than *E. coli* and its close relatives is not known. The finding that FtsH is not needed for viability in the alphaproteobacterium *Caulobacter crescentus* (14) suggests that the requirement of the protease in control of LPS biosynthesis is not entirely conserved.

Our present study was motivated by three interesting observations. (i) The degradation tag of *E. coli* LpxC ($LpxC_{Ec}$) is entirely missing in the *Aquifex aeolicus* protein (Fig. 1) whose three-dimensional structure has been solved (3, 19, 62). (ii) The C-terminal ends of alphaproteobacterial LpxC proteins differ substantially from the *E. coli* sequence (Fig. 1). (iii) Several differences in the susceptibility toward chemical LpxC inhibitors have been described between the *E. coli*, *Pseudomonas aeruginosa*, *A. aeolicus*, and *Rhizobium leguminosarum* enzymes (3, 4, 30, 32, 41, 42, 48). These findings raised the

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organism	C-terminal sequence of LpxC	length	class
E. coli	-HALNNKLLQAVLAKQEAWEYVTFQDDAELP**LA**FKAPS**AVLA**	305	gamma-proteo-bacteria
S. enterica	-HALNNKLLQAVLAKQEAWEFVTFQDDAELP**LA**FKAPST**VLA**	305	
Y. pseudotuberculosis	-HALNNKLLQAVLAKQEAWEFVTFQDEAEMP**LA**FKAPST**VLA**Y	307	
V. cholerae	-HGLNNQLLRAVLADQEAWEWATFEEEVGSP**VA**FAEPNM**VLA**	305	
P. aeruginosa	-HALNNQLLRTLIADKDAWEVVTFEDARTAP**I**SYMRP**AAAV**	303	
A. tumefaciens	-HKVNANALKALLSDPSAYEIVEAPAARNQVRAREFVAVNMPEFAPWSA	318	alpha-proteo-bacteria
R. capsulatus	-HALTNKLLRALFADPDAWIWETCSPEQAHRLPGAGVLRGSMAAR	314	
A. aeolicus	-QKLTRDLPHLPSVQAL	282	aquificeae

FIG. 1. Comparison of the C-terminal sequences of LpxC from selected Gram-negative bacteria. Sequences identical to $LpxC_{Ec}$ are underlined, and residues conforming to the C-terminal degradation signal of $LpxC_{Ec}$ are shown in bold. The length of the LpxC proteins is given as the number of amino acids.

possibility that the mechanisms controlling LPS biosynthesis differ in Gram-negative bacteria. Therefore, we set out to analyze the stability of LpxC enzymes from representative Gram-negative species, including various human or plant pathogens, a photosynthetic bacterium, and *A. aeolicus* as the most distant relative of *E. coli* (Fig. 1). We provide evidence that FtsH-dependent proteolysis of LpxC is a widespread but not entirely conserved mechanism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 2. *E. coli* W3110, BL21(DE3), RH166, and Δ*lon* strains and *Salmonella enterica* cells were cultivated in liquid LB medium or on LB agar plates at 37°C. Main cultures were grown at 30°C. Cultivation of *E. coli* Δ*ftsH* and AR5088 strains was routinely performed at 30°C. *P. aeruginosa* cells were cultivated on *Pseudomonas* isolation agar plates (PIA; Difco) or in liquid LB medium at 37°C. When needed, antibiotics were used in the following concentrations: 100 μg ml^{-1} ampicillin (Amp), 50 μg ml^{-1} kanamycin (Kan), and 200 μg ml^{-1} chloramphenicol (Cm) for *E. coli* and *S. enterica*; 500 μg ml^{-1} carbenicillin (replaces Amp), 25 μg ml^{-1} Cm, and 200 μg ml^{-1} gentamicin (Gm) for *P. aeruginosa*.

Construction of LpxC expression plasmids. Plasmids used in this study are listed in Table 2, and oligonucleotides and restriction sites used for construction are shown in Table S1 in the supplemental material. All recombinant DNA techniques were performed using standard protocols (56) with *E. coli* DH5α as a cloning host.

Heterologous expression of LpxC in *E. coli*. *E. coli* cells transformed with the corresponding plasmids coding for full-length LpxC or LpxC variants from *A. aeolicus* ($LpxC_{Aa}$), *Rhodobacter capsulatus* ($LpxC_{Rc}$), *Agrobacterium tumefaciens* ($LpxC_{At}$), *P. aeruginosa* ($LpxC_{Pa}$), *Vibrio cholerae* ($LpxC_{Vc}$), *Yersinia pseudotuberculosis* ($LpxC_{Yp}$), or *S. enterica* ($LpxC_{Se}$) were inoculated to an optical density at 580 nm (OD_{580}) of 0.05 in liquid LB medium. Cultures were grown in a water bath shaker (180 rpm) at 30°C to an OD_{580} of 0.5. To gain equal and soluble amounts of LpxC, inducer concentrations and plasmids used were as follows: 0.2 to 0.25 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) with pBO1173 for the P_{T7} expression system, 200 ng ml^{-1} anhydrotetracycline (AHT) with pBO1718 and pBO2381 for the P_{tet} expression system, and 0.05% arabinose with pBO1142, 0.05 to 0.1% arabinose with pBO1172, 0.05 to 0.5% arabinose with pBO1177, 0.1% arabinose with pBO1146, 0.1 to 0.5% arabinose with pBO1144, and 0.3% arabinose with pBO1187 for the P_{BAD} expression system.

LpxC activity assays. Activity of LpxC from different Gram-negative bacteria in *E. coli* cells was determined by phenotypic analysis in liquid LB medium or on MacConkey agar plates and by measurement of 3-deoxy-D-manno-oct-2-ulopyranosonic acid (KDO) amounts. Growth of LpxC-expressing cells in LB medium (30°C) was monitored with three different concentrations of arabinose as inducer (0, 0.1, and 0.5%) by measuring the OD_{580}. As a comparison, growth of cells with the corresponding vector control or an $lpxC_{Ec}$ expression plasmid was analyzed likewise. For phenotypic analysis on solid medium, 2 μl of serial 1:10 dilutions of cultures adjusted to an OD_{580} of 0.5 were spotted on MacConkey agar plates with 0 and 0.1 mM IPTG or with 0 and 100 ng ml^{-1} AHT, respectively. Growth was monitored after incubation at 30°C for 1 to 2 days. KDO amounts in the membranes of LpxC-expressing cells were analyzed using a KDO assay as described previously (16, 34).

Heterologous *in vivo* degradation of LpxC in *E. coli*. Stability of plasmid-encoded LpxC proteins in *E. coli* was measured using *in vivo* degradation experiments. Cells carrying the corresponding plasmids were grown at 30°C in a water bath shaker (180 rpm) in liquid LB medium until an OD_{580} of 0.5 was reached. Depending on the vector, expression was induced by addition of arabinose, IPTG, or AHT at the concentrations described above. After 30 min, translation was blocked by addition of Cm. Samples were taken at different time points and were frozen in liquid nitrogen.

***In vivo* degradation experiments in *S. enterica* or *P. aeruginosa*.** Cells of *S. enterica* were electroporated with pBO1142 ($LpxC_{Se}$) or pBO1172 (for $LpxC_{Se}$ with a deletion of 5 amino acids [aa] in the C terminus [$LpxC_{Se}$ΔC5]). Degradation experiments were performed as described for *E. coli* with addition of 0.05% arabinose for 10 min to induce LpxC expression. For protein expression in *P. aeruginosa*, the shuttle vector pBO1745 ($LpxC_{Pa}$) was transferred by conjugational transfer using *E. coli* S17I-λpir as a donor. Growth of *E. coli* on PIA plates was inhibited by addition of Cm. LpxC expression was induced by addition of 1% arabinose for 30 min. Translation was blocked by addition of Gm.

Protein preparation and LpxC detection. To gain protein extracts, cell pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) according to their optical densities (100 μl for an OD_{580} of 1). After addition of protein sample buffer (final concentration: 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, 10% glycerol, 50 mM Tris-HCl, pH 6.8), cells were heated (at 100°C for 10 min) and centrifuged (for 1 min at 16,000 × *g*). The protein extract was subjected to SDS gel electrophoresis and Western transfer following standard protocols (56). LpxC proteins were detected either with a polyclonal LpxC antibody ($LpxC_{Ec}$ and $LpxC_{Se}$ [15]) combined with a secondary goat anti-rabbit horseradish peroxidase (HRP) conjugate (Bio-Rad) or with a His_5-HRP conjugate (Qiagen) (all other LpxC proteins). Chemiluminescence signals were visualized with an ECL chemiluminescence detection system (Amersham) and a ChemiImager Ready (Alpha Innotec). Half-lives of LpxC were calculated with the AlphaEaseFC software (version 4.0.0; Alpha Innotec).

TABLE 1. Sequence identity of LpxC, FtsH, and Lon from selected Gram-negative bacteria compared to *E. coli*

Organism	Sequence identity to the corresponding *E. coli* protein (%)[a]		
	LpxC	FtsH	Lon
S. enterica	98	97	99
Y. pseudotuberculosis	92	91	91
V. cholerae	73	77	82
P. aeruginosa	57	67	69
A. tumefaciens	39	58	62
R. capsulatus	39	50	58
A. aeolicus	30	46	48

[a] Identities were measured using Clustal W2 (39).

RESULTS

LpxC proteins from various Gram-negative bacteria can be expressed as active enzymes in *E. coli*. As a basis for further

TABLE 2. Bacterial strains, genomic DNA, and plasmids used in this study

Strain, genomic DNA source, or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
E. coli		
DH5α	λ^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	56
S17I-λpir	*recA pro thi hsdR*$^-$*M*$^+$ RP4-2 Tc::*Mu*-Km::Tn*7*-λ*pir*	12
W3110	F^- IN(*rrnD-rrnE*)1	1
Δ*ftsH* strain	W3110 *zad220*::Tn*10 sfhC21* Δ*ftsH3*::*kan*	60
RH166	MC4100 Δ*ara* Δ*leu lac* mutant	6
Δ*lon* strain	RH166 Δ*lon*::Tn*10*	5
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	57
AR5088	BL21(DE3) *zad220*::Tn*10 sfhC21* Δ*ftsH3*::*kan*	33
S. enterica serovar Typhimurium M556	SL1344 *sseD*::*aphT*	26
P. aeruginosa PAO1	Wild type	13
Origins of genomic DNA		
E. coli K12	Wild type	1
S. enterica serovar Typhimurium M556	SL1344 *sseD::aphT*	26
P. aeruginosa PAO1	Wild type	13
A. aolicus VF5	Wild type	11
R. capsulatus B10S	Wild type	37
A. tumefaciens C58	Wild type	61
V. cholerae O1 serovar El Tor strain N16961	Wild type	27
Y. pseudotuberculosis YPIII	*Inv*$^+$ (pIB1)	18
Plasmids		
pBAD24	Amp^r; P_{BAD} *araC*	25
pBO110	pBAD24 derivative coding for $LpxC_{Ec}$	15
pBO197	pBAD24 derivative coding for N-terminal His_6 tag fusions	H. Baumann
pET19b	Amp^r; P_{T7}; codes for N-terminal His_{10} tag fusions	Novagen
pASK-IBA5(+)	Amp^r; P/O_{tet} *tetR;* codes for N-terminal Strep tag fusions	IBA GmbH
pBO1721	pASK-IBA5(+) derivative; the coding region for the Strep tag was replaced by a His_6 sequence	S. Hagen
pHERD20T	Amp^r; P_{BAD} *araC*; *E. coli-P. aeruginosa* shuttle vector	52
pBO1702	pET19b derivative coding for His_{10}-$LpxC_{Ec}$	This study
pBO2382	pASK-IBA5(+) derivative coding for His_{10}-$LpxC_{Ec}$	This study
pBO2381	pASK-IBA5(+) derivative coding for His_{10}-$LpxC_{Aa}$	This study
pBO1718	pASK-IBA5(+) derivative coding for His_6-$LpxC_{Rc}$	This study
pBO1144	pBAD24 derivative coding for His_6-$LpxC_{At}$	This study
pBO1187	pBAD24 derivative coding for His_6-$LpxC_{At}$ΔC13	This study
pBO1177	pBAD24 derivative coding for His_6-$LpxC_{Pa}$	This study
pBO1146	pBAD24 derivative coding for His_6-$LpxC_{Vc}$	This study
pBO1173	pET19b derivative coding for His_{10}-$LpxC_{Yp}$	This study
pBO1142	pBAD24 derivative coding for $LpxC_{Se}$	This study
pBO1172	pBAD24 derivative coding for $LpxC_{Se}$ΔC5	This study
pBO1745	pHERD20T derivative coding for His_6-$LpxC_{Pa}$	This study

[a] Amp^r, ampicillin resistance. For truncated proteins, the number of C-terminally deleted residues is indicated (ΔC).

analysis, it was important to establish whether the LpxC enzymes from the model organisms used were active enzymes in *E. coli* as a host. To this end, the *lpxC* genes from *S. enterica*, *Y. pseudotuberculosis*, *V. cholerae*, *P. aeruginosa*, *A. tumefaciens*, *R. capsulatus*, and *A. aeolicus* were cloned and expressed in *E. coli* to moderate levels using either the arabinose-inducible pBAD system, the IPTG-inducible pET system, or the AHT-inducible pASK-IBA system, depending on which system worked best (Table 2). Except for LpxC from *S. enterica* ($LpxC_{Se}$), which can be detected by the antiserum raised against *E. coli* LpxC, all other LpxC variants carried an N-terminal hexa- or decahistidine tag to facilitate protein detection. All LpxC enzymes were expressed as soluble proteins (see Fig. S1 in the supplemental material).

Three different assays were used to monitor the activity and subsequent phenotypic consequences of heterologously expressed LpxC proteins in *E. coli*. Figure 2 illustrates representative results for three different phenotypic assays. Figures S2 to S5 in the supplemental material summarize the data for all three activity assays for all LpxC variants used in this study. Figures S2 to S5 additionally include the microscopic analysis of cells expressing the LpxC variants showing that overexpression of active LpxC leads to the formation of elongated cells (16). In the first two assays shown in Fig. 2, we took advantage of the fact that overproduction of active LpxC is toxic in *E. coli* due to the formation of abnormal membrane stacks in the periplasm (47, 59). The effect of LpxC from *S. enterica* and *V. cholerae* ($LpxC_{Vc}$) on growth in liquid LB medium is depicted in Fig. 2A. As shown previously (15), induction of plasmid-encoded *E. coli* LpxC ($LpxC_{Ec}$) expression in *E. coli* caused severe growth defects. Nontreated cultures or cells harboring the empty vector grew normally. The observed growth effects are not due to overexpression of plasmid-encoded proteins but are related to LpxC activity because production of an inactive

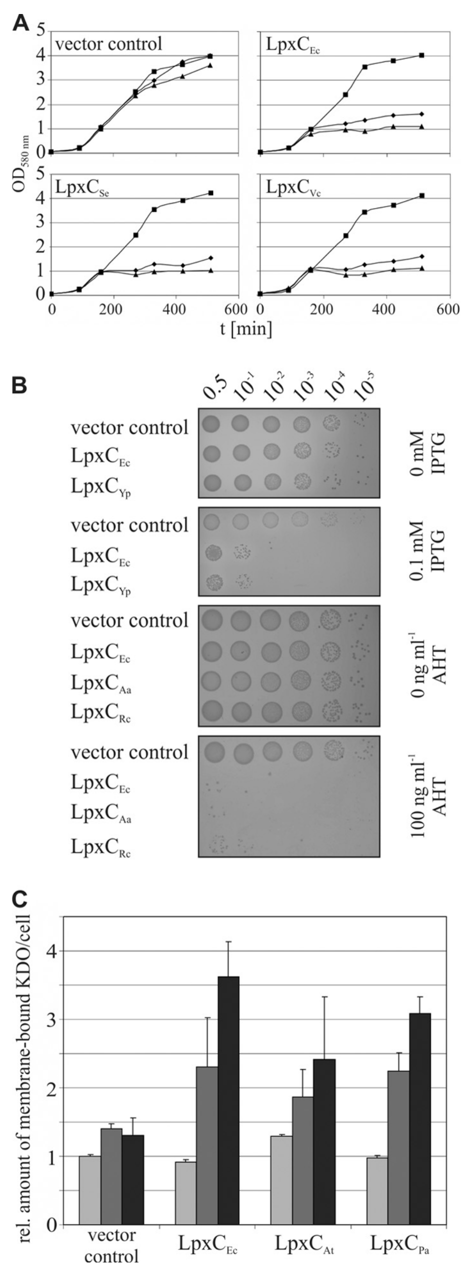

FIG. 2. Functional expression of LpxC variants from Gram-negative bacteria in *E. coli*. (A) Expression of LpxC from *S. enterica* ($LpxC_{Se}$; pBO1142) and *V. cholerae* ($LpxC_{Vc}$; pBO1146) impaired growth of *E. coli* W3110 cells as effectively as the expression of *E. coli* LpxC ($LpxC_{Ec}$; pBO110). Growth of cells containing the vector control (pBO197) is given as a reference. Cultures carrying the corresponding plasmids were grown in LB medium at 30°C supplemented with 0 (■), 0.1 (◆), or 0.5% arabinose (▲) for protein induction. (B) *E. coli* BL21 or W3110 cells showed increased sensitivity against bile salts in MacConkey agar when LpxC from *Y. pseudotuberculosis* ($LpxC_{Yp}$; pBO1173) or *A. aeolicus* ($LpxC_{Aa}$; pBO2381) and *R. capsulatus* ($LpxC_{Rc}$; pBO1718) was expressed. Serial dilutions of cultures (OD_{580} of 0.5) containing the corresponding plasmids were spotted on MacConkey agar plates with 0 and 0.1 mM IPTG or 0 and 100 ng ml^{-1} AHT. Cells harboring the plasmids pET19b or pBO1721 were used as vector controls, and pBO1702 or pBO2382 was used as a positive control. (C) Induction of active LpxC from *A. tumefaciens* ($LpxC_{At}$; pBO1144) and *P. aeruginosa* ($LpxC_{Pa}$; pBO1177) with 0 (light gray), 0.1 (gray), and 0.5% arabinose (black) resulted in accumulating KDO amounts per cell compared to the vector control (pBO197), as has been shown for $LpxC_{Ec}$ (pBO110) (16). *t*, time.

LpxC variant ($LpxC_{Ec}$ with an N-terminal deletion of 2 amino acids [$LpxC_{Ec}$ΔN2]) does not impair growth (see Fig. S1 in the supplemental material) (15, 16). Protein expression was validated by Western transfer and immunodetection (see Fig. S1). Expression of $LpxC_{Se}$ and $LpxC_{Vc}$ was toxic in *E. coli*, suggesting that both proteins were expressed as active enzymes. Alternatively, the toxic effect of LpxC overproduction can be monitored on MacConkey agar plates due to increased sensitivity against bile salts (15). The toxic effect of LpxC expression can also be monitored on LB agar plates, yet the growth defect is not as evident as on MacConkey plates, as shown in Fig. S2 to S5. Serial dilutions of *E. coli* cultures expressing LpxC from *Y. pseudotuberculosis* ($LpxC_{Yp}$), *A. aeolicus* ($LpxC_{Aa}$), or *R. capsulatus* ($LpxC_{Rc}$) were spotted on MacConkey agar plates (Fig. 2B). Cultures carrying the corresponding empty vector or a plasmid coding for $LpxC_{Ec}$ were used as a reference. Growth of all cultures was comparable on plates without inducer. While the vector control was unaffected by addition of 1 mM IPTG or 100 ng ml^{-1} AHT, growth of *E. coli* was compromised upon expression of each of the four heterologous LpxC proteins.

In the third assay, we measured the amounts of KDO since production of active LpxC is known to result in KDO accumulation (16). Expression of LpxC from *A. tumefaciens* ($LpxC_{At}$) and *P. aeruginosa* ($LpxC_{Pa}$) led to increased amounts of KDO in *E. coli* membranes (Fig. 2C). While addition of 0.1 and 0.5% of arabinose did not significantly alter KDO amounts in the presence of the empty vector, expression of $LpxC_{At}$ and $LpxC_{Pa}$ increased KDO amounts almost as efficiently as expression of $LpxC_{Ec}$. This was true for all other LpxC variants tested in this study (see Fig. S2 to S5 in the supplemental material). Finally, the toxic effect of LpxC was documented by the filamentous growth of *E. coli* strains expressing recombinant *lpxC* genes (see Fig. S2 to S5). Using four different approaches, all LpxC proteins used in this study were shown to be active when expressed in *E. coli*.

Some, but not all, LpxC proteins are subject to FtsH-mediated degradation in *E. coli*. To gain insights into the susceptibility of the chosen LpxC proteins toward FtsH, their half-lives were determined in *E. coli* wild-type (WT) and Δ*ftsH* strains as described in Materials and Methods. LpxC proteins from *S. enterica*, *Y. pseudotuberculosis*, and *V. cholerae* were degraded in an FtsH-dependent manner with half-lives comparable to the half-life of *E. coli* LpxC (8 to 12 min) (Fig. 3) (15). $LpxC_{Pa}$ appeared stable within the documented time period of 60 min (Fig. 3) but turned out to be degraded slowly, with a half-life of 78 ± 9.9 min when stability was monitored for longer time periods (data not shown).

$LpxC_{Aa}$ was stable both in the *E. coli* wild-type and Δ*ftsH* strains, indicating that this protein is not degraded by FtsH or any other protease present in *E. coli*. $LpxC_{Rc}$ and $LpxC_{At}$ were degraded with half-lives of about 68 min and 20 min, respectively. The turnover was independent of the FtsH protease because these LpxC proteins were not stabilized in the absence of FtsH (Fig. 3). To determine which protease is responsible for proteolysis of $LpxC_{At}$ and $LpxC_{Rc}$ in *E. coli*, half-lives were determined in various protease-deficient strains. Degradation of $LpxC_{At}$ and $LpxC_{Rc}$ was not affected by loss of either HslUV or ClpP (data not shown). However, the absence of

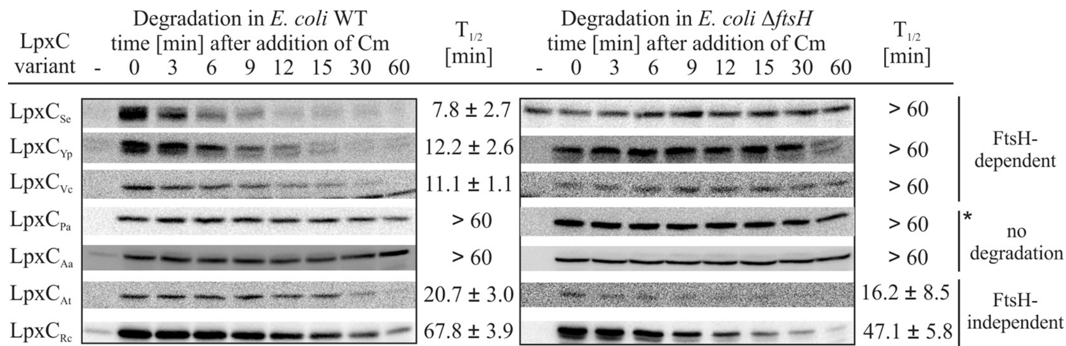

FIG. 3. Degradation experiments of LpxC proteins from Gram-negative bacteria in *E. coli*. Stability of plasmid-encoded LpxC from different bacteria was measured in *E. coli* W3110 or BL21 (WT) and the corresponding Δ*ftsH* strain depending on the expression system. LpxC was encoded on pBO1142 ($LpxC_{Se}$), pBO1173 ($LpxC_{Yp}$), pBO1146 ($LpxC_{Vc}$), pBO1177 ($LpxC_{Pa}$), pBO1718 ($LpxC_{Rc}$), pBO2381 ($LpxC_{Aa}$), and pBO1144 ($LpxC_{At}$). Half-lives ($T_{1/2}$) of LpxC variants were analyzed using *in vivo* degradation experiments and Western blot analysis. Chloramphenicol (Cm) was used to block translation. The sample taken before induction of LpxC expression is indicated by a minus sign. Standard deviations were calculated from at least three independent experiments. The asterisk indicates that $LpxC_{Pa}$ turned out to be a poor protease substrate with a half-life of 78 ± 9.9 min when degradation experiments were performed for 120 min.

Lon stabilized both $LpxC_{At}$ and $LpxC_{Rc}$, indicating that these proteins are substrates of the *E. coli* Lon protease (Fig. 4).

The C terminus of LpxC serves as a degradation signal for FtsH and Lon substrates. The C-terminal residues of $LpxC_{Ec}$ have been characterized as the pivotal signal needed for degradation in *E. coli* (15, 16). LpxC proteins from *S. enterica*, *Y. pseudotuberculosis*, and *V. cholerae* share significant sequence similarities in their C terminus and a comparable overall length with $LpxC_{Ec}$(Fig. 1 and Table 1). To analyze whether the C terminus of LpxC also mediates degradation in other organisms, several LpxC variants were constructed. Both solubility and activity of these proteins were verified (see Fig. S1 in the supplemental material). The stability of a C-terminally truncated LpxC from *S. enterica* ($LpxC_{Se}$ΔC5) was measured in *E. coli*. The C terminus was found to be essential for degradation, as $LpxC_{Se}$ΔC5 was completely stable (Fig. 5). $LpxC_{At}$ was used as example of a Lon substrate. Although its C terminus does not resemble that of $LpxC_{Ec}$, a C-terminal truncation of 13 amino acids ($LpxC_{At}$ΔC13), making it the same length as the *E. coli* enzyme (305 aa), stabilized the protein (Fig. 5).

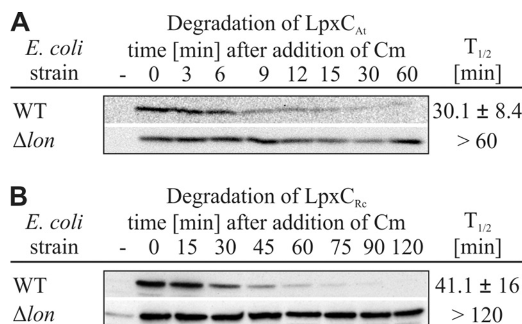

FIG. 4. LpxC proteins from *A. tumefaciens* and *R. capsulatus* are Lon substrates in *E. coli*. Stabilities of $LpxC_{At}$ and $LpxC_{Rc}$ were measured in the *E. coli* Δ*lon* strain and the corresponding parental strain (RH166; WT). The half-lives were analyzed using *in vivo* degradation experiments and Western blot analysis. Chloramphenicol (Cm) was used to block translation. The sample taken before induction of LpxC expression is indicated by a minus sign. Standard deviations were calculated from three independent experiments.

Proteolysis of $LpxC_{Se}$ and $LpxC_{Pa}$ in *E. coli* reflects the situation in the homologous background. To verify that the stability of LpxC from different Gram-negative bacteria in *E. coli* as a heterologous host agrees with the homologous systems, degradation experiments with $LpxC_{Se}$ and $LpxC_{Pa}$ were performed in *S. enterica* and *P. aeruginosa*, respectively. Activity and solubility of $LpxC_{Se}$ and $LpxC_{Se}$ΔC5 in the homologous system were determined (see Fig. S6 and S7 in the supplemental material). In full agreement with the results obtained in *E. coli*, $LpxC_{Se}$ was turned over with a half-life of ~10 min. Likewise, the C terminus of $LpxC_{Se}$ was also crucial for degradation in *S. enterica* as $LpxC_{Se}$ΔC5 was stabilized (Fig. 6).

Comparable to its stability in *E. coli*, $LpxC_{Pa}$ was degraded only slowly, with a half-life of 93 min in *P. aeruginosa* (Fig. 7A). $LpxC_{Pa}$ was confirmed to be an active enzyme in *E. coli* because overproduction was toxic for the cells and because $LpxC_{Pa}$ overproduction led to KDO accumulation in the membranes (Fig. 2; see also Fig. S3 in the supplemental material). Overproduction of $LpxC_{Pa}$ in the homologous background was not toxic as growth of *P. aeruginosa* was not impaired when increasing amounts of inducer were added (Fig. 7B and C). $LpxC_{Pa}$ overexpression in *P. aeruginosa* did not elevate KDO amounts and had no effect on cell morphology (see Fig S8). This suggests a different mode of LPS biosynthesis control in this organism.

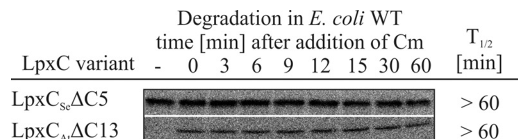

FIG. 5. The LpxC C terminus is conserved as a degradation signal. Truncation of 5 or 13 amino acids from the C terminus from $LpxC_{Se}$ (pBO1172) or $LpxC_{At}$ (pBO1187), respectively, led to stabilization of these proteins in *E. coli*. Half-lives of LpxC variants were analyzed using *in vivo* degradation experiments and Western blot analysis. Chloramphenicol (Cm) was used to block translation. The sample taken before induction of LpxC expression is indicated by a minus sign. Standard deviations were calculated from at least three independent experiments.

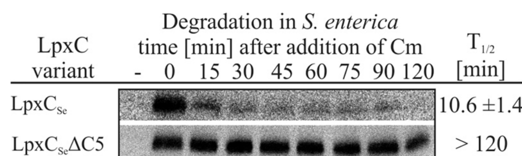

FIG. 6. $LpxC_{Se}$ is a protease substrate in *S. enterica*, and the C terminus is crucial for degradation. $LpxC_{Se}$ or $LpxC_{Se}\Delta C5$ encoded on pBO1142 or pBO1172, respectively, was expressed in *S. enterica* cells. Chloramphenicol (Cm) was used to block translation. Half-lives were analyzed using *in vivo* degradation experiments and Western blot analysis. The sample taken before induction of LpxC expression is indicated by a minus sign. Standard deviations were calculated from at least three independent experiments.

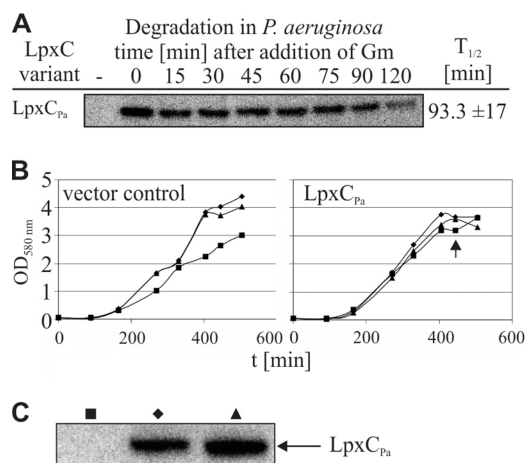

FIG. 7. $LpxC_{Pa}$ is a poor protease substrate in *P. aeruginosa*, and overexpression of this enzyme is not toxic in this organism. (A) Stability of $LpxC_{Pa}$ was measured in *P. aeruginosa* using gentamicin (Gm) to block translation. The sample taken before induction of LpxC expression is indicated by a minus sign. (B) Overexpression of $LpxC_{Pa}$ in *P. aeruginosa* is not toxic. Cultures carrying pBO1745 were grown in LB medium at 30°C supplemented with 0 (■), 0.5 (◆), or 1% arabinose (▲) for $LpxC_{Pa}$ protein induction. Growth of cells containing the vector control (pHERD20T) is given as a reference. Samples of *P. aeruginosa* cells harboring pBO1745 were taken after 445 min (indicated by the arrow), and $LpxC_{Pa}$ production was verified using Western blot analysis (C).

DISCUSSION

FtsH-dependent proteolysis of LpxC is conserved in enterobacteria. FtsH is the only essential AAA protease in *E. coli*. The indispensable activity of FtsH is the turnover of LpxC, the key enzyme of lipid A biosynthesis. LpxC belongs to the constitutive enzymes of the lipid A pathway, shows no sequence similarity to other deacetylases or amidases, and is encoded by a highly conserved single-copy gene (55). To fully exploit its potential as a target for the design of novel antibiotics, detailed understanding of the regulation of this protein in Gram-negative bacteria is required.

The high conservation of LpxC proteins from organisms used in this study is reflected by the activity of these enzymes in *E. coli*, as shown by four different *in vivo* phenotypic analyses (Fig. 2; see also Fig. S2 to S5 in the supplemental material). *In vivo* degradation experiments with *E. coli* as a host provided the first evidence that turnover of LpxC by FtsH is a common mechanism in enterobacteria (Fig. 3). The *E. coli* Δ*ftsH* strain is viable due to a suppressor mutation in the *fabZ* gene. This mutation improves the outbalanced ratio of LPS to phospholipids when LpxC degradation is abolished (43, 47). No such mutant is available in *S. enterica*, and specific inhibitors of FtsH *in vivo* are not established. Yet we confirmed that $LpxC_{Se}$ is degraded in *S. enterica* in an ATP-dependent manner as addition of arsenate stabilized the protein (see Fig. S9). The high conservation of both LpxC and FtsH (Table 1) and the comparable turnover rates of $LpxC_{Se}$ in *Salmonella* and in *E. coli* (Fig. 6) strongly suggest that FtsH-dependent proteolysis is a conserved strategy to ensure balanced biosynthesis of LPS in these species.

LpxC proteins from alphaproteobacteria are degraded by the Lon protease. It has been reported previously that during evolution, degradation of certain proteins has switched to a different protease. Polypeptides trapped in stalled ribosomes are labeled with the SsrA tag and are commonly degraded by the ClpXP machinery (21, 63). The genes for *clpXP* in *Mycoplasma* were lost during genome reduction, and both the SsrA tag and the Lon protease have coevolved to allow a switch to this substrate-protease pair (23).

Here, we provide evidence that turnover of LpxC in alphaproteobacteria like *A. tumefaciens* and *R. capsulatus* is coupled to proteolysis as in enterobacteria but has switched from FtsH to the Lon protease. Given that FtsH is not responsible for degradation of $LpxC_{At}$, it might not be an essential protease in this organism, as has been reported for another alphaproteobacterium, namely, *C. crescentus* (14). To the best of our knowledge, construction of an *ftsH* knockout strain in *A. tumefaciens* has not yet been attempted. On the other hand, an *A. tumefaciens lon* mutant is available (58) although Lon is expected to be essential due to its potential role in LpxC degradation in this organism. It is important to note, however, that the mutant displayed severe defects and heterogeneity in cell morphology. This might indicate the accumulation of suppressor mutations, which ensured viability of these cells, as has been demonstrated for the *E. coli* Δ*ftsH* strain (43). Interestingly, absence of Lon impairs virulence of *A. tumefaciens*. This effect was ascribed to the slower growth of the protease mutant (58). Another possibility raised by our study is an imbalance in LPS production due to the absence of the Lon protease. It is known that LPS is important for plant-microbe interactions (45).

The extended C terminus of LpxC is a degradation signal for various proteases. The exceptionally short *A. aeolicus* LpxC protein does not carry a C-terminal extension and was resistant to proteolysis in *E. coli*, lending weight to the importance of the C terminus for turnover of LpxC enzymes. LpxC from *A. aeolicus* is the evolutionarily oldest LpxC variant analyzed in this study. The Gram-negative membranes of this hyperthermophilic organism contain variants in lipid A that might confer thermal tolerance (51, 55). Although unknown, it is likely that unbalanced amounts of lipid A are toxic in *Aquifex*. Novel regulatory mechanisms might be responsible for control of LPS biosynthesis in this organism. Since LpxC, FtsH, and Lon share only limited identity with the corresponding *E. coli* proteins (Table 1), it cannot be excluded that in *Aquifex* these proteins have evolved to establish proteolytic control of LPS biosynthe-

sis by a mechanism independent of a C-terminal degradation signal.

Degradation of *E. coli* LpxC depends on a sequence- and length-specific, nonpolar C-terminal degradation signal (15, 16). Consistent with this, the five C-terminal amino acids of $LpxC_{Ec}$ have recently been shown by nuclear magnetic resonance (NMR) analysis to be highly dynamic and disordered (2). Enterobacterial LpxC enzymes have C termini of similar lengths and sequences (Fig. 1). Individual point mutations in the C terminus of *E. coli* LpxC still permitted FtsH-dependent degradation (15, 16). In agreement with these reports are variations in the C termini of enterobacterial LpxC proteins which did not interfere with proteolysis. Compared to the *E. coli* LpxC sequence, $LpxC_{Se}$ and $LpxC_{Vc}$ sequences carry single amino acid substitutions (T302 and M302, respectively, instead of A302). Nonpolar residues are exchanged in $LpxC_{Vc}$(V295 instead of L295), and $LpxC_{Yp}$ is extended by a Y residue.

The C termini of alphaproteobacterial LpxC proteins are even longer than the enterobacterial ones and share no significant sequence homology with them. Interestingly, shortening of the C terminus of $LpxC_{At}$($LpxC_{At}\Delta C13$) and $LpxC_{Se}$ ($LpxC_{Se}\Delta C5$) led to drastic stabilization of these proteins (Fig. 5) without affecting the activity of the enzymes (see Fig. S2 in the supplemental material). This suggests that C-terminally elongated LpxC proteins are subject to proteolytic control independent of the executing protease. Since Lon is known to recognize aromatic residues in both native and nonnative substrates (20, 24), it is tempting to speculate that the C-terminal residues W316 and F313 in $LpxC_{At}$ are crucial for Lon degradation.

Control of LPS biosynthesis in *P. aeruginosa* is independent of proteolysis. $LpxC_{Pa}$ was a poor protease substrate both in *E. coli* and *P. aeruginosa* (Fig. 3 and 5). If FtsH is not involved in LpxC control, the protease should not be essential in *P. aeruginosa*. In support of this assumption, a *P. aeruginosa* transposon insertion mutant for *ftsH* (strain 18778) is viable (31). Transposon mutants for all additional AAA proteases known so far (HslV, Lon, ClpP, ClpP2, and ClpP3) also have been reported (31, 40, 53), strongly suggesting that LPS biosynthesis is not controlled by proteolysis.

The presence of LPS is critically important for *P. aeruginosa* because LpxC activity is essential for growth (42). Notably, $LpxC_{Pa}$ accumulation was toxic for *E. coli* but not for *P. aeruginosa* (Fig. 2 and 7). This indicates that lipid A biosynthesis by LpxC in *P. aeruginosa* is kept in balance by a yet unknown mechanism not available in *E. coli.* The *E. coli* deacetylase does not seem to be subject to this control mechanism since overexpression of $LpxC_{Ec}$ in *P. aeruginosa* resulted in sick cells with a strong tendency to aggregate (see Fig. S8C in the supplemental material).

The divergent control strategies acting on LpxC from *E. coli* and *P. aeruginosa* suggest significant structural differences between both proteins despite a sequence identity of 57% and a similarity of 82%. In fact, several differences have already been reported. Many β-strands in $LpxC_{Ec}$ are predicted to be longer than those in $LpxC_{Pa}$ (2). Clearly indicative of conformational deviations are the different responses to synthetic inhibitors. Except for CHIR-090 (41), most LpxC inhibitors active against $LpxC_{Ec}$ did not effectively inhibit $LpxC_{Pa}$ (10, 36, 42, 48). Most likely, the structure, catalytic activity, and regulation of LpxC proteins are dictated not only by their sequence but also by intracellular molecules, such as proteins (42) or the metals Zn and Fe (17, 29). Revealing the complexity of LpxC regulation will be a challenging task of future research. Comparative analyses of LpxC proteins from *P. aeruginosa* and *E. coli* will largely depend on insights from the not yet determined three-dimensional structure of the *E. coli* LpxC enzyme.

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