Purification and Characterization of the Lipid A Disaccharide Synthase (LpxB) from *Escherichia coli*: a Peripheral Membrane Protein

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Supporting Information

Supporting Table 1. Primers used to construct LpxB over-producing strains.

Name	Gene	5' restriction site	3' restriction site	Mutation conferred	Primer sequence (5' to 3' orientation)
EC_*ndeI*	*EC lpxB*	*ndeI*			CGCGCGCATATGACTGAACAGCGTCCATTAAC
EC_*hindIII*	*EC lpxB*	*hindIII*			CGCGCGAAGCTTACTGAACAGCGTCCATTAAC
EC_*xhoI_stop*	*EC lpxB*		*xhoI*		CGCGCTCGAGTCATTGTGCTAACTCCAG
EC_*xhoI*	*EC lpxB*		*xhoI*	Removal of EC *lpxB* stop codon	CGCGCTCGAGTTGTGCTAACTCCAG
EC_*kpnI*	*EC lpxB*		*kpnI*	Removal of EC *lpxB* stop codon	CGCGCGGTTACCTTGTGCTAACTCCAGA
HI_*ndeI*	*HI lpxB*	*ndeI*			CGCGCGCCATATGATGAACAAAACAAATCC
HI_*xhoI*	*HI lpxB*		*xhoI*		CGCGCGCTCGAGTCAAACATTTTCTTTTCC
EC_TEV_FW	*EC lpxB*			Factor Xa to TEV protease cleavage site	CATCATCATCATCATCACAGCGAAAACCTGTACTTCCAGAGCCATATGACTGAACAGCGTCCATTAAC
EC_TEV_RV	*EC lpxB*			Factor Xa to TEV protease cleavage site	GTTAATGGACGCTGTTCAGTCATATGGCTCTGGAAGTACAGGTTTTCGCTGTGATGATGATGATGATG
HI_TEV_FW	*HI lpxB*			Factor Xa to TEV protease cleavage site	CATCATCATCATCATCACAGCGAAAACCTGTACTTCCAGAGCCATATGATGAACAAAACAAATCC
HI_TEV_RV	*HI lpxB*			Factor Xa to TEV protease cleavage site	GGATTTGTTTTGTTCATCATATGGCTCTGGAAGTACAGGTTTTCGCTGTGATGATGATGATGATG
E15A_FW	*EC lpxB*			E15A	CGATTGCCCTGGTCGCCGGAGCGACCTCCGGCGATATCCTG
E15A_RV	*EC lpxB*			E15A	CAGGATATCGCCGGAGGTCGCTCCGGCGACCAGGGCAATCG
S17A_FW	*EC lpxB*			S17A	CTGGTCGCCGGAGAAACCGCGGGCGATATCCTGGGGGCC
S17A_RV	*EC lpxB*			S17A	GGCCCCCAGGATATCGCCCGCGGTTTCTCCGGCGACCAG
D98A_FW	*EC lpxB*			D98A	CCAGATGTTTTTGTTGGTATTGCGGCGCCTGACTTCAATATTAC
D98A_RV	*EC lpxB*			D98A	GTAATATTGAAGTCAGGCGCCGCAATACCAACAAAAACATCTGG
F102A_FW	*EC lpxB*			F102A	GTTGGTATTGATGCGCCTGACGCGAATATTACTCTTGAAGGTAAC
F102A_RV	*EC lpxB*			F102A	GTTACCTTCAAGAGTAATATTCGCGTCAGGCGCATCAATACCAAC
W126A_FW	*EC lpxB*			W126A	CATTACGTCAGTCCGTCAGTCGCGGCGTGGCGACAGAAACGTG
W126A_RV	*EC lpxB*			W126A	CACGTTTCTGTCGCCACGCCGCGACTGACGGACTGACGTAATG
R210A_FW	*EC lpxB*			R210A	CTGGCGTTGCTACCGGGGAGCGCGGGTGCAGAAGTTGAAATGC
R201A_RV	*EC lpxB*			R210A	GCATTTCAACTTCTGCACCCGCGCTCCCCGGTAGCAACGCCAG
L314A_FW	*EC lpxB*			L314A	GTGAAAACTGATTATGTCTCGGCGCCAAATCTGCTGGCGGGCAG
L314A_RV	*EC lpxB*			L314A	CTGCCCGCCAGCAGATTTGGCGCCGAGACATAATCAGTTTTCAC
N316A_FW	*EC lpxB*			N316A	CTGATTATGTCTCGCTGCCAGCGCTGCTGGCGGGCAGAGAG
N316A_RV	*EC lpxB*			N316A	CTCTCTGCCCGCCAGCAGCGCTGGCAGCGAGACATAATCAG

Supporting Table 2. Comparison of the specific activities and NiNTA resin binding properties of various *E. coli* LpxB constructs

Plasmid in *E. coli* C41(DE3)	Affinity tag	Specific activity* Crude lysate (μmol/min/mg)	Specific activity* Purified protein‡ (μmol/min/mg)	Relative binding to NiNTA resin
pECLpxB23	*C*-terminal uncleavable His_6	37.2	228	+
pECLpxB30	*C*-terminal enterokinase-cleavable His_8	1.8	Not determined	Not bound
pECLpxB19	*N*-terminal enterokinase-cleavable His_{10}	19.2	183	+ +
pECLpxB16	*N*-terminal factor Xa-cleavable His_{10}	26.6	205	+ +
pECLpxB-TEV	*N*-terminal TEV protease-cleavable His_{10}	42	180	+ +

* Specific activities were determined using standard, optimized assay conditions, as described in *Methods*.

‡These proteins were purified to at least 90% homogeneity as determined by SDS-PAGE analysis.

Supporting Table 3. Purification of *H. influenzae* LpxB from C41(DE3)/pHILpxB-TEV.

Step	Total Protein (mg)	Total volume (ml)	Total Units (mmol/min)	Specific activity (μmol/min/mg)	Yield (%)	Fold-purification
Membrane-free lysate	1200	225	7.5	6.1	100	1.0
Ni-NTA column fractions	34	90	4.4	129	58	20
TEV-digested Ni-NTA flow-through	20	100	2.5	125	32	19
Size exclusion chromatography	15	20	1.6	107	21	16

Supporting Table 4. Relative specific activities of purified *E. coli* LpxB point mutants.

Substitution	Conservation of mutated residue among LpxB orthologs	Wild-type specific activity %
E15A	highly conserved	0.99
S17A	highly conserved	2.70
D98A	absolutely conserved	< 0.01 (none detected)
F102A	highly conserved	3.20
W126A	absolutely conserved	3.72
R201A	absolutely conserved	< 0.01 (none detected)
L314A	highly conserved	9.47
N316A	absolutely conserved	0.10

Supporting Table 5. ESI/MS quantification of the three most abundant phosphatidylethanolamine species associated with *E. coli* LpxB

PE species	Exact mass	Peak area (counts)	Estimated PE (pmols)
PE: 31:1 (internal standard)	675.48	4.83×10^4	3.00*
PE: 34:1	717.53	8.82×10^4	6.21
PE 32:1	689.50	4.48×10^4	3.15
PE 33:0 cyclopropane form	703.52	1.90×10^4	1.34

*The amount of internal standard contained in the portion of the extracted phospholipids loaded onto the Zorbax SB-C8 reverse-phase column.

Supporting Table 6. ^{32}P-phospholipid counts in whole cell pellets and purified *E. coli* LpxB fractions.

Sample	^{32}P (cpm ± range* x 10^{-6})
C41(DE3)/pET19b whole cell pellet	12.2 ± 1.1
C41(DE3)/pET19b Ni-NTA fractions	0.003 ± 0.001
C41(DE3)/pECLpxB19 whole cell pellet	23.0 ± 2.2
C41(DE3)/pECLpxB19 Ni-NTA fractions	0.29 ± 0.06

*Average deviations from the mean were determined from two experiments.

Supporting Table 7. Composition of ^{32}P-labeled phospholipids purifying with *E. coli* LpxB.

Source of phospholipid	PE*	PG*	CL*
C41(DE3)/pET19b whole cell pellet	74 ± 1	22 ± 2	3 ± 2
C41(DE3)/pECLpxB19 whole cell pellet	65 ± 1	17 ± 3	15 ± 2
LpxB purified from C41(DE3)/pECLpxB19	72 ± 2	21 ± 1	7 ± 3

* % of total ± average deviations from the mean were determined from two experiments.

Supporting Methods

Expression testing of E. coli and H. influenzae LpxB. The various LpxB constructs (Supporting Table 2) were transformed by heat-shock into the *E. coli* expression strain C41(DE3). This BLR(DE3)-derived strain has been optimized for the expression of membrane proteins (*1*). Single colonies of the resulting transformants were used to inoculate 5 ml LB medium cultures supplemented with either 100 μg/ml ampicillin or 25 μg/ml kanamycin, as appropriate. Outgrowth was performed overnight (14-16 hours) at 37°C in a shaker rotating at 220 rpm. Expression testing was performed in 250 ml flasks containing media identical to that used in the over-night cultures, and was commenced by inoculation from the overnight growths to an initial A_{600} of 0.02. Test growths continued at 220 rpm until the cells reached A_{600} of ~0.5, whereupon the DE3 strains were induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, cells were harvested at various times by centrifugation at 3,000 *x g*, washed with PBS, and stored at -80 °C. Cell lysis was accomplished by re-suspension of the thawed pellets in PBS with three ml PBS per ml cell pellet, followed by two passages through an ice-cold French pressure cell at 18,000 *psi*. Cell debris was removed by centrifugation at 10,000 *x g* for 20 minutes in a Beckman J2-HC centrifuge (Beckman Coulter, Inc., Fullerton, CA) at 4°C. Testing of the various strain and plasmid combinations for optimal LpxB expression involved variation of growth temperature and induction time. Over-expression in comparison to each construct's vector control was determined by SDS-PAGE analysis of crude lysates, treated with Laemmli buffer at 100°C for 10 minutes. Specific activities of the various constructs' crude lysates were determined using the TLC-based *in vitro* radiographic assay described in the main text.

Optimized expression of E. coli and H. influenzae LpxB. Optimized expression of both the *E. coli* and *H. influenzae* constructs was accomplished by growth from an initial A_{600} of 0.02 in ampicillin-supplemented (100 μM) LB media at 37°C in a rotary shaker, followed by induction with 1 mM IPTG when the A_{600} of the cultures reached ~0.5. Induction was continued for 4 hours at 37°C, whereupon the cells were harvested, washed, and frozen as described above.

Small-scale trial purifications of various E. coli and H. influenzae LpxB constructs. *E. coli* LpxB in pET23b, which contained a carboxy-terminal His_6 tag (Supporting Table 2), was purified by metal affinity chromatography using Ni-NTA Fastflow™ resin (Qiagen, Valencia, CA). PBS-washed C41(DE3) *E. coli* pellets expressing this construct were rapidly thawed from -80°C in a 25°C water bath. The pellets were then suspended in a 4°C pre-chilled buffer containing 50 mM sodium phosphate buffer, pH 8.0, 250 mM NaCl, and 20% v/v glycerol, typically at 1:3 wet pellet:buffer (v/v). Suspension was accomplished by gentle vortexing. The re-suspended cell pellets were lysed using a French pressure cell, and the cell debris removed as described above. Membranes were separated by ultracentrifugation for 45 min, 4°C, at 100,000 *x g* using a Beckman L8-70M ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). In cell lysates made in PBS, the activity of this construct (as measured by the *in vitro* assay), partitioned primarily to the membranes. However, in lysates made in buffers containing 300 mM (or more) salt and 20% v/v glycerol, ~70% of the activity was

retained in the supernatant. Therefore, for the purification of this and other poly-histidine-tagged LpxB constructs, the membranes were discarded.

The resultant membrane-free supernatant was loaded onto a pre-chilled (4°C) NiNTA Fast-Flow™ (column, diameter 3.6 cm), at a ratio of 1 ml of NiNTA resin per 100 mg protein present in the membrane-free lysate. Prior to use, the NiNTA column was washed with 5 column volumes of distilled water, and equilibrated in 5 column volumes of buffer identical to that used for the cell lysis. Sample loading was allowed to proceed at the natural flow rate of the column. The resin was washed with 5 column volumes of lysis/load buffer at a flow rate of ~1 ml/min. Next, the columns was washed with lysis buffer containing 10 mM imidazole applied at the same flow rate. The final wash consisted of 5 column volumes of lysis buffer containing 25 mM imidazole. Elution was accomplished with 8 column volumes of lysis buffer supplemented with 300 mM imidazole. The eluate was collected in 0.5 column volume fractions. As judged by both SDS-PAGE and *in vitro* activity assays, most of the LpxB eluted in fractions 3-9. The resulting partially-purified protein was ~90% pure as judged by SDS-PAGE. Similar purification trials performed upon various constructs revealed that those possessing an *N*-terminal His_{10} tag were able to bind to the NiNTA resin with significantly greater affinity than that with the *C*-terminal His_6 tag.

Small-scale purification of E. coli LpxB for analysis of bound lipids and characterization of point mutants. Cells from 50 to 750 ml culture were lysed in a French pressure cell at 18,000 psi in 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 20% v/v glycerol (typically at 1:3 wet pellet:buffer (v/v). Membranes were

removed by ultracentrifugation for 1 h at 100,000 x *g*. Membrane-free supernatant was loaded onto a NiNTA Fast-Flow™ column (diameter 1.8 cm), at a ratio of 1 ml of NiNTA resin per 100 mg protein present in the membrane-free lysate, which had been equilibrated with lysis buffer. Following sample application by gravity, the column was washed with 50 column volumes of lysis buffer, followed by 20 column volumes of lysis buffer supplemented with 25 mM imidazole, and then with 20 column volumes of lysis buffer containing 50 mM imidazole. Elution was accomplished with a 10-column volume step consisting of lysis buffer supplemented with 300 mM imidazole.

The membrane-free supernatant was loaded NiNTA Fast-Flow™ (column, diameter 1.8 cm), with a ratio of 1 ml of NiNTA resin per 100 mg of membrane-free lysate total protein. Prior to use, the NiNTA column was washed with 5 column volumes of distilled water, and equilibrated in 5 column volumes of buffer identical to that used in the cell lysis. Sample loading was allowed to proceed at the natural flow rate of the column. The resin was washed with 5 column volumes of lysis/load buffer at a flow rate of ~1 ml/min. An identical wash, this time with lysis buffer containing 25 mM imidazole, was applied at the same flow rate. A final wash, consisting of 5 column volumes of lysis buffer containing 50 mM imidazole, was applied. Elution was accomplished by the application of 10 column volumes of lysis buffer supplemented with 250 mM imidazole, and the eluate collected in a single fraction.

References

1. Miroux, B., and Walker, J. E. (1996) Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels., *J. Mol. Biol. 260*, 289-298.