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A continuous fluorescent enzyme assay for early steps of lipid A biosynthesis

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ABSTRACT

UDP-*N*-acetylglucosamine acyltransferase (LpxA) and UDP-3-*O*-(*R*-3-hydroxyacyl)-glucosamine acyltransferase (LpxD) catalyze the first and third steps of lipid A biosynthesis, respectively. Both enzymes have been found to be essential for survival among gram-negative bacteria that synthesize lipopolysaccharide and are viable targets for antimicrobial development. Catalytically, both acyltransferases catalyze an acyl-acyl carrier protein (ACP)-dependent transfer of a fatty acyl moiety to a UDP-glucosamine core ring. Here, we exploited the single free thiol unveiled on holo-ACP after transfer of the fatty acyl group to the glucosamine ring using the thiol-specific labeling reagent, ThioGlo. The assay was continuously monitored as a change in fluorescence at λ_{ex} = 379 nm and λ_{em} = 513 nm using a microtiter plate reader. This assay marks the first continuous and nonradioactive assay for either acyltransferase.

The outer cell wall of gram-negative bacteria is an asymmetrical bilayer composed of phospholipids in the inner monolayer and lipopolysaccharide (LPS)[1] in the outer monolayer [1–3]. LPS contains the glycolipid lipid A, which anchors it to the membrane through fatty acyl chains, a core polysaccharide region, and an *O*-antigen repeat. Lipid A is an essential moiety necessary for survival of the bacterium [4] and further plays a crucial role in natural antibacterial resistance and bacterial sepsis [5]. Thus, lipid A biosynthesis should provide optimal targets for antimicrobial chemotherapeutic discovery [6–8].

Lipid A biosynthesis entails nine constitutive enzymatic processes using UDP-*N*-acetylglucosamine (UDP-GlcNAc) as the precursor [9]. UDP-GlcNAc is acylated at the 3-hydroxyl through a thermodynamically unfavorable reaction catalyzed by the type II acyl carrier protein (ACP)-dependent UDP-*N*-acetylglucosamine acyltransferase (LpxA) [10]. UDP-3-*O*-(*R*-3-hydroxyacyl)-GlcNAc is subsequently deacetylated by UDP-3-*O*-(*R*-3-hydroxyacyl)-GlcNAc deacetylase (LpxC), providing the first committed step of lipid A biosynthesis [11,12]. The third step in the pathway involves acylation of the free amine of the glucosamine ring by UDP-*O*-3-(*R*-3-hydroxyacyl)-glucosamine acyltransferase (LpxD), which is also an ACP-dependent acyltransferase [13]. Six downstream enzymes catalyze the formation of the mature hexaacylated Kdo_2-lipid A moiety of LPS [2].

LpxA and LpxD are structurally homologous, as demonstrated by their unique left-handed β-helix (LβH) fold, which stems from an extensive hexapeptide repeat motif in their respective primary amino acid sequences [14–16]. In addition, both are functionally similar, as displayed by a common mechanism whereby conserved histidine residues mediate the deprotonation of the 3-hydroxyl (LpxA) or the 2-amine (LpxD) of the core glucosamine ring, allowing a subsequent nucleophilic attack on the thioester of acyl-ACP [17,18]. Holo-ACP is generated on acyl group transfer that contains a single free thiol on its phosphopantetheine prosthetic arm [19].

Although the LPS biosynthetic pathway has been established as a prime target for the development of novel antimicrobials, very few of the essential enzymes in the pathway have been subjected to high-throughput screening (HTS) efforts in an attempt to find novel inhibitors. Established assays for enzymes in this pathway are radioactivity based and, as such, are not welcomed in many HTS facilities. Here, we report the first nonradioactive assay for LpxA and LpxD. The assay uses a thiol-specific chemical reporter, ThioGlo, to continuously label and monitor holo-ACP generation over the course of LpxA and LpxD catalysis (Fig. 1).

Materials and methods

Materials

R-3-Hydroxymyristic acid was purchased from Wako Chemicals. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP),

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[1] *Abbreviations used:* LPS, lipopolysaccharide; UDP-GlcNAc, UDP-*N*-acetylglucosamine; ACP, acyl carrier protein; LpxA, UDP-*N*-acetylglucosamine acyltransferase; LpxC, UDP-3-*O*-(*R*-3-hydroxyacyl)-GlcNAc deacetylase; LpxD, UDP-*O*-3-(*R*-3-hydroxyacyl)-glucosamine acyltransferase; LβH, left-handed β-helix; HTS, high-throughput screening; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; IPTG, isopropyl β-D-1-thiogalactopyranoside; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UV, ultraviolet; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; Ni-NTA, nickel-nitrilotriacetic acid; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; HPLC, high-performance liquid chromatography; RFU, relative fluorescence units.

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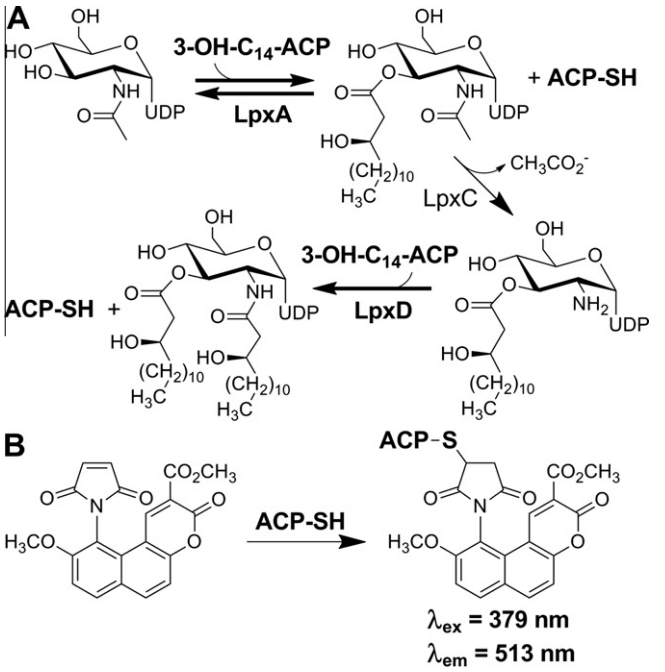

Fig.1. (A) Early enzymatic steps of LPS biosynthesis in *E. coli.* (B) Generation of ThioGlo–ACP conjugate from holo-ACP produced in the acyltransferase-catalyzed reactions.

isopropanol, magnesium chloride, and buffer reagents were purchased from Thermo Fisher Scientific. L-Arabinose, ATP, and UDP-*N*-acetylglucosamine were purchased from Sigma–Aldrich. Benzonase, ThioGlo 1, and *Escherichia coli* Rosetta(DE3)/pLysS cells were purchased from EMD Chemicals (Novagen). *E. coli* XL-1 Blue cells were purchased from Stratagene. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and *E. coli* BL21-AI cells were purchased from Invitrogen. Bio-Gel P2 was purchased from Bio-Rad. All DNA modifying and restriction enzymes were purchased from New England Biolabs.

Cloning of E. coli lpxA, lpxC, lpxD, acpP, and acpS and V. harveyi aasS

Polymerase chain reaction (PCR) protocols were carried out under standard conditions using Pfu DNA polymerase and DNA obtained from the *E. coli* K-12 strain MG1655 or the *Vibrio harveyi* ATCC 14126 strain. To perform the amplifications of the individual genes, the following primers were used: *E. coli lpxA* (forward 5′-G CGCCATATGATTGATAAATCCGCCTTTGTGCATCCAACCGC, reverse 5′-CGCGCTCGAGTTAACGAATCAGACCGCGCGTTGAGCG); *lpxC* (forward 5′-GCGCCATATGATGATCAAACAAAGGACACT, reverse 5′-GCGCCTCGAGTGCCAGTACAGCTGAAGGCG); *lpxD* (forward 5′-CA TCACC ATCACCATCACGCTCAATTCGACTGGCTGATTTAGCG, reverse 5′-CGC GCTCGAGTTAGTCTTGTTGATTAACCTTGCGCTC); *acpP* (forward 5′-GCGCCATATGAGCACTATCGAAGAACGCGTTAAGAAAATTATC, reverse 5′-GCGCCTCGAGTTAACTTTCAATAATTACCGTGGCA C); *acpS* (forward 5′-CGCGTGGCATATGGCAATATTAGGTTTAG, reverse 5′-GCGCCTCGAGACTTTCAATAATTACCGTGGCACAAGC); *V. harveyi aasS* (forward 5′-GCGCCATATGAACCAGTATGTAAAT, reverse 5′-GCGCCTCGAGCAGATGAAGTTTACGCAG).

The PCR products for both *lpxA* and *acpP* were cloned into pET24a using *Nde*I and *Xho*I restriction sites (underlined in the previous paragraph). The *Xho*I-restricted PCR product for *lpxD* was cloned into pET23d, which had been *Nco*I restricted, T4 DNA polymerase filled in, and then restricted with *Xho*I. Each of the PCR products for *lpxC*, *acpS*, and *aasS* was cloned into pET23a using *Nde*I and *Xho*I restriction sites. All plasmids were transformed into *E. coli* XL-1 Blue cells for amplification, and plasmids isolated from these cell lines were sequenced at the University of Michigan Sequencing Core Facility. From these confirmed plasmids, the following *E. coli* expression strains were constructed: BL21-AI/pET24a::*lpxA*, Rosetta (DE3)/pLysS/pET23a::*lpxC-his*$_6$, Rosetta (DE3)/pLysS/pET23d::-*his*$_6$-*lpxD*, BL21-AI/pET24a::*acpP*/pET23a::*acpS-his*$_6$, and BL21-AI/pET23a::*aasS-his*$_6$. Genes containing a histidine-6 tag coding region are indicated by *his*$_6$ in the above construct names. The *his*$_6$ in front of the gene name denotes a 5′ histidine coding region, whereas the *his*$_6$ after the gene name denotes a 3′ histidine coding region.

Cell cultures

Strains of interest were used to inoculate 500 ml of Luria–Bertani (LB) medium (Lennox) or Terrific Broth (TB) medium containing the appropriate antibiotic(s) and were incubated while shaking (250 rpm) at 37 °C until an OD_{600} of 0.6 to 1.0 was reached. The cultures were then induced with either 1 mM IPTG (Rosetta DE3/pLysS strains) or 0.2% L-arabinose/1 mM IPTG (BL21-AI strains). Unless otherwise noted, cells were induced at 37 °C and allowed to incubate at 37 °C for 4 h postinduction. Cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C, suspended in 10 ml of buffer, and stored at −80 °C. Cell suspensions were thawed and disrupted by French press at 20,000 psi. Cellular debris was removed by centrifugation at 20,000*g* for 30 min at 4 °C, and the resultant crude cytosol was used for protein purification.

Purification of LpxA

For LpxA purification, 10 ml of crude cytosol in 20 mM potassium phosphate (KPhos) buffer and 20% glycerol (pH 7.0) was applied to a 10-ml Reactive Green 19 column that had been preequilibrated in the same buffer. The column was washed successively with 50 ml of loading buffer containing 0, 0.5, and 1 M NaCl. LpxA eluted with the 1-M NaCl fractions and was dialyzed overnight at 4 °C against 4 L of 20 mM Tris–HCl and 10% glycerol (pH 8.0). The enzyme was then loaded onto an 8-ml Source 15Q column, washed with 24 ml of loading buffer, and eluted with a gradient from 0 to 500 mM NaCl. LpxA was desalted on a Bio-Gel P2 column equilibrated in 20 mM Hepes (pH 8.0). The purified LpxA was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and its concentration was determined by ultraviolet (UV) absorbance at 280 nm (ε = 9190 M^{-1} cm^{-1}). The molecular weight of LpxA was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry (MS) at the University of Michigan Protein Structure Facility.

Purification of LpxC-His$_6$ *and His*$_6$*-LpxD*

For His_6-LpxD purification, Benzonase was added after cell lysis and the lysate was incubated for 30 min on ice prior to centrifugation at 20,000*g*. Then, 10 ml of crude cytosol in 20 mM Hepes and 50 mM imidazole (pH 8.0) was loaded onto 3 ml of nickel-nitrilotriacetic acid (Ni–NTA) resin (Qiagen) equilibrated in the same buffer. The resin was washed with 10 column volumes of loading buffer containing 500 mM NaCl and then eluted with 20 mM Hepes and 250 mM imidazole (pH 8.0). Purified His_6-LpxD and LpxC-His_6 were desalted on a Bio-Gel P2 column and analyzed by SDS–PAGE. Concentrations were determined by UV absorbance at 280 nm (ε = 22,920 M^{-1} cm^{-1} for LpxC, ε = 27,305 M^{-1} cm^{-1} for LpxD).

Purification of holo-ACP

A slightly modified protocol from Broadwater and Fox was used to prepare holo-ACP [20]. Holo-ACP was produced in cells