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# Overexpression of *Pseudomonas aeruginosa* LpxC with its inhibitors in an *acrB*-deficient *Escherichia coli* strain

Ning Gao [a,*,1], Sarah M. McLeod [b,1], Laurel Hajec [b], Nelson B. Olivier [a], Sushmita D. Lahiri [b], D. Bryan Prince [a], Jason Thresher [b], Philip L. Ross [a], James D. Whiteaker [b], Peter Doig [a], Amanda Haixi Li [c], Pamela J. Hill [c], Mark Cornebise [c], Folkert Reck [c,2], Michael R. Hale [c]

[a] Discovery Sciences, Infection Innovative Medicine Unit, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, USA
[b] Department of Bioscience, Infection Innovative Medicine Unit, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, USA
[c] Department of Chemistry, Infection Innovative Medicine Unit, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, USA

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ABSTRACT

In Gram-negative bacteria, the cell wall is surrounded by an outer membrane, the outer leaflet of which is comprised of charged lipopolysaccharide (LPS) molecules. Lipid A, a component of LPS, anchors this molecule to the outer membrane. UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) is a zinc-dependent metalloamidase that catalyzes the first committed step of biosynthesis of Lipid A, making it a promising target for antibiotic therapy. Formation of soluble aggregates of *Pseudomonas aeruginosa* LpxC protein when overexpressed in *Escherichia coli* has limited the availability of high quality protein for X-ray crystallography. Expression of LpxC in the presence of an inhibitor dramatically increased protein solubility, shortened crystallization time and led to a high-resolution crystal structure of LpxC bound to the inhibitor. However, this approach required large amounts of compound, restricting its use. To reduce the amount of compound needed, an overexpression strain of *E. coli* was created lacking *acrB*, a critical component of the major efflux pump. By overexpressing LpxC in the efflux deficient strain in the presence of LpxC inhibitors, several structures of *P. aeruginosa* LpxC in complex with different compounds were solved to accelerate structure-based drug design.



## Introduction

The evolution of multidrug resistant bacteria, particularly among Gram-negative pathogens, has created an urgent need for new antibiotics [1,2]. A distinguishing feature of Gram-negative pathogens is the outer membrane, an asymmetric bilayer in which the inner leaflet is composed of phospholipids and the outer leaflet is comprised mainly of lipopolysaccharide. LPS[3] provides a formidable permeability barrier for hydrophobic inhibitors in Gram-negative bacteria [3–5]. Moreover, Lipid A, a key component of LPS, is responsible for the endotoxic effect of LPS [6]. The LpxC gene (*envA*) of *Pseudomonas aeruginosa* encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC), a $Zn^{2+}$-dependent enzyme that performs the second reaction and first committed step in biosynthesis of Lipid A [7]. LpxC is required for cell viability and is highly conserved among Gram-negative bacteria [6]. Additionally, LpxC has limited sequence homology to known human deacetylases or amidases, making it an attractive target for the development of novel antibiotics [8–10]. X-ray crystal structures of *P. aeruginosa* LpxC with inhibitors bound in the active site are extremely valuable to drive structure-based drug discovery. Crystal structures of LpxC from *Escherichia coli*, *Aquifex aeolicus* and *P. aeruginosa* with small molecule inhibitors have been reported previously [11–14]. Although crystals have been shown to form under various conditions, no robust crystallization systems have been described for *P. aeruginosa* LpxC. Due to aggregation of *P. aeruginosa* LpxC in the traditional *E. coli* overexpression systems [15], it has been challenging to prepare sufficient amounts of high-quality *P. aeruginosa* LpxC protein to support structure-based drug design.

Often, overexpression of recombinant proteins in *E. coli* results in insoluble aggregates that cause buildup of inclusion bodies in the cell or form soluble aggregates as partially misfolded protein intermediates [16,17]. Large-scale expression of *P. aeruginosa* LpxC in *E. coli* resulted in mostly aggregated protein with little soluble

* Corresponding author. Tel.: +1 (781) 839 4559; fax: +1 (781) 839 4600.
*E-mail address:* ning.gao@astrazeneca.com (N. Gao).
[1] Authors with equal contributions.
[2] Current address: Infection Disease Chemistry, Novartis Institutes for Biomedical Research, 4560 Horton Street, Emeryville, CA 94608-2916, USA.
[3] *Abbreviations used:* LPS, lipopolysaccharide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC–MALS, size exclusion chromatography and multi-angle light scattering; RND, resistance nodulation cell division.

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LpxC. While several methods were tried to improve *P. aeruginosa* LpxC solubility, the most successful approach was to overexpress LpxC in the presence of an LpxC-specific inhibitor. One limitation of this approach is the requirement for a compound to penetrate to the cytoplasm of the *E. coli* expression strain. This restricted the selection of compounds for structural studies and necessitated a large-scale synthesis of each compound for each LpxC-inhibitor co-crystal system. *E. coli*, like many Gram-negative organisms, has an efflux system that is capable of transporting toxic molecules, such as antibiotics, from the interior of the cell to the exterior environment [18–20]. To reduce the amount of compound and expand the range of usable compounds during expression of *P. aeruginosa* LpxC, an overexpression strain of *E. coli* with a deletion of *acrB* gene, a component of the major efflux pumps, was developed. By using this strain to prepare *P. aeruginosa* LpxC, we obtained multiple high-quality crystal structures of *P. aeruginosa* LpxC in complex with a wide range of compounds and substantially reduced the protein-to-structure turnaround time.

## Materials and methods

### *Cloning P. aeruginosa LpxC(1-299)C40S expression construct*

The *P. aeruginosa lpxC* gene encoding residues 1–299 was cloned from *P. aeruginosa* PAO1 genomic DNA using the polymerase chain reaction (PCR) as previously reported [14]. Amplification was performed using High Fidelity PCR Master (Roche Applied Science, Indianapolis, IN) and the following primers, encoding NdeI and EcoRI restriction sites (EuroFins MWG Operon, Huntsville, AL)

5′GTACGACATATGATCAAACAACGCACC3′ and 5′GTATCGGAATTCTTACGGGCGCATATAGGA3′.

The PCR product was purified using the QuickStep 2 PCR Purification Kit (EDGE Biosystems, Gaithersburg, MD). The resulting fragment was digested with the restriction endonucleases NdeI and EcoRI (Roche Applied Science) and cloned into the appropriate sites of the expression vector pET-21b (Novagen Biosciences, Madison, WI). The DNA sequence of the cloned *lpxC* gene was confirmed by sequencing on an ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Foster City, CA) using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Computer analysis of DNA sequences was performed with Sequencer (Gene Codes Corp., Ann Arbor, MI). The mutation, C40S, was subsequently introduced into the *lpxC* expression plasmid by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions utilizing the following mutagenic primers (EuroFins MWG Operon)

5′GGACACCGGTATCGTGTTCTCCCGCACCGACCTGGATCCGG3′ and 5′CCGGATCCAGGTCGGTGCGGGAGAACACGATACCGGTGTCC3′.

The resulting plasmid was designated pJT638. The sequence of the *lpxC* gene containing the C40S change was confirmed by sequencing as described above.

### *Development of an efflux mutant expression strain*

To create an efflux mutant strain that would express *P. aeruginosa* LpxC from pJT638, a deletion internal to the coding region of *acrB* was created on the chromosome of *E. coli* BL21(DE3) using lambda Red-mediated recombination, as previously described [21]. Briefly, the DNA deletion construct was created by PCR using primers that anneal to the kanamycin resistance cassette of pKD4 and that contain 36 bp regions of homology to the *acrB* coding region on the 5′ ends. The forward primer was 5′AGTCCAAGTCTTAACTTAAACAGGAGCCGTTAAGACGTGTAGGCTGGAGCTGCTTCG3′ and reverse primer was 5′AAGGCCGCTTACGCGGCCTTAGTGATTACACGTTGTACATATGAATATCCTCCTTA3′. The resulting PCR product was purified and electroporated into *E. coli* BW25113 strain containing the lambda Red system on plasmid pKD46 as previously described [21]. Recombinants were selected on LB agar containing 25 μg/ml kanamycin. Deletion of *acrB* from the chromosome was verified by PCR. The *acrB* gene deletion was then moved by P1 phage transduction [22] into *E. coli* BL21(DE3), selecting for kanamycin resistance. The kanamycin resistance gene was then excised from the chromosome using the FLP recombinase expressed from plasmid pCP20 as previously described [21], resulting in *E. coli* strain ARC5328. For tighter control of expression from pJT638, the pLysS plasmid (Novagen) was transformed into *E. coli* ARC5328 to create the strain *E. coli* ARC5329.

### *Expression of P. aeruginosa LpxC in the absence of LpxC inhibitors*

To express LpxC, plasmid pJT638 was transformed into *E. coli* BL21(DE3) pLysS, plated on Luria-Bertani (LB) medium containing 100 μg/ml ampicillin and incubated at 37 °C overnight. A single colony of BL21(DE3) pLysS/pJT638 was inoculated into a 100-ml culture of LB medium containing 100 μg/ml ampicillin and 0.2% glucose and grown overnight at 37 °C. This overnight culture was diluted to $OD_{600}$ = 0.1 in 6 × 1 L of LB containing 100 μg/ml ampicillin and 0.2% glucose and grown at 30 °C with aeration to mid-logarithmic phase ($OD_{600}$ = 0.5). The culture was incubated on ice for 30 min and transferred to 18 °C. IPTG was then added to a concentration of 0.5 mM. After overnight induction at 18 °C, the cells were harvested by centrifugation at 5000*g* for 15 min at 25 °C. Cell pastes were stored at −20 °C.

### *Expression of P. aeruginosa LpxC in the presence of LpxC inhibitors*

To express LpxC in the presence of inhibitors, plasmid pJT638 was transformed into *E. coli* strain BL21(DE3) Δ*acrB* pLysS or ARC5329, and plated on Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 37 °C overnight. A single colony of ARC5329/pJT638 was used to inoculate a 5-ml culture of LB medium containing 100 μg/ml ampicillin and 0.2% glucose and incubated overnight at 37 °C. This overnight culture was diluted to $OD_{600}$ = 0.1 in 6 × 5-ml of LB containing 100 μg/ml ampicillin and 0.2% glucose and grown at 30 °C with aeration to mid-logarithmic phase ($OD_{600}$ = 0.5). The culture was incubated on ice for 30 min and 5 μl of 1000-fold serial dilutions of LpxC inhibitors in DMSO were added to each of the 6 tubes. After 30 min, the cell culture was transferred to 18 °C and IPTG was added to each culture at a final concentration of 0.5 mM. After overnight induction at 18 °C, the cells were harvested by centrifugation at 14,000*g* for 10 min at 25 °C. Cell pellets were resuspended in 1 ml of 25 mM Tris–HCl, pH 8.0 and 5% (v/v) glycerol and disrupted by two passages through a French press (Thermo Fisher Scientific, Wilmington, DE) operated at 18,000 psi. The crude extract was centrifuged at 14,000*g* for 30 min at room temperature. The solubility of LpxC was determined on 4–20% SDS–PAGE (Invitrogen, Grand Island, NY). The concentration of inhibitor that displayed the highest percent of soluble LpxC was then subsequently used for a scaled up expression in a 1 L volume of LB culture.

### *Purification of recombinant P. aeruginosa LpxC*

Frozen cell paste was suspended in 50 ml of Lysis Buffer [25 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 5% (v/v) glycerol and 1 Protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN)]. All purification steps were performed at 4 °C. Cells were disrupted by passing them twice through a French press operated at 18,000 psi, and the crude extract was centrifuged at 120,000*g* for 30 min at 4 °C. The supernatant was loaded at a flow