Lipopolysaccharide Is a 4-Aminoarabinose Donor to Exogenous Polyisoprenyl Phosphates through the Reverse Reaction of the Enzyme ArnT

Beth A. Scarbrough, Colleen R. Eade, Amanda J. Reid, Tiffany C. Williams, and Jerry M. Troutman*

Cite This: ACS Omega 2021, 6, 25729−25741

ABSTRACT: Modification of the lipid A portion of LPS with cationic monosaccharides provides resistance to polymyxins, which are often employed as a last resort to treat multidrug-resistant bacterial infections. Here, we describe the use of fluorescent polyisoprenoids, liquid chromatography-mass spectrometry, and bacterial genetics to probe the activity of membrane-localized proteins that utilize the 55-carbon lipid carrier bactoprenyl phosphate (BP). We have discovered that a substantial background reaction occurs when B-strain E. coli cell membrane fractions are supplemented with exogenous BP. This reaction involves proteins associated with the arn operon, which is necessary for the covalent modification of lipid A with the cationic 4-aminoarabinose (Ara4N). Using a series of arn operon gene deletion mutants, we identified that the modification was dependent on ArnC, which is responsible for forming BP-linked Ara4N, or ArnT, which transfers Ara4N to lipid A. Surprisingly, we found that the majority of the Ara4N-modified isoprenoid was due to the reverse reaction catalyzed by ArnT and demonstrate this using heat-inactivated membrane fractions, isolated lipopolysaccharide fractions, and analyses of a purified ArnT. This work provides methods that will facilitate thorough and rapid investigation of bacterial outer membrane remodeling and the evaluation of polyisoprenoid precursors required for covalent glycan modifications.

INTRODUCTION

Bactoprenyl phosphate (BP, also known as undecaprenyl phosphate) is an essential precursor required to synthesize vital surface polysaccharides including peptidoglycan, capsules, and teichoic acids. BP is also required to glycosylate a variety of outer membrane components. Notably, the 55-carbon BP lipid carrier is necessary for the covalent modification of lipid A with cationic glycoses such as 4-aminoarabinose (Ara4N), glucosamine, and galactosamine in many species of Gram-negative pathogens.1−6 These outer membrane modifications are frequently associated with resistance to cationic antimicrobial peptides (CAMPs) such as polymyxin B and colistin (polymyxin E) and in certain species are required for cell viability.4,7,8 Currently, polymyxin and colistin are being used as last-resort treatments against multidrug-resistant Gram-negative bacterial infections.3 Consequently, enzymes within lipid A modification pathways are being targeted for the development of inhibitors to resensitize CAMP-resistant Gram-negative pathogens.10,11

In Salmonella enterica and Escherichia coli, CAMP resistance is due to covalent modification of the lipid A portion of LPS with Ara4N (reviewed by Raetz et al.).12 In those species, incorporation of Ara4N into the outer membrane begins in the cytosol with the biosynthesis of UDP-L-4-formamido-arabinose (UDP-Ara4FN) from UDP-glucose via the well-characterized enzymes Ugd, ArnA (PmrI), and ArnB (PmrH).13−17 Membrane-bound ArnC (PmrF) will then append Ara4FN to BP to produce bactoprenyl monophosphate-4-formamido-arabinose (BP-Ara4FN) (Figure 1).14 Subsequently, ArnD (PmrJ) will deformylate 4-formamido-arabinose to produce bactoprenyl monophosphate-4-amino-arabinose (BP-Ara4N), which is then flipped into the periplasm by the proposed ArnE/F (PmrM/L) flippase heterodimer.18,19 The deformylation of the BP-Ara4FN intermediate likely commits this pathway to lipid A modification and prevents reversal of the ArnC reaction.12,14,19 In the periplasm, ArnT (PmrK) then transfers Ara4N from BP-Ara4N to lipid A.20

Limitations in procuring and detecting native BP-linked substrates have hindered characterization of enzymes involved in lipid A modifications. Recently, our group demonstrated the utility of fluorescent bactoprenyl phosphate to rapidly investigate the activity and specificity of bacterial glycosyl-
transferrases including several enzymes within the initiating phospho-glycosyltransferase (PGT) protein family.\textsuperscript{21–23} Importantly, we found that the initiating PGT WecA from \textit{E. coli}, which is responsible for appending GlcNAc-phosphate (GlcNAc-P) to BP, was present in cell envelope fractions in high enough quantities without overexpression to catalyze exogenous BP modification. In this report, we focused our efforts on determining whether these fluorescent probes could be used to characterize additional important endogenous proteins within the bacterial cell envelope, such as those required for polymyxin resistance.

## RESULTS

**Utilization of 2CN-BP by \textit{E. coli} Cell Envelope Fractions.** In our previous analysis of PGT enzyme activity, we consistently observed that upon incubation of fluorescent 2-nitrileanilinobactoprenyl phosphate (2CN-BP) with membrane fractions prepared from B-strain \textit{E. coli}, an unidentified fluorescent product was formed.\textsuperscript{21} The presumed modified 2CN-BP was formed without the addition of nucleotide-linked sugar (NDP-sugar), which suggested that the donor substrate for the reaction was contained within the membrane fraction. In addition, the formation of this product was considerably lower and in some cases undetectable when a PGT (e.g., WecA) and its NDP-sugar substrate were added to the reactions exogenously to compete for available 2CN-BP (Figure 2).\textsuperscript{21} RP-HPLC analysis
of C41(DE3) membrane fraction reactions exempt of NDP-sugar demonstrated that 0.5 mg/mL (total protein concentration) of the membrane fraction consumed approximately up to 100 pmol of 2CN(6Z)BP (six isoprenes in the Z-
configuration) (Figure S1). As a vehicle control, we found that product formation was not impacted by the presence of DMSO (up to 12%) (Figure S1).

Notably, the retention time of this product on a conventional C18 column (Agilent Zorbax) with an ammonium bicarbonate/n-propanol mobile phase (condition A) was intermediate to 2CN-BP and 2CN-BPP-GlcNAc, as evidenced by the 2.7 min difference in retention time when compared to the product of WecA (Figure 2A).21 However, using a high pH-resistant C18 column (Waters XBridge) with an ammonium hydroxide/n-propanol mobile phase (condition B), the retention time of the unidentified product was greater than both 2CN-BP and 2CN-BPP-GlcNAc (Figure 2B). The chromatographic behavior of the unidentified product under both described conditions was comparable to a 2CN-BP-monosaccharide that we have observed during our investigation of another lipid A modification system (unpublished data), suggesting that the unidentified product, too, could be a polysoprenoid monophosphate-linked sugar.

In support of this, Trent et al. demonstrated that B-strain E. coli such as C41(DE3) appear to be inherently resistant to polymyxin and modify lipid A with Ara4N.24 Since lipid A modification systems are characterized by the requirement of a BP-glycosyl donor, we viewed this as a possible source of a 2CN-BP-monosaccharide (reviewed by Mann and Whittenbury).25 Lipid A modification and expression of the arn operon (arnBCAD-TEF) in E. coli has previously been attributed to the activation of the PmrA/B two-component regulatory system, including constitutively activating mutations in pmrA or pmrB.26-28 Unlike B-strains, K-strains do not modify lipid A with Ara4N and are thus sensitive to polymyxin unless induced by various environmental cues such as exposure to Fe3+ or metavanadate.29-31 Therefore, if the unidentified product was associated with Ara4N lipid A modification, no such modification of 2CN-BP should occur with a membrane fraction prepared from a polymyxin-sensitive K-12 strain such as MG1655 grown under standard conditions, but perhaps we could induce this modification by culturing in arn-inducing conditions.26

To test this, we prepared two separate membrane fractions of the E. coli K-strain MG1655, the first prepared from cells cultured in LB and the second prepared from cells cultured in LB supplemented with 200 μM Fe3+. When 2CN-BP was incubated with a membrane fraction prepared from MG1655 cells cultured in standard conditions, no product was formed (Figure 3A). However, when compared to the product of the C41(DE3) membrane fraction, a new product with an identical retention time was formed only by MG1655 membrane fraction prepared from cells cultured with Fe3+ (Figure 3A). These results indicated that the formation of this product could be induced by preparing membrane fractions from cells cultured with Fe3+ and that it is likely associated with Ara4N lipid A modification. Additionally, we noted that a new product was formed by the MG1655 membrane fraction (cultured without Fe3+) when incubated with UDP-glucose (Figure 3A). This product possessed a similar retention time (6.07 min) to that of the unknown product (6.20 min) and an m/z corresponding to the [M+H]+ ion of a 2CN-BP-hexose as evidenced by ESI-MS in negative ion mode (Figure 3B). Of the known possible gene products in E. coli that may append glucose to 2CN-BP, we attributed this activity to the pghe-encoded glucosyltransferase YfdH (GtrB), which is not present in B-strains.32,33

To aid in the identification of the unknown product from C41(DE3) membrane fraction reactions, we prepared a reaction containing the C41(DE3) membrane fraction and 100 nmol 2CN-BP that was subsequently separated and isolated via RP-HPLC. Analysis of the unidentified product with ESI-MS in negative ion mode indicated a predominant ion with an m/z of 888.60, which corresponds to a predicted [M−H]− m/z of 2CN(6Z)BP-Ara4N (888.57) (Figure 4A). Upon collision-induced fragmentation, we observed an m/z of 757.59, which is consistent with the [M−H]− ion of the fluorescent 2CN(6Z)BP (expected m/z 757.51), and a loss of an Ara4N unit (Figure 4B). We then used ESI-IC-MS with SIM in negative ion mode to evaluate crude membrane fraction reactions for 2CN-BP-Ara4N (2CN-BP-PentN) (Figure 4C). As expected, the MG1655 membrane fraction produced the purported 2CN-BP-Ara4N product only when cells were cultured in the presence of Fe3+. These results demonstrated that the unidentified product was likely a modification of 2CN-BP with Ara4N or another modification with an identical mass.

Membrane-Bound Enzymes in Ara4N Lipid A Modification Pathway Contribute to the Membrane Fraction-Mediated Formation of 2CN-BP-Ara4N. We envisioned two possibilities as the source of the membrane fraction-mediated Ara4N modification of 2CN-BP: (1) membrane-bound ArnC transfers Ara4N to 2CN-BP and is subsequently deformylated by membrane-associated ArnD or (2) membrane-bound ArnT transfers Ara4N from either native BP-Ara4N or LPS-Ara4N to 2CN-BP (Figure 5). To evaluate each of these possibilities, we created deletions of arnC and arnT in C41(DE3). We reasoned that a non-polar deletion of arnC would not impede the formation of UDP-Ara4N and thus preclude the synthesis of endogenous BP-Ara4N and LPS-Ara4N. Thus, we expected that deleting arnC would abolish the production of 2CN-BP-Ara4N in membrane fraction reactions by preventing transfer from ArnC or ArnT. In the same regard, we anticipated that if ArnCD was responsible for mediating the transfer of Ara4N to 2CN-BP, the deletion of arnT would not affect the production of 2CN-BP-Ara4N but would preclude the formation of LPS-Ara4N.

To evaluate the activity of ΔarnT and ΔarnC mutant membrane fractions, we prepared reactions containing either a wild-type or mutant membrane fraction incubated with 2CN-BP and analyzed the crude reactions for 2CN-BP-Ara4N using RP-HPLC (condition A) (Figure 6). As predicted, RP-HPLC analysis of the ΔarnC membrane fraction with 2CN-BP did not demonstrate the formation of 2CN-BP-Ara4N. Extrachromosomal complementation of arnC with a high copy number
plasmid partially restored the activity of ΔarnC pArnC membrane fraction compared to the wild type. Interestingly, we found that the ΔarnT membrane fraction also did not produce 2CN-BP-Ara4N, suggesting that ArnT is required for the modification of exogenous 2CN-BP with Ara4N (Figure 6B). Complementation of arnT restored that activity, while expression of an empty plasmid control in either mutant did not (Figure 6C). To confirm expression of arnC and arnT in our complementation analyses, we confirmed the presence of 6XHis-tagged ArnC and ArnT in membrane fractions with an anti-His Western blot (Figure S2). These results further support our hypothesis that Arn enzymes in the membrane fraction are involved in this new product formation and that the modification is indeed Ara4N (i.e., not another modification with an identical mass).

**Modification of 2CN-BP by ΔarnC Membrane Fraction Is Facilitated by the Addition of Exogenous Substrate.**

We next evaluated potential sources of Ara4N modification by supplying an exogenous donor substrate in the form of a heat-inactivated wild-type membrane fraction to either the ΔarnC or ΔarnT mutant membrane fraction (Figure 7). The inactivated wild-type membrane fraction would harbor the donor substrate, but the proteins (ArnC or ArnT) are inactive. Supplementation of the exogenous substrate to a reaction containing the ΔarnC membrane fraction would enable us to evaluate the activity of ArnT. Similarly, by using the ΔarnT membrane fraction, we could then evaluate the activity of ArnC. To do so, each active

---

**Figure 6.** Deletion of arnC or arnT in C41(DE3) abolishes cell envelope production of 2CN-BP-Ara4N. Reactions with 2CN(6Z)BP and membrane fractions prepared from wild-type or mutant C41(DE3) were separated by RP-HPLC (condition A) and evaluated for product formation with fluorescence detection. Chromatograms were offset by 100 FLU increments. (A) Schematic of potentially available intermediates in each mutant membrane fraction. (B) ΔarnC C41(DE3) membrane fraction compared to wild-type membrane fraction incubated with 2CN(6Z)BP. (C) ΔarnT C41(DE3) membrane fraction compared to wild-type membrane fraction incubated with 2CN(6Z)BP.

---

**Figure 7.** arnT, but not arnC, is required for the modification of 2CN-BP in the presence of an exogenous substrate. Mutant membrane fractions (0.5 mg/mL) were incubated with 5 μM 2CN(6Z)BP both with and without 0.5 mg/mL of the inactivated wild-type membrane fraction (WTi). Crude reactions were separated by RP-HPLC (condition A) and analyzed with fluorescence detection. Chromatograms are offset by 100 FLU increments.
membrane fraction was incubated with or without equivalent quantities of inactivated wild-type membrane fraction. Wild-type or inactive wild-type membrane fractions with 2CN-BP were used as controls. When reactions containing the 2CN-BP and \( \Delta \text{arnC} \) membrane fraction were supplemented with the inactive membrane fraction, a new product was formed. The retention time of this new product corresponded to the same retention time as 2CN-BP-Ara4N (4.83 min, condition A) produced by the wild-type membrane fraction. No such product was formed under these conditions with the \( \Delta \text{arnT} \) membrane fraction. These results suggest that ArnT is responsible for transferring Ara4N to 2CN-BP.

To address whether native BP-Ara4N could serve as the Ara4N donor substrate for the ArnT-catalyzed formation of 2CN-BP-Ara4N, we first determined whether native BP-Ara4N would accumulate in the C41(DE3) strain. Accumulation and detection of BP-Ara4N have been well established in polymyxin-resistant \( E. \ coli \) and \( S. \ enterica \).\(^{19,24} \) Similar methods typically employ lipid extraction from cell lysates followed by MALDI-MS or ESI-MS.\(^{19,24,34} \) Similarly, we used ESI-LC-MS to typically employ lipid extraction from cell lysates followed by SIM in negative ion mode, we were able to detect native BP and BP-Ara4N in total lipid extracts of \( E. \ coli \) cell lysates.\(^{36} \) Using SIM in negative ion mode, we were able reliably detect endogenous BP (\([M-H]^- m/z 845.7\)) in wild-type C41(DE3) (condition B) and mutant lipid extracts. BP-Ara4N (BP-PentN, \([M-H]^- m/z 976.7\)) was detected only in wild-type C41(DE3) and \( \Delta \text{arnT} \) C41(DE3) but not in \( \Delta \text{arnC} \) C41(DE3) (Figure 8), a result that is corroborated by a previous characterization of BP-Ara4N biosynthesis.\(^{14,24} \) Interestingly, triplicate analysis of equally prepared lipids demonstrated a consistent 2.5-fold increase in the abundance of BP in lipids prepared from \( \Delta \text{arnC} \) C41(DE3).

Complementation of the \( \Delta \text{ ArnC} \) mutant with a high copy number plasmid restored the accumulation of BP-Ara4N but only to a minor extent when compared to the wild type (Figure S3). This result is akin to the previous complementation analysis with RP-HPLC, in which full conversion of 2CN-BP was not achieved by complementation of the \( \Delta \text{arnC} \) mutant (Figure 6). Complementation of the \( \Delta \text{arnT} \) mutant did not significantly alter the intensity of either BP or BP-Ara4N relative to the wild type. A signal corresponding to BP-Ara4FN was not detected in either wild-type or mutant lipid extracts (BP-PentN, \([M-\text{MH}^+]^+ 1004.71\)). However, we were able to detect BP-Ara4FN in lysates prepared from the complemented \( \Delta \text{arnC} \) mutant (Figure S3).

Based on the analysis of native BP and BP-Ara4N in C41(DE3) lipids, the \( \Delta \text{arnT} \) membrane fraction would likely harbor both BP and BP-Ara4N but not lipopolysaccharide (LPS)-Ara4N.\(^{24} \) To rule out the possibility of native BP-Ara4N as the donor substrate for the modification of 2CN-BP, we then prepared the inactivated \( \Delta \text{arnT} \) membrane fraction and supplied it to wild-type and mutant membrane fractions as a source of BP-Ara4N. HPLC analysis of crude reactions did not indicate the production of 2CN-BP-Ara4N when the \( \Delta \text{arnC} \) membrane fraction was incubated with the inactivated \( \Delta \text{arnT} \) membrane fraction (Figure S4). These results suggest that endogenous BP-Ara4N does not supply the Ara4N donor for 2CN-BP modification and collectively indicate that the aberrant production of 2CN-BP-Ara4N is mediated through the reverse reaction of ArnT, in which Ara4N is transferred from LPS-Ara4N to exogenously supplied 2CN-BP.

**ArnT-Catalyzed Reverse Transfer of Ara4N Occurs with Fluorescent and Native BP.** To evaluate whether LPS could serve as an Ara4N donor substrate, a fraction of LPS was prepared from wild-type C41(DE3) that was expected to contain Ara4N-modified LPS. Using a shortened procedure for lipid A isolation, C41(DE3) cells were lysed in a single-phase Bligh and Dyer solution and crude LPS was collected as an insoluble precipitate.\(^{36,37} \) Samples prepared using this method are expected to be relatively free of any BP and BP-linked intermediates, as phospholipids and prenyl lipids are extracted in the soluble fraction (see Experimental Methods).\(^{38} \) A sample of isolated material was visualized with SDS-PAGE, which showed minimal contamination and a banding pattern consistent with the anticipated truncated LPS of B-strain \( E. \ coli \) (Figure S5).\(^{38,39} \) The isolated material was then added to reactions containing 2CN-BP and wild-type or mutant membrane fractions (Figure 9A). In this analysis, we found that only the \( \Delta \text{arnC} \) membrane fraction produced 2CN-BP-Ara4N when incubated with isolated LPS fractions. Additionally, a consistent, linear increase in the turnover of 2CN-BP was observed in reactions containing the \( \Delta \text{arnC} \) membrane fraction when the amount of LPS (approximately 20 \( \mu \)g) was increased up to 4-fold (Figure 9B and Figure S6).

Based on our analysis of BP and BP-Ara4N levels in wild-type and mutant C41(DE3) cell lysates (Figure 8), we determined that ArnC cells have an abundance of available BP. To ensure that the transfer of Ara4N from LPS-Ara4N also occurred with native BP and not just 2CN-BP, we prepared reactions containing the \( \Delta \text{arnC} \) membrane fraction supplemented with LPS, in which the \( \Delta \text{arnC} \) membrane fraction would serve as a source of both BP and ArnT (Figure 10A). Lipids were then extracted with \( n \)-butanol, dried under vacuum, and resuspended in \( n \)-propanol/10 mM ammonium hydroxide prior to ESI-LC-MS analysis. As anticipated, BP-Ara4N was observed only when
the ΔarnC membrane fraction was incubated with LPS but not in the ΔarnC membrane fraction or LPS alone.

To demonstrate that reverse transfer is catalyzed by ArnT and not another enzyme within the membrane fraction, ArnT bearing a 6xHis-tag was overproduced in C41(DE3) and solubilized as described in the Experimental Methods (Figure S7). To evaluate the activity of purified ArnT, we incubated ArnT and 2CN-BP both with and without LPS and monitored for 2CN-BP-Ara4N (Figure 10B). RP-HPLC analysis of these reactions indicated that purified ArnT without added LPS catalyzed the conversion of 2CN-BP, albeit full-turnover was not achieved. This result was not surprising since isolated proteins are typically contaminated with LPS. When purified LPS from C41(DE3) was added to ArnT reactions, 2CN-BP was nearly consumed as indicated by the increase in 2CN-BP-Ara4N and reduction in 2CN-BP.

Characterization of E. Coli ArnD with Native BP-Ara4FN Substrate. Based on the utility of ESI-LC-MS methods described above to detect native BP-linked intermediates, we next sought to apply these methods to characterize the function of ArnD with its native substrate, BP-Ara4FN. In similar lipid A modification pathways, deacetylation or deformylation is required to finalize the transfer of cationic glycoses to lipid A (reviewed by Mann and Whitfield). Recent analysis of an ArnD homolog from Burkholderia cenocepacia with a synthetic analogue of BP-Ara4FN demonstrated ArnD deformylase activity. To bypass the need for syntheses of BP-Ara4FN or UDP-Ara4FN, we used a genetic approach to promote the accumulation of native BP-Ara4FN in E. coli, which could then be collected and used for functional assays in vitro. To do so, we created a ΔarnD mutation in the K-12 MG1655 background and tested whether the accumulation of BP-Ara4FN could be promoted by culturing these cells in the presence of Fe³⁺. ESI-LC-MS analysis of total lipid extracts from wild-type MG1655 demonstrated a minor accumulation of BP-Ara4N when cells were cultured in the presence of Fe³⁺ (Figure 11A). Accordingly, analysis of lipid extracts from ΔarnD MG1655 cells demonstrated that BP-Ara4FN was only detected when cells were grown in the presence of Fe³⁺ (Figure 11,BA).

Using lipid extracts of ΔarnD MG1655 cells containing BP-Ara4FN, we then assessed the deformylase activity of E. coli ArnD. To express and purify ArnD, we used a purification...
procedure recently reported by Adak et al.18 The presence of ArnD was confirmed with SDS-PAGE and anti-His Western blotting (Figure S8). Purified ArnD was then incubated in buffered reactions with lipids prepared from MG1655 ΔarnD cells cultured with Fe3+. Deformylase activity was monitored with ESI-LC-MS using SIM in negative ion mode and evidenced by the formation of BP-Ara4N (Figure 11).

## DISCUSSION

Previously established methods for evaluating polyprenyl-phosphate glycosyltransferases have relied on the specific detection of radiolabeled glycoses. Here, we have highlighted additional polyprenoid-linked products that are formed due to the reversibility of these reactions and likely go undetected. In events of poor recombinant protein production or function, exogenously added isoprenoid substrate may be consumed by these reactions, significantly reducing the availability of BP and preventing glycosylation or detection of the intended product. On the other hand, we also note that the availability of BP glycos substrates is limited and that reverse reactions demonstrated here may be used to generate these substrates in vitro.

Accordingly, this research directly addresses the deficit of polyprenoid substrates by demonstrating the utility of two efficient methods used to obtain both native and tagged BP-glycoses. Specifically, we demonstrate the unique advantage of 2CN-BP used to selectively probe the activity of endogenous BP--utilizing proteins within membrane fractions of both K- and B-strains of E. coli. This method may also prove effective for the evaluation of other Gram-negative pathogens, particularly, without the need for gene overexpression and protein purification. Previously, such findings have enabled the characterization of early stages of bacterial glycan biosynthesis such as the enterobacterial common antigen in E. coli.43

Similarly, our methods revealed an unestablished activity of ArnT, for which prior characterization of the forward reaction has required the isolation of small quantities of native BP-Ara4N or the use of shorter, synthetically prepared Ara4N-linked isoprenoids.24,42 Reverse catalysis of PGT enzymes, where UDP-sugar is formed from BPP-sugar in the presence of UMP, has been demonstrated in vitro with various initiating PGT and glycosyltransferase enzymes.44,45 Research has also been directed at harnessing these reactions to obtain complex NDP-linked sugars, akin to our generation of fluorescent BP-linked Ara4N here.46 We show that the reverse transfer of ArnT may be harnessed to provide ample fluorescent BP-Ara4N donor substrate for further analysis of ArnT activity in vitro.

It is interesting to consider whether reverse transfer of Ara4N from LPS to BP may occur in the cell. LPS transport from the

Figure 11. Induced accumulation of native BP-Ara4FN and deformylation of BP-Ara4FN by E. coli ArnD. (A) Lipids prepared from MG1655 and ΔarnD MG1655 cells cultured in LB with or without Fe3+ were analyzed with ESI-LC-MS (condition B) with SIM in negative ion mode for BP, BP-PentN, and BP-PentFN ([M−H]− m/z 1004.7). (B) Mass spectrum obtained from the total ion scan of ΔarnD MG1655Δ from 12.0 to 12.6 min (C) Reactions containing lipids prepared from MG1655 ΔarnD cultured with Fe3+ and 0.05 mg/mL ArnD were monitored for BP-Ara4N (BP-PentN) with SIM. Control reactions contained lipids in reaction buffer with no ArnD.
inner membrane to the outer membrane requires an ATP-binding cassette (ABC) transporter and a protein bridge to shuttle the large glycolipid through the periplasmic space to the outer leaflet of the outer membrane.\(^{25-27}\) Recently, Owens et al. demonstrated that ATP hydrolysis promotes LPS movement onto the protein bridge and prevents the backward transport of LPS, providing substantial evidence of unidirectional LPS transport.\(^2\) With the exception of reaction equilibriums at the periplasmic inner membrane, the ArnT-catalyzed reverse transfer of Ara4N to BP would require LPS to be translocated back to the periplasmic face of the inner membrane where ArnT is situated.\(^20\) We conclude that reverse transfer of Ara4N to BP after LPS has been modified and translocated to the outer membrane is unlikely. Additionally, in the cell, the rapid translocation of modified LPS to the outer membrane likely pushes the reaction forward. It is more likely that reverse transfer is an artifact of cell membrane fractionation. Nonetheless, this report reveals considerations that must be made during in vitro analyses of glycosyltransferases and substrate utilization, where this unfavored reaction may consume, as evidenced by our analyses, a large portion of exogenously added isoprenoid.

In addition to analyses with fluorescent BP, our methods enabled the rapid detection of native BP and BP intermediates in lipids collected from constitutive and Fe\(^{3+}\)-induced polymyxin-resistant \textit{E. coli}. Considering the unlikely reversibility of the BP-Ara4N intermediate, we noted a consistent increase in the Ara4N intermediate in the Arn pathway and the resultant sequestration of the vital BP precursor would make ArnT an ideal candidate for characterizing and quantifying the effects of BP sequestration in various culturing conditions and among environmental or host-mediated stressors. Ultimately, the sequestration of BP through reverse transfer of Ara4N to BP would require LPS to be translocated back to the periplasmic face of the inner membrane where ArnT is situated.\(^20\) We conclude that reverse transfer of Ara4N to BP after LPS has been modified and translocated to the outer membrane is unlikely. Additionally, in the cell, the rapid translocation of modified LPS to the outer membrane likely pushes the reaction forward. It is more likely that reverse transfer is an artifact of cell membrane fractionation. Nonetheless, this report reveals considerations that must be made during in vitro analyses of glycosyltransferases and substrate utilization, where this unfavored reaction may consume, as evidenced by our analyses, a large portion of exogenously added isoprenoid.

**EXPERIMENTAL METHODS**

**Materials and Bacterial Strains.** The fluorescent 2CN-(6Z)BP substrate was prepared as previously described (Ex: 340, Em: 390 nm).\(^{21,55}\) \textit{E. coli} MG1655 was generously provided by the Young/Jorgenson Laboratory at the University of Arkansas for Medical Sciences. C41(DE3) was purchased from Novagen.

**Cell Envelope Fractionation.** To evaluate the activity of \textit{E. coli} membrane proteins with the 2CN-(6Z)BP, we prepared membrane fractions from wild-type and mutant \textit{E. coli} in both C41(DE3) and MG1655. Starter cultures were prepared from isolated colonies and incubated overnight at 37 °C. Cultures containing 250 mL of LB Miller broth were inoculated with a 1:1000 dilution of starter culture and incubated at 37 °C at 220 rpm until an OD\(_{595}\) of 0.8–1.0. Cells were then pelleted at 5000 RCF for 15 min. Cell pellets were resuspended in 0.8% NaCl, pelleted at 5000 RCF for 15 min, and stored at −80 °C for about 1 week prior to the preparation of the membrane fraction. To prepare membrane fractions, cells were resuspended in buffer A (50 mM Tris pH 8.0, 300 mM NaCl) and lysed with sonication (Fisher Sonic Dismembrator). Lysates were centrifuged at 5000 RCF for 20 min to remove unlysed cells and cellular debris. The supernatant was then spun at 100,000 RCF for 1 h. The resulting pellets were resuspended in buffer B (50 mM Tris, 200 mM NaCl) and stored at −80 °C until use. The total protein content of membrane fractions was determined with a Bradford assay at 595 nm using bovine serum albumin (BSA) as a standard.

**RP-HPLC.** All RP-HPLC (condition A) was performed with an Agilent 1100 HPLC system equipped with a variable wavelength and fluorescence detector (FLD) using an Agilent Zorbax XBD-C18 column (4.6 mm × 50 mm, 3 μm, 80 Å). The mobile phase comprised n-propanol (solvent C) and 10 mM ammonium hydroxide (solvent D) with a flow rate of 1 mL/min. For all analyses, the column temperature was set to 30 °C. MS parameters for each analysis included a nebulizer pressure of 50 psi, drying gas flow of 12.0 L/min, drying gas temperature of 350 °C, and capillary voltage of 4000 V. All analyses were performed in negative ion mode.

For analysis of native BP, BP-Ara4N, and BP-Ara4PN with ESI-LC-MS, each of the four channels analyzed three SIM ions and one scan from 400–1500 m/z. Scan parameters included a fragmentation voltage 350 of V, gain of 1.0, threshold of 150, step size of 0.1, and scan speed of 2600 u/s. SIM parameters included a fragmentation voltage of 350 V, gain of 1.0, and dwell time of 215 ms. The predicted m/z value for the [M−H]\(^+\) ion of each analyte used for SIM are as follows: bactoprenyl phosphate (BP) 845.66, bactoprenyl phosphate-4-aminoarabinose (BP-Ara4N) 976.72, bactoprenyl phosphate-4-formamido-arabinose (BP-Ara4PN) 1004.71. For each analysis, 5–10 μL of total lipids were analyzed with a linear gradient starting with 15% solvent C, raising to 95% solvent C over 1 min and holding for 1 min, then decreasing to 15% solvent C over 3 min with a post run of 2 min. Each analysis included a 3 s needle wash.

For the analysis of fluorescent 2CN-BP and enzyme products with LC-MS, a TEE connector was used to split the solvent flow through both the MSD and FLD. Each of the four channels analyzed three SIM ions and one scan from 500–2000 m/z. MS parameters were the same as described above except the fragmentation voltage, which was set to 50 V. The predicted m/z value for the [M−H]\(^+\) ion of each analyte used for SIM are as follows: 2CN-(6Z)BP 757.51, 2CN-(6Z)BP-PentN 888.57, 2CN-(6Z)BP-Hex 919.56. For each analysis, 100–200 pmol of the starting material (2CN-(6Z)BP) was analyzed with a linear gradient method starting with 25% solvent C/75% solvent D, raising to 65% solvent C over 10 min then decreasing back to 25% solvent C over 2 min with a 2 min post run.

**ESI-MS-MS.** To obtain the purified C41(DE3) membrane fraction product, a reaction containing 10 nmol 2CN-(6Z)BP and 0.5 mg/mL C41(DE3) of the membrane fraction were incubated in buffered reaction conditions (100 mM Bicine pH 3.5, 25737

ACS Omega 2021, 6, 25729−25741

https://doi.org/10.1021/acsomega.1c04036

ACS Omega 2021, 6, 25729−25741

https://doi.org/10.1021/acsomega.1c04036
8.0, 200 mM KCl, 15 mM cholate, and 5 mM MgCl₂ overnight at 37 °C. The unidentified product was then purified from the reaction mixture with RP-HPLC (condition A) using an Agilent Zorbax XBD-C18 column (9.4 mm × 250 mm, 5 μm, 80 Å), dried under vacuum, and resuspended in n-propanol and 25 mM ammonium bicarbonate (1:1). The isolated protein was then analyzed by MS using a VELOS Pro Dual-Pressure Linear Ion Trap with direct infusion (100 μL/min) in negative ion mode. MS was performed by CID of the parent ion with an m/z of 888.60.

Cell Envelope Fraction Assays with 2CN-BP. To assess the transferase activity of E. coli C41(DE3) and MG1655 membrane fractions with 2CN(6Z)BP, the membrane fractions (0.5 mg/mL total protein) were incubated with 2CN(6Z)BP in reaction buffer at 37 °C for 2 h. Reactions contained 5 μM 2CN(6Z)BP unless otherwise indicated. The turnover of 2CN-BP was monitored by RP-HPLC and fluorescence detection. Inactivated wild-type C41(DE3) membrane fractions were prepared by heating wild-type membrane fractions to 95 °C for 20 min. In experiments with inactivated membrane fractions, equivalent amounts (0.5 mg/mL total protein) of inactive and active membrane fractions were used.

Preparation of Total Lipids. To identify accumulated BP and BP-linked intermediates in wild-type and mutant E. coli MG1655 or C41(DE3), samples of total lipids were separated and analyzed by ESI-LC-MS. To prepare samples of total lipids, cultures consisting of 100 mL of Miller LB were prepared from a 1:1000 dilution of an overnight starter culture and grown to an OD₅₉₅ of 1.0. In experiments with MG1655 and MG1655ΔarnD, cells were cultured with or without 100 μM FeSO₄. Cells were then pelleted at 15,000 RCF for 15 min, resuspended in 1.6 mL of deionized water and transferred to glass centrifuge tubes. To aid in cell lysis and solubilization of prenyl and phospholipids, each cell suspension was mixed with 2 mL of chloroform and 4 mL of methanol to form a single-phase Bligh and Dyer solution.36 Tubes were vortexed and incubated for 20 min at 5000 RCF and washed with 25 mL of 10 mM phosphate buffered saline (PBS) pH 7.4. The cells were then resuspended in 3.2 mL PBS and divided equally between two 15 mL glass centrifuge tubes (1.6 mL of cell resuspension in each tube). To each glass centrifuge tube, 2 mL of chloroform and 4 mL of methanol was added to create a single-phase Bligh and Dyer solution.56 Tubes were vortexed and incubated for 20 min at room temperature. LPS along with insoluble proteins and nucleic acids were precipitated from the single-phase solution by centrifugation at 2000 RCF for 20 min. The supernatant was discarded, and the crude LPS pellet was washed with a second single-phase Bligh and Dyer solution (see above). The supernatant was discarded and the crude LPS pellet was dried under vacuum to remove any remaining solvent. The LPS pellet was then resuspended in 15 mL of 10 mM Tris–HCl pH 7.5 and 15 mM NaCl with vigorous vortexing and sonication. The LPS pellet was then treated with 100 μg/mL of RNase A and incubated at 65 °C for 30 min. Once the solution cooled to room temperature, 1.5 mM MgCl₂, 0.5 mM CaCl₂, and 50 U of DNase I were added and the solution was incubated at 37 °C for 30 min. After 30 min, 100 μg/mL of proteinase K was added and the solution was incubated at 37 °C for 60 min. Digested proteins and nucleic acids were removed from the LPS samples by converting the solution to a single-phase Bligh and Dyer solution and centrifugation at 5000 RCF for 10 min. The supernatant containing the digested proteins and nucleic acids was discarded, and the insoluble LPS precipitate was dried under vacuum. Finally, the dried LPS was resuspended in 1× PBS to a concentration of approximately 10 mg/mL. Protein and nucleic acid contamination was evaluated by SDS-PAGE and staining with either Coomassie Blue, ethidium bromide, or silver stain.57

Testing Purified LPS as a Donor Substrate for ArnT. Reactions to test LPS as a Ara4N donor substrate contained 200 mM Bicine pH 8.0, 100 mM KCl, 5 mM MgCl₂, 15 mM sodium cholate, 5.0 μM 2CN(6Z)BP, 0.5 mg/mL (total protein) of membrane fraction, and approximately 20 μg of LPS unless otherwise specified. Reactions were incubated for 2 h at 37 °C and monitored by fluorescence using RP-HPLC as previously described for the presence of 2CN(6Z)BP-Ara4N. In a second experiment, incremental amounts of purified LPS (20–80 μg) were added to reactions consisting of the same buffering conditions and incubated for 2 h prior to RP-HPLC analysis. To evaluate the activity of purified ArnT, 0.005 mg/mL of protein was incubated with 5 μM 2CN(6Z)BP in reaction buffer (described above) both with and without approximately 20 μg of LPS. The reactions were monitored by fluorescence using RP-HPLC (condition A) as previously described.

Transferase Assays with Native BP. To assess transferase activity of the ΔarnC membrane fraction with native BP using wild-type LPS as a donor substrate, the ΔarnC membrane fraction (0.5 mg/mL) was incubated in buffered reaction conditions (described above) with 500 μg of LPS in a total volume of 500 μL. Reactions were then incubated for 2 h at 37 °C and extracted twice with 500 μL of n-butanol. The upper phase was collected, dried under vacuum, and resuspended in 50 μL of n-propanol/10 mM ammonium hydroxide (1:1). The reactions were monitored for the formation of BP-Ara4N using ESI-LC-MS with SIM in negative ion mode.

Bacterial Strain Construction. Bacterial mutants (Table S1) were constructed by using the lambda red recombinase method of Datenson and Wanner.58 In brief, parent strains MG1655 or C41 (DE3) were transformed with the plasmid pKD46. Transformants were grown to the log phase at 30 °C in LB supplemented with 50 μg/mL carbenicillin and then induced by the addition of 100 mM arabinose. After 1 h induction, bacteria were made electrocompetent by washing 3–6 times with ice-cold 0.3 M sucrose and then resuspending in 0.3 M sucrose at ~1/50 the original culture volume. This preparation was either transformed immediately or aliquoted and stored at ~80 °C. To transform electrocompetent cells, 50 μL cells were
combined with purified PCR amplicon (~2 μg), generated as described below. This mixture was electroporated at 2500 V followed by the immediate addition of LB media. After overnight recovery at 30 °C, cells were plated on LB agar with the appropriate selective media (chloramphenicol at 10–20 μg/mL for ΔarnC::camR and ΔarnT::camR, kanamycin at 50 μg/mL for ΔarnD::kanR). Resistant colonies were evaluated by colony PCR to confirm the desired insertion. Primers for these reactions are listed in the Supporting Information (Table S2). To remove antibiotic resistance genes, strain CE356 or CE391 was transformed with pCP20, and streaked on LB agar with 50 μg/mL carbenicillin for 30 min. Cloning expression was induced at an OD595 of 1.0 with 1 mM IPTG.

**Molecular Cloning.** Vector, restriction enzyme, and epitope tags used for each plasmid construct are listed in the Supporting Information (Table S3). Gene inserts were amplified from E. coli MG1655 using Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific). Purified inserts and vector were ligated using T4 DNA ligase (Invitrogen) at room temperature for 30 min. Cloning efficiency of arnT in pET-28b was very poor and required incubation with T4 DNA ligase at 16 °C for 16 h. Ligation reactions were chemically transformed into E. coli DH5α cells. Positive clones were confirmed by sequencing (Eurofins). For complementation assays, plasmids were transformed into chemically competent mutants. Cultures were supplemented with 0.1 mM IPTG and 50 μg/mL of kanamycin from the time of inoculation.

**Purification of ArnT.** To solubilize the ArnT protein from the membrane fraction, we followed a previously reported procedure for the extraction of ArnT from Salmonella typhimurium expressed in E. coli. All steps of this protein purification occurred at 4 °C unless otherwise noted. A 5 mL culture of Miller LB broth supplemented with 50 μg/mL of kanamycin was inoculated from a single colony of C41(DE3) cells harboring the pArnT plasmid. Starter cultures were incubated overnight at 37 °C with shaking at 220 rpm. Terrific broth (24 g yeast extract, 20 g tryptone, 0.4% glycerol, and 10 mM PBS in a total volume of 1 L) containing 50 μg/mL of kanamycin were inoculated with a 1:1000 dilution of the starter culture and incubated at 37 °C with shaking at 220 rpm. Protein expression was induced at an OD595 of 1.0 with 1 mM IPTG. Cultures were then incubated overnight at 16 °C with shaking at 220 rpm. Cells were pelleted at 5000 RCF for 15 min and resuspended in buffer A containing 0.1% n-dodecyl-maltoside (DDM). Resuspended cells were lysed with sonication (Fisher Sonic Dismembrator). Lysates were clarified by centrifugation at 100,000 RCF for 60 min. Insoluble fractions were resuspended in buffer A with 1.0% DDM and placed on a spinner overnight at 4 °C. Homogenate was clarified by ultracentrifugation at 100,000 RCF for 60 min. The resulting supernatant was purified using 1 mL of Ni-NTA (Thermo Scientific). The fractions were then analyzed by SDS-PAGE and anti-His (GenScript) Western blotting.

**Purification of ArnD.** To overproduce and isolate ArnD, a 5 mL culture of Miller LB supplemented with 50 μg/mL of kanamycin was inoculated with a single colony of C41(DE3) cells harboring pArnD and grown overnight. Terrific broth (1 L) supplemented with 50 μg/mL of kanamycin was inoculated with a 1:1000 dilution of the overnight culture and incubated at 37 °C with shaking at 220 rpm. Protein expression was induced with 1 mM IPTG at an OD595 of 0.6. Cells were then incubated at 30 °C with shaking at 300 rpm overnight. Cells were harvested at 15,000 RCF for 15 min and resuspended in lysis buffer containing 1% Triton X-100. Resuspended cells were lysed by sonication (Fisher Sonic Dismembrator). Lysates were clarified by centrifugation at 5000 RCF for 20 min. The supernatant was then spun for 1 h at 150,000 RCF. ArnD was isolated from the supernatant with Ni-NTA affinity chromatography and assessed for protein content with SDS-PAGE and anti-His Western blotting. Fractions containing ArnD were dialyzed in buffer B and stored at ~80 °C until use.

**ArnD Activity Analyses with Native BP-Ara4FN.** To enable the characterization of E. coli ArnD, we used crude lipids prepared from MG1655 ΔarnD cultured with 100 μM FeSO4 as a source of native BP-Ara4FN substrate. To prepare lipids from MG1655 ΔarnD, single colonies cultured on LB/agar supplemented with 50 μg/mL of kanamycin were used to prepare 5 mL overnight cultures. Total lipids were prepared from 100 mL of cells at an OD595 of 1.0 as described above. To evaluate the activity of ArnD, 0.20 mg/mL of protein was added to reactions consisting of 50 mM Hepes pH 7.0, 100 mM NaCl, 5 mM MnCl2, 20 mM DTT, and 2 μL of MG1655 ΔarnD lipids in a total volume of 40 μL. Reactions were incubated at 30 °C for 2 h and evaluated for the formation of BP-Ara4N using ESI-LC-MS with 20 μL injections as previously described.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04036.

- HPLC and ESI-LC-MS chromatograms, SDS-PAGE and Western blots of recombinant proteins, SDS-PAGE of LPS, strain list, primers used for mutagenesis, and primers and restriction sites used to prepare plasmid constructs (PDF)

### Accession Codes

ArnC, P77757; ArnT, P76473; ArnD, P76472.

#### AUTHOR INFORMATION

**Corresponding Author**

Jerry M. Troutman — Department of Chemistry and Nanoscale Science Program, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001, United States; orcid.org/0000-0002-8340-462X; Phone: 704-687-5180; Email: jtroutm3@uncc.edu

**Authors**

Beth A. Scarbrough — Nanoscale Science Program, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001, United States

Colleen R. Eade — Department of Chemistry, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001, United States

Amanda J. Reid — Nanoscale Science Program, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001, United States

Tiffany C. Williams — Department of Chemistry, The University of North Carolina at Charlotte, Charlotte, North Carolina 28223-0001, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c04036
Glucosamine found as a substituent of both phosphate groups in lipid A modifications in polymyxin-resistant Salmonella typhimurium and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J. Biol. Chem. 2001, 276, 43122–43131.

(19) Yan, A.; Guan, Z.; Raetz, C. R. H. An undecaprenyl phosphate-aminobiose flipase required for polymyxin resistance in Escherichia coli. J. Biol. Chem. 2007, 282, 36077–36089.

(20) Trent, M. S.; Ribeiro, A. A.; Lin, S.; Cotter, R. J.; Raetz, C. R. H. An inner membrane enzyme in Salmonella and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J. Biol. Chem. 2001, 276, 43122–43131.

(21) Reid, A. J.;Scarborough, B. A.; Williams, T. C.; Gates, C. E.; Eade, C. R.; Troutsam, J. M. General Utilization of Fluorescent Polysaccharides with Sugar Selective Phosphoglycosyltransferases. Biochemistry 2020, 59, 615–626.

(22) Scott, P. M.; Erickson, K. M.; Troutsam, J. M. Identification of the Functional Roles of Six Key Proteins in the Biosynthesis of Enterobacteriaceae Colanic Acid. Biochemistry 2019, 58, 1818–1830.

(23) Sharma, S.; Erickson, K. M.; Troutsam, J. M. Complete Tetrasaccharide Repeat Unit Biosynthesis of the Immunomodulatory Bacteroides fragilis Capsular Polysaccharide A. ACS Chem. Biol. 2017, 12, 92–101.

(24) Trent, M. S.; Ribeiro, A. A.; Doerrler, W. T.; Lin, S.; Cotter, R. J.; Raetz, C. R. H. Accumulation of a polysaccharide-linked amino sugar in polymyxin-resistant Salmonella typhimurium and Escherichia coli: structural characterization and transfer to lipid A in the periplasm. J. Biol. Chem. 2001, 276, 43132–43144.

(25) Mann, E.;Whitfield, C. A widespread three-component mechanism for the periplasmic modification of bacterial glycoconjugates. Antimicrob. Agents Chemother. 2015, 59, 2051–2061.

(26) Fredrich, J. M.; Tran, K.;Wall, D. A pmrA constitutive mutant sensitizes Escherichia coli to deoxycholic acid. J. Bacteriol. 2006, 188, 1180–1183.

(27) Groisman, E. A. The pleiotropic two-component regulatory system PmrP-PmrQ. J. Bacteriol. 2001, 183, 1835–1842.

(28) Cannatelli, A.; Giani, T.; Azeza, N.; Di Pilato, V.; Principe, L.; Luzzaro, F.; Galeotti, C. L.; Rossolini, G. M. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an Escherichia coli strain of clinical origin. Sci. Rep. 2017, 7, 5071.
(30) Westen, M. M.; Kox, L. F.; Chammongpol, S.; Soncini, F. C.; Groisman, E. A. A signal transduction system that responds to extracellular iron. *Cell 2000*, 103, 113−125.

(31) Zhou, Z.; Lin, S.; Cotter, R. J.; Raetz, C. R. H. Lipid A modifications characteristic of Salmonella typhimurium are induced by NH4VO3 in Escherichia coli K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. *J. Biol. Chem. 1999*, 274, 18503−18514.

(32) Hagiwara, D.; Yamashino, T.; Mizuno, T. A Genome-wide view of the Escherichia coli BasS-BasR two-component system implicated in iron-responses. *Biosci., Biotechnol., Biochem. 2004*, 68, 1758−1767.

(33) Gibbons, H. S.; Kalb, S. R.; Cotter, R. J.; Raetz, C. R. H. Role of Mg2+ and pH in the modification of Salmonella lipid A after endocytosis by macrophage tumour cells. *Mol. Microbiol. 2005*, 55, 425−440.

(34) Song, F.; Guan, Z.; Raetz, C. R. H. Biosynthesis of undecaprenyl phosphate-galactosamine and undecaprenyl phosphate-glucose in Francisella novicida. *Biochemistry 2009*, 48, 1173−1182.

(35) Guan, S.; Bastin, D. A.; Verma, N. K. Functional analysis of the O antigen glucosylation gene cluster of Shigella flexneri. *Amino-4-deoxy-l-arabinose Derivatives Demonstrate that ArnT is an endocytosis by macrophage tumour cells. Mol. Microbiol. 2005*, 55, 425−440.

(36) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 1959*, 37, 911−917.

(37) Henderson, J. C.; O’Brien, J. P.; Brodbelt, J. S.; Trent, M. S. Isolation and chemical characterization of lipid A from gram-negative bacteria. *J. Visualized Exp. 2013*, 79, No. e50623.

(38) Jeong, H.; Barbe, V.; Lee, C. H.; Vallenet, D.; Yu, D. S.; Choi, S.-H.; Couloux, A.; Lee, S. W.; Toon, S. H.; Cattolico, L.; Hur, C. G.; Park, H. S.; Ségurens, B.; Kim, S. C.; Oh, T. K.; Lenski, R. E.; Studier, F. W.; Daegelen, P.; Kim, J. F. Genome sequences of Escherichia coli B strains REL606 and BL21(D3E3). *J. Mol. Biol. 2009*, 394, 644−652.

(39) Jansson, P. E.; Lindberg, A. A.; Lindberg, B.; Wollin, R. Structural studies on the hexose region of the core in lipopolysaccharides from Enterobacteriaceae. *Eur. J. Biochem. 1981*, 115, 571−577.

(40) Bretscher, L. E.; Morrell, M. T.; Funk, A. L.; Klug, C. S. Purification and characterization of the L-Ara4N transferase protein AmT from Salmonella typhimurium. *Protein Expression Purif. 2006*, 46, 33−39.

(41) Petsch, D.; Anspach, F. B. Endotoxin removal from protein solutions. *J. Biotechnol. 2000*, 76, 97−119.

(42) Olagon, C.; Monjaras Feria, J.; Gruenwald-Gruber, C.; Blaukopf, M.; Valvano, M. A.; Kosma, P. Synthetic Phosphodiester-Linked 4-Amino-4-deoxy-l-arabinose Derivatives Demonstrate that AmT is an Inverting Aminoarabinosyl Transferase. *ChemBioChem 2019*, 20, 2936−2948.

(43) Barr, K.; Rick, P. D. Biosynthesis of enterobacterial common antigen in Escherichia coli. In vitro synthesis of lipid-linked intermediates. *J. Biol. Chem. 1987*, 262, 7142−7150.

(44) Cartee, R. T.; Forsee, W. T.; Bender, M. H.; Ambrose, K. D.; Yother, J. CPS E from type 2 Streptococcus pneumoniae catalyzes the reversible addition of glucose-1-phosphate to a polypropenyl phosphate acceptor, initiating type 2 capsule repeat unit formation. *J. Bacteriol. 2005*, 187, 7425−7433.

(45) Osborn, M. J.; Tze-Yuen, R. Y. Biosynthesis of bacterial lipopolysaccharide. VII. Enzymatic formation of the first intermediate in biosynthesis of the O-antigen of Salmonella typhimurium. *J. Biol. Chem. 1968*, 243, 5145−5152.

(46) Gantt, R. W.; Pellett-Pain, P.; Singh, S.; Zhou, M.; Thorson, J. S. Broadening the scope of glycosyltransferase-catalyzed sugar nucleotide synthesis. *Proc. Natl. Acad. Sci. U. S. A. 2013*, 110, 7648−7653.

(47) Chng, S. S.; Grönenberg, L. S.; Kahne, D. Proteins required for lipopolysaccharide assembly in Escherichia coli form a transenvelope complex. *Biochemistry 2010*, 49, 4565−4576.

(48) Sherman, D. J.; Xie, R.; Taylor, R. J.; George, A. H.; Okuda, S.; Foster, P. J.; Needleman, D. J.; Kahne, D. Lipopolysaccharide is transported to the cell surface by a membrane-to-membrane protein bridge. *Science 2018*, 359, 798−801.

(49) Whitfield, C.; Trent, M. S. Biosynthesis and export of bacterial lipopolysaccharides. *Annu. Rev. Biochem. 2014*, 83, 99−128.

(50) Owens, T. W.; Taylor, R. J.; Pahil, K. S.; Bertani, B. R.; Ruiz, N.; Kruse, A. C.; Kahne, D. Structural basis of unidirectional export of lipopolysaccharide to the cell surface. *Nature 2019*, 567, 550−553.

(51) Paradis-Bléau, C.; Krittik, G.; Ornola, K.; Typas, A.; Bernhardt, T. G. A genome-wide screen for bacterial envelope biogenesis mutants identifies a novel factor involved in cell wall precursor metabolism. *PLoS Genet. 2014*, 10, No. e1004056.

(52) Jorgenson, M. A.; Kannan, S.; Laubacher, M. E.; Young, K. D. Dead-end intermediates in the enterobacterial common antigen pathway induce morphological defects in Escherichia coli by competing for undecaprenyl phosphate. *Mol. Microbiol. 2016*, 100, 1−14.

(53) Jorgenson, M. A.; Young, K. D. Interrupting Biosynthesis of O Antigen or the Lipopolysaccharide Core Produces Morphological Defects in Escherichia coli by Sequestering Undecaprenyl Phosphate. *J. Bacteriol. 2016*, 198, 3070−3079.

(54) Kahler, C. M.; Sarkar-Tyson, M.; Kibble, E. A.; Stubbs, K. A.; Vrielink, A. Enzyme targets for drug design of new anti-virulence therapeutics. *Curr. Opin. Struct. Biol. 2018*, 53, 140−150.

(55) Troutman, J. M.; Erickson, K. M.; Scott, P. M.; Hazel, J. M.; Martinez, C. D.; Dodbele, S. Tuning the production of variable length, fluorescent polyisoprenoids using surfactant-controlled enzymatic synthesis. *Biochemistry 2015*, 54, 2817−2827.

(56) Hankins, J. V.; Madsen, J. A.; Needham, B. D.; Brodbelt, J. S.; Trent, M. S. The outer membrane of Gram-negative bacteria: lipid A isolation and characterization. *Methods Mol. Biol. 2013*, 966, 259−258.

(57) Fomsgaard, A.; Freudenberg, M. A.; Galanos, C. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *J. Chromatogr. A. 1990*, 28, 2627−2631.

(58) Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A. 2000*, 97, 6640−6645.