Lipid A

9

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Contents

Abstract

Diverse lipid A structures have been observed in a multitude of Gram-negative bacteria, but the metabolic logic of lipid A biosynthesis is widely conserved. This chapter will start by describing the nine constitutive enzymes of the Raetz pathway, which catalyze conserved lipid A biosynthetic reactions that depend on cytoplasmic cofactors. Concomitant with lipid A export and assembly on the cell surface, a number of regulated covalent modifications of lipid A can occur in the extracytoplasmic compartments. The narrow phylogenetic distribution of the lipid A modification enzymes, combined with the diverse regulatory signals governing their expression, is responsible for most of the lipid A structural diversity that is observed in nature. By focusing on *E. coli* as a model system,

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the general principles of lipid A biosynthesis and assembly are revealed to inform related processes that occur in more divergent organisms.

1 Introduction

In 1892, Richard Pfeiffer first defined endotoxin as a heat-stable toxic substance that was released upon disruption of microbial envelopes (Beutler and Rietschel 2003). The bioactive lipid A component of lipopolysaccharide (LPS) is arguably the most potent of the substances that fit Pfeiffer's endotoxin definition. Gram-negative bacteria can modulate the structure of lipid A in order to evade detection by the host immune system (Raetz et al. 2007). This chapter summarizes the recently elucidated pathways for the biosynthesis and transport of lipid A in *Escherichia coli*, which provides a framework for understanding lipid A structure and function in all Gram-negative bacteria.

2 Endotoxin Biosynthesis

The molecular genetics and enzymology of the conserved steps of lipid A biosynthesis are best characterized in *E. coli*, as shown in Fig. 1. The Raetz pathway begins with the key precursor molecule UDP-GlcNAc, which is also the first substrate for peptidoglycan biosynthesis (Raetz and Whitfield 2002). The first enzyme in lipid A biosynthesis is a cytoplasmic acyltransferase LpxA, which selectively transfers thioester-activated 3-OH-14:0 from acyl carrier protein (ACP) to the 3-OH of UDP-GlcNAc. The crystal structure of LpxA revealed a homotrimeric molecule, which self-associates by a distinctive left-handed parallel β-helix motif (Raetz and Roderick 1995). *E. coli* LpxA is extraordinarily selective for 3-OH-14:0-ACP as the acyl donor substrate, while the *Pseudomonas aeruginosa* LpxA prefers 3-OH-10:0-ACP. However, mutating certain key residues lining the active site cleft could modulate acyl chain selection. For example, the specificity for the G173M mutant of *E. coli* LpxA was shifted to 3-OH-10:0-ACP. In contrast, the specificity of *P. aeruginosa* LpxA could be extended to accommodate 3-OH-14:0-ACP in the corresponding M169G mutant (Wyckoff et al. 1998). These findings demonstrated the existence of precise hydrocarbon rulers in LpxAs, which can explain variations in lipid A acylation that are observed between different organisms.

The acylation of UDP-GlcNAc by LpxA is thermodynamically unfavorable, and the first committed step in lipid A biosynthesis is the subsequent deacetylation catalyzed by LpxC (EnvA). LpxC is a Zn^{2+}-dependent enzyme that is an established target for antibiotic development (Onishi et al. 1996). The crystal and NMR structures of *Aquifex* LpxC revealed two slightly different models for the mechanism of catalysis, but both include a critical role for Zn^{2+} (Coggins et al. 2003; Whittington et al. 2003). Most LpxC inhibitors are hydroxamate compounds that interact with the

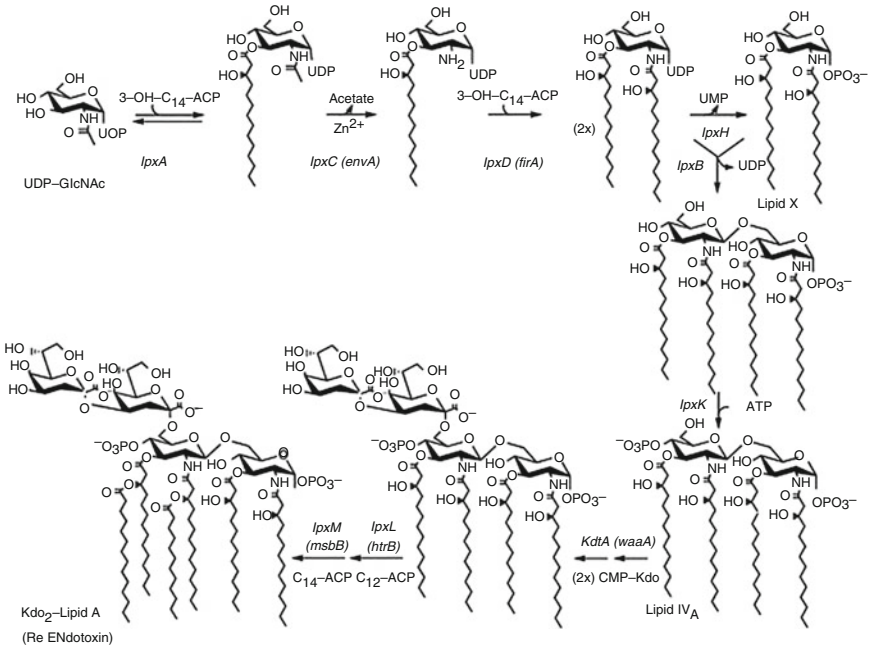

Fig. 1 The Raetz pathway for synthesis of Kdo_2-lipid A. LpxA catalyzes the addition of 3-OH-14:0 to position 3 of UDP-GlcNAc. LpxC then hydrolyzes the acetamido group at position 2, which allows LpxD to add a second 3-OH-14:0 group. LpxH cleaves the nucleotide to generate lipid X, which is then condensed with UDP-diacyl-GlcN to generate the disaccharide 1-phosphate. The 4′-kinase LpxK then generates lipid IV_A, which is converted into Kdo_2-lipid IV_A by a bifunctional Kdo transferase KdtA. Kdo_2-lipid IV_A is a substrate for the LpxL and LpxM acyltransferases, which generate the acyloxyacyl linkages at positions 2′ and 3′, respectively (Originally published in Bishop (2010), published with kind permission of ©Springer Science+ Business Media New York, 2003. All rights reserved)

catalytic Zn^{2+} ion. Current challenges are aimed at the development of inhibitors with the ability to evade efflux pumps that provide resistance, particularly in pseudomonads (Lee et al. 2016). Following deacetylation, an *N*-linked 3-OH-14:0 moiety is incorporated from ACP by LpxD (FirA) to generate UDP-2,3-diacylglucosamine (Kelly et al. 1993; Bartling and Raetz 2009). A highly selective pyrophosphatase LpxH then cleaves UDP-2,3-diacylglucosamine to form lipid X (Babinski et al. 2002; Cho et al. 2016). Next, the disaccharide synthase, LpxB, condenses UDP-2,3-diacylglucosamine and lipid X to generate the β-1′,6-linkage found in all lipid A molecules. The membrane-bound 4′ kinase LpxK then phosphorylates the disaccharide 1-phosphate to produce lipid IV_A, which is an important pharmacological agent because it functions as an endotoxin antagonist in human cell lines (Garrett et al. 1997; Emptage et al. 2012). Next, two 3-deoxy-D- *manno*-2-octulosonic acid (Kdo) sugars are incorporated by a Kdo transferase, which is encoded by the *kdtA* (*waaA)* gene, using the labile nucleotide CMP-Kdo as the

Kdo donor (Schmidt et al. 2012). The final lipid A biosynthetic steps that occur on the cytoplasmic side of the inner membrane depend on the prior addition of the Kdo sugars and involve the transfer of lauroyl (12:0) and myristoyl (14:0) groups from ACP to the distal glucosamine unit to produce acyloxyacyl linkages; the reactions are catalyzed at the at the 2'-position by LpxL (HtrB) and at the 3′-position by LpxM (MsbB), respectively (Six et al. 2008; Dovala et al. 2016). Under conditions of cold growth at 12 °C, LpxL is replaced by LpxP, which has a preference for palmitoleate (16:1cisΔ^9) in ACP (Carty et al. 1999). The incorporation of an unsaturated acyl chain into lipid A likely increases membrane fluidity under cold growth conditions. Viable mutants that lack acyloxyacyl linkages in lipid A are attenuated for virulence and reveal the importance of the lipid A acylation pattern in inflammation (Vorachek-Warren et al. 2002). All other enzymatic steps of the Raetz pathway, and those for the biosynthesis of CMP-Kdo, are essential for cell viability and, thus, provide potential targets for antibiotic development.

3 The Outer Membrane Permeability Barrier

LPS contains phosphate and acidic sugars and is therefore negatively charged. In order to reduce the electrostatic repulsion between LPS molecules at the cell surface, the bacterial outer membrane (OM) sequesters divalent cations, mainly Mg^{+2}, which neutralize the negative charges and maintain the integrity of the OM. The presence of hydrogen-bond donors and acceptors in the lipid A molecule allows for additional lateral interactions that cannot occur between phospholipid molecules (Nikaido 2003). Moreover, the six or seven saturated acyl chains of lipid A serve to reduce the fluidity of the OM bilayer compared with the inner membrane. The tight lateral interactions between LPS, combined with low membrane fluidity, provide a permeability barrier in the OM to lipophilic solutes and detergents. However, cationic antimicrobial peptides (CAMPs) can displace Mg^{+2} ions from the cell surface to promote their uptake by the bacterial cell (Hancock et al. 1995).

4 Lipid A Modifications

Considering the importance of Mg^{+2} in maintaining the OM permeability barrier, it is not surprising that Mg^{+2} limitation and CAMPs can regulate the covalent structure of lipid A by triggering the PhoP/PhoQ and PmrA/PmrB signal transduction pathways (Bader et al. 2005; Groisman 2001). Several covalent modifications of lipid A have been characterized in *E. coli* (Fig. 2). PagP is a transacylase that incorporates a palmitate chain at position 2 (Bishop et al. 2000). Moreover, the phosphate groups at positions 1 and 4′ of the lipid A disaccharide backbone can be modified with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (pEtN), which serve to reduce the overall negative charge of lipid A (Gunn et al. 1998). Lipid A modifications occur in the extracytoplasmic compartments and help to restore the OM permeability barrier and provide resistance to CAMPs.

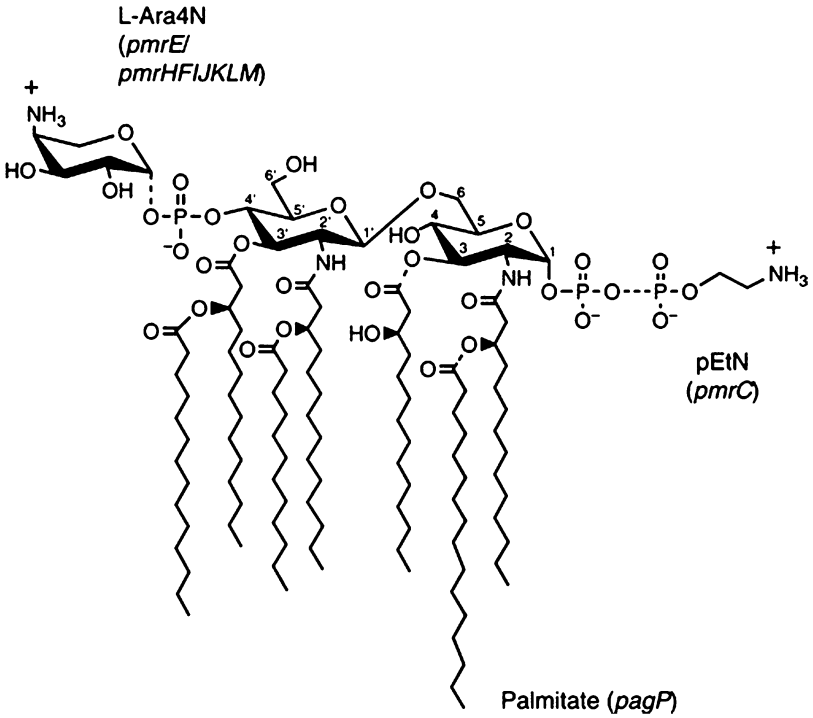

Fig. 2 Regulated covalent lipid A modifications in *E. coli*. The conserved lipid A nucleus can be modified by the addition of L-Ara4N and pEtN to the phosphate groups and by the addition of a palmitate chain at position 2. Palmitoylation of lipid A is under the direct control of PhoP/PhoQ, while PmrA/PmrB controls the phosphate modifications (Originally published in Bishop (2010), published with kind permission of ©Springer Science+Business Media New York, 2003. All rights reserved)

5 L-Ara4N Cluster

PmrA/PmrB is only one of several clusters of *pmr* genes that were originally identified in *p*oly*m*yxin-*r*esistant mutants of *E. coli*. The *pmrF* (*pbgP*) locus encodes an operon of seven open reading frames *pmrHFIJKLM*, which, together with the unlinked *pmrE* (*ugd*), are required for L-Ara4N synthesis (Gunn et al. 1998). The first step involves the conversion of UDP-glucose into UDP-glucuronic acid catalyzed by a dehydrogenase encoded by *pmrE*. Complex regulation of dehydrogenase gene expression reflects the fact that UDP-glucuronic acid is a precursor for both colanic acid-containing capsules and L-Ara4N. Next, PmrI (ArnA) catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate a novel UDP-4-keto-pyranose intermediate (Breazeale et al. 2002; Genthe et al. 2016). PmrH (ArnB) then catalyzes a transamination reaction using glutamate as the amine donor to generate UDP-L-Ara4N (Breazeale et al. 2003). The crystal structure of PmrH has verified

that a pyridoxal phosphate cofactor contributes to the catalytic mechanism (Noland et al. 2002). Interestingly, PmrI contains a second domain that formylates the 4-amine of UDP-L-Ara4N (Breazeale et al. 2005). The resultant UDP-L-Ara4-Formyl-N is transferred by PmrF (ArnC) to the membrane-anchored undecaprenyl phosphate, forming undecaprenyl phosphate-L-Ara4-Formyl-N. The formylation step may drive forward the equilibrium of the transamination step, which is thermodynamically unfavorable. The necessity of a subsequent deformylation step catalyzed by PmrJ (ArnD) is dictated by the fact that undecaprenyl phosphate-L-Ara4N is the substrate for PmrK (ArnT), which catalyzes the final transfer of L-Ara4N to lipid A at the periplasmic surface of the inner membrane (Trent et al. 2001a, b; Petrou et al. 2016). Roles for PmrL and PmrM (ArnE and ArnF) in flipping undecaprenyl phosphate-L-Ara4N across the inner membrane have been confirmed (Yan et al. 2007).

6 EptA

The pEtN-adding enzyme EptA has been cloned from *E. coli* (Lee et al. 2004), and a homologous gene from *Neisseria* has been associated with the addition of pEtN to lipid A (Cox et al. 2003; Wanty et al. 2013). The EptA-encoding gene is the upstream open reading frame that is part of the *pmrAB* operon and is also known as *pmrC* (*pagB*). The EptA active site is located on the periplasmic side of the inner membrane. Phosphatidylethanolamine is the reported pEtN donor, and several EptA homologues are likely responsible for pEtN addition to other cell envelope components including the inner core sugars of LPS (Reynolds et al. 2005). It is noteworthy that roughly one third of *E. coli* lipid A carries a diphosphate moiety instead of the monophosphate at position 1 (Touzé et al. 2008) and that the responsible phosphorylating enzyme shares with EptA the ability to generate a phosphoanhydride bond at the same position in lipid A.

7 PagP

PagP functions to transfer a palmitate chain from a phospholipid to the hydroxyl group of the *N*-linked 3-OH-14:0 chain on the proximal glucosamine unit of lipid A (Bishop 2005). PagP is regulated by PhoP/PhoQ and was the first enzyme of lipid A biosynthesis shown to be localized in the OM (Bishop et al. 2000; Jia et al. 2004). Since thioester-containing substrates are not available in the extracellular compartments, PagP uses a phospholipid as the palmitoyl donor instead. PagP was first identified in the salmonellae due to its role in providing resistance to cationic antimicrobial peptides (Guo et al. 1998) and was subsequently purified from *E. coli* (Bishop et al. 2000). In addition to these enteric pathogens, PagP homologues are present in the respiratory pathogens *Legionella pneumophila* and *Bordetella bronchiseptica*, where PagP has been shown to be necessary for disease causation in animal models of infection (Preston et al. 2003; Robey et al. 2001). PagP

homologues are also found in *Yersinia*, *Photorhabdus*, and *Erwinia* species, which adopt pathogenic lifestyles in animals, insects, and plants, respectively. The solution and crystal structures of PagP indicate that the enzyme is activated in the OM in response to perturbations of lipid asymmetry (Ahn et al. 2004; Hwang et al. 2002). Phospholipids must gain access to the PagP hydrocarbon ruler after first migrating into the OM external leaflet (Khan et al. 2007, 2009, 2010a, b; Cuesta-Seijo et al. 2010). Some evidence indicates PagP can function as a sensory transducer, which is triggered by perturbations to OM lipid asymmetry (Smith et al. 2008). PagP also displays a unique regiospecificity for the lipid A 3′-position in *Pseudomonas* (Thaipisuttikul et al. 2014) and the ability to palmitoylate the headgroup of phosphatidylglycerol in *Salmonella* (Dalebroux et al. 2014). The lysophospholipid by-product of the PagP reaction is rapidly transported to the inner membrane and reacylated (Hsu et al. 1989). PagP is the only enzyme of lipid A biosynthesis known to be located in the outer membrane of *E. coli*, but other outer membrane lipid A modification enzymes include the latent *Salmonella* lipid A deacylases PagL and LpxR, which are functional during heterologous expression in *E. coli* (Bishop 2008).

8 LPS Transport

LPS is an elaborate phospholipid that is extensively decorated with sugars, and its presence provides a barrier against harmful detergents and lipophilic antibiotics. LPS transport to the outer membrane is essential for survival and thus provides a target for new antibiotics. Structural studies have now disclosed a remarkable molecular mechanism for LPS delivery and insertion into the external leaflet of the outer membrane (Bishop 2014; Okuda et al. 2016). LPS molecules are mostly assembled by biosynthetic enzymes exposed to the cytoplasm, but the activity of several LPS-modifying enzymes in the extracytoplasmic compartments can be used to monitor the molecule on its journey to the outer membrane. First, the lipid A, along with its attached core sugars, is translocated by the ABC transporter MsbA to the external leaflet of the inner membrane. Here, a terminal O-sugar chain can be incorporated before the seven Lpt proteins of the LPS transport system take over (Fig. 3a). Next, the ABC transporter complex $LptB_2FG$ in the inner membrane detaches LPS from the external leaflet and ushers it along a protein filament, made up of LptA and LptC, which tracks through the periplasmic space. Finally, a complex between LptD and LptE in the outer membrane functions as a station to complete the track for delivering and inserting LPS into the external leaflet of the outer membrane.

LptD is built primarily from β-strands that fold into two domains: a β-jellyroll and a β-barrel. The smaller of the two, the β-jellyroll, extends away from the membrane and contains a lipophilic slot adapted to bind the lipid A while leaving the LPS sugar chain exposed. The same β-jellyroll fold is found in LptA and LptC, which interconnect to align their slots in an extended lipophilic groove that spirals across the periplasmic space between the inner and outer membranes. Thus, it seems that the LptD β-jellyroll is the terminal track for delivering LPS to the outer membrane (Fig. 3b). The structure of this LptD platform is positioned to deliver the LPS directly

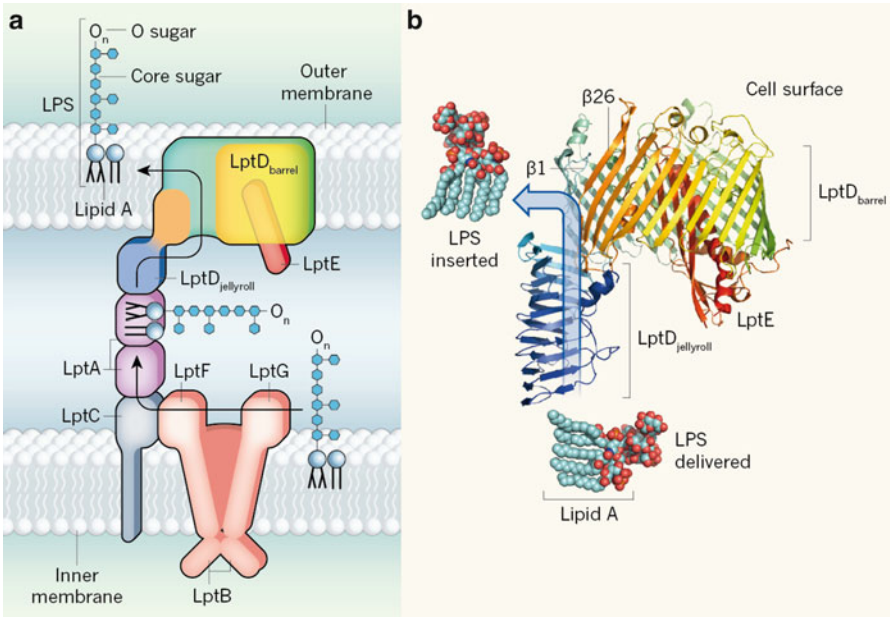

Fig. 3 Organization of the Lpt transport system and structure of the LptD–LptE complex. **a** LPS comprises a lipid A membrane anchor, a core sugar unit, and a terminal O-sugar chain. Following lipid A-core assembly in the cytoplasm and its export to the external leaflet of the inner membrane, the O-antigen can be attached before the seven Lpt proteins transport LPS from the inner to the outer membrane. **b** The crystal structure of the complex formed between LptD and LptE proteins in the outer membrane reveals the route for LPS delivery and insertion into the external leaflet (*blue arrow*). The structure shows how the sugars of LPS are positioned by the β-jellyroll region of LptD to engage first with LptE, which probably reorients the LPS into a vertical position. Delivery of the LPS into the outer membrane external leaflet then occurs by lateral migration through an opening between the first (1) and final (26) β-strands in the LptD β-barrel (First published in Nature, 511, 37–38, 2014 by Nature Publishing Group)

into the cavity of the much larger domain, the β-barrel, which is composed of 26 membrane-spanning β-strands, the greatest number of these strands ever described. The LptD β-barrel takes the shape of a kidney bean, with the lobe opposite the β-jellyroll plugged with a completely different outer membrane protein. The LptE lipoprotein is positioned almost entirely inside the LptD β-barrel so as to interact most readily with the sugar chain of the delivered LPS; it has only its lipid-modified end anchored outside the β-barrel and within the inner leaflet of the outer membrane. The other lobe, located adjacent to the β-jellyroll, remains empty on the periplasmic side so that it can transiently accommodate LPS on its passage into the external leaflet of the outer membrane. The empty lobe of the LptD β-barrel is closed at the cell surface by the L4 loop, which likely repositions itself in order to create a transitory opening where the LPS sugar chain can emerge. The LPS lipid A region then diffuses laterally into the external leaflet of the outer membrane through a weakly hydrogen-bonded gate located between β-strands 1 and 26.

LptF and LptG each display their own periplasmic β-jellyroll domains that likely help to propel LPS into the LptA–LptC filament during LptB-catalyzed ATP hydrolysis, but it remains to be determined what impels LPS to emerge from the LptD–LptE complex so it inserts into the outer membrane. LPS carries a strong net negative charge, and electrostatic repulsion between neighboring molecules might help to move them along the filament; bridging of LPS by positively charged magnesium ions in the outer membrane might then facilitate its lateral egress from LptD. Roughly 200 LptD–LptE complexes are evenly distributed over the surface of a typical bacterium, and each complex delivers about five LPS molecules to the cell surface every second. A synthetic cyclic-peptide antibiotic prevents growth of *Pseudomonas aeruginosa* by targeting its LptD and blocking LPS transport. The structures of the Lpt transport system components thus facilitate the design of better antibiotics, in addition to further clarifying the mechanism by which LPS quite literally rolls out the barrel.

9 Research Needs

Lipid A and its regulated covalent modifications exhibit profound effects on bacterial and human physiology. Novel endotoxin antagonists and immune adjuvants have already been developed from modified lipid A structures (Christ et al. 1995; Ulrich and Myers 1995). By revealing the biochemical details of lipid A structure and function, we hope to understand its role in bacterial pathogenesis and to intervene with novel treatments for infection. However, we must remind ourselves that multiple molecular subtypes of lipid A are acting in concert in the bacterial cell. The need to unravel the interactions between individual lipid A modifications will provide fertile ground for future research.

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