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COMMENTARY

The Importance of Being an Antagonist as Well as Persistent

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ABSTRACT The current work by Jain et al. (S. Jain, A. M. Chang, M. Singh, J. S. McLean, et al., J Bacteriol 201:e00683-18, 2019, https://doi.org/10.1128/JB.00683-18) reports the cloning of the lipid A deacylase gene of *Porphyromonas gingivalis* and the phenotypic characterization of the enzyme. Attempts to clone the gene and thus provide proof of the existence of this enzyme had gone on for 2 decades. The enzyme is central to the bacterium's ability to modify and tailor the structure of its lipid A, changing a lipid A that is a moderate Toll-like receptor 4 (TLR4) agonist to an antagonist or silencer and thereby potentially changing the course of infection.

KEYWORDS LPS, *Porphyromonas gingivalis*, TLR4, deacylation, lipid A

Bacterial lipopolysaccharide (LPS) has been among the most well-studied bacterial virulence factors during the past decades and continues to be the favorite bacterial antigen/modulator employed in multitudes of studies of host responses. The existence of LPS was first described a century ago, and it was first isolated about 60 years ago. It has taken half a century to understand its structure, biosynthesis, pathogenesis, and role in bacterial cell morphology, membrane organization, and survival, as well as its very diverse and far-ranging effects on hosts and host systems. For example, septic infection with a Gram-negative bacterium can cause death in a matter of 2 to 3 h in a susceptible host (1). To add insult to injury, antibiotics which result in death of the infecting bacteria may only make matters worse, as LPS toxicity is not dependent on a live bacterium and lysis of a bacterium may actually enhance its effects by promoting the formation of blebs containing LPS that more quickly enter host cells. It is also resistant to autoclaving.

The structure of this complex amphipathic macromolecule is unique to Gram-negative bacteria and is very well conserved among bacterial genera and species. There are three chemical/structural components of LPS: the lipid A, the core oligosaccharide, and the O antigen. The lipid A structure is the toxic moiety, is anchored in the bacterial outer membrane, and is the relatively invariant component. The remainder of the macromolecule, the core and O antigen, extends distally from the bacterial cell surface. The O antigen is made up of repeating units of sugars, sometimes becoming so long that the molecular weight is much greater than those of most proteins. In contrast to the lipid A portion, this part of the LPS molecule is highly variant and is the basis of many serotyping schemes for bacteria. For example, there are more than 1,000 different serotypes of *Salmonella enterica* serovar Typhimurium singularly based on the O antigen. In addition, there are a few bacterial species, such as *Neisseria* spp., that make little or no O-antigen polysaccharide.

During the 1970s and 1980s, much of the biosynthesis and genetics of LPS was deciphered by M. J. Osborn and others using *S.* Typhimurium as the model organism (2–4). It was known that LPS exists only in the outer leaflet of the Gram-negative outer membrane but is synthesized inside the cell in the cytoplasm and inner membrane. This provided a conundrum as to how such a large and amphipathic macromolecule is synthesized inside the cell but exists exclusively on the cell surface, traversing two membranes. After many years of work by multiple labs, the long list of genes encoding

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the LPS biosynthetic enzymes and the location and method of putting the pieces of the molecule together were identified. This biosynthetic pathway of LPS, and especially lipid A, is conserved among Gram-negative bacteria. However, it is now recognized that some bacterial species modify the initially synthesized lipid A. For reviews, see references 5 and 6.

Coincident with the goal of biochemists and bacteriologists to define the LPS structure, biochemistry, and biosynthesis, immunologists were captivated by its biological properties and how it interacted with and affected host cells and systems. Defining the mechanisms of interplay between LPS and the host was deemed so important to medicine that the discoverers of the mechanism of host recognition of an LPS molecule were awarded a Nobel Prize for their work (7). The mechanism of toxicity of lipid A is not direct but is through the induction of arms/molecules of innate immunity. Specifically, lipid A is recognized by Toll-like receptor 4 (TLR4), which is present on the surfaces of macrophages, monocytes, neutrophils, and dendritic cells. With the assistance of other cytoplasmic (adapter) proteins, a signal which turns on components of the inflammatory system is generated and amplified. A very strong signal results in pathological effects, which can include death (see above), whereas a more moderate signal induces nonspecific resistance to pathogenic intracellular bacteria.

More recently, it has been recognized that intracellular LPS, via lipid A, also activates inflammatory caspases (8, 9). There is thus no doubt that lipid A functions as an important and critical immunomodulator. For a more complete review of the biological properties of LPS/lipid A, see reference 6.

Porphyromonas gingivalis, the keystone pathogen of human chronic periodontal disease (10), produces multiple lipid A structures, depending on growth conditions. Interestingly, different labs had reported different structures of *P. gingivalis* lipid A, which all turned out to be correct. These multiple structures allowed the Darveau laboratory to combine both fine structural and host response studies with the goal of determining which moieties of lipid A are responsible for its biological activity, specifically regarding innate immunity and inflammation. Using a novel technique combining both positive- and negative-mode matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), 4 different lipid A moieties were established, differing in phosphorylation and/or acylation status (11, 12). *P. gingivalis* lipid A is initially synthesized as a structure that contains 2 phosphate moieties at the 1 and 4′ positions of the diglucosamine backbone and is penta-acylated. Consequently, these "tailoring" modifications are made to the lipid A molecule postsynthesis.

The existence of these tailored lipid A molecules then made it possible to establish that minor differences among the lipid A structures greatly affect the interactions of LPS with the host, specifically TLR4, and therefore host response outcomes. The Darveau group hypothesized that *P. gingivalis* must have 3 lipid A-modifying enzymes, since these four lipid A structures differed by the presence or absence of 2 different phosphate groups and/or the presence or absence of one acyl group. Using this approach, they determined that the diphosphorylated, penta-acylated lipid A that is initially synthesized is a weak agonist, whereas the monophosphorylated, tetra-acylated lipid A is a TLR4 antagonist (13). They hypothesized that *P. gingivalis* must have lipid A phosphatase enzymes that remove the phosphate groups from the initially synthesized lipid A structure. When the sequence of the *P. gingivalis* genome and suitable DNA sequence comparison software became available, they identified and cloned 2 such enzymes by their homology to the C-1- and C-4′-phosphatases, LpxE and LpxF, in *Francisella novicida* (14, 15). This was the first report that a loss of a phosphate group was responsible for a loss of lipid A toxicity.

Following the construction of deletion mutations in each of these genes, the mutants were used to confirm that *P. gingivalis* uses a novel strategy of immune evasion by being able to modify its lipid A, thus converting the normally agonistic effect of TLR4 activation to one that is antagonistic and thereby preventing TLR4 activation.

Such a change in host response would be predicted to drastically modify the course of inflammatory disease.

This was not the complete story, however. As mentioned above, *P. gingivalis* lipid A is modified by not only phosphatase but also deacylase activity. In contrast to the phosphatases, no amount of homology analyses of the two known deacylase enzymes PagL and LpxR (13, 14) produced any information suggesting that *P. gingivalis* contained a lipid A deacylase gene. Twenty years after the initial suggestion of the requirement for a lipid A deacylase gene in *P. gingivalis*, and a decade after the cloning of the lipid A phosphatases, the identification of the deacylase gene is reported by Jain et al. (16).

This breakthrough was accomplished by the creation of a novel assay using a transposon mutant library combined with a functional assay to identify mutants capable of increased TLR4 stimulation. Once the screening was completed and slow-growing mutants were eliminated, one mutant, J5-c5, exhibited the desired phenotype. Structural analysis of the lipid A from the mutant confirmed that its lipid A contained no tetra-acylated moieties, and functional analysis determined that it stimulated TLR4-dependent signaling, including a proinflammatory response. The mutation mapped to PGN_1123, which encodes a novel deacylase present in all *P. gingivalis* strains sequenced to date and in some closely related species. For example, an ortholog is present in *Bacteroides fragilis* but not in *Bacteroides thetaiotaomicron*. Analysis of mRNA from the wild-type strain revealed PGN_1123 to be the third gene in a 3-gene operon. The group then constructed a new deletion mutant that was confirmed to retain the J5-c5 phenotype and lipid A structure, i.e., penta-acylated and nonphosphorylated. Complementation of the PGN_1123 mutant resulted in the complemented strain producing tetra-acylated lipid A, thereby proving that PGN_1123 indeed encodes the lipid A deacylase. To add further proof that PGN_1123 encodes the lipid A deacylase, the gene was cloned in *trans* into *B. thetaiotaomicron*, a related species that does not synthesize a tetra-acylated lipid A, resulting in the synthesis of tetra-acylated moieties by this host species. This is the ultimate proof that PGN_1123 encodes a lipid A deacylase.

This work is significant from multiple perspectives. First, the identification of PGN_1123 as a lipid A deacylase gene establishes a third distinct class of bacterial lipid A deacylase enzymes and suggests that there may be other, as-yet-undiscovered classes in other Gram-negative species. In addition, remodeling the outer membrane by changing the acylation pattern of lipid A has previously been shown to affect membrane curvature. This in turn greatly influences the formation of outer membrane vesicles (OMVs), so it is not surprising that deacylation promotes the formation of these vesicles (17). *P. gingivalis* produces copious amounts of OMVs, which have multiple roles in pathogenesis, including heme acquisition, coaggregation of oral bacterial species, and invasion of host cells. Further, *P. gingivalis* OMVs contain an enriched amount of deacylated lipid A (18, 19).

In addition to its interactions with TLR4, recent studies have demonstrated that *P. gingivalis* penta-acylated lipid A is also an agonist of inflammasome activation, a pathway that kills intracellular bacteria (20). Others have shown that deacylated lipid A fails to induce activation of inflammasomes (8, 9). It thus seems likely that the deacylation state of *P. gingivalis* lipid A also contributes to the viability of *P. gingivalis* that has invaded host cells. This ability of *P. gingivalis* to invade, survive in, and reprogram host cells has been proposed as a mechanism of maintaining a state of chronic infection, a hallmark of *P. gingivalis* (21, 22).

The lipid A acylation state has been shown to greatly modify virulence by moderating the host response in other pathogens, such as *S.* Typhimurium and *Pseudomonas aeruginosa* (23, 24). Deacylation occurs in these species only under specific environmental conditions. For *S.* Typhimurium, the conditions are those that would be found in phagolysosomes, and for *P. aeruginosa*, the condition is growth in the lungs of cystic fibrosis patients.

There is significant evidence to indicate that the expression of PGN_1123 is also

induced *in vivo* during (periodontal) disease. As stated above, PGN_1123 is the third gene of a 3-gene operon. This operon in *P. gingivalis* W83 has been linked to expression *in vivo* in that the second gene, homologous to PGN_1124, was identified using *in vivo*-induced antigen technology (IVIAT) selection and shown to be expressed in *P. gingivalis* in the human oral cavity but not under conditions of laboratory growth (25). Furthermore, the expression of PGN_1124 was found to be significantly higher in diseased pockets than in healthy sites in patients, suggesting that expression of the operon is determined by specific environmental signals that are present during active disease but not in the healthy gingiva (25).

Now that the long-sought-after lipid A deacylase gene of *P. gingivalis* has been discovered, one of the remaining significant questions is what conditions regulate its expression. Its regulation appears to be more complex than just regulation of the operon, as data from reverse transcription-PCR (RT-PCR) analysis of mRNA, as reported in the present work, reveal that not each of the 3 genes is expressed at the same level. mRNA for the second and third genes of the operon was more abundant than mRNA for only the first 2 genes. Thus, other factors and signals not yet known may be involved in this regulation. It is likely that these factors contribute to the *in vivo* expression of PGN_1123 in response to host defense molecules. Increased expression of PGN_1123 corresponding to an increased inflammatory assault by the host would promote bacterial cell survival. Furthermore, if PGN_1123 is involved in the silencing of the TLR4 cascade, PGN_1123 would be protective of the various other Gram-negative bacteria in the periodontal microbiota as well.

In conclusion, *P. gingivalis* synthesizes four different lipid A structures in response to environmental cues. The present work reports the cloning, identification, and function of the deacylase gene and enzyme, therefore completing the cloning of the entire repertoire of genes encoding lipid A-modifying enzymes in this organism. Combinations of the 3 modifying enzymes produce lipid A species that determine the interactions with and effects on TLR4 signaling. These effects, depending on lipid A modification, can be agonistic, inert, or antagonistic. Strong evidence suggests that the deacylase is induced *in vivo* during disease, resulting in TLR4 antagonism or silencing, likely increased production of OMVs, and an increase in virulence. The antagonism is hypothesized to promote survival and persistence of not only *P. gingivalis* but likely other bystander/biofilm species as well. *P. gingivalis* is another pathogen that proves that the lipid A biosynthetic pathway in Gram-negative bacteria is severely constrained but not absolutely conserved.

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