

## Editorial: O-specific polysaccharide confers lysozyme resistance to extraintestinal pathogenic *Escherichia coli*

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**ARTICLE HISTORY** Received 27 March 2018; Accepted 28 March 2018

**KEYWORDS** *Escherichia coli*; LPS; lysozyme; lipopolysaccharide; lysozyme resistance; O-specific polysaccharide

Bao *et al.* [1]. report on the lipopolysaccharide (LPS, endotoxin) structure relevance for the resistance of extraintestinal pathogenic *Escherichia coli* (ExPEC) to lysozyme (LZ). Both LPS and LZ play a key role in host–pathogen interactions within the immune system. Moreover the authors demonstrate how LPS structure influences its inhibitory activity against enzymatic and bactericidal activity of LZ against Gram-positive and Gram-negative bacteria.

Lysozyme (muramidase, N-acetylmuramide glycanhydrolase) is a key element of innate immunity characterized by antibacterial activity. It is evolutionarily conserved enzyme found in variety of organisms such as bacteria, viruses, plants, invertebrates, and animals [2]. The protein was discovered by famous bacteriologist Sir Alexander Fleming in 1922 who demonstrated an ability of human nasal mucus to lyse of bacteria present on agar plate [3]. Even earlier, Nicolle (1907) and Laschtschenko (1909) observed lytic factors produced by *Bacillus subtilis* and enzymatic-like bactericidal action of hen egg white LZ (HEWLZ), respectively [4]. Both reported effects were attributed to LZ and resulted in its discovery. Moreover, studies of Fleming led to the identification of *Micrococcus lysodeikticus* – Gram-positive species that is highly sensitive to LZ and serves as a model microorganism for determination of LZ muramidase activity.

Lysozymes are widely spread, since they represent first line defense factors against bacterial invasion. Three major LZ types have been distinguished to date within the animal kingdom: (i) the c-type (conventional or chicken type) represented also by human LZ; (ii) the g-type (goose-type), and (iii) the i-type (invertebrate type) LZ [2]. For example, human LZ belongs to c-type muramidases and is present in various body fluids such as tears, saliva, airway fluid, breast milk, urine, serum, cerebrospinal fluid, cervical mucus and amniotic fluid, respiratory tract, intestinal tract, and in

the lysosomal granules of neutrophils and macrophages [2]. Regardless of the origin, LZ are small and relatively conserved proteins that share basic tertiary structure of the catalytic site [5]. Their enzymatic activity is attributed to hydrolysis of  $\beta$ -1,4 glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in peptidoglycan (PG, murein), the main cell envelope component of Gram-positive and Gram-negative bacteria [2,6]. PG degradation catalyzed by LZ leads to cell wall permeabilisation, lysis, and killing of bacteria [6]. Gram-positive bacteria represent the most sensitive group of microorganisms due to their cell envelope architecture characterized by highly accessible PG layers. However, only a minority of Gram-positive bacteria are directly susceptible to hydrolytic activity of LZ under physiological conditions, what suggests that the major role of LZ might be attributed to degradation of cell envelope debris resulted from previous action of antimicrobial peptides, serum complement, and other factors of immune system [7].

Generally Gram-negative bacteria are resistant to hydrolytic activity of LZ due to multi-layered cell envelope hindering internal PG layers. On the other hand, both Gram-negative and Gram-positive bacteria are sensitive for muramidase-independent bactericidal activities of LZ. For widely studied HEWLZ, several nonenzymatic mechanisms of action were suggested such as DNA and RNA synthesis impairment, an activation of autolysin production [8–11], membrane permeabilization, depolarization, and finally cytosol leakage [8,9]. LZ-induced cell wall permeabilization was suggested as a result of its cationic antimicrobial protein (CAMP) activity attributed to general LZ structure characterized by highly positive charge characteristic for CAMP [12].

Recently several reports put forward modulatory role of LZ in the host immune response to infection. The lysis

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of bacterial cells leads to the release of variety of pathogen associated molecular patterns (PAMP) such as PG, LPS or lipoteichoic acid, recognized by pattern recognition receptors (PRR) in host cells (for example Toll-like receptors, cytosolic receptors NOD). As an illustration, NOD1 recognizes low-molecular-weight PG fragments containing meso-diaminopimelic acid, whereas NOD2 recognizes the minimal bioactive PG motif – muramyl dipeptide [13,14]. Interaction between PAMP and PRR leads to stimulation of pro-inflammatory signaling and cytokine production [6]. Nowadays, the most intriguing aspects concerning LZ are about its dual activity and the interplay with other components of immune system.

Bacteria developed various resistance strategies targeting different mechanisms of LZ activity [6]. PG is being modified by *N*-deacetylation of *N*-acetylglucosamine (NAG), *O*-acetylation and *N*-glycolylation of *N*-acetylmuramic acid (NAM), and cross-linking of PG peptide stem to avoid hydrolytic activity of LZ [6]. C-type LZ resistance is also conferred in Gram-negative bacteria by expression of inhibitors such as Ivy (*E. coli*, *Yersinia enterocolitica*), MliC/PliC family (*E. coli*), and PliI (*Aeromonas hydrophila*) proteins [14]. Finally, alterations in cell envelope net negative charge and integrity significantly reduce binding of LZ and other antimicrobial proteins.

Cell wall integrity of Gram-negative bacteria is provided by outer membrane constituents. LPS represents predominant surface antigen and important virulence factor [15–17]. It is a glycolipid built up of three regions: lipid A, core oligosaccharide, and the O-specific polysaccharide (O-PS). Lipid A anchors LPS in the external leaflet of the outer membrane, whereas long O-PS constitutes external region of LPS. Rough strains of bacteria synthesize R-LPS consisting of the lipid A and core OS only, whereas smooth strains synthesize full length LPS containing all three regions (S-LPS). The O-PS, called O-antigen represents the most variable and unique LPS region and determines strain's O serotype within the species. Since LPS outnumbers other outer membrane constituents, its presence, structure and modifications largely provide for outer membrane integrity. This makes LPS important resistance factor to lipophilic antibiotics and innate host defenses such as bile salts, complement system, and CAMP, where O-PS plays predominant role [18].

It is known that LPS interacts with LZ and is able to inhibit its enzymatic activity. In the late 1980s, Ohno and colleagues demonstrated formation of LZ-LPS complexes without regard to the source of LZ (human and avian) and LPS (various Gram-negative species) [19–21]. Moreover they showed for the first time that the LPS binding by LZ resulted in loss of muramidase activity. Additionally this pioneering research demonstrated the significance of carbohydrate regions of LPS, since weaker inhibition

effect was observed for deep-rough LPS (the lipid A substituted by two Kdo residues) and isolated lipid A in comparison with S-LPS [21]. Brandenburg *et al.* also demonstrated direct interaction between HEWLZ and LPS isolated from deep-rough mutant (Re) and its lipid A, pointed out a remodeling of LPS and lipid A aggregates upon LZ action [22]. During *et al.* showed that heat-inactivated bacteriophage T4 LZ and HEWLZ with abolished enzymatic activity still revealed bactericidal activity against Gram-positive (*M. lysodeikticus*) and Gram-negative (*E. coli*) bacteria, fungi, and plants [23]. Membrane permeability was suggested as a mechanism of LZ action. Ibrahim *et al.* provided first direct genetic evidence and confirmed previous observations using catalytically inactive mutants of HEWLZ showing its bactericidal effect against *Staphylococcus aureus* and *B. subtilis*. Moreover the heat-inactivation of the mutated protein not only impaired but also increased its bactericidal activity [7]. Further the observed effects were confirmed *in vivo* by Nash *et al.* for transgenic mice expressed muramidase-deficient recombinant LZ in in the respiratory epithelium that were still protected against *S. aureus*, *Pseudomonas aeruginosa* and *K. pneumoniae* infection [24]. Described results clearly indicated that muramidase activity of LZ is not required for Gram-positive and Gram-negative bacterial killing *in vitro* and *in vivo*.

Even though LZ-LPS interaction was discovered in the late 1980s, details and mechanism of bactericidal activity remains unclear. Ohno and colleagues clearly showed correlation between LZ binding and LPS structure, particularly the significance of the O-PS [21]. Recently Derde *et al.* used model of *E. coli* LPS monolayers to simulate the mode of muramidase-independent HEWLZ activity against Gram-negative bacteria. Using biophysical methods they demonstrated high affinity of LZ towards LPS monolayer and ability of LZ to insert into the monolayer as long as O-PS was present in LPS structure. As results the reorganization of the LPS monolayer and pore formation were reported [25].

Some insights on molecular basics of LPS binding by LZ were recently provided by Zhang *et al.* who used precise methodology (NMR spectroscopy, molecular modeling, and X-ray crystallography) to study interactions between human LZ and synthetic oligosaccharides representing repeating unit(s) of *K. pneumoniae* O1 O-PS [26]. The LZ molecules underwent structural rearrangements after the interaction with synthetic oligosaccharides. Moreover a direct involvement of some crucial amino acids of LZ was identified within the binding site that provides residue-specific, direct or water-mediated hydrogen bonds and hydrophobic contacts. Interestingly, some flexibility of LZ binding site for O-PS fragments was suggested, which might be adapted to various

ligands, other than *K. pneumoniae* O-PS. It pointed out for LZ's lectin-like features that may facilitate host immune response.

In terms of described observation, the most recent study of Bao *et al.* responds to previously suggested specificity and flexibility of LZ towards O-PS [1]. The authors found the differences in HEWLZ-resistance between the clinical ExPEC isolates and LZ-sensitive laboratory strain. They screened 15,000 transposon mutants of the lysozyme-resistant ExPEC strain NMEC38 and identified a few LZ-resistance-related genes that were involved in O-PS biosynthesis. Indeed, the LZ-sensitive laboratory strain of *E. coli* represented rough strain expressing R-LPS devoid of the O-PS. Deletion of identified genes in selected ExPEC strain resulted in expression of truncated R-LPS and significantly decreased the resistance of the mutant to LZ. Moreover the observed sensitivity or resistance to LZ does not depend on the protein inhibitors such Ivy and MliC/PliC. Furthermore, the authors demonstrated that muramidase-activity of LZ towards *M. lysodeikticus* was inhibited by S-LPS and isolated O-PS. Instead, nonenzymatic bactericidal activity of LZ against Gram-negative bacterium was inhibited solely by the complete S-LPS containing O-PS. Discrepancies in inhibitory activities of LPS, R-LPS, and isolated O-PS against LZ observed between hydrolytic- and muramidase-independent activities of LZ supported different modes of LZ action and are in accordance with observations of Derde *et al.* [25]. Only supramolecular structures of S-LPS containing O-PS (*e.i.* aggregates, monolayers) are able to bind LZ, thereby inhibit its activity against Gram-negative bacteria. This was not the case in muramidase-dependent activity, where both LPS and O-PS have inhibitory activity. Bao *et al.* confirmed broader specificity of LZ towards various O-PS. The set of smooth ExPEC strains used in their study clearly confirmed that LPS-LZ binding does not depend on LPS O-serotype therefore suggesting that different O-PS structures are capable to inhibit hydrolytic activity of LZ. However, the conclusion assigning observed effects particularly to ExPEC strains goes much too far and needs further examination within variable group of pathogenic smooth strains of *E. coli*. Finally, the authors supported the need for further investigation of LZ carbohydrate specificity at molecular level. Moreover, duality of activity and possible involvement of LZ in cross-talks within immune system make LZ an intriguing molecule, even a century after its discovery.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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