Adv. Exp. Med. Biol. 2007; **603**: 88-96.

7
Relationship of the Lipopolysaccharide Structure of *Yersinia pestis* to Resistance to Antimicrobial Factors

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Abstract. Disruption of lipopolysaccharide (LPS) biosynthesis genes in an epidemiologically significant *Yersinia pestis* strain showed that the ability to synthesize the full inner core of the LPS is crucial for resistances to the bactericidal action of antimicrobial peptides and to complement-mediated serum killing. Resistance to polymyxin B also requires a high content of the cationic sugar, 4-amino-4-deoxy-L-arabinose, in lipid A.

7.1 Introduction

For constant circulation in natural foci, the plague pathogen, *Yersinia pestis*, must penetrate into the host organism, counteract its protective bactericidal systems and reproduce to ensure bacteremia that is essential for further transmission of the infection by fleas to a new host. Each of these stages in the cyclic existence of *Y. pestis* is insured by numerous properties of the plague pathogen, including multi-functional pathogenicity factors such as lipopolysaccharide (LPS), which possesses a pleiotropic ability to counteract the defense mechanisms of insect vectors and mammalian hosts. In mammals, the LPS induces endotoxic shock, determines resistance to the bactericidal action of cationic peptides and normal sera, may partake in adhesive activity, and is necessary for the enzymatic activities of the plasminogen activator of *Y. pestis* (Anisimov et al. 2004; Bengoechea et al. 1998; Brubaker 1991; Butler 1983; Dmitrovskii 1994; Kukkonen et al. 2004; Porat et al. 1995; Straley 1993).

Owing to a frame-shift mutation in the O-antigen gene cluster inherited from *Yersinia pseudotuberculosis* (Skurnik et al. 2000), *Y. pestis* possesses a rough(R)-type LPS composed of an oligosaccharide core and lipid A, with no O-polysaccharide chain present. A single major LPS core glycoform is synthesized at 37°C (mammalian body temperature) whereas multiple glycoforms differing in terminal monosaccharides are produced at 25°C (flea temperature) (Knirel et al. 2005). The degree of acylation and the level of the cationic sugar, 4-amino-4-deoxy-L-arabinose (Ara4N), in lipid A increases with lower growth temperatures (Kawahara et al. 2002; Knirel

et al. 2005; Rebeil et al. 2004). While a biological role for the structural variations in the LPS core remains to be determined, the modifications to the lipid A structure are evidently a part of the mechanism of optimal adaptation of *Y. pestis* to significantly different conditions in insect vectors and mammalian hosts. The less immunostimulatory LPS synthesized at mammalian temperature (37°C) compromises the host's ability to rapidly respond with a proper inflammatory response to infection (Kawahara et al. 2002; Rebeil et al. 2004). The LPS structure synthesized at flea temperatures confers resistance to antimicrobial peptides (Anisimov et al. 2005; Rebeil et al. 2004).

The aims of this work are to unravel the impact of particular LPS components on resistance of the bacteria to various antimicrobial factors and to understand better the biological significance of the temperature-dependent LPS structural variations. For these purposes, we identified genes that are involved with biosynthesis of the *Y. pestis* LPS, generated the corresponding nonpolar mutants and studied the resultant LPS structures and biological properties.

7.2 Methods and Results

7.2.1 Generation of Mutants

Parental strain *Y. pestis* 231 (ssp. *pestis*, bv. antiqua) was isolated in the Aksai focus, Kirghizia. When cultivated at 25°C, the pCD$^-$ plasmidless attenuated derivative named KM260(11) was resistant to polymyxin B and normal human serum (NHS) (Anisimov et al. 2005).

The *Y. pestis* chromosome was found to harbor the *waa* gene cluster containing five genes (64469..69941, locus YPO0054-YPO0058) that are most likely involved in biosynthesis of the LPS inner core. *In silico* analysis of the *Y. pestis* strain CO92 genome sequence revealed several other putative genes for glycosyl transferases presumably involved in LPS biosynthesis that are located in different parts of the chromosome. Among them there are YPO0416 (434938..435708), YPO0417 (435822..437054), YPO0186 (203866..204846), YPO0187 (204939..205928), YPO3866 (4340131..4341228), and YPO2421 (2722822..2722328) genes. The YPO0654 (714210..715640) gene was suggested to encode ADP-L-*glycero*-D-*manno*-heptose synthase. Dispersion of *Y. pestis* LPS core biosynthesis genes among different regions of the chromosome indicates that they were acquired during different events of horizontal transfer.

Mutant strains with impaired LPS biosynthesis pathway were generated by one-step inactivation of the chromosomal genes mentioned above by λ Red recombination technology (Datsenko and Wanner 2000). Repeated electroporations of the PCR products into *Y. pestis* strain, KM260(11)/pKD46, gave each time single recombinant bacteria disrupted for the target LPS loci. The proper insertion of the *kan* cassette and subsequent deletion of the central parts of the target genes were verified by PCR.

The parental and mutant strains were grown in a New Brunswick Scientific fermentor in Brain Heart Infusion (BHI; Himedia Laboratories, Mumbai, India) at 25°C; kanamycin was used at final concentration of 40 $\mu g \cdot mL^{-1}$.

7.2.2 Structure of Mutant Lipopolysaccharides

The LPSs were isolated by the phenol/chloroform/light petroleum procedure (Galanos, Lüderitz, and Westphal 1969) and purified by treatment with DNAse, RNAse, and Proteinase K followed by ultracentrifugation. Each LPS was degraded with dilute acetic acid to cleave the linkage between the core and lipid A moieties. The resultant water-soluble supernatant was fractionated by gel-permeation chromatography on Sephadex G-50 (S) to yield core oligosaccharides.

The whole LPSs and core oligosaccharides were analyzed by electrospray ionization Fourier transform ion-cyclotron resonance mass spectrometry in the negative ion mode using an Apex II instrument (Bruker Daltonics, MT) equipped with a 7 T actively shielded magnet. The data of the products from mutant strains were compared to those of the parental *Y. pestis* strain.

In the mass spectrum of the isolated core from the parental strain (Fig. 1A), there were several major series of ions due to a structural heterogeneity of the following types (Fig. 2). i) Alternation of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) at one of the terminal positions. These resulted in the appearance of two series with a 236-Da mass difference for ions that either contain or lack Ko. The latter came from compounds with terminal Kdo, which, in contrast to Ko, was cleaved upon mild acid hydrolysis of the LPS. The series for Ko-lacking ions was split into two for compounds with either Kdo or anhydro-Kdo at the reducing end (mass difference 18 Da). ii) Alternation of D-*glycero*-D-*manno*-heptose (DD-Hep) and D-Gal at another terminal position of the oligosaccharide. As a result, two series were observed with a mass difference of 30 Da. iii) Non-stoichiometric substitution with terminal D-GlcNAc giving rise to two series of ions with a 203-Da mass difference.

The mass spectrum of the core from YPO0186::*kan* mutant (Fig. 1B) showed no Gal-containing compounds and that from YPO0187::*kan* mutant (Fig. 1C) no compounds with DD-Hep. Therefore, the functions of the corresponding glycosyl transferases were assigned to the mutated genes. Mutation in either YPO0417 or YPO3866 resulted in the same product lacking GlcNAc (Fig. 1D). These and homology data enabled assignment of their functions as genes for ligase WaaL and UndP:GlcNAc transferase WecA, which transfer GlcNAc to LD-Hep II or undecaprenyl-phosphate, respectively. Similarly, based on the data of the corresponding mutants having a truncated inner core, it was concluded that YPO0416 (Fig. 1E), YPO0057 (Fig. 1F), and YPO0654 encode transferase WaaQ for distal L-*glycero*-D-*manno*-heptose (LD-Hep III), LD-Hep II transferase WaaF, and LD-Hep synthase, respectively. GlcNAc is present in the core of YPO0416::*kan* mutant but in a significantly lower content than in those with the inner core unaffected. Therefore, although not strictly necessary, LD-Hep III is important for effective incorporation of GlcNAc. No Glc is present in the LPS of YPO0057::*kan* mutant and, hence, addition

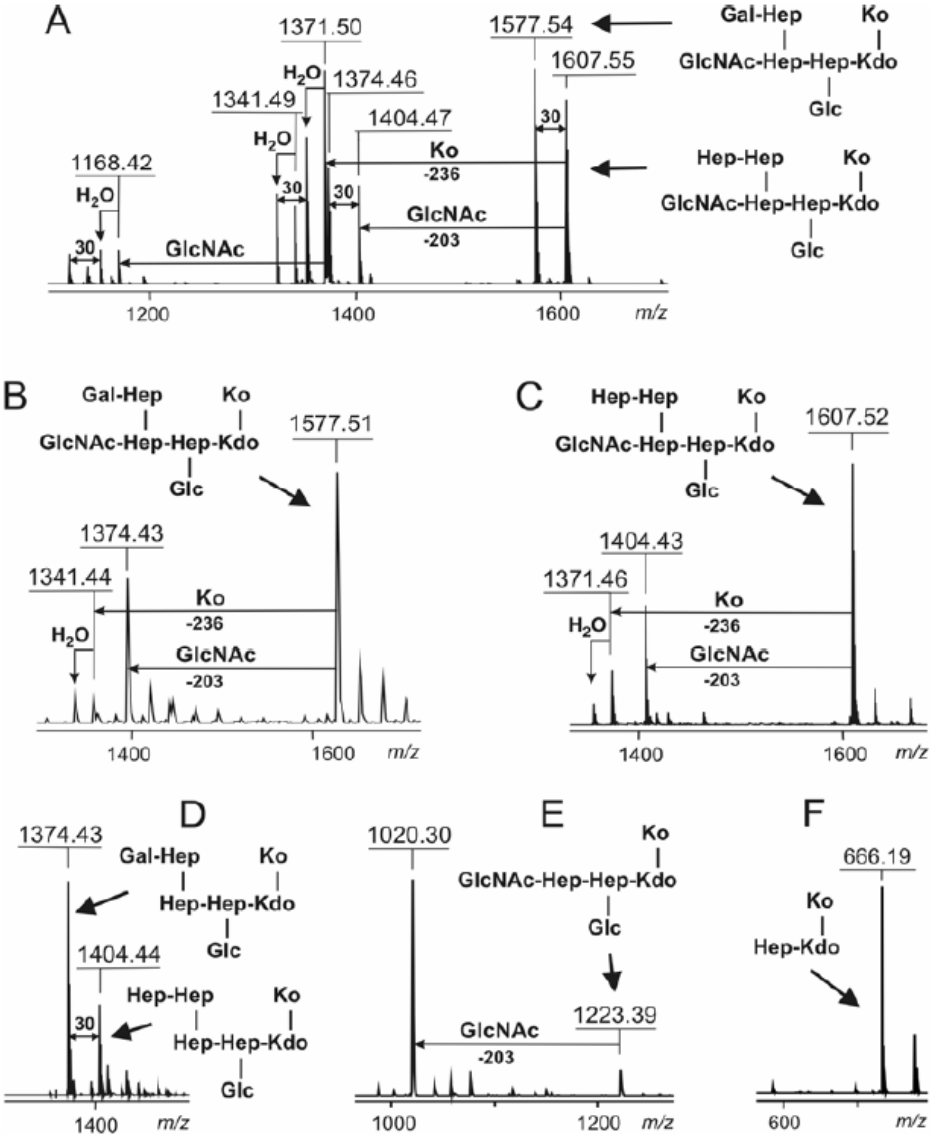

Fig. 1. High-resolution negative ion electrospray ionization mass spectra of the core oligosaccharides from the parental *Y. pestis* strain KM260(11) (A) and derived mutants with a mutation in the YPO0186 (B), YPO0187 (C), YPO0417 (D), YPO0416 (E), and YPO0057 (F) genes. The corresponding core structures are schematically shown in the insets.

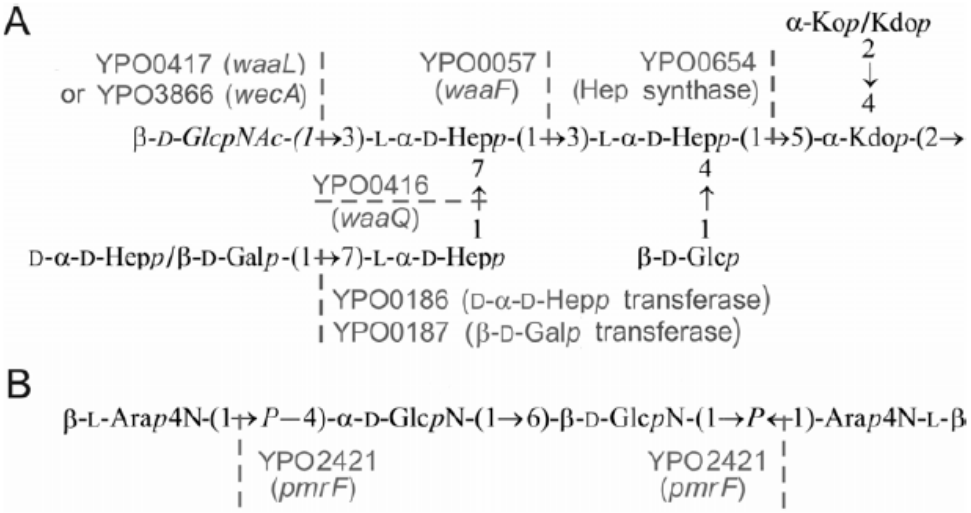

Fig. 2. Structure of the LPS core (A) and lipid A backbone (B) of *Y. pestis* (Knirel et al. 2005) and assignment of some gene functions involved in biosynthesis of the LPS. Abbreviations: L-α-D-Hep and D-α-D-Hep, L-*glycero*- and D-*glycero*-α-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko, D-*glycero*-D-talo-oct-2-ulosonic acid; Ara4N, 4-amino-4deoxy-L-arabinose. A non-stoichiometric content of GlcNAc is indicated by italics.

of Glc requires prior incorporation of LD-Hep II. The assigned core biosynthesis gene functions are summarized in Fig. 2.

MS studies of the whole LPSs showed that the mutant strains possess essentially the same lipid A moiety as the parental strain. The only exception was that the content of Ara4N in lipid A is lower in deeply truncated core mutants as compared with those having the complete inner core. While LPS molecules with two Ara4N residues prevail in the parental strain (Fig. 3A), most LPSs molecules in YPO0416::*kan*, YPO0057::*kan* (Fig. 3B), and YPO0654::*kan* mutants contain one or no Ara4N residue. The YPO2421::*kan* mutant is fully unable to incorporate Ara4N into the LPS (Fig. 3C), and it was concluded that YPO2421 encodes Ara4N transferase PmrF. The mutation in this gene had no influence on the LPS core structure.

7.2.3 Resistance of Mutants to Bactericidal Action of Polymyxin B

The sensitivity of *Y. pestis* strains to polymyxin B (PMB) was tested using different doses of this cationic antimicrobial peptide to calculate the minimum inhibitory concentration (MIC) as described previously (Anisimov et al. 2005). Table 1 shows that when grown at 25°C the YPO0416, YPO0057, YPO0654, and YPO2421 deficient derivatives were from 31 to 250 times less resistant to PMB (MIC <20 U·mL^{-1}) as compared with the wild-type parent strain and its derivatives carrying mutations in YPO0186, YPO0187, and YPO0417 loci (MIC >625 U·mL^{-1}).

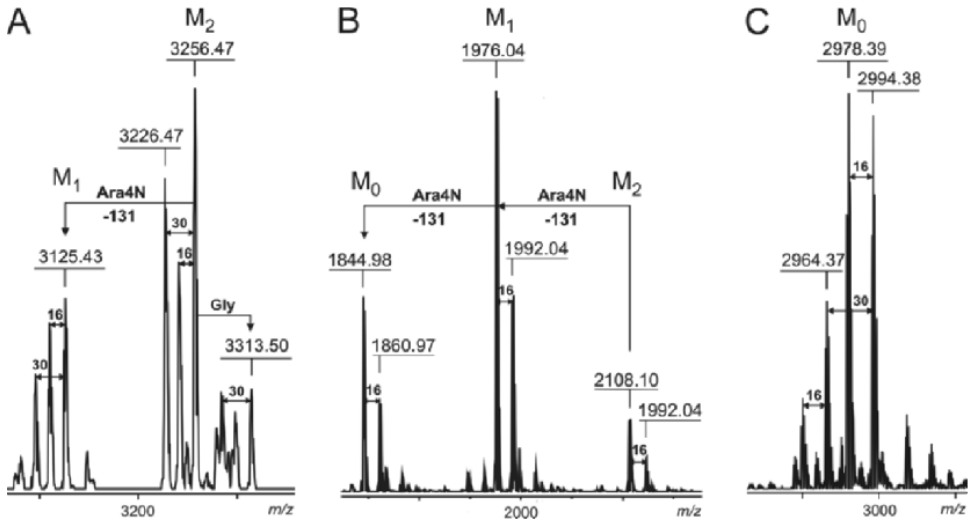

Fig. 3. Negative ion electrospray ionization mass spectra of the whole LPSs from *Y. pestis* strain KM260(11) (A), YPO0057::*kan* mutant (B), and YPO2421::kan mutant (C). Shown is the region of tetraacylated LPS ions with four 3-hydroxymyristoyl groups. Ions containing no, one, and two Ara4N residues are marked with M0, M1, and M2, respectively. Mass differences of 30 and 16 Da correspond to Hep/Gal and Ko/Kdo alternations, respectively.

7.2.4 Resistance of Mutants to Bactericidal Action of Human Serum

A pool of normal human serum (NHS) was obtained from ten non-immunized healthy volunteers. The complement was inactivated by incubating NHS at 56°C for 30 min. Bactericidal properties of NHS were studied by incubation of bacteria with serum for 1 h as described earlier (Anisimov et al. 2005). When grown at 25°C, YPO0416, YPO0057, and YPO0654 deficient derivatives were highly sensitive to the bactericidal action of NHS but not heat-inactivated NHS (Table 1), whereas the other mutants were almost as resistant as the parent strain.

7.3 Conclusions

A single mutation in the gene for Ara4N transferase significantly reduces the polymyxin B resistance of *Y. pestis*, thus demonstrating a role of the cationic sugar in protection of the bacteria. At the same time, Ara4N has no influence on serum resistance. A mutation in either of YPO0186 and YPO0187, which are involved in biosynthesis of the variable outer core region of the *Y. pestis* LPS, does not affect polymyxin B and serum resistances of *Y. pestis*. In contrast, the susceptibility to both antimicrobial factors of the innate immune system is induced by mutation in either of the genes involved in synthesis of the inner heptose region of the LPS core, which is

Table 1. Susceptibility to antimicrobial factors of *Y. pestis* KM260(11) and derived mutants with defined LPS core structures. In LPS of all strains, there is also a core glycoform with the lateral Kdo instead of Ko. LA, wild-type lipid A; LA*, Ara4N-lacking lipid A. Dotted line indicates non-stoichiometric substitution with GlcNAc. MIC, minimum inhibitory concentration; CFU, colony-forming unit; PMB, polymyxin B; NHS, normal human serum; HIS, heat-inactivated serum. Data for susceptible strains are shown in bold face

Y. pestis strain	Core glycoform (schematic view)	MIC of PMB ($U \cdot mL^{-1}$)	Viable cells after 1-h incubation ($lg\ CFU \cdot mL^{-1}$)	
			NHS	HIS
Parental strain KM260(11)	Gal/DD-Hep–Hep Glc Ko GlcNAc---Hep–Hep–Kdo–LA	1250	7.0 ± 0.81	7.2 ± 0.73
YPO0186	Gal–Hep Glc Ko GlcNAc---Hep–Hep–Kdo–LA	2500	6.4 ± 0.65	6.8 ± 0.43
YPO0187	DD-Hep–Hep Glc Ko GlcNAc---Hep–Hep–Kdo–LA	1250	6.3 ± 0.51	6.1 ± 0.72
YPO0417 (*waaL*)	Gal/DD-Hep–Hep Glc Ko Hep–Hep–Kdo–LA	625	6.3 ± 0.71	6.8 ± 0.62
YPO0416 (*waaQ*)	Glc Ko GlcNAc---Hep–Hep–Kdo–LA	**20**	**3.6** ± 0.53	6.7 ± 0.70
YPO0057 (*waaF*)	Ko Hep–Kdo–LA	**20**	**2.3** ± 0.19	6.1 ± 0.56
YPO0654 (*rfaE*)	Ko Kdo–LA	**10**	**2.1** ± 0.23	6.7 ± 0.41
YPO2421 (*pmrF*)	Gal/DD-Hep–Hep Glc Ko GlcNAc---Hep–Hep–Kdo–LA*	**20**	6.5 ± 0.55	6.7 ± 0.71

thus crucial for the bacterial resistance. The susceptibility to polymyxin B of mutants with a deeply truncated LPS core may be accounted for by a poorer incorporation of Ara4N into mutant lipid A.

7.4 Acknowledgments

This work was supported by the International Science and Technology Center/ Cooperative Threat Reduction Program of the US Department of Defense Partner Project

#1197p, the Russian Foundation for Basic Research (grant 06-04-49280), and the Council on Grants at the President of the Russian Federation for Support of Young Russian Scientists (grant MK-3998.2005.4 for O.V.B.).

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Picture 11. Yuriy Knirel presents research on LPS structural variations and their biological effects. Photograph by A. Anisimov.