Chapter 8
Yersinia pestis Lipopolysaccharide in Host-Pathogen Interactions

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Abstract Isogenic *Y. pestis* strains with a single mutation in 14 genes of lipopolysaccharide (LPS) biosynthetic pathways were constructed. Using high-resolution electrospray ionization mass spectrometry, the full LPS structures were elucidated in each mutant, and the sequence of monosaccharide transfers in the assembly of the LPS core was inferred. Truncation of the core decreased significantly the resistance of bacteria to normal human serum (NHS) and polymyxin B. Impairing of LPS biosynthesis resulted also in reduction of LPS-dependent enzymatic activities of plasminogen activator. A gradual truncation of the LPS core was accompanied by a decrease of virulence in mice and guinea pigs. However, the reduction in virulence remained behind the decrease of bacterial resistance to innate immunity factors. E.g., *waaQ* mutant deficient in HepIII transferase was highly susceptible to polymyxin B and NHS but was as virulent as the parental strain in both animal models. *Y. pestis* mutants with two or less sugar residues in the core were not only susceptible to antimicrobial cationic peptides and NHS but also avirulent in animal infection models. This finding demonstrated that the LPS structure is crucial for the lethality of plague infection, and *waaC*, *hldE* and *waaA* or their protein products can be considered as promising candidates for targeting *Y. pestis* virulence using specific inhibitors.

Keywords Host-pathogen interactions · Lipopolysaccharide · *Yersinia pestis*

8.1 Introduction

Lipopolysaccharide (LPS, endotoxin) is an important factor of pathogenicity common for most Gram-negative bacteria. The full LPS molecule (S-LPS) consists of three well-defined domains: (i) lipid A composed of sugars, fatty acids and phosphate; it represents the endotoxic principle of the LPS and anchors it in the

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outer leaflet of the outer membrane; (ii) a core oligosaccharide containing charged groups; and (iii) an O-chain polysaccharide (O-antigen), which protrudes from the cell surface to the environment and carries immunodeterminant groups eliciting specific antibodies in animals. The Gram-negative bacterium *Yersinia pestis*, the etiological agent of bubonic and pneumonic plague, is a clone evolved from the enteric pathogen *Yersinia pseudotuberculosis* 1,500–20,000 years ago (Achtman et al., 1999). In the course of the divergence *Y. pestis* lost by deletion or inactivation a lot of genes useful for enteropathogenic yersiniae but unnecessary for the vector-borne lifestyle of *Y. pestis*. Particularly, it lost the ability to synthesize O-chain (Skurnik et al., 2000), and, as a result, the LPS of *Y. pestis* is restricted to the core and lipid A moieties (R-LPS).

The O-antigen-deficient LPS plays a role in resistance of *Y. pestis* to serum-mediated lysis (Anisimov et al., 2005; Porat et al., 1995) that is necessary for survival of the bacteria in mammalian blood and for transmission between insects and mammals (Perry and Fetherston, 1997). It determines also bacterial resistance to cationic antimicrobial peptides (Anisimov et al., 2005; Bengoechea et al., 1998), a key component of the innate immunity in both mammals and insects (Dimopoulos, 2003). Reduction in the degree of acylation of lipid A of *Y. pestis* at the mammalian host temperature (37°C) (Kawahara et al., 2002) makes this pathogen-associated molecule invisible for pattern recognition Toll-like receptor 4 (Montminy et al., 2006) and enables uncontrolled multiplication of *Y. pestis* in the host organism resulting in septic shock and death necessary for further transmission of the pathogen by fleas (Brubaker, 1991).

Recently, the fine LPS structures have been reported in representatives of various intraspecies groups of *Y. pestis* grown at 37, 21–28 or 6°C to mimic the conditions in warm-blooded hosts, insects and animals during winter hibernation, respectively (Dentovskaya et al., 2008; Knirel et al., 2006, 2008). Variations in the LPS structure of *Y. pestis* have been documented and found to depend on the growth temperature, composition of the nutrient media and belonging of *Y. pestis* strains to a certain intraspecies group. However, structural elements of *Y. pestis* LPS responsible for different pathogenic properties of this macromolecule are characterized scarcely. This work aims at unraveling the impact of particular LPS components on *Y. pestis* virulence, including 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 non-polar mutants and studied their LPS structures and virulence-related biological properties.

8.2 Materials and Methods

Parental strain *Y. pestis* 231 (bv. antiqua) was isolated in the Aksai focus, Kirghizia. Search for LPS associated genes was performed through annotated genome sequences of *Y. pestis* strains (http://www.ericbrc.org/portal/eric/yersiniapestis?id=enteropathogens&subid=yersiniapestis). When relevant information was absent,

genes with already revealed functions from other bacteria were used as templates for search of orthologs in genome sequence of *Y. pestis* strain CO92 by using the BLAST network service at the National Center for Biotechnology Information. The nomenclature proposed by Reeves et al. (1996) is used for designation of bacterial polysaccharide synthesis genes. 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). Structure of mutant LPSs was determined as described earlier (Knirel et al., 2005, 2008). 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). 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). Coagulation and fibrinolysis were assayed as described previously (Bahmanyar and Cavanaugh, 1976).

BALB/c mice (∼20 g each) and guinea pigs (∼260 g each) of both sex were used in animal experiments that were approved by the ethical committee of the State Research Center for Applied Microbiology and Biotechnology. Animals were kept in cages in groups at the most of five and three, respectively, and allowed to feed and drink *ad libitum* during experiment terms. Six naïve groups of mice (each of 40) and five naïve groups of guinea pigs (each of 12) were administered subcutaneously (s.c.) in the right inguinal region with serial 10-fold dilutions (10^7 to 1 cfu, five mice for one dose; 10^4 to 10^1 cfu, three guinea pigs for one dose, respectively) of 2-day *Y. pestis* agar cultures grown at 25°C. Humane endpoints were strictly observed. Animals that succumbed to infection were sacrificed and examined bacteriologically. The remaining animals were observed for 21 days. The animals that survived were humanely killed by carbonic gas inhalation. Mortality times were recorded and the LD_{50} and 95% confidence intervals (CI) were determined according to the method of Kärber (Finney, 1978).

8.3 Results and Discussion

8.3.1 Generation of Mutants in LPS Biosynthesis Genes

Each single gene with a predicted function in LPS biosynthesis was successfully inactivated using PCR products with kanamycin and/or chloramphenicol resistance cassettes. Each single mutant was complemented *in trans* by the corresponding wild type gene (data not shown) to rule out the possibility that the phenotype observed is caused by a polar effect or a spontaneous mutation elsewhere in the genome. All generated mutants were shown to be non-polar.

8.3.2 Structures of Mutant LPSs and Confirmation of LPS Biosynthesis Gene Functions

The LPS of each mutant was degraded with mild acid to yield a lipid A precipitate and a core oligosaccharide; the latter was purified by gel chromatography on Sephadex G-50. The isolated core and lipid A samples or/and the whole undegraded LPSs were studied by electrospray ionization Fourier transform ion-cyclotron resonance mass spectrometry in the negative ion mode. Mass spectra of the whole LPS of *arnT* mutant, core oligosaccharides from *waaL*, *wabC, wabD, waaQ* and *waaF* mutants (Knirel et al., 2007) and lipid A of *lpxM* mutant (Anisimov et al., 2007) have been reported earlier. The data obtained were compared with the data of wild-type *Y. pestis* LPS with known structure (Dentovskaya et al., 2008, Kawahara et al., 2002, Knirel et al., 2005, 2006, 2008), and functions of the inactivated genes were confirmed (Fig. 8.1).

waaL *(YPO0417) mutant.* A sample from *waaL* mutant showed essentially the same MS pattern as the wild type LPS except that 2-acetamido-2-deoxy-β-D-glucose (GlcNAc)-containing compounds were absent from the mutant LPS. As in wild type, there were present oligosaccharides having either β-D-galactose (Gal) or D-*glycero*-α-D-*manno*-heptose-IV (DD-HepIV) at the non-reducing terminus. The absence of GlcNAc from *waaL* mutant suggested that ligase WaaL encoded by the inactivated gene is responsible for adding GlcNAc to the LPS core. The same GlcNAc-lacking LPS chemotype was detected in *wecA* (YPO3866) mutant, which is unable to synthesize undecaprenyl diphosphate derivative of GlcNAc, a donor substrate for WaaL.

wabC *(YPO0186) and* wabD *(YPO0187) mutants.* Core samples from *wabC* and *wabD* mutants were distinguished by the absence of either terminal DD-HepIV

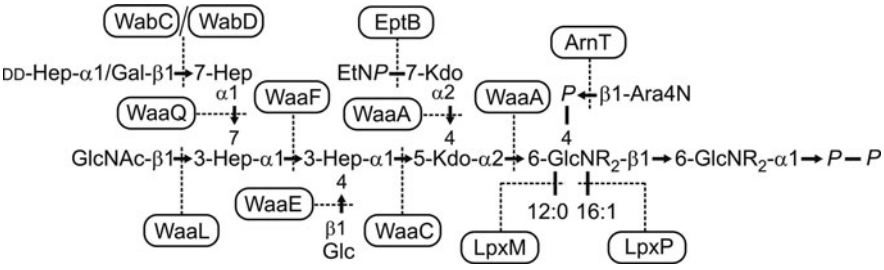


Fig. 8.1 Structure of *Y. pestis* LPS and functional assignment of glycosyl- and acyl-transferases involved with LPS biosynthesis. Ara4N, 4-amino-4-deoxy-L-arabinose; DD-Hep and Hep, D-*glycero*- and L-*glycero*-D-*manno*-heptose, respectively; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid; EtNP, phosphoethanolamine; 12:0, lauroyl; 16:1, palmitoleoyl (hexadec-9-enoyl); R stands for 3-hydroxymyristoyl. In cultures grown at 25°C, DD-HepIV and Gal, KdoII and Ko alternate pairwise whereas at 37°C DD-HepIV and KdoII predominate. EtNP in the core is mainly expressed in cultures grown at 6°C. Diphosphate in lipid A is expressed at 37°C and replaced with Ara4NP at 25°C. Glycine linked to HepI (authors' unpublished data) is not shown

or terminal Gal, and, therefore, the encoded enzymes are heptosyltransferase IV and galactosyltransferase, respectively. The inability to incorporate either of these monosaccharides does not interfere with biosynthesis of the remaining part of the LPS core.

waaQ *(YPO0416) mutant.* The core of *waaQ* mutant was devoid of L-*glycero*-α-D-*manno*-heptose-III (HepIII). As expected, neither DD-HepIV nor Gal was present as these monosaccharides are carried by HepIII. GlcNAc that is linked to L-*glycero*-α-D-*manno*-heptose-II (HepII) occurs only in a minority of molecules. Therefore, *waaQ* encodes heptosyltransferase III, and WaaL transfers GlcNAc to the core in the absence of HepIII inefficiently. The inner core region was not affected by inactivation of *waaQ*.

waaE *(YPO0054) mutant.* The mass spectrum of the whole LPS from *waaE* mutant showed the major species with the core moiety containing D-*glycero*-D-*talo*-oct-2-ulososnic acid (Ko), 3-deoxy-D-*manno*-oct-2-ulososnic acid-I (KdoI), L-*glycero*-α-D-*manno*-heptose-I (HepI) and HepII and the lipid A moiety consisting of bisphosphorylated glucosamine disaccharide backbone with four 3-hydroxymyristic (3HO14:0) groups and one 4-amino-4-deoxy-L-arabinose (Ara4N) residue. The lack from the *waaE* mutant LPS of both β-D-glucose (Glc) and HepIII suggested that *waaE* encodes glucosyltransferase and that incorporation of HepIII into the core requires the prior addition of Glc to HepI. As in *waaQ* mutant, only a small proportion of GlcNAc-containing core variants were present in *waaE* mutant.

waaF *(YPO0057) mutant.* A single heptose residue (HepI) was found in the LPS of *waaF* mutant, and, hence, the inactivated gene encodes heptosyltransferase II. The lack of Glc from *waaF* mutant suggested that its addition to the core requires the prior transfer of HepII onto HepI.

waaC *(YPO0056) mutant.* Only Kdo (Ko) mono- and disaccharides were obtained by mild acid degradation of the *waaC* mutant LPS. The mass spectrum of the whole LPS revealed the major compounds having KdoII→KdoI or Ko→KdoI disaccharide core linked to lipid A containing four 3HO14:0 groups and one Ara4N residue. The lack of any heptose from the core suggested that *waaC* encodes heptosyltransferase I. The same LPS chemotype was found in *hldE* (YPO0654) mutant, which is unable to synthesize the activated Hep nucleotide derivative.

waaA *(YPO0055) mutant*. No core component was isolated after mild acid degradation of the *waaA* mutant LPS. The mass spectrum of the whole LPS showed that it is restricted to the lipid A moiety with four (major) and three (minor) 3HO14:0 groups. Therefore, it was suggested that the inactivated gene encodes Kdo transferase WaaA, a bifunctional enzyme that catalyzes transfer of both KdoI to lipid A and KdoII to KdoI (Raetz and Whitfield, 2002).

Only a minority of LPS molecules in *waaA* mutant contain Ara4N. A relatively low content of Ara4N (on the average not more than one residue per molecule) was observed also in lipid A of other *Y. pestis* LPS mutants with deeply truncated core, including *waaE* and *waaC* mutants. In contrast, in wild type LPS (Knirel et al., 2005) as well as in *waaL, wabC* and *wabD* mutants both phosphate groups in lipid A are almost completely glycosylated with Ara4N. These findings indicate a lower

efficiency of Ara4N transfer to lipid A in the LPS having an incomplete inner core region or, especially, no core as in *waaA* mutant.

eptB *(YPO4013) mutant*. A core variant containing phosphoethanolamine (EtNP) on terminal Ko has been found to be abundant in the wild-type LPS grown at 6°C (Knirel et al., 2006). No EtNP was present in the LPS of *eptB* mutant cultivated at the same temperature, while the carbohydrate core backbone and lipid A in the mutant and wild type were the same. Therefore, *eptB* encodes EtNP transferase.

arnT *(YPO2421) mutant.* The mass spectrum of the whole LPS showed that *arnT* mutant is fully unable to incorporate Ara4N to lipid A and, therefore, the inactivated gene encodes Ara4N transferase ArnT. Inactivation of *arnT* had no influence on the LPS core structure.

lpxM *(YPO2063) mutant.* No hexaacyl form was found in lipid A from *lpxM* mutant but a pentaacyl form with four 3HO14:0 groups and one palmitoleoyl (16:1) group. None of lower acylated variants present included a lauroyl group either. Other significant changes were observed neither in lipid A nor in the core of the mutant LPS. Therefore, it was concluded that *lpxM* encodes lauroyl transferase LpxM.

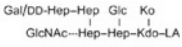
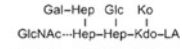
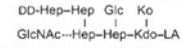
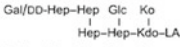
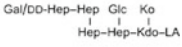
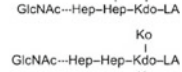
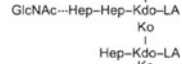
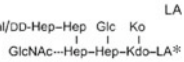
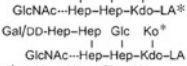
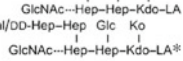
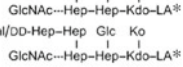
lpxP *(YPO3632) mutant.* As in lipid A from *lpxM* mutant, no hexaacyl form was found in lipid A from *lpxP* mutant but in this case the highest acylated variant contained four 3HO14:0 groups and one lauroyl (12:0) group. None of lower acylated variant included 16:1 group either, and, therefore, *lpxP* encodes palmitoleoyl transferase LpxP.

8.3.3 Resistance of Mutants to Bactericidal Action of Polymyxin B and Normal Human Serum

Resistance of *Y. pestis* to cationic antimicrobial peptides depends on the content of Ara4N, whose temperature-dependent incorporation to lipid A is regulated by the two-component PhoP/PhoQ regulatory system (Rebeil et al., 2004). Accordingly, we found that *Y. pestis arnT* mutant deficient in Ara4N was highly susceptible to PMB, as reported for *arnT* mutants of some other bacteria (Nizet, 2006). *waaQ*, *waaE*, *waaF*, *waaC, hldE* and *waaA* mutants with deeply truncated LPS core were 31– 250 times less resistant to PMB (MIC < 20 U mL^{-1}) than the wild-type strain (Table 8.1). The drop of the resistance may be accounted for by a low content of Ara4N in the LPS having an incomplete inner core region. Strains carrying mutations in genes responsible for adding the lateral core monosaccharides (*wabC, wabD, wecA* and *waaL*) were as resistant to PMB as the parental strain (MIC >625 U mL^{-1}).

waaQ, *waaE*, *waaF*, *waaC* and *waaA* deficient strains with an impaired inner core region were highly susceptible to the bactericidal action of normal human serum (NHS) (Table 8.1). The serum killing was complement-mediated as these mutants were resistant to heat-inactivated serum. All other generated mutants, including *arnT* mutant with an Ara4N-lacking LPS, were almost as resistant as the parental strain.

Table 8.1 Biological properties of wild-type strain *Y. pestis* 231 and derived LPS mutants

Mutation in gene	Core oligosaccharide structure	Relevant characteristics
Wild type		PMB^R, NHS^R, Fib^{++}, Coa^{++}, mLD_{50} = 2 (1–4) cfu, $gpLD_{50}$ 7 (2–27) cfu
YPO0186 (*wabC*)		PMB^R, NHS^R, Fib^{++}, Coa^{++}
YPO0187 (*wabD*)		PMB^R, NHS^R, Fib^{++}, Coa^{++}, mLD_{50} = 8 (1–32) cfu, $gpLD_{50}$ 7 (2–27) cfu
YPO3866 (*wecA*)		PMB^R, NHS^R, Fib^{+}, Coa^{+}
YPO0417 (*waaL*)		PMB^R, NHS^R, Fib^{+}, Coa^{+}, mLD_{50} = 13 (2–50) cfu, $gpLD_{50}$ 32 (8–126) cfu
YPO0416 (*waaQ*)		**PMB^S**, **NHS^S**, Fib^{+}, Coa^{+}, mLD_{50} = 5 (1–16) cfu, $gpLD_{50}$ 15 (4–58) cfu
YPO0054 (*waaE*)		**PMB^S**, **NHS^S**, Fib^{+}, Coa^{+}, mLD_{50} = 32 (8–126) cfu, $gpLD_{50}$ **> 10^4** cfu
YPO0057 (*waaF*)		**PMB^S**, **NHS^S**, **Fib^{-}**, **Coa^{-}**
YPO0056 (*waaC*)		**PMB^S**, **NHS^S**, **Fib^{-}**, **Coa^{-}**
YPO0654 (*hldE*)		**PMB^S**, **NHS^S**, **Fib^{-}**, **Coa^{-}**, mLD_{50} = **2.0×10^5** (5.0×10^4–7.9×10^5) cfu, $gpLD_{50}$ **> 10^4** cfu
YPO0055 (*waaA*)		**PMB^S**, **NHS^S**, **Fib^{-}**, **Coa^{-}**
YPO2421 (*arnT*)		**PMB^S**, NHS^R, Fib^{++}, Coa^{++}
YPO4013 (*eptB*)		PMB^R, NHS^R, Fib^{+}, Coa^{+}
YPO2063 (*lpxM*)		PMB^R, NHS^R, Fib^{++}, Coa^{++}
YPO3632 (*lpxP*)		PMB^R, NHS^R, Fib^{++}, Coa^{++}

LA, wild-type lipid A; LA∗, lipid A deficient in Ara4N, lauroyl (12:0) or palmitoleoyl (16.1) group; Ko∗, core deficient in PEtN. *Dotted line* indicates a non-stoichiometric amount of GlcNAc. Significant changes in biological properties are indicated in *bold face.* PMB^R, minimal inhibitory concentration (MIC) of polymyxin B (PMB) = 625–1250 U ml^{-1}; PMB^S, MIC of PMB ≤ 20 U ml^{-1}; NHS^R, resistant to normal human sera; NHS^S, after incubation of mutants grown at 25°C with NHS serum for 1 h the amounts of viable bacteria were three to five lg cfu ml^{-1} lower when compared with the wild type or mutants incubated in heat-inactivated NHS; Fib and Coa, coagulase and fibrinolytic activities of plasminogen activator; Fib^{++} or Coa^{-}, the complete clot lysis or absence of clotting, respectively; Fib^{+}, Coa^{+}, any degree of clot lysis or incomplete clotting (from a loose clot to a solid clot in liquid plasma); Fib^{-}, Coa^{++}, a solid clot; mLD_{50} and $gpLD_{50}$, LD_{50} for mice and guinea pigs, respectively; cfu, colony forming unit.

Serum resistance is necessary for survival and multiplication of causative agents of vector-borne diseases in mammal blood for further transmission by insects to new hosts (Perry and Fetherston, 1997). *Y. pestis* R-LPS incorporated into liposomes was shown to ensure serum resistance in contrast to S-LPS from serum-susceptible yersiniae phenotypes (Porat et al., 1995). On the other hand, serum resistance in *Y. pestis* is mediated by an outer membrane protein Ail (OmpX), and it has been hypothesized that changes in the LPS structure may influence the Ail conformation and specific activity (Bartra et al., 2008). Our data of *Y. pestis* LPS mutants show

that the full inner core composed of six sugar residues (KdoI, KdoII or Ko, HepI, HepII, HepIII and Glc) is required for the serum resistance, whereas the outer core monosaccharides (DD-HepIV, Gal and GlcNAc) are of little importance.

8.3.4 The Full LPS Core is Necessary for Enzymatic Activity of Plasminogen Activator

Fibrinolytic and plasmocoagulase activities of Pla were tested in *Y. pestis* strains with mutations in the genes of the LPS core biosynthetic pathway (Table 8.1). It was found that both activities are first decreased (in *wecA, waaL, waaQ* and *waaE* mutants) and then totally abolished (in *waaF, waaC, hldE* and *waaA* mutants) with a gradual reduction of the core.

Plasminogen activator Pla, a transmembrane protease belonging to omptins, is one of the recognized *Y. pestis* pathogenicity factors responsible for systemic spread of bacteria in the host organism (Kukkonen and Korhonen, 2004). In some *Y. pestis* strains, Pla expression is obligatory for full subcutaneous virulence. In contrast, in all representatives of subspecies *caucasica*, which are naturally deficient in plasmid pPst (pPla, pPCP1 or pYP) coding for Pla, or in a number of artificial Pla^- mutants of subspecies *pestis* strains, the absence of Pla does not affect the high subcutaneous virulence, which is comparable to that of classical wild-type Pla^+ strains (for review see Anisimov et al., 2004). Recently, it has been demonstrated that the presence of an S-type LPS with a long-chain O-antigen inhibits the action of *Y. pestis* Pla, whereas R-LPS is necessary for proper folding of Pla and manifestation of its enzymatic activities (Pouillot et al., 2005).

Our data on Pla fibrinolytic and plasmocoagulase activities in *Y. pestis* strains with gradual truncation of the core suggest that all eight wild-type *Y. pestis* core constituent sugars are necessary for maximal enzymatic activities of Pla. A smaller core consisting of seven to five sugar residues was still able to ensure the fibrinolytic and coagulase activities, though at noticeably reduced levels, whereas a deeper truncation resulted in inactivation of Pla.

8.3.5 Virulence of Mutants in Mice and Guinea Pigs

A gradual truncation of the LPS core was accompanied by a decrease of bacterial virulence in mice and guinea pigs (Table 8.1). In all cases the animal death was dose-related. However, the reduction in virulence remained behind the decrease of the bacterial resistance to the innate immunity factors. For instance, *waaQ* mutant deficient in HepIII transferase synthesis was highly susceptible to PMB and NHS but was as virulent as the parental strain in both animal models. In mice, further reduction of the core, e.g. as in *waaE* mutant deficient in synthesis of glucosyltransferase, was accompanied by a slight but reliable increase in LD_{50} values ($2 \rightarrow 32$ cfu) and mean times to death ($4.7 \rightarrow 7.9$ days). In any case such LD_{50} is extremely low

and *waaE* mutant was still highly virulent (for mice). The least virulent for mice was *hldE* mutant deficient in synthesis of Hep and possessing a core restricted to a Kdo→Kdo or Ko→Kdo disaccharide. In guinea pig, neither *waaE* nor *hldE* mutant caused animal death during 21 days of experiment.

Bacteriological examination of spleen from animals succumbed to infection indicated that they were all positive for *Y. pestis* during experiment terms. Indeed, a heavy growth of bacteria was observed on plates inoculated with homogenized spleen specimens. Organs from survivors infected with strain 231 and derived *wabD*, *waaL*, *waaQ* (mice and guinea pigs) or *waaE* (mice) mutants on the day 22 postinfection were all negative for *Y. pestis*. However, 60– 90% spleen specimens from survivors of both animals challenged with *hldE* mutant and guinea pigs infected with *waaE* mutant on the day 22 postinfection were positive for *Y. pestis*: solitary colonies of bacteria were observed on plates inoculated with homogenized spleen specimens.

wabD, *waaL* and *waaQ* mutants of *Y. pestis* subsp. *pestis* were not attenuated in a biologically significant way as each was still extraordinarily virulent in both mice and guinea pigs (Table 8.1). Only truncation of the core down to five sugar residues caused a noticeable decrease in subcutaneous virulence of *Y. pestis* subsp. *pestis* for guinea pigs ($=10^3$ cfu). A further truncation of the core to two sugar residues abolished virulence for both mice and guinea pigs.

8.4 Conclusion

To sum up, *Y. pestis* knock-out mutants with two or less sugar residues in the LPS core were not only susceptible to antimicrobial cationic peptides and NHS but also avirulent in murine and guinea pig infection models. This finding demonstrated that the LPS structure is crucial for the lethality of plague infection, and *waaC*, *hldE* and *waaA* or their protein products can be considered as promising candidates for targeting *Y. pestis* virulence using specific inhibitors.

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