

# Identification of the Lipopolysaccharide Core of *Yersinia pestis* and *Yersinia pseudotuberculosis* as the Receptor for Bacteriophage $\phi$ A1122<sup>∇</sup>

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$\phi$ A1122 is a T7-related bacteriophage infecting most isolates of *Yersinia pestis*, the etiologic agent of plague, and used by the CDC in the identification of *Y. pestis*.  $\phi$ A1122 infects *Y. pestis* grown both at 20°C and at 37°C. Wild-type *Yersinia pseudotuberculosis* strains are also infected but only when grown at 37°C. Since *Y. pestis* expresses rough lipopolysaccharide (LPS) missing the O-polysaccharide (O-PS) and expression of *Y. pseudotuberculosis* O-PS is largely suppressed at temperatures above 30°C, it has been assumed that the phage receptor is rough LPS. We present here several lines of evidence to support this. First, a rough derivative of *Y. pseudotuberculosis* was also  $\phi$ A1122 sensitive when grown at 22°C. Second, periodate treatment of bacteria, but not proteinase K treatment, inhibited the phage binding. Third, spontaneous  $\phi$ A1122 receptor mutants of *Y. pestis* and rough *Y. pseudotuberculosis* could not be isolated, indicating that the receptor was essential for bacterial growth under the applied experimental conditions. Fourth, heterologous expression of the *Yersinia enterocolitica* O:3 LPS outer core hexasaccharide in both *Y. pestis* and rough *Y. pseudotuberculosis* effectively blocked the phage adsorption. Fifth, a gradual truncation of the core oligosaccharide into the Hep/Glc (L-glycero-D-manno-heptose/D-glucofuranose)-Kdo/Ko (3-deoxy-D-manno-oct-2-ulopyranosonic acid/D-glycero-D-talo-oct-2-ulopyranosonic acid) region in a series of LPS mutants was accompanied by a decrease in phage adsorption, and finally, a *waaA* mutant expressing only lipid A, i.e., also missing the Kdo/Ko region, was fully  $\phi$ A1122 resistant. Our data thus conclusively demonstrated that the  $\phi$ A1122 receptor is the Hep/Glc-Kdo/Ko region of the LPS core, a common structure in *Y. pestis* and *Y. pseudotuberculosis*.

*Yersinia pestis*, the etiologic agent of bubonic plague and pneumonic plague, is one of the most potent bacterial pathogens known and is transmitted by a bite from infected fleas to their mammalian hosts (56). The present number of human plague cases in the world is relatively stable, with approximately two thousand reported incidents each year. This number may, however, be largely underestimated due to inadequate diagnostics and reporting in some countries of disease endemicity.

$\phi$ A1122 is a T7-related bacteriophage that is used as a diagnostic phage by the Centers for Disease Control and Prevention for the identification of *Y. pestis*. It infects most isolates of *Y. pestis* grown both at 20°C and at 37°C. *Yersinia pseudotuberculosis* is also infected but only when grown at 37°C (30).

The receptor used by phage  $\phi$ A1122 for adsorption has not been identified yet.

Gram-negative bacteria are surrounded by two membranes, the inner membrane (IM) and the outer membrane (OM). The IM is a bilayer composed of phospholipids, and the OM is asymmetric, having phospholipids in its inner leaflet and, in most Gram-negative bacteria, lipopolysaccharides (LPS) in its outer leaflet (13, 14). Embedded in the OM are also a number of outer membrane proteins (OMPs) that function in solute and protein translocation, pathogenesis, structural linkers, and signal transduction (13, 43). Both OMPs and LPS may function as specific phage receptors (43, 77).

LPS is an amphipathic molecule that is anchored to the OM by lipid A (71). Core oligosaccharide is attached to lipid A, and the outermost structure of LPS is O-polysaccharide (O-PS). In *Y. pestis*, the LPS is rough, containing no O-PS (19, 21, 34, 37, 66); however, *Y. pseudotuberculosis* strains express O-PS (65), and the expression is optimal at temperatures below 30°C. For a long time it was believed that mutants of LPS-containing Gram-negative bacteria without a minimal core structure (i.e., one residue of 3-deoxy-D-manno-oct-2-ulonic acid [Kdo]) are not viable (29, 63); thus, the core-lipid A unit was thought

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TABLE 1. Bacteriophages, bacterial strains, and plasmids used in this work

Bacteriophage/strain/plasmid	Genotype/relevant feature <sup>a</sup>	Source/reference(s)
<b>Bacteriophages</b>		
φA1122	Reference phage used by the CDC to identify <i>Y. pestis</i>	30
φYeO3-12	Phage using <i>Y. enterocolitica</i> serotype O:3 O-PS as receptor	2, 54, 55
φR1-37	Phage using <i>Y. enterocolitica</i> serotype O:3 OC as receptor	39, 70
<b>Bacterial strains</b>		
<i>Yersinia pestis</i>		
KIM D27	Nonpigmented isolate of KIM10.Lcr <sup>+</sup> Pgm <sup>-</sup> Pst <sup>+</sup>	31
KIM D27-ΔwabD	ΔwabD::nptII Kan <sup>r</sup>	This work
KIM D27-ΔwaaL	ΔwaaL::nptII Kan <sup>r</sup>	This work
KIM D27-ΔwaaQ	ΔwaaQ::nptII Kan <sup>r</sup>	This work
KIM D27-ΔwaaE	ΔwaaE::nptII Kan <sup>r</sup>	This work
KIM D27-ΔwaaA	ΔwaaA::nptII Kan <sup>r</sup>	This work
KIM D27Nar	Nal <sup>r</sup>	39
EV76-c	70-kb virulence plasmid-cured derivative of EV76	10, 60
231	pFra <sup>+</sup> pCD <sup>+</sup> pPst <sup>+</sup> ; bv. antiqua, subsp. <i>pestis</i> ; wild type	6
KM260(11)	pFra <sup>+</sup> pCD <sup>-</sup> (Lcr <sup>-</sup> ) pPst <sup>-</sup> ; derived from wild-type bv. antiqua subsp. <i>pestis</i> strain 231; avirulent	41, 42
KM260(11)pKD46	KM260(11) harboring plasmid pKD46; Amp <sup>r</sup>	5
KM260(11)ΔwabC	ΔwabC::nptII; derived from strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwabD	ΔwabD::nptII; derived from strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwabC/waaD	ΔwabC ΔwabD::nptII; derived from strain KM260(11)pKD46; Kan <sup>r</sup>	This work
KM260(11)ΔwabC/waaL	ΔwabC::nptII ΔwaaL::cat; derived from strain KM260(11)ΔwabCpKD46; Kan <sup>r</sup> Clm <sup>r</sup>	This work
KM260(11)ΔwabD/waaL	ΔwabD::nptII ΔwaaL::cat; derived from strain KM260(11)ΔwabDpKD46; Kan <sup>r</sup> Clm <sup>r</sup>	This work
KM260(11)ΔwaaL	ΔwaaL::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwaaQ	ΔwaaQ::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwaaQ/waaL	ΔwaaQ ΔwaaL::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	This work
KM260(11)ΔwaaE	ΔwaaE::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwaaF	ΔwaaF::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwaaC	ΔwaaC::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
KM260(11)ΔwaaA	ΔwaaA::nptII; derived from the strain KM260(11)pKD46; Kan <sup>r</sup>	5
<i>Y. pseudotuberculosis</i>		
PB1	Serotype O:1a	59
1	Serotype O:1a; Lcr <sup>-</sup>	65
43	Serotype O:3; Lcr <sup>-</sup>	65
32	Serotype O:4a; Lcr <sup>-</sup>	65
YPIII	Serotype O:3	12
PB1Δwb	Serotype O:1a; O-PS-negative derivative of PB1; Kan <sup>r</sup>	This work
PB1Δwb-R4	Spontaneous φA1122-resistant derivative of PB1Δwb	This work
PB1Δwb-R7	Spontaneous φA1122-resistant derivative of PB1Δwb	This work
PB1Δwb-R12	Spontaneous φA1122-resistant derivative of PB1Δwb	This work
<i>Y. enterocolitica</i>		
8081	Serotype O:8	61
8081-R2	Serotype O:8; O-PS-negative derivative of 8081	80
6471/76-c (YeO3-c)	Serotype O:3; virulence plasmid-cured derivative of 6471/76	68
YeO3-R1	Spontaneous O-PS-negative derivative of 6471/76-c	70
YeO3-c-OC	Δ(wzx-wbcQ) outer core-negative derivative of 6471/76-c	11
YeO3-OC-R	Δ(wzx-wbcQ) outer core- and O-PS-negative derivative of 6471/76	11
<i>E. coli</i>		
DH10B	F <sup>-</sup> mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara leu)7697 galU galK λ <sup>-</sup> rpsL nupG λ <sup>-</sup> tonA	Life Technologies
C600	thi thr leuB tonA lacY supE	7
HB101	F <sup>-</sup> Δ(gpt-proA)62 leuB6 glnV ara-14 galK2 lacY1 Δ(mcr-mrr) rpsL20 (Str <sup>r</sup> ) xyl-5 mtl-1 recA13	15
Sm10λpir	thi thr leuB tonA lacY supE recA::RP4-2-Ycc::Mu-Kan (λpir)	67
<b>Plasmids</b>		
pUC18	Cloning vector; Amp <sup>r</sup>	79
pAY100.1	O-PS gene cluster of YeO:3 cloned in pBR322; Amp <sup>r</sup>	53
pRV16NP	Outer core gene cluster of YeO:3 cloned in pTM100; Clm <sup>r</sup>	39
pRK2013	Helper plasmid for conjugation; Kan <sup>r</sup>	25
p34S-Km	Cloning vector; Amp <sup>r</sup> Kan <sup>r</sup>	23
pCVD442	Suicide vector; Amp <sup>r</sup>	26
pCVD442-wabD	Suicide vector with wabD::nptII; Amp <sup>r</sup> Kan <sup>r</sup>	5
pCVD442-waaL	Suicide vector with waaL::nptII; Amp <sup>r</sup> Kan <sup>r</sup>	5
pCVD442-waaQ	Suicide vector with waaQ::nptII; Amp <sup>r</sup> Kan <sup>r</sup>	5
pCVD442-waaE	Suicide vector with waaE::nptII; Amp <sup>r</sup> Kan <sup>r</sup>	5
pCVD442-waaA	Suicide vector with waaA::nptII; Amp <sup>r</sup> Kan <sup>r</sup>	5
pUCwbup	Upstream region of O-PS gene cluster of YPIII cloned in pUC18; Amp <sup>r</sup>	This work
pUCwbdel	Up- and downstream regions of the YPIII O-PS gene cluster cloned in pUC18; Amp <sup>r</sup>	This work
pUCwbGB	Kan <sup>r</sup> gene of p34S-Kan cloned between the up- and downstream regions of the YPIII O-PS gene cluster in pUCwbdel; Amp <sup>r</sup> Kan <sup>r</sup>	This work
pCVDwbGB	Suicide vector carrying the PvuII fragment of pUCwbGB, including the Kan <sup>r</sup> gene and the up- and downstream regions of O-PS gene cluster of YPIII; Amp <sup>r</sup> Kan <sup>r</sup>	This work

<sup>a</sup> Lcr, low calcium response; Pgm, pigmentation; Pst, pesticide resistance.

TABLE 2. Primers used for verification of the *Y. pestis* D27 LPS mutants

Primer	Gene	Primer sequence
YPO0187-F	<i>wabD</i>	AATGCGGTACGTTGTGGTGA
YPO0187-R	<i>wabD</i>	TTTGCCGATGGTGATGATTG
YPO0417-F	<i>waaL</i>	AGTTGATTCTGGCGAGTTG
YPO0417-R	<i>waaL</i>	CTCCCCTGCCTATCCTCACC
YPO0416-F	<i>waaQ</i>	GCTGCGTGTATGCTCCGTTGACTG
YPO0416-R	<i>waaQ</i>	ATCCGGGCCATAGCTGTTGTTTG
YPO0054-F	<i>waaE</i>	TAGTAATGGGATCAAATGTC
YPO0054-R	<i>waaE</i>	ACTCTATCGCTGGTAAAAG
YPO0055-F	<i>waaA</i>	TTTGCTGCGTTTACTATTA
YPO0055-R	<i>waaA</i>	ACACCGTATTCTTTTACC

to be important for bacterial viability and membrane function. However, it has been proven in recent years that mutants synthesizing only lipid A or a precursor thereof are viable (49, 50, 74). Together with other LPS-free Gram-negative bacteria (33, 35, 36, 38, 47, 73, 78), *Neisseria meningitidis* can also survive without any LPS (72).

The T7 group phages T7 and T3, infecting *Escherichia coli* (45, 76), and *Yersinia enterocolitica* phage  $\phi$ YeO3-12 (2, 3), all use LPS as a receptor. The receptor recognition of these phages is dependent on the tail fiber protein gp17. Since the gp17 of  $\phi$ A1122 is highly similar to its counterparts in T7 and T3, it was assumed that  $\phi$ A1122 also may bind to LPS (30).

The lipid A-core oligosaccharide structures of *Y. pestis* and *Y. pseudotuberculosis* LPS are more or less identical, with both expressing several glycoforms that show temperature-dependent variations (41). The main core structure is heptasaccharide, which contains hexoses (Glc [D-glucopyranose] and Gal [D-galactopyranose]), heptoses (DD-Hep [D-glycero-D-mannoheptopyranose] and Hep [L-glycero-D-manno-heptopyranose]), and octulosonic acids (Kdo [3-deoxy-D-manno-oct-2-ulopyranosonic acid] and Ko [D-glycero-D-talo-oct-2-ulopyranosonic acid]). In bacteria grown at 25°C, the core main chain (from nonreducing end to lipid A) is a pentasaccharide, DD-Hep(IV)-Hep(III)-Hep(II)-Hep(I)-Kdo, with branching Ko from Kdo and Glc from Hep(I), while in bacteria grown at 37°C, DD-Hep is replaced by Gal, and Ko is replaced by Kdo (24). In this work, we present conclusive evidence demonstrating that the  $\phi$ A1122 receptor is the Hep/Glc-Kdo/Ko region of *Y. pestis* and *Y. pseudotuberculosis* LPS.

MATERIALS AND METHODS

**Bacterial strains, phages, plasmids, and media.** The bacteriophage, bacterial strains, and plasmids used in this work are described in Table 1. Unless otherwise stated, *Yersinia* and phage cultures were incubated at room temperature (RT; 22°C) and *E. coli* cultures at 37°C. Tryptone soya broth (TSB) medium (Oxoid) was used for bacterial liquid cultures, and soft agar medium included an additional 0.4% (wt/vol) agar (Biokar Diagnostics). For transconjugant selections, *Yersinia* selective agar (CIN agar; Oxoid) plates supplemented with the appropriate antibiotics were used. Luria agar (64) was used as solid medium for bacteria, and lambda agar (tryptone at 10 g/liter, NaCl at 2.5 g/liter, agar at 15 g/liter) for phage plates. Plates were supplemented with ampicillin (Amp; 100 µg/ml), kanamycin (Kan; 100 µg/ml), nalidixic acid (Nal; 100 µg/ml), or chloramphenicol (Cm; 30 µg/ml) when required.

**Construction of mutants.** Standard recombinant DNA techniques were applied as described previously (64). All the enzymes were used as recommended by the suppliers. To construct the strain PB1Δwb, a region upstream of the O-PS gene cluster was amplified by PCR using primers P1 (5'-CCGGAATTCGAGCTCATGCGTATCATTCTGCTGGGC-3') and P2 (5'-CGGGTACCTTATAT

ATTATGTGCGAAT-3'), and chromosomal DNA of the *Y. pseudotuberculosis* strain YPIII (Table 1) was used as template. This fragment was cloned into pUC18 digested with EcoRI-KpnI to obtain plasmid pUCwbup. A region downstream of the O-PS gene cluster was amplified by PCR using primers P3 (5'-CGGGGTACCTTTAGTTGAGCACTTTGT-3') and P4 (5'-AAACTGCAGAGCTCTATGCTGTTTCGACCCAATGAT-3') and was cloned into pUCwbup digested with KpnI and PstI to give pUCwbdel. A kanamycin gene cassette was obtained by digestion of p34S-Km (Table 1) with KpnI and gel purified. This cassette was cloned into the KpnI site of pUCwbdel to give pUCwbGB. A PvuII fragment from pUCwbGB was cloned into the SmaI site of plasmid pCVD442 (Table 1) to give pCVDwbGB. Mutants were selected after mating *E. coli* Sm10λpir/pCVDwbGB with *Y. pseudotuberculosis* strain PB1 (Table 1) by following the protocol previously described (9). Mutant genotypes were confirmed by PCR and Southern blot hybridization with appropriate DNA probes (data not shown).

A series of *Y. pestis* D27 mutant strains with truncated LPS were generated by allelic exchange using pCVD442-based suicide vectors (Table 1). The suicide vectors were introduced into the parental *Y. pestis* D27 strain from *E. coli* S17-λpir (Table 1) by conjugation, and Kan<sup>r</sup> Amp<sup>r</sup> merodiploid transconjugants were selected using CIN(Suc) agar for counterselection of the donor. The selected merodiploids were plated onto brain heart infusion (BHI) agar with 10% sucrose and grown at RT for 2 days. The correct allelic exchange in the resultant Suc<sup>r</sup> Kan<sup>r</sup> Amp<sup>s</sup> colonies was confirmed using PCR with corresponding primers (Table 2). The *Y. pestis* KM260(11) LPS mutants, on the other hand, were generated by allelic exchange based on homologous recombination between genomic DNA and PCR products (5, 20).

$\phi$ A1122-resistant derivatives of *Y. pestis* D27 and *Y. pseudotuberculosis* PB1Δwb were isolated by spreading bacterial suspension on agar plates and pipetting drops of phage lysate on a dry bacterial lawn. Phage-resistant colonies were picked from within the lysis zone after 2 to 4 days.

Plasmid pRV16NP was mobilized to *Y. pseudotuberculosis* by triparental conjugation using helper strain HB101/pRK2013 (Table 1) (32).

**Phage adsorption assays and calculation of efficiency of plating (EOP).** Approximately  $8 \times 10^5$  PFU of  $\phi$ A1122 in 100 µl was mixed with a 500-µl sample of bacteria ( $A_{600} = 1.2$ ). For *Y. enterocolitica* and *Y. pseudotuberculosis* overnight cultures and for *Y. pestis*, a 48-h culture was used. The suspension was incubated at RT for 5 min and centrifuged at  $16,000 \times g$  for 3 min, and the phage titer remaining in the supernatant, i.e., the residual PFU percentage, was determined. TSB was used as a nonadsorbing control in each assay, and the phage titer in the control supernatant was set to 100%. Each assay was performed in duplicate and repeated at least twice.

To assay the adsorption kinetics and eliminate the effect of reversible adsorption, the following protocol was used (1). The phage and bacteria were mixed in a total volume of 2 ml (ca.  $2 \times 10^6$  PFU/ $10^8$  CFU). A TSB tube without bacteria was used as a negative control. At different time points, duplicate 10-µl samples

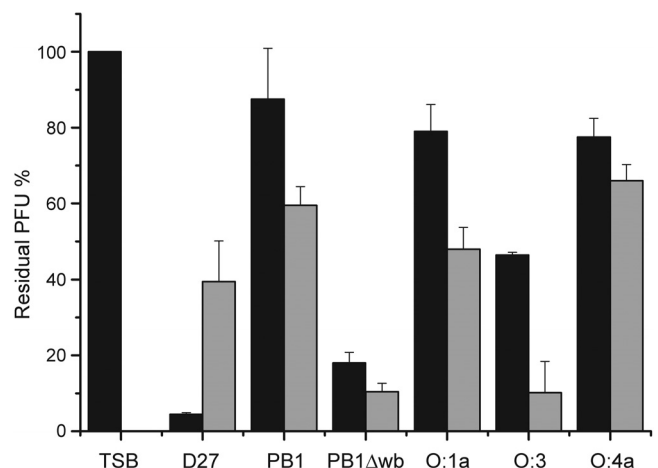


FIG. 1. Effect of temperature on  $\phi$ A1122 adsorption to *Y. pestis* and *Y. pseudotuberculosis*. Black bars show the residual PFU percentages after adsorption of phage on bacteria grown at RT, and gray bars show the residual PFU percentages after adsorption of phage on bacteria grown at +37°C. The control (TSB) and strains used for adsorptions are indicated below the columns. Error bars indicate ranges.

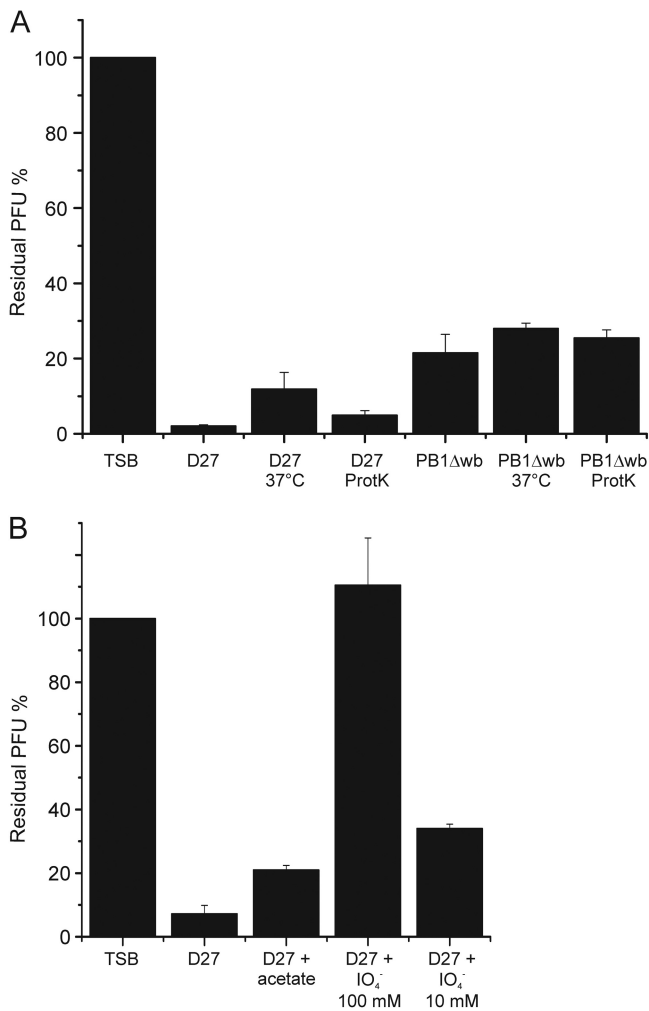


FIG. 2. Effects of different treatments of bacteria on  $\phi$ A1122 adsorption, shown as residual PFU percentages. (A) The effect of proteinase K treatment on adsorption of  $\phi$ A1122 to *Y. pestis* and *Y. pseudotuberculosis*. (B) The effect of periodate treatment on adsorption of  $\phi$ A1122 to *Y. pestis*. The control, strains, and treatments used for adsorptions are indicated below the columns. Error bars indicate ranges.

were withdrawn from the tube and mixed by vortexing with 990  $\mu$ l of TSB in an Eppendorf tube to release reversibly adsorbed phage. After 2 min of centrifugation at  $16,000 \times g$ , 0.5 ml of the supernatant was withdrawn and stored on ice for titration of the PFU. The residual PFU percentage was calculated as described above. The adsorption rate constants were calculated as described previously (1).

To determine the efficiency of plating (EOP), 100  $\mu$ l of wild-type (WT) and mutant *Y. pestis* cultured bacteria ( $A_{600} = 1.2$ ) was mixed with 50  $\mu$ l of  $\phi$ A1122 ( $2 \times 10^3$  PFU/ml) in 3 ml of 0.4% soft agar and poured onto LB plates. The number of PFU was counted after 24 to 48 h. Each strain was tested in triplicate. The EOP was calculated using the following formula: EOP = (number of PFU on mutant strain)/(number of PFU on the wild type).

**Periodate and proteinase K treatments.** To test how proteinase K treatment affects  $\phi$ A1122 adsorption, 2 ml of D27 and PB1 $\Delta$ wb cultures was treated with proteinase K (0.2 mg/ml; Promega) at 37°C for 3 h and washed with 2 ml of TSB, and the phage adsorption assay was performed as described above. To confirm that the possible effect was not due to incubation at 37°C, a control without proteinase K addition was included.

In order to study whether periodate can destroy the phage receptor, 1.5 ml of D27 culture was centrifuged at  $16,000 \times g$  for 1 min, and the bacterial pellet was suspended into 1.5 ml sodium acetate (50 mM; pH 5.2) or sodium acetate

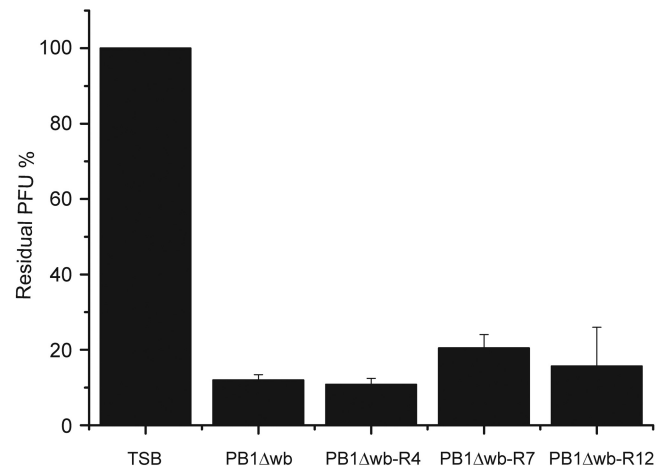


FIG. 3.  $\phi$ A1122 adsorption to phage-resistant derivatives of *Y. pseudotuberculosis* PB1 $\Delta$ wb, shown as residual PFU percentages. The control and strains used for adsorptions are indicated below the columns. Error bars indicate ranges.

containing either 10 or 100 mM IO<sub>4</sub><sup>-</sup>. The cells were incubated for 2 h (protected from light), centrifuged as described above, washed with 1.5 ml TSB, centrifuged, and suspended in TSB. Finally, the  $A_{600}$  of the bacterial suspension was adjusted to 1, and the phage adsorption assay was carried out.

**Isolation and analysis of lipopolysaccharide.** Small-scale isolation and deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) analysis of LPS were performed as described earlier (70, 81). Large-scale isolation and compositional analyses of LPS were performed as described previously (22).

## RESULTS

**Effect of temperature on the expression of the  $\phi$ A1122 receptor.** The earlier observation that  $\phi$ A1122 infects *Y. pestis* grown at 22°C and at 37°C but *Y. pseudotuberculosis* only when grown at 37°C (30) led us to study the effect of temperature on the expression of the phage receptors of these species more closely. To this end, the phage adsorption to *Y. pestis* D27 and different *Y. pseudotuberculosis* strains cultured at RT and 37°C was examined (Fig. 1). A slightly surprising finding was that D27 seemed to adsorb more phage at RT than at 37°C. All the smooth *Y. pseudotuberculosis* strains tested, representing serotypes O:1a, O:1b, O:3, and O:4a (Table 1), adsorbed more phage at 37°C than at RT, even though for serotype O:4a, the difference was not so apparent. On the other hand, strain PB1 $\Delta$ wb, a rough derivative of serotype O:1b strain PB1, adsorbed  $\phi$ A1122 equally well at both temperatures (Fig. 1). These results may suggest that the phage receptor is the LPS core that is sterically blocked by O-PS expression in *Y. pseudotuberculosis* at temperatures below 30°C. However, the possibility that the receptor is an OMP blocked by O-PS cannot be rigorously excluded.

**Periodate but not proteinase K destroys phage receptors.** Since bacteriophage can exploit both LPS and OMPs as receptors (43, 77), it was important to test whether the degradation of cell surface proteins or LPS could destroy the  $\phi$ A1122 receptor. For this reason, bacteria were treated with proteinase K or periodate prior to the phage adsorption assay. As seen in Fig. 2A, the proteinase K treatment of *Y. pestis* D27 or *Y. pseudotuberculosis* PB1 $\Delta$ wb did not reduce the adsorption capacity of the cells, suggesting that the functional receptor does



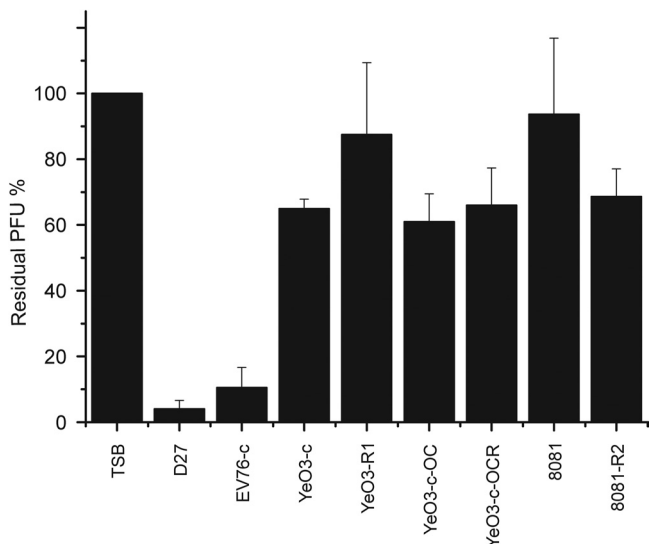


FIG. 4.  $\phi$ A1122 adsorption to *Y. pestis* and *Y. enterocolitica* serotype O:3 and O:8 wild-type and LPS mutant strains, shown as residual PFU percentages. The control and strains used for adsorptions are indicated below the columns. Error bars indicate ranges.

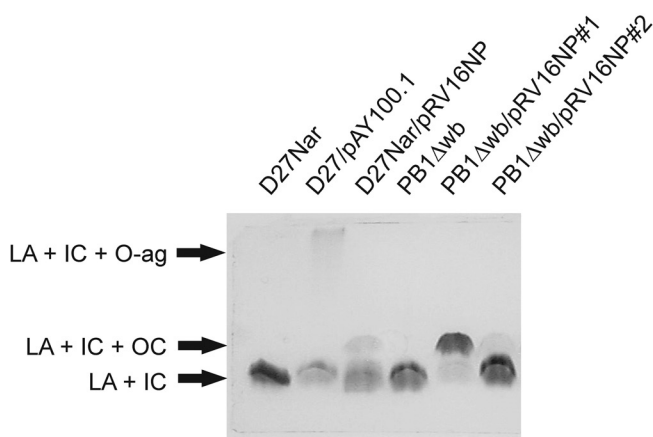
not contain a protein structure. The possibility that the receptor is a protein resistant to proteinase K is unlikely, considering the broad substrate specificity of this enzyme (27).

To study how the degradation of carbohydrates affects the phage adsorption, the effect of periodate (degrades carbohy-

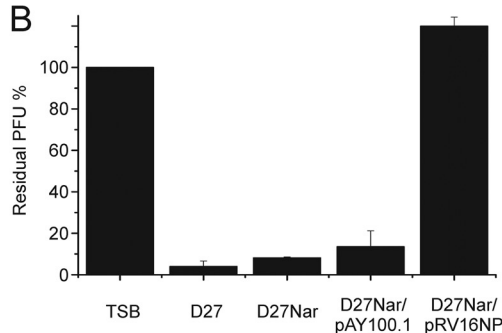
drates containing a 1,2-diol motif in their structure) on the phage receptor was tested. Incubation of *Y. pestis* D27 in the presence of 100 mM periodate abolished  $\phi$ A1122 binding completely, whereas incubation in 10 mM periodate or acetate buffer alone did not (Fig. 2B). This result thus confirmed that a carbohydrate structure, most likely LPS, is the receptor for  $\phi$ A1122.

**$\phi$ A1122-resistant bacterial mutants.** In order to study  $\phi$ A1122 adsorption more thoroughly, we aimed to isolate phage-resistant mutants of *Y. pestis* D27 and *Y. pseudotuberculosis* PB1 $\Delta$ wb. LPS biosynthesis involves tens of genes; therefore, isolation of spontaneous LPS mutants is usually easy, but that was not the case here. After substantial efforts, we isolated a few D27 mutants but only with reduced infectivity; the EOP of  $\phi$ A1122 on the most resistant mutant obtained was still ca. 0.2 (data not shown). With PB1 $\Delta$ wb we were more successful; strain PB1 $\Delta$ wb-R7 was fully  $\phi$ A1122 resistant, and strains PB1 $\Delta$ wb-R4 and PB1 $\Delta$ wb-R12 were highly resistant, having EOPs of ca.  $10^{-3}$  (Table 1). Surprisingly, when  $\phi$ A1122 adsorption to these mutants was measured, they all adsorbed the phage as efficiently as the parental strain PB1 $\Delta$ wb (Fig. 3). The same was true for the moderately resistant derivatives of D27 (data not shown). Thus, even though the phage propagation on these mutants was restricted, the phage receptor was not affected. In conclusion, these results indicated that under the experimental conditions applied, the intact phage receptor was essential for the growth of *Y. pestis* and *Y. pseudotuberculosis*. The LPS sugar compositions of the resistant mutants and those

A



B



C

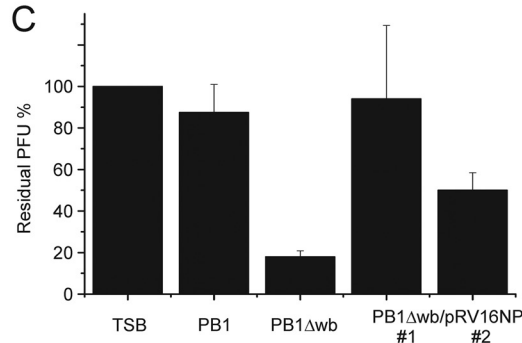


FIG. 5. Expression of *Y. enterocolitica* O:3 O-PS and outer core hexasaccharide in *Y. pestis* and in *Y. pseudotuberculosis*. (A) Analysis of LPS by DOC-PAGE and silver staining. LA, lipid A; IC, inner core; OC, outer core; O-ag, O-PS. (B) Effect of *Y. enterocolitica* O:3 O-PS and outer core hexasaccharide expression on  $\phi$ A1122 adsorption to *Y. pestis*, shown as residual PFU percentages. (C) Effect of *Y. enterocolitica* O:3 outer core hexasaccharide expression on  $\phi$ A1122 adsorption to *Y. pseudotuberculosis*, shown as residual PFU percentages. The control and strains used for adsorptions are indicated below the columns. TSB was used as a no-bacteria control. Error bars indicate ranges.

of wild-type bacteria were analyzed, and no significant differences were detected (data not shown).

**Absence of the  $\phi$ A1122 receptor in *Y. enterocolitica*.** The LPS core structures of *Y. enterocolitica* and *Y. pestis* are similar but not identical (16, 52, 75). To study whether *Y. enterocolitica* contains the  $\phi$ A1122 receptor, the phage adsorption assay was conducted with *Y. enterocolitica* strains belonging to serotypes O:3 and O:8 either expressing wild-type LPS or missing the O-PS, outer core (OC), or both (Table 1). *Y. pestis* strains D27 and EV76-c (Table 1) were used as positive controls. As seen in Fig. 4, none of the *Y. enterocolitica* strains, not even the rough or deep rough derivatives, specifically adsorbed  $\phi$ A1122. The phage receptor therefore is most likely a structure of the LPS cores of *Y. pestis* and *Y. pseudotuberculosis* that is not present in *Y. enterocolitica*.

**Blocking the  $\phi$ A1122 receptor.** In *Y. enterocolitica* serotype O:3, O-PS and OC hexasaccharide are linked to the LPS inner core, with the latter specifically linked to Hep(II) (28, 57, 69). To obtain more information about the carbohydrate residues forming the  $\phi$ A1122 receptor, the effect of heterologous expression of *Y. enterocolitica* O:3 O-PS and OC on *Y. pestis* D27 and OC on *Y. pseudotuberculosis* PB1 $\Delta$ wb for phage adsorption was studied. To this end, plasmids pAY100.1 and pRV16NP (Table 1), containing *Y. enterocolitica* O:3 O-PS and OC gene clusters, respectively, were used. The expression of heterologous LPS was verified by DOC-PAGE analysis (Fig. 5A) and by using O-PS- and OC-specific bacteriophages  $\phi$ YeO3-12 and  $\phi$ R1-37 (Table 1), respectively (data not shown). The adsorption assay for these strains showed that the expression of *Y. enterocolitica* O:3 O-PS on *Y. pestis* had no effect on  $\phi$ A1122 binding, whereas *Y. enterocolitica* O:3 OC blocked the phage receptor completely (Fig. 5B). Consistently, strain D27/pAY100.1 was sensitive to  $\phi$ A1122, while strain D27Nar/pRV16NP was resistant (data not shown).

In the case of *Y. pseudotuberculosis* PB1 $\Delta$ wb, two different clones were obtained after the conjugation, named PB1 $\Delta$ wb/pRV16NP#1 and PB1 $\Delta$ wb/pRV16NP#2. These clones differed from each other by the expression level of *Y. enterocolitica* O:3 OC, since OC was more strongly expressed in PB1 $\Delta$ wb/pRV16NP#1 than in PB1 $\Delta$ wb/pRV16NP#2 or in D27Nar/pRV16NP (Fig. 5A). Coherently with the *Y. enterocolitica* O:3 OC expression level, strain PB1 $\Delta$ wb/pRV16NP#1 was resistant to  $\phi$ A1122 and did not adsorb it at all, while PB1 $\Delta$ wb/pRV16NP#2 was sensitive and adsorbed the phage moderately (Fig. 5C). The OC overexpression of PB1 $\Delta$ wb/pRV16NP#1 is most probably due to a mutation in plasmid pRV16NP#1, since the overexpressing phenotype transfers with the plasmid (data not shown). The nature of this mutation is not yet known.

**Mapping the  $\phi$ A1122 receptor by using LPS mutants.** After establishing LPS as the probable receptor for phage  $\phi$ A1122, we deleted the *wabD*, *waaL*, *waaQ*, *waaE*, and *waaA* genes (Fig. 6A) of the LPS core biosynthetic pathway in *Y. pestis* D27 in order to pinpoint the role of LPS core in the phage receptor. The deletions caused the predicted LPS truncations (5), as verified by DOC-PAGE analysis (data not shown). Most of the deletion mutants grew normally under the applied conditions; however, the growth rate of the  $\Delta$ *waaA* mutant was severely decreased (data not shown). We measured the EOPs and adsorption kinetics of the D27 wild type and the mutant strains.

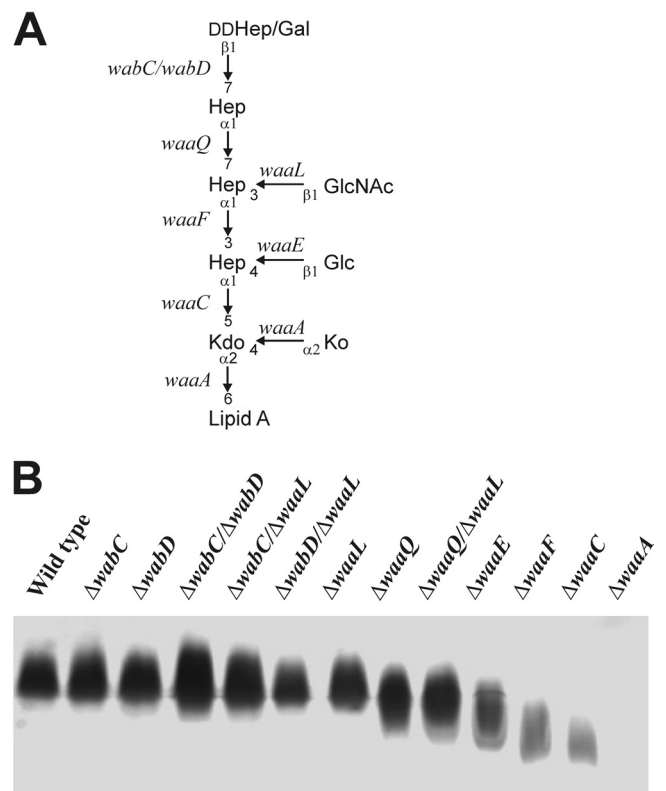


FIG. 6. Structure of *Y. pestis* LPS and characteristics of LPS mutants. (A) Schematic structure and the relevant genes involved in the biosynthesis of the core oligosaccharide. The glycosidic bonds between different residues are indicated. (Modified from reference 5.) (B) Silver-stained SDS-PAGE of LPS isolated from wild-type KM260(11) and LPS mutants. The deleted genes are indicated at the top of the gel.

Both approaches revealed that the phage infected the D27  $\Delta$ *wabD*,  $\Delta$ *waaL*, and  $\Delta$ *waaQ* strains at a level comparable to that of the wild-type strain; i.e., the EOPs were 0.97 to 0.98, and the residual PFU percentage in the adsorption kinetics assay at the 10-min time point was 8 to 10%, compared to 17% for the wild type (Fig. 7A). However, the D27  $\Delta$ *waaE* strain was less sensitive (residual PFU percentage at 10 min was  $\sim$ 40%, with an EOP of 0.28), and finally, the D27  $\Delta$ *waaA* strain was completely resistant. These results provided direct evidence showing LPS as the receptor of the phage. To study the roles of the individual sugar residues in the Hep/Glc-Kdo/Ko region, we used a set of deep rough mutants of *Y. pestis* KM260(11) (Table 1; Fig. 6). In general, the *Y. pestis* KM260(11) mutants behaved similarly to the corresponding *Y. pestis* D27 mutants (Table 3; Fig. 7B). With the KM260(11) mutants, infection and adsorption defect became visible with the  $\Delta$ *waaE* strain missing the Glc residue, after which the phage sensitivity gradually decreased with further losses of the Hep(II) and Hep(I) residues (Table 3). As with D27, the KM260(11)  $\Delta$ *waaA* strain missing the Kdo/Ko region was completely resistant (Table 3).

The adsorption kinetics assay (Fig. 7) demonstrated that irreversible adsorption to wild-type bacteria was extremely rapid; most of the adsorption (over 70%) took place within the first 2 min, and by 5 min, the adsorption rate leveled off. On the

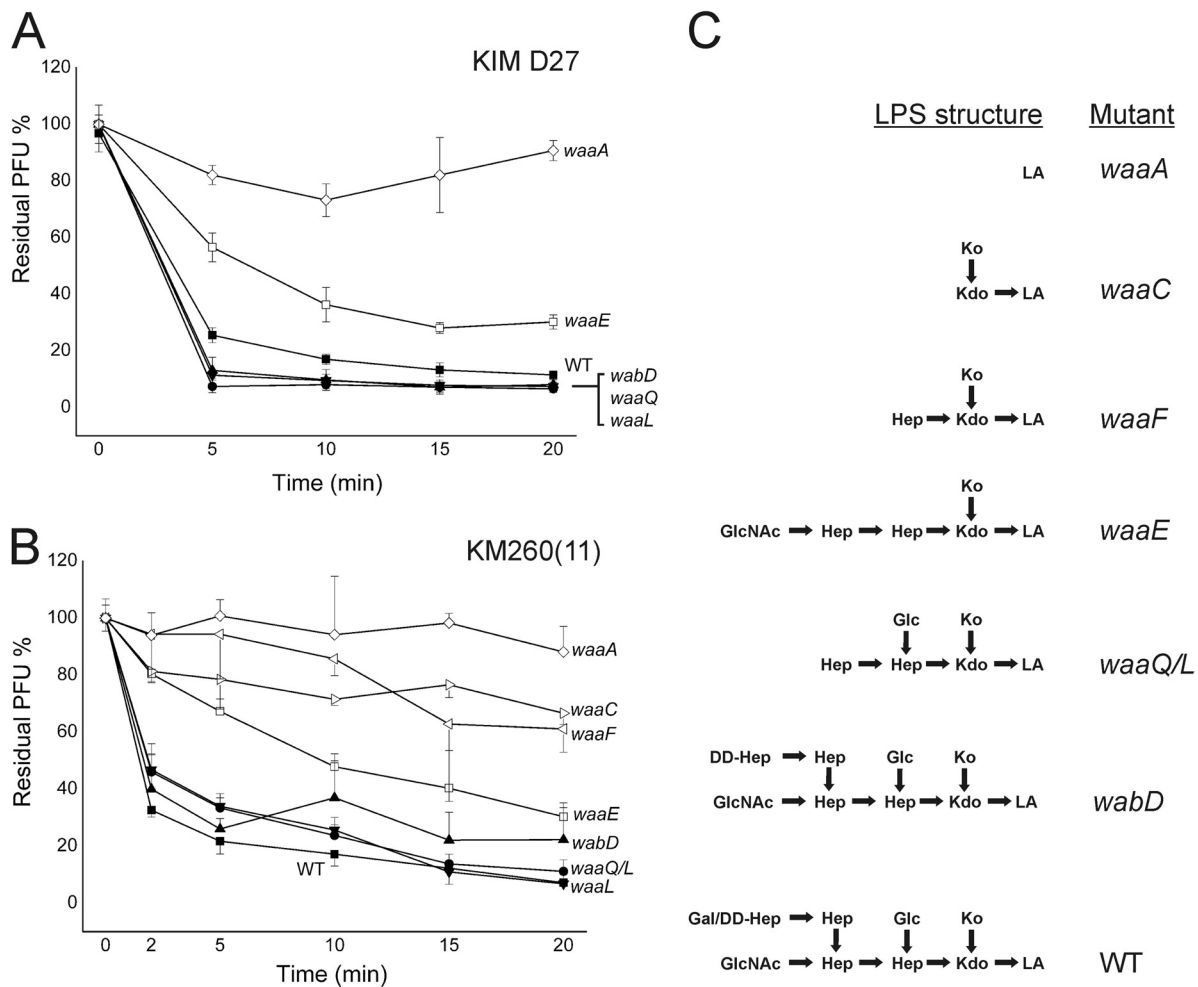


FIG. 7. Adsorption kinetics of  $\phi$ A1122 to *Y. pestis* LPS mutants, shown as residual PFU percentages. (A) *Y. pestis* KIM D27 derivatives. (B) *Y. pestis* KM260 (11) derivatives. Standard errors in panels A and B are indicated by vertical lines (in panel B, only in one direction). (C) LPS core structures of the mutants (Fig. 6). (Modified from reference 5.)

other hand, adsorption to the  $\Delta waaE$ ,  $\Delta waaF$ , and  $\Delta waaC$  strains was much slower and continued over the duration of the experiment. The  $\Delta waaA$  bacteria adsorbed no or very few phage particles. The adsorption rate constants were calculated for the strains based on the data of the 2- and 10-min time points presented in Fig. 7B (Table 3). As expected, the wild-type strain had the highest adsorption rate constant, and the deep rough mutants had the lowest. Interestingly, for all strains, the 10-min constants were lower than the respective 2-min constants, indicating that a subfraction of phages adsorbed slower (44). Nevertheless, these numbers further demonstrated that the phage receptor was affected in the  $\Delta waaE$ ,  $\Delta waaF$ , and  $\Delta waaC$  strains.

The above-described experiments were all carried out with bacteria grown at RT. Interestingly, when the experiments were carried out with bacteria grown at 37°C, we observed reduced adsorption efficiencies for  $\Delta wabC$ ,  $\Delta wabD$ ,  $\Delta wabC \Delta wabD$ ,  $\Delta wabC \Delta waaL$ , and  $\Delta wabD \Delta waaL$  mutants (data not shown), while there were no significant differences in their plating efficiencies (Table 3). This could be attributed to some conformational changes occurring in the *Y. pestis* core struc-

ture, surface charge changes causing repulsion, or expression of loosely blocking surface structures. All these could be at least partially due to any of the temperature-dependent structural variations taking place in *Y. pestis* LPS structure at 37°C, i.e., replacement of Ko with Kdo(II) or of Gal with DD-Hep(IV), or to the change of lipid A acylation from penta/hexa acylated to tetra acylated and the decrease in 4-amino-4-deoxy-L-arabinose substitution of lipid A (40).

### DISCUSSION

The susceptibility of a bacterium to bacteriophage infection is dependent primarily on whether or not the bacteriophage can find its specific attachment sites, i.e., the receptors on the cell. The recognition of the receptor is a highly specific process and is part of the natural mechanism of host recognition. During the last decade, the cell envelopes of both Gram-negative and Gram-positive bacteria have been studied intensively from structural, biosynthetic, genetic, and functional viewpoints. As a result of these studies, information of considerable value for investigations of phage receptor sites has





As an attempt to further characterize which core sugar residues are needed for  $\phi$ A1122 adsorption, the potential of *Y. enterocolitica* serotype O:3 O-PS or OC to inhibit the phage binding when expressed on the *Y. pestis* or *Y. pseudotuberculosis* core oligosaccharide was studied. The O:3 O-PS expressed in *Y. pestis* had no effect on  $\phi$ A1122 adsorption, in contrast to OC, which blocked it efficiently (Fig. 5B). In fact, partial substitution of LPS molecules seemed to be sufficient, as the phage adsorption to both *Y. pestis* and *Y. pseudotuberculosis* was blocked even when unsubstituted LPS molecules were present (Fig. 5). An interesting finding was that while the *Y. pseudotuberculosis* O-PS blocked the phage receptor, the expression of *Y. enterocolitica* O:3 O-PS in *Y. pestis* had no effect on the phage binding. The location of the attachment site of *Y. enterocolitica* O:3 O-PS in the core is not known, but this study implies that it is different from that of *Y. pseudotuberculosis*, which is at O-3 of Hep(II).

Finally, to decipher the minimal portion of LPS required as a receptor for the phage, adsorption studies with truncated LPS mutants of *Y. pestis* D27 and *Y. pestis* strain KM260(11) were carried out. The mutants with outermost core truncations missing Hep(III), DD-Hep(IV), and GlcNAc had EOPs comparable to those of the wild type (WT) (Table 3) and supported the phage binding in the adsorption experiments (Fig. 7). The  $\Delta waaE$  mutants of both the D27 and KM260(11) strains showed a small defect, as both had decreased EOPs and reduced adsorption (Table 3; Fig. 7), and the defect increased with increasing truncation of the core ( $\Delta waaF$  and  $\Delta waaC$  mutants). The only fully  $\phi$ A1122-resistant derivatives were the  $\Delta waaA$  mutants of both strains. Effectively, it seems that since the removal of the Hep(I)-linked Glc residue in the  $\Delta waaE$  mutant reduced the phage adsorption by  $\sim 30\%$ , the Glc residue is part of the receptor. The phage infection assays (EOPs) with the  $\Delta waaE$  mutant strain point to the same conclusion. In summary, our data revealed that the complete phage receptor contains two Hep residues with a Glc branching from Hep(I) attached to lipid A via Kdo/Ko.

The  $\phi$ A1122 receptor was not present in any of the *Y. enterocolitica* strains studied (Fig. 3). The core structures of different *Yersinia* species are rather similar, and in fact, the *Y. enterocolitica* serotype O:3 and O:8 cores resemble those of *Y. pestis* and *Y. pseudotuberculosis*, with the major differences being an additional Glc residue  $\beta$ -(1 $\rightarrow$ 2) linked to Hep(II) and the presence of Kdo(II) instead of Ko  $\alpha$ -(2 $\rightarrow$ 4) linked to Kdo(I). In addition, *Y. enterocolitica* O:3 deep rough mutants (*hldE*, *waaF*, and *galU* mutants) were fully  $\phi$ A1122 resistant (data not shown). In the *hldE*, *waaF*, and *galU* mutants, the core is truncated at Kdo(II), Hep(I), and Hep(II), respectively. As corresponding *Y. pestis* KM260(11) mutants were infected by  $\phi$ A1122, the presence of Ko appears to be of central importance to the phage receptor. Interestingly, the Kdo dioxygenase gene of *Y. pestis* involved in converting Kdo to Ko was recently identified (18). The homolog in *Y. enterocolitica* is a pseudogene, explaining the absence of Ko in this species. Finally, the distinct host range difference between T3 and  $\phi$ A1122 (30), despite the 98.9% identity between their tail fibers, could be due to Ko, which is also absent in *E. coli*; the presence of an additional hydroxyl group in Ko at C-3 instead of a hydrogen changes the chemical microenvironment from hydrophobic to more hydrophilic, and it might require a major

change in the tail fiber binding pocket surface to accommodate this difference. Interestingly, out of the six replacements between the tail fibers of  $\phi$ A1122 and T3 (Y333H, L350V, K468M, G478S, L523S, S544A), the most dramatic change is provided by the K468M replacement. Future work will be needed to elucidate this question.

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