Outer Membrane Proteins Ail and OmpF of *Yersinia pestis* Are Involved in the Adsorption of T7-Related Bacteriophage Yep-phi

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Yep-phi is a T7-related bacteriophage specific to *Yersinia pestis*, and it is routinely used in the identification of *Y. pestis* in China. Yep-phi infects *Y. pestis* grown at both 20°C and 37°C. It is inactive in other *Yersinia* species irrespective of the growth temperature. Based on phage adsorption, phage plaque formation, affinity chromatography, and Western blot assays, the outer membrane proteins of *Y. pestis* Ail and OmpF were identified to be involved, in addition to the rough lipopolysaccharide, in the adsorption of Yep-phi. The phage tail fiber protein specifically interacts with Ail and OmpF proteins, and residues 518N, 519N, and 523S of the phage tail fiber protein are essential for the interaction with OmpF, whereas residues 518N, 519N, 522C, and 523S are essential for the interaction with Ail. This is the first report to demonstrate that membrane-bound proteins are involved in the adsorption of a T7-related bacteriophage. The observations highlight the importance of the tail fiber protein in the evolution and function of various complex phage systems and provide insights into phage-bacterium interactions.

Yersinia pestis has caused three major pandemics of plague during human history. It is one of several virulent microorganisms known to have changed human civilization pathways and has attracted great attention due to its bioterrorism potential (1–3). Naturally occurring and genetically engineered antibiotic-resistant *Y. pestis* strains increase our concerns about the threat of its misuse as a biological weapon or bioterrorism agent. For example, the isolation of a multidrug-resistant (MDR) *Y. pestis* strain (IP275) in 1995 caused considerable alarm in the public health and biodefense communities (4). Thus, there is an urgent need for improved treatments and detection methods. Phage therapy has been proposed since their discovery in the early 20th century (5). However, the advent of antibiotics led to this therapeutic approach being largely abandoned (5, 6). Nevertheless, renewed interest in phage therapy has grown over the past decade due to the rapid emergence of antibiotic resistance in bacterial pathogens (7, 8), with several phages shown to be effective in treating various bacterial infections (5, 9).

Five *Y. pestis* phages have been fully sequenced, including L-413C (GenBank accession number NC004745) (10), PhiA1122 (AY247822) (11), Yep-phi (HQ333270) (12), Berlin (AM183667) (12), and Yepe2 (EU734170) (12). L-413C is a temperate phage belonging to the *Myoviridae* family (10). The other four phages are classified as members of the *Podoviridae* family, with genomes that are colinear with those of the *Escherichia coli* phages T7 and T3, as well as the *Y. enterocolitica* phage PhiYeO3-12 (12, 13). Whole-genome phylogenies have indicated that the phage Yep-phi is most closely related to the Berlin and Yepe2 phages; thus, Berlin, Yepe2, and Yep-phi are classified in the Yep-phi subgroup, while PhiA1122 belongs to the other subgroup (12).

Yep-phi is a T7-related bacteriophage that is routinely used as a diagnostic agent for the identification of *Y. pestis* in China. It is thought that the third pandemic of plague originated from China (14), and there are several epidemic foci of *Y. pestis* in China (12). The phage forms clear plaques on all isolates of *Y. pestis* in China but is inactive on other *Yersinia* species irrespective of the growth temperature. Based on the spectra of lytic activity, Yep-phi is much more specific for *Y. pestis* than the other diagnostic phage,

PhiA1122, which is used by the Centers for Disease Control and Prevention of the United States for the identification of *Y. pestis*. *Y. pseudotuberculosis* is also infected by PhiA1122 when grown at 37°C (15). The PhiA1122 receptor is demonstrated to be the common lipopolysaccharide (LPS) core of *Y. pestis* and *Y. pseudotuberculosis*. The receptor used by phage Yep-phi for adsorption has not yet been identified (15).

The interaction of a phage with the bacterial surface (adsorption) is the first step in the infection process (16). This step involves the recognition of, and binding to, one or more cell envelope constituents, which leads to the ejection of the phage DNA from the capsid (17). The implementation of efficient phage therapies to target pathogenic bacteria requires detailed knowledge of the bacterial receptors recognized by the phages (18). Phages tend to use structures that are exposed on the outer membrane of the host bacteria as receptors, since they are easily accessible (8). Porins, affinity transporters, and LPS are the most common outer membrane components acting as receptors for tailed phages that infect Gram-negative bacteria (19, 20). Outer membrane proteins (OMPs), known to act as phage receptors, all share a channel-forming β-barrel region organized by a variable number of antiparallel β-strands. Such proteins can function as monomeric (FhuA) or trimeric (OmpA, OmpC, and LamB) proteins (19, 21). LPS is the major component of the outer membrane of Gram-negative bacteria and is a surface structure encountering the surrounding environment. The LPS of *Y. pestis* is O antigen deficient and restricted to the core and lipid A moieties (22). Much of the available detailed information about the receptors of *Y. pestis* phages focuses on the LPS (15, 23). The LPS of *Y. pestis* has been

TABLE 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics
Y. pestis strain	
201 (wild type)	Avirulent to humans, highly lethal to mice
201pKD46	201 harboring pKD46 vector, Amp^r
201Δ*waaA*(*YPO0055*)	Δ*waaA*, derived from strain 201pKD46, Km^r cassette replacement of *waaA* gene
201Δ*lamB*(*YPO3711*)	Δ*lamB*, derived from strain 201pKD46, Km^r cassette replacement of *lamB* gene
201Δ*ompF*(*YPO1411*)	Δ*ompF*, derived from strain 201pKD46, Km^r cassette replacement of *ompF* gene
201Δ*ompA*(*YPO1435*)	Δ*ompA*, derived from strain 201pKD46, Km^r cassette replacement of *ompA* gene
201Δ*ail*(*YPO2905*)	Δ*ail*, derived from strain 201pKD46, Km^r cassette replacement of *ail* gene
201*C-lamB*	Complemented Δ*lamB* strains, transformed with a pACYC184 vector with cloned *lamB*
201*C-ompF*	Complemented Δ*ompF* strains, transformed with a pACYC184 vector with cloned *ompF*
201*C-ompA*	Complemented Δ*ompA* strains, transformed with a pACYC184 vector with cloned *ompA*
201*C-ail*	Complemented Δ*ail* strains, transformed with a pACYC184 vector with cloned *ail*
201*C-waaA*	Complemented Δ*waaA* strains, transformed with a pACYC184 vector with cloned *waaA*
201Δ*ail*Δ*ompA*	Δ*ail* Δ*ompA*, derived from strain 201Δ*ompA*, Cm^r cassette replacement of *ail* gene
201Δ*lamB*Δ*ail*	Δ*lamB* Δ*ail*, derived from strain 201Δ*ail*, Cm^r cassette replacement of *lamB* gene
201Δ*ompF*Δ*ail*	Δ*ompF* Δ*ail*, derived from strain 201Δ*ail*, Cm^r cassette replacement of *ompF* gene
201Δ*ompF*Δ*ompA*	Δ*ompF* Δ*ompA*, derived from strain 201Δ*ompA*, Cm^r cassette replacement of *ompF* gene
201Δ*ompF*Δ*lamB*	Δ*ompF* Δ*lamB*, derived from strain 201Δ*lamB*, Cm^r cassette replacement of *ompF* gene
201Δ*ompA*Δ*lamB*	Δ*ompA* Δ*lamB*, derived from strain 201Δ*lamB*, Cm^r cassette replacement of *ompA* gene
201Δ*ompF*Δ*ail*Δ*ompA*	Δ*ompF* Δ*ail* Δ*ompA*, derived from strain 201Δ*ail*Δ*ompA*, Gm^r cassette replacement of *ompF*gene
201Δ*ail*Δ*ompA*Δ*lamB*	Δ*ail* Δ*ompA* Δ*lamB*, derived from strain 201Δ*ompA*Δ*lamB*, Gm^r cassette replacement of *ail* gene
201Δ*ompF*Δ*ompA*Δ*lamB*	Δ*ompF* Δ*ompA* Δ*lamB*, derived from strain 201Δ*ompA*Δ*lamB*, Gm^r-cassette replacement of *ompF* gene
201Δ*ail*Δ*ompF*Δ*waaA*	Δ*ail* Δ*ompF* Δ*waaA*, derived from strain 201Δ*ompF*Δ*ail*, Gm^r cassette replacement of *waaA* gene
Y. pseudotuberculosis	Pa3606 Human, Japan Yp 1b
Plasmids	
pKD46	Red recombinase plasmid; Amp^r
pET28a::*gp17*	Expression vector with a PCR-amplified *gp17* gene in the NheI/HindIII site; Km^r
pET28a::*lamB*	Expression vector with a PCR-amplified *lamB* gene in the NheI/HindIII site; Km^r
pET32a::*ompF*	Expression vector with a PCR-amplified *ompF* gene in the BamHI/SalI site; Amp^r
pET32a::*ompA*	Expression vector with a PCR-amplified *ompA* gene in the BamHI/SalI site; Amp^r
pET32a::*ail*	Expression vector with a PCR-amplified *ail* gene in the BamHI/SalI site; Amp^r
pACYC184::*waaA*	Cloning vector with a PCR fragment covering a region from 500-bp fragment upstream to 300-bp fragment downstream of the *waaA* gene in the BamHI/HindIII site;Cm^r
pACYC184::*lamB*	Cloning vector with a PCR fragment covering a region from 500-bp fragment upstream to 300-bp fragment downstream of the *lamB* gene in the BamHI/HindIII site; Cm^r
pACYC184::*ompF*	Cloning vector with a PCR fragment covering a region from 500-bp fragment upstream to 300-bp fragment downstream of the *ompF* gene in the BamHI/HindIII site; Cm^r
pACYC184::*ompA*	Cloning vector with a PCR fragment covering a region from 500-bp fragment upstream to 300-bp fragment downstream of the *ompA* gene in the BamHI/HindIII site; Cm^r
pACYC184::*ail*	Cloning vector with a PCR fragment covering a region from 500-bp fragment upstream to 300-bp fragment downstream of the *ail* gene in the BamHI/HindIII site; Cm^r

studied extensively in terms of structure, activity, composition, modification, virulence, and assembly (15, 22–24). The receptor was identified as the LPS of *Y. pestis* for eight bacteriophages, including L-413C, P2 vir1, PhiJA1, PhiA1122, Pokrovskaya, T7$_{Yp}$, Y, and PST (23). The phages were demonstrated to employ different components of the LPS core as a specific receptor (23).

On the other hand, little attention has been devoted to the interactions between phages and *Y. pestis* OMPs. In the current report, we analyzed the host factors attached by Yep-phi. Although most of the *Y. pestis* phages require only a single bacterial receptor (LPS) (23), Yep-phi infection requires *Y. pestis* OMPs Ail and OmpF as well as the LPS receptor. Furthermore, amino acid residues critical for the specific binding of the tail fiber protein gene product (gp) 17 to Ail and OmpF were identified. To the best of our knowledge, the current study is the first to report T7-related phages employing OMPs in the adsorption process, thereby pro-

viding novel insights into the interactions between the host and these phages.

MATERIALS AND METHODS

Bacterial strains, phages, and media. *Y. pestis* and phage cultures were incubated at 26°C, whereas *Y. pseudotuberculosis* and *E. coli* cultures were incubated at 37°C (Table 1). The wild-type (WT) *Y. pestis* biovar microtus strain 201, which is avirulent to humans but highly lethal to mice (25), was used. Brain heart infusion and Luria-Bertani (LB) media were used for bacterial liquid cultures, and soft-agar medium included an additional 0.4% (wt/vol) agar.

Phage adsorption assays. Approximately 8×10^5 PFU of Yep-phi in 100 μl was mixed with a 500-μl sample of bacteria ($A_{600} = 1.2$). *Y. pseudotuberculosis* was cultured overnight, and *Y. pestis* was cultured for 48 h. The suspension was incubated at room temperature for 5 min and centrifuged at $16,000 \times g$ for 3 min, after which the phage titer remaining in the supernatant (i.e., residual PFU%), was determined. LB was used as a non-

adsorbing control in each assay, and the phage titer in the control supernatant was set to 100%. Each assay was performed in duplicate and repeated twice (15).

Periodate and proteinase K treatments. To test the effects of proteinase K treatment on Yep-phi adsorption, the protocol described here was used (15). *Y. pestis* cultures (2 ml) were treated with proteinase K (0.2 mg/ml; Promega) at 37°C for 3 h and then washed with 2 ml of LB. The phage adsorption assay was performed as described above. A control without proteinase K was used to ensure that the possible effect was not due to the incubation at 37°C.

To test the effects of periodate, *Y. pestis* culture (1.5 ml) was centrifuged at 16,000 × *g* for 1 min, and the bacterial pellet was suspended in 1.5 ml sodium acetate (50 mM, pH 5.2), or sodium acetate containing 100 mM IO$_4^{-}$, to determine whether periodate can destroy the phage receptor (15). The cells were incubated for 2 h (protected from light), centrifuged according to the above-mentioned process, washed with 1.5 ml of LB, and then centrifuged again and suspended in LB. The A_{600} of the bacterial suspension was adjusted to 1, and the phage adsorption assay was carried out.

Identification of the bacterial targets of phage-encoded tail fiber protein gp17. Tail fiber protein gp17 was expressed as His$_6$ fusions and purified from *E. coli* BL21(λDE3) (26). Recombinant protein was covalently coupled to Affi-Gel resin (Bio-Rad) using HNG buffer (20 mM HEPES-KOH, pH 7.5, 150 mM sodium chloride, 10% [vol/vol] glycerol). The unreacted sites were blocked with 10 mM ethanolamine, with a pH of 7.5, at 4°C for 30 min. Lysates were prepared from exponential-phase *Y. pestis* cells, resuspended in 20 ml of HNG buffer (1 mM EDTA, 1% [vol/vol] Triton X-100, 1 mM [each] dithiothreitol and phenylmethylsulfonyl fluoride), followed by sonication. Cell debris was removed by centrifugation for 0.5 h at 12,000 rpm. Affinity chromatography was performed by mixing 100 μl of resin with 1 ml of *Y. pestis* lysate at 4°C overnight. The resin was washed extensively with HNG buffer with increasing concentrations (0.1 M to 1 M) of NaCl. Bound proteins were eluted with 1% (wt/vol) SDS, resolved by SDS-PAGE, and stained with Coomassie brilliant blue. Specific bands were excised, and proteins were digested with trypsin prior to being subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry, in conjunction with postsource decay and collision-induced decay for the determination of protein identity (27, 28).

Preparation and verification of mutants. The genes that can encode the possible receptors of Yep-phi from *Y. pestis* were subjected to single, double, or triple deletion, by replacing the target gene(s) with kanamycin (single), chloramphenicol (double), and/or gentamicin (triple) resistance cassettes (29). Mutant genotypes were confirmed by PCR. A PCR-generated DNA fragment containing the target gene-coding region, with its promoter-proximal region (~500 bp upstream from the coding sequence) and transcriptional terminator (~300 bp downstream), was cloned into the pACYC184 vector (30) and then verified by DNA sequencing. The recombinant plasmid was subsequently introduced into the mutants, yielding the respective complemented mutant strains. All the DNA sequences mentioned in this study were derived from the genomic data of strain CO92. The characteristics of the strains and the plasmids are presented in Table 1. All the primers used in this study, designed using Primer Premier 5.0 software, are listed in Table 2.

Phage plaque formation assays. The lysis activity of each phage was examined using spot tests on *Y. pestis* strains (9, 31). A series of exponential-phase cultures were prepared for each mutant strain. One hundred-microliter cultures were concentrated by centrifugation, dissolved in 10 ml LB, and then mixed with 3 ml of liquefied soft-agar medium. The mixture was then vortexed and poured onto individual LB plates to produce double-layer agar plates. When the medium solidified, the serial dilutions of parental phage stocks (3 μl) were spotted onto the surface of the plates. The plates were inverted, incubated at 26°C overnight, and then examined and photographed.

Anti-OMP antibodies and immunoblot analysis. The OMPs were expressed as His$_6$ fusions and purified from *E. coli* BL21(λDE3) cells (26). For polyclonal antibody production, New Zealand White rabbits were subcutaneously injected with 1 mg of purified Ail or OmpF proteins mixed in 0.5 ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA) for a total of three immunizations. Three days after the final boost, all rabbits were exsanguinated by heart puncture under general anesthesia. Sera were separated from blood cells by centrifugation at 12,000 × rpm for 15 min at 4°C. This crude serum was purified using saturated ammonium sulfate precipitation and stored at −80°C. For immunoblot analysis, SDS-PAGE and Western blotting were performed according to standard protocols (32). The captured proteins were subjected to electrophoresis by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The PVDF membranes were blocked in PBST (PBS and 0.2% Tween 20) containing 5% skimmed milk for 2 h and subsequently incubated with our polyclonal antibody for 1 h at room temperature or overnight at 4°C. The membranes were washed with PBST 3 times and probed by goat anti-rabbit secondary antibody, conjugated with horseradish peroxidase (Roche, USA), for 1 h at room temperature. After washing the membranes 3 times with PBST, the immunoblots were developed with the enhanced chemiluminescence system SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

RESULTS

Destruction of phage receptors by both periodate and proteinase K. The surface of Gram-negative bacteria, exposed to the environment, consists mainly of proteins and polysaccharide structures (24). It is important to test whether the degradation of cell surface proteins, or LPS, can destroy the Yep-phi receptor, as the bacteriophage could exploit both LPS and OMPs as receptors (15). Bacteria were treated with either proteinase K (for destroying surface proteins) or periodate (for destroying surface carbohydrates), prior to the phage adsorption assay, in order to determine the possible nature of the phage receptor (15). Yep-phi exhibited high infection efficiency when mixed with *Y. pestis* cells (Fig. 1); 5 min after mixing, most phages were removed from the suspension by binding to *Y. pestis* cells. When the phage was incubated with proteinase K or periodate-treated *Y. pestis*, the majority of phages remained in the supernatant. The significant increase of free phage particles indicated that the virions were unable to gain efficient adsorption on the treated bacteria. Therefore, the *Y. pestis* receptors (which were recognized and bound by Yep-phi) likely contain both protein and carbohydrate structures. In this respect, they differ notably from the features of PhiA1122 and T7 (11, 15, 19).

Alignment of RBP tail fiber protein gp17. For T7-related phages, the primary determinant of the host receptor is the tail fiber protein gp17 (11). The six T7 tail fibers, which are the homotrimers of gp17, are responsible for the first specific, albeit reversible attachment to *E. coli* LPS (11). As a T7-related phage, Yep-phi has a unique host specificity that can be attributed to its tail fiber gp17 protein. It has been reported that the receptor binding protein (RBP) of phages in the same species has a conserved N-terminal moiety due to its involvement in interactions with other tail proteins, whereas the C-terminal portion diverges in the region that was proposed to recognize the host (33). This pattern of sequence conservation is also observed in the alignment of gp17 sequences of *Y. pestis* phages (Yep-phi, PhiA1122, Yepe2, and Berlin) and *E. coli* phages (T7, T3) (Fig. 2A). The alignment shows a highly conserved N-terminal moiety (amino acids 1 to 149), which belongs to domain family PHA00430 (34), indicating that they have the same mechanism of attaching the fiber to the phage as numerous

TABLE 2 Oligonucleotides used in this work

Target	Primer (forward/reverse, 5′–3′)[a]
Protein expression	
lamB	CTAGCTAGCCATGGTTATGCGCGT/CCCAAGCTTTTACCACCAGGCTTCG
ail	GGGATCCAGTATTTCTATTGGATATGC/GGTCGACTTAACCCACTTTCACATCATCG
ompF	GGGATCCTACGGTAAAGTTGACGC/GGTCGACTTAGGACTGAACATAACCCAG
ompA	GGGATCCGGTTTAGTATGGCGTGC/GGTCGACTTAAGCCTGTGGCTGAGTC
gp17	CTAGCTAGCATGGCTAACAAAATTTC/CCCAAGCTTTCAGTTTCGAACTAAG
gp17(1–469)	CTAGCTAGCATGGCTAACAAAATTTC/CCCAAGCTTTCAATCGACCATCTTG
gp17(1–119)	CTAGCTAGCATGGCTAACAAAATTTC/CCCAAGCTTTCAAGTATCCGCAG
gp17(470–569)	CTAGCTAGCGGACTACTCACTACCG/CCCAAGCTTTCAGTTTCGAACTAAG
Construction of mutants	
Kanamycin resistance cassette	
waaA	ATGAATAACCACGGCTCAATGTGGTGCTCCGCCCTCCTAGGTGTAGGCTGGAGCTGCTTC/
	TTAGTGGCTCCGTTGTGGCAGATAAGGCTCCAACAAATGTCATATGAATATCCTCCTTA
lamB	ATGATTACTCTGCGCAAGTTACCAATAGCACTGGCTGTTGGTGTAGGCTGGAGCTGCTTC/
	TTACCACCAGGCTTCGAATTGCGCACCGAAGGTGACTTCACATATGAATATCCTCCTTA
ail	ATGGTTTTTATGAATAAGACATTACTGGTCTCTTCTTTAAGTGTAGGCTGGAGCTGCTTC/
	TTAGAACCGGTAACCCGCGCCAAGCATCCAAGTACCCACTCATATGAATATCCTCCTTA
ompF	ATGAGGGTAATAATAATGATGAAGCGCAATATTCTTGCAGGTGTAGGCTGGAGCTGCTTC/
	TTAGAACTGGTAAACCAAGCCAACAGCAACAACGTTGTCGCATATGAATATCCTCCTTA
ompA	TTGCCTATTTGGATGATAATGAGGCGTAAAATGAAAAAGAGTGTAGGCTGGAGCTGCTTC/
	TTAAGCCTGTGGCTGAGTCACAACTTCTTTGTAGCCTTTCCATATGAATATCCTCCTTA
Chloromycetin resistance cassette	
lamB	ATGATTACTCTGCGCAAGTTACCAATAGCACTGGCTGTTGAGATTGCAGCATTACACGTC/
	TTACCACCAGGCTTCGAATTGCGCACCGAAGGTGACTTCATGTAACGCACTGAGAAGC
ail	ATGGTTTTTATGAATAAGACATTACTGGTCTCTTCTTTAAAGATTGCAGCATTACACGTC/
	TTAGAACCGGTAACCCGCGCCAAGCATCCAAGTACCCACTTGTAACGCACTGAGAAGC
ompF	ATGAGGGTAATAATAATGATGAAGCGCAATATTCTTGCAGAGATTGCAGCATTACACGTC/
	TTAGAACTGGTAAACCAAGCCAACAGCAACAACGTTGTCGTGTAACGCACTGAGAAGC
ompA	TTGCCTATTTGGATGATAATGAGGCGTAAAATGAAAAAGAAGATTGCAGCATTACACGTC/
	TTAAGCCTGTGGCTGAGTCACAACTTCTTTGTAGCCTTTCTGTAACGCACTGAGAAGC
Gentamicin resistance cassette	
lamB	ATGATTACTCTGCGCAAGTTACCAATAGCACTGGCTGTTGTTAGGTGGCGGTACTTGGG/
	TTACCACCAGGCTTCGAATTGCGCACCGAAGGTGACTTCACGTCAGGTGGCACTTTTCG
ail	ATGGTTTTTATGAATAAGACATTACTGGTCTCTTCTTTAATTAGGTGGCGGTACTTGGG/
	TTAGAACCGGTAACCCGCGCCAAGCATCCAAGTACCCACTCGTCAGGTGGCACTTTTCG
ompF	ATGAGGGTAATAATAATGATGAAGCGCAATATTCTTGCAGTTAGGTGGCGGTACTTGGG/
	TTAGAACTGGTAAACCAAGCCAACAGCAACAACGTTGTCGCGTCAGGTGGCACTTTTCG
Verification of the mutants	
waaA	CTGCCGGCAGGTTACAAGAAG/ATGTGTGCTAGTCGCAATCC
lamB	TACTCAAGAAGGTTACTCG/GTTGGCATAAGTTGCGAAG
ail	ACTGGGGAGTGATAGGTTC/TTACTTCTACTCTCTGATTG
ompF	CGGCAACGCAACCCGTCTG/CTTCAGAACGGTCATTTTTG
ompA	GGGTGGTTTAGTATGGCGTG/TCCTGACCAAAGCGGTAAG
Complementation of mutants	
lamB	CGCGGATCCGCCCAGAACATC/CCCAAGCTTGTACCAGACAGAGTG
ompF	CGCGGATCCCCGTTGCGGCGATGGATG/CCCAAGCTTTTTTTCAAACATGTCAGAG
ail	CGCGGATCCCCATTGAGCGTGTGAAAC/CCCAAGCTTCAACTTTACCGTCTCAAC
ompA	CGCGGATCCTACAGTGTTGTTCTGGG/CCCAAGCTTTACTGAACCCCC
waaA	CGCGGATCCGTGCAAAAGCCGTAGTC/CCCAAGCTTCGATTTTAAGCTCTGGG

[a] Underlining indicates endonuclease restriction sites.

T7-related phages. Aside from this region, the other sequence of the Yep-phi subgroup diversified from that of the other three (T7, T3, and PhiA1122). It is known that T7, T3, and PhiA1122 tail fibers adsorb to the host surface LPS (11, 15), so we speculate that the Yep-phi subgroup adsorbs to different receptors.

Identification of cell wall-anchored protein targets of tail fiber protein gp17. Considering that the initial binding of phage to

bacteria is mediated by the interaction of its six gp17 tail fibers with bacterial surface receptors, gp17 protein of Yep-phi was expressed and purified as a His$_6$ fusion (11, 34). Consistent with a previous report of T7 (34), we found that the purified recombinant gp17 protein of Yep-phi existed mainly as a multimer in solution. The multimer fraction was SDS resistant unless previously boiled (Fig. 2B). The bacterial targets of phage tail fiber
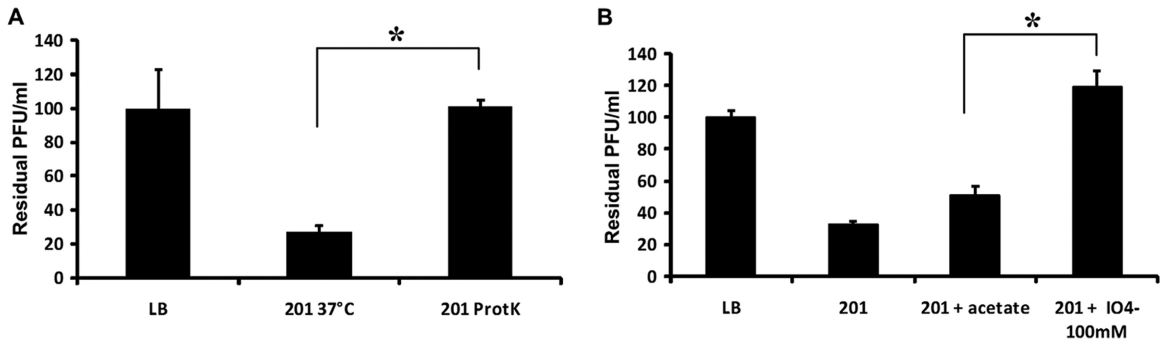
FIG 1 Effects of the different treatments of bacteria on Yep-phi adsorption, which are shown as residual PFU percentages. (A) Effect of proteinase K treatment on the adsorption of Yep-phi to *Yersinia pestis* strain 201. (B) Effect of periodate treatment on the adsorption of Yep-phi to *Y. pestis* strain 201. The control (LB and "201+acetate"), untreated strain (strain 201), and treatment ("201 ProtK" for proteinase K treatment and "201+IO$_4^-$" for periodate treatment) groups were tested for adsorptions as indicated in the *x* axes. Error bars denote statistical variations. Significance was determined by one Student *t* test for comparison between the treated group and the WT group. *, $P < 0.05$.

protein gp17 were identified by affinity chromatography of *Y. pestis* lysates using a gp17-coupled Affi-Gel resin column (Fig. 2C). The protein bands were recovered specifically from a *Y. pestis* lysate compared with other controls, including *Y. pseudotuberculosis* lysate. Trypsinolysis and mass spectrometry revealed that the captured polypeptides were the *Y. pestis* OMPs LamB, OmpF, OmpA, and Ail (Table 3). The common band in all lanes was UreE protein, which was nonspecifically bound to the nickel column.

LamB is found in numerous species of Gram-negative bacteria and is an abundant integral outer membrane porin responsible for maltose uptake. LamB also serves as the receptor for bacteriophage lambda (35). OmpA protein plays a structural role in the integrity of the bacterial cell surface (36). It is proposed to interact specifically with the peptidoglycan layer (36) and serves as a receptor for several T-even phages infecting *E. coli* (31). OmpF is often regarded as a classical porin, which is present in large quan-
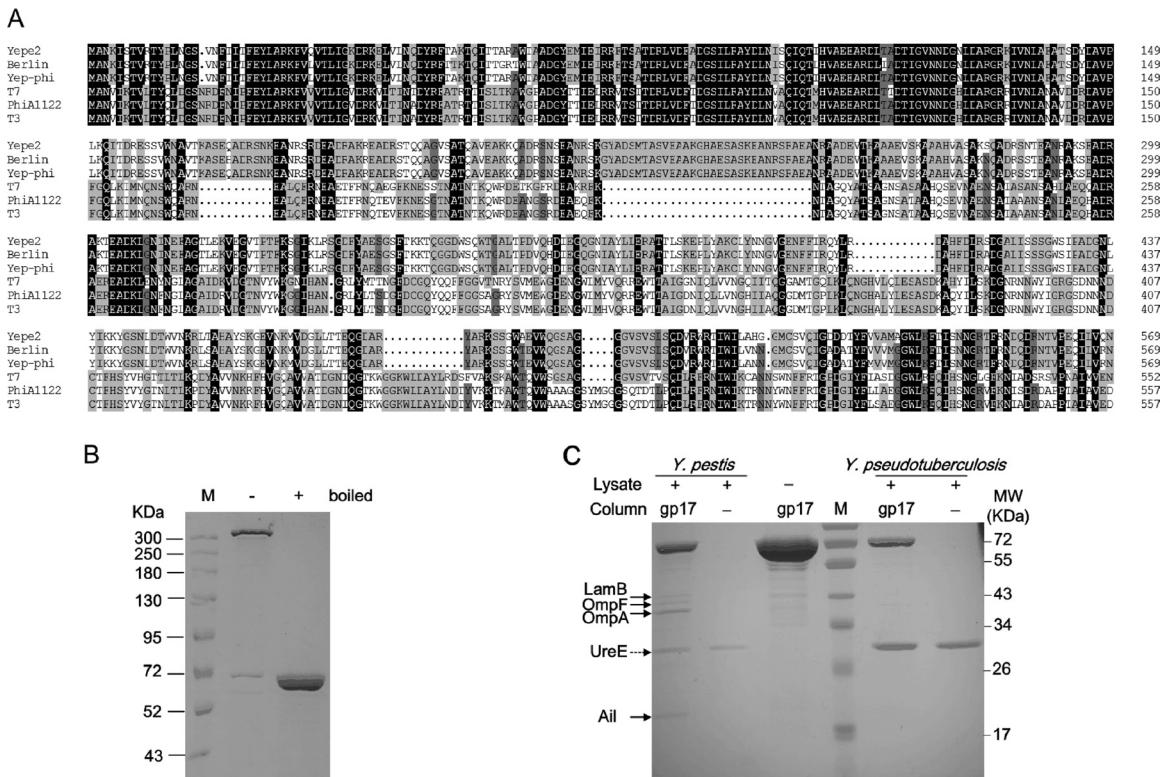

FIG 2 (A) Alignment of tail fiber proteins. Identical residues are shaded in black, residues sharing >75% homology are shaded in deep gray, and those sharing >50% homology are shaded in light gray. Gaps (indicated by dotted lines) were introduced into the sequences to maximize the alignments. Numbering is based on the N-terminal methionine. Multiple sequence alignment was analyzed and edited with DNAman software (Lynnonon Biosoft, Quebec, Canada). The names of phages are indicated on the left. (B) SDS-PAGE gel showing the SDS-resistant multimer formed by gp17. Symbols "+" and "−" above the gel denote the purified protein boiled previously and unboiled, respectively. (C) Identification of cell wall-anchored protein targets of gp17 by affinity chromatography. The protein bands found to interact specifically with gp17 are indicated by black arrows. UreE protein binding to the nickel column is denoted by a dotted arrow.

TABLE 3 Functional features of the proteins on polyacrylamide gels

| Protein | MW[a] | Locus tag | | Product | Function in phage receptor(s): |
		CO92	201		
LamB	46,783	YPO3711	YP_3073	Maltoporin	λ
OmpF	40,362	YPO1411	YP_1182	Outer membrane porin protein F	K20, RB69, SV76, RB51, T2
OmpA	39,193	YPO1435	YP_1326	Outer membrane protein A	K3, M1, Ox2, TuII, RB32, PST
Ail	20,153	YPO2905	YP_2550	Attachment invasion locus protein	ND[b]
UreE[c]	25,943	YPO2668	YP_2469	Urease accessory protein ureE	ND

[a] MW, molecular weight.
[b] ND, not determined.
[c] UreE protein exists both in *Y. pestis* and *Y. pseudotuberculosis* and is able to bind the nickel column.

tities, and is also required as a receptor by several phages of *E. coli* (31, 36, 37). Ail protein is essential for *Y. pestis* to resist complement-mediated killing, and Ail-mediated resistance to human serum depends on the LPS core (38, 39). However, the function of Ail in phage receptors has yet to be fully explained. It is worth mentioning that we employed the single nucleotide polymorphism (SNP) method to scan the sequence polymorphisms of the proteins in the available 133 sequenced *Y. pestis* strains (40). The results indicated that the *ail* gene displayed the highest variability. Information for four SNP loci, i.e., a T-to-G change at position 136 encoded by *ail* [$ail_{136(T-G)}$], $ail_{238\ (T-G)}$, $ail_{240\ (C-A)}$, and $ail_{253\ (G-T)}$, were acquired in the relatively small 549-bp coding region. The mutation rate was unusually higher than that of the other genes. Ail was predicted to consist of eight transmembrane β-sheets and four cell surface-exposed loops (41, 42). In the current work, the resulting amino acid alteration $Ail_{46\ (Y-D)}$ was located at loop 1, whereas $Ail_{80\ (V-F/L)}$ and $Ail_{85\ (Y-D)}$ were located at loop 2. The results are consistent with the notion that the external loops of the OMPs, between the transmembrane β-strands, undergo rapid mutational alterations as they interact with the elements of the external world, including phages (36, 43).

Phage propagation on the various mutant host strains. Single-, double-, or triple-gene inactivation was accomplished in *Y. pestis* using PCR products with kanamycin, chloramphenicol, and/or gentamicin resistance cassettes, in order to test the respective roles of the four proteins in Yep-phi infection (Table 1). The *waaA* gene of the LPS core biosynthetic pathway was eliminated to confirm the role of LPS core in the phage receptor, as previously shown (15). Each single gene mutant was complemented in *trans* by the corresponding wild-type gene to avoid the phenotype caused by a polar effect, or a spontaneous mutation, elsewhere in the genome (Table 1).

The results show that the *waaA* mutant lost most of its phage-binding activity during the adsorption assay (Fig. 3A), thereby confirming that the rough LPS is a critical part of the receptor (15). This result is in agreement with the notion that the closely related phages T7 and T3 utilize the LPS core as their receptors (16, 19). ΔlamB and ΔompA mutants had phage adsorption rates comparable to that of the WT strain, while the ΔompF mutant showed a small defect, and the defect increased with Δail. The ΔompF Δail double mutant was accompanied by a decrease at a level comparable to that of the *waaA* mutant. The results indicated that the *Y. pestis* LPS core was a major target of the phage, but Ail and OmpF were also involved in the phage infection. We speculate that the loss of the *ail* and *ompF* products may result in defective phage receptors.

It is likely that there are at least three molecules participating in

the adsorption process; therefore, we constructed the Δ*ail* Δ*ompF* Δ*waaA* mutant. To test whether the mutant could express antiphage activity, the plaquing efficiency of Yep-phi on a series of mutants was measured (Fig. 3B). Significant differences were observed in the ability of the phage to lyse the mutants. Additionally, Yep-phi formed plaques very robustly on the Δ*ail* Δ*ompF* Δ*waaA* mutant compared to the WT strain (Fig. 3B). The results suggest that under the same culture conditions, the antiphage activity is the highest on the Δ*ail* Δ*ompF* Δ*waaA* mutant, followed by the Δ*waaA* and the Δ*ompF* Δ*ail* mutants. None of the defined mutants completely prevented the phage attachment or altered the ability of the phage to lyse the mutant bacteria, hinting at the complexity of the adsorption process that has yet to be uncovered. However, the mutation appeared to alter a subsequent step, which was measured by a reduction in plaque-forming efficiency (Fig. 3B). The adsorption kinetics assay (Fig. 3C) further demonstrated that the phage receptors were affected in the Δ*ail*, Δ*ompF* Δ*ail*, and Δ*waaA* strains. It is clear that LPS is the major contributor to the phage fibers' adsorption. It seems likely that the OmpF protein did not affect the adsorption alone but may be able to cooperate with Ail to make a contribution to the attachment. Fibers "walk" across the cell surface to find the right receptor for the tail (44), but binding of fibers to bacterial receptors is weak. Hypothetically, the two OMPs could cooperate with LPS to assist the fibers in binding to the correct sites as soon as possible. That is why, when the two OMPs are not available, the phage can still infect the cell, but with a lower efficiency. Effective adsorption needs cooperative binding among fibers (44). Perhaps the six fibers bind different sites, with some of the fibers binding OMPs, and some binding LPS. Normally, the fibers bind to both sites, making the binding stable and fast, which results in higher infection efficiency. However, when either OMPs or LPS are deficient, the fibers can bind only one site. This causes the binding to be weak, ultimately reducing infection efficiency.

To the best of our knowledge, the OMPs of Gram-negative bacteria have not exhibited receptor activity for T7-related phages. In this study, we found that a T7-related phage, Yep-phi, required not only LPS but also Ail and OmpF for its efficient infection. Deficiencies of the three molecules exhibited a remarkable decrease in adsorption efficiency, although it was not enough to cause complete resistance against phage infection. The failure to inhibit the infection implies that other factors remain to be discovered in order to gain a better understanding of the phage system.

Specific binding with the C-terminal region (510 amino acids [aa] to 529 aa) of gp17. To analyze the locations of the OMPs' binding region, three fragments spanning different regions of
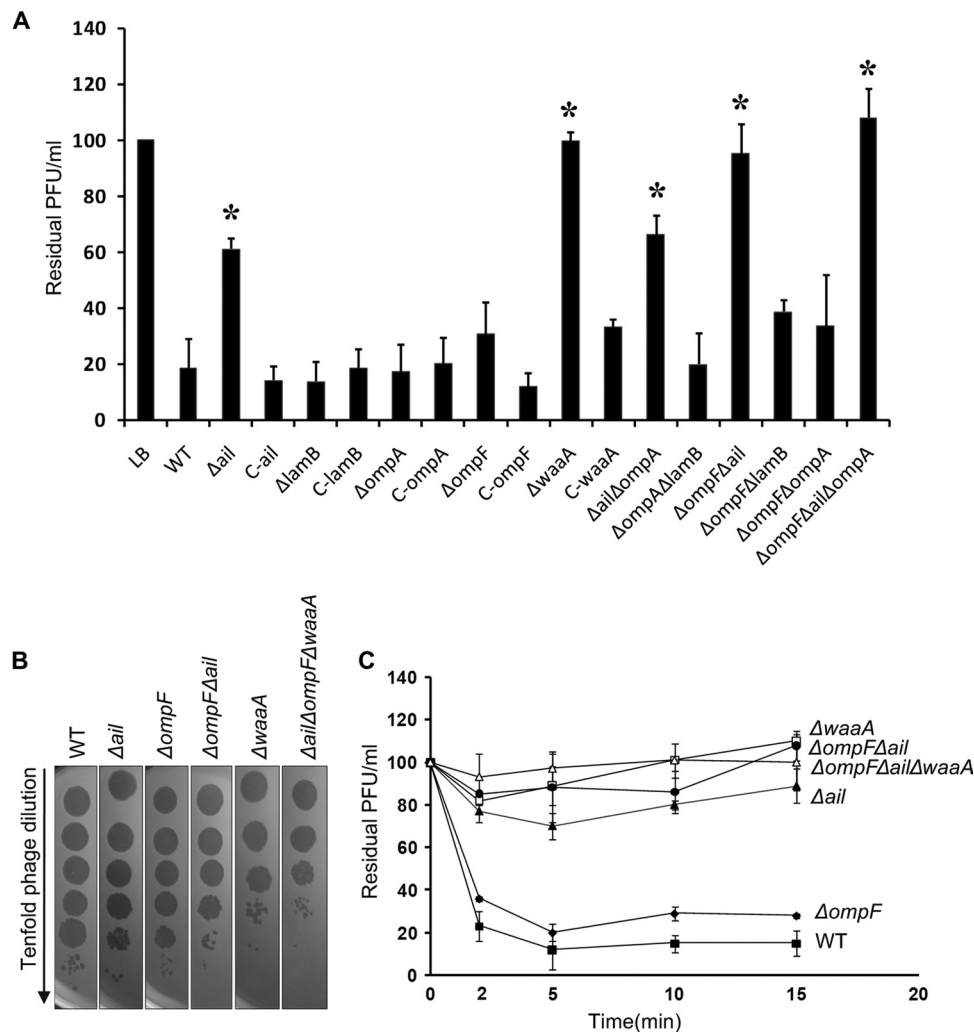
FIG 3 (A) Yep-phi adsorption to *Y. pestis* outer membrane protein and lipopolysaccharide mutants, shown as residual PFU percentages. The control and strains used for adsorptions are presented below the columns (complete strain names and descriptions can be found in Table 1). Error bars denote ranges. Significance was determined by one Student *t* test for comparison between the mutant group and the WT group; *, $P < 0.05$. (B) Tenfold dilution of lysates of Yep-phi applied to bacterial lawns of wild-type and mutant *Y. pestis* strains. (C) Adsorption kinetics of Yep-phi to *Y. pestis* LPS and OMP mutants, shown as residual PFU percentages. Standard errors are indicated by vertical lines.

gp17 protein were expressed as His$_6$ fusions and purified from *E. coli* BL21(λDE3) (Fig. 4A, upper part). The purified fusions were used as baits to capture OmpF and Ail proteins from the lysates of Δail ΔompA ΔlamB and ΔompF ΔompA ΔlamB mutants, respectively. The triple gene mutants were used to eliminate the interplay between the OMPs, and the captured proteins were examined by immunoblotting, using rabbit anti-OmpF or anti-Ail polyclonal antibodies. The result showed that fragment gp17$_{470-569}$ was responsible for the interaction with Ail and OmpF, while no interactions were observed with fragments gp17$_{1-469}$ and gp17$_{1-119}$ (Fig. 4B). Fragment gp17$_{470-569}$ contains the tip domain of the tail fiber protein. The tip domain plays an important role in determining the host range for the T7 phage. The structure of the tip domain has been resolved and is composed of an eight-stranded β-sandwich (BCDEFGHI) and four loops (i.e., BC, DE, FG, and HI loops) at the top, which are thought to interact with the receptors (34). We performed alignment of the tip domains of the Yep-phi subgroup, T7, PhiA1122, and T3 gp17 proteins. The align-

ment showed conserved hydrophobic positions, indicating a common fold (Fig. 5A). Of special interest is the mosaic nature of the T7 tip domain; the BC loop displayed significant similarity with the Yep-phi subgroup, whereas the DE, FG, and HI loops had the highest similarity to T3 and PhiA1122.

To decipher the minimal portion of the tip domain required to bind the two OMPs, fragment gp17$_{470-569}$ was divided into nine N-terminally 6×His-tagged peptides (the lower part of Fig. 4A). Immunoblotting was performed to test the interaction, and the results showed that one of the peptides (510 aa to 529 aa), with only 20 amino acids, was sufficient for the interaction with the OMPs, which is located at the DE loop of the tip domain. We thus speculate that the DE loop may provide a suitable location for specific binding. We noticed that β-strand E of the Yep-phi subgroup consisted of unique hydrophobic residues that were distantly diverged from PhiA1122, T7, and T3. The dissimilarities existing between them may account for different receptor specificities.
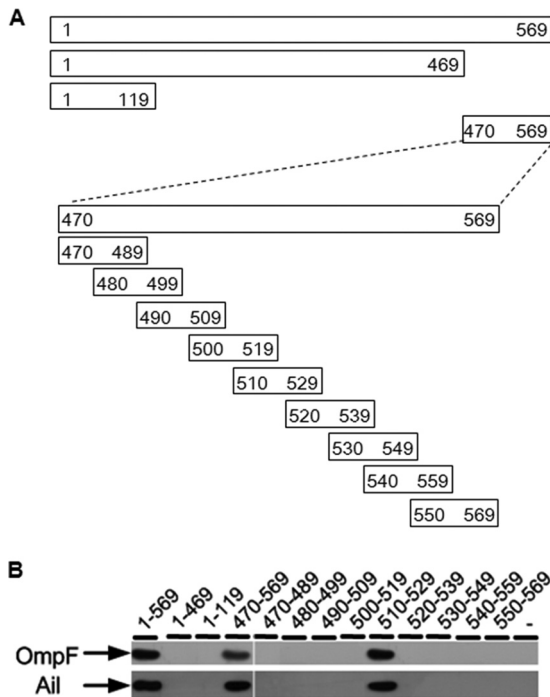
FIG 4 (A) Positions of the fragments on the gp17 protein. The thick line represents the fragments, and the numbers on the two ends of each fragment denote the starting and ending amino acid positions. (B) OmpF and Ail detected by Western blotting using either rabbit anti-OmpF or anti-Ail polyclonal antibody after affinity chromatography.

In order to localize the residue(s) necessary for the binding activity, 12 deletion/replacement mutations (designated M1 to M12, respectively [Fig. 5B]), located at the polarity residues throughout the β-strand E region, as well as the loop region, were

constructed. Mutational analysis suggested that both the DE loop and the β-strand E regions were involved in the binding. Mutations at residues 518N, 519N, or 523S were completely deficient in terms of binding to OmpF, whereas residues 518N, 519N, 522C, or 523S were found to be essential for the interaction with Ail (Fig. 5C).

DISCUSSION

Phages bind to unique host-specific structures, allowing them to recognize a suitable host in a mixed bacterial population (18). LPS is currently recognized as the receptor for T7 and T3 phages (34). Four T7-related phages have been sequenced in prior studies of *Y. pestis*-specific phages (11, 12). However, only the receptor of PhiA1122 has been identified (15). The other three phages, represented by Yep-phi (used in a phage lysis assay in China for the identification of *Y. pestis* isolates), have significantly different characteristics, either at the genome or at the phenotype level, from those of PhiA1122. To date, the molecular interactions between Yep-phi and *Y. pestis* have not been fully understood.

In this study, the host components that interact with Yep-phi were found to be the rough LPS and two OMPs (Ail and OmpF). First, the different specificities of the infection on *Y. pseudotuberculosis* indicate that Yep-phi and PhiA1122 adsorb different surface structures. Second, both periodate and proteinase K treatments of *Y. pestis* inhibited phage binding. Third, the spontaneous Yep-phi resistance mutants of *Y. pestis* could not be isolated, indicating that the phage had more complex behavior. Fourth, affinity chromatography revealed that tail fiber protein of Yep-phi is likely responsible for binding to four OMPs of *Y. pestis*, among which two (Ail and OmpF) were identified to be involved in the adsorption process. Fifth, an LPS mutant and a series of OMP mutants were accompanied by a decrease of phage adsorption efficiency.

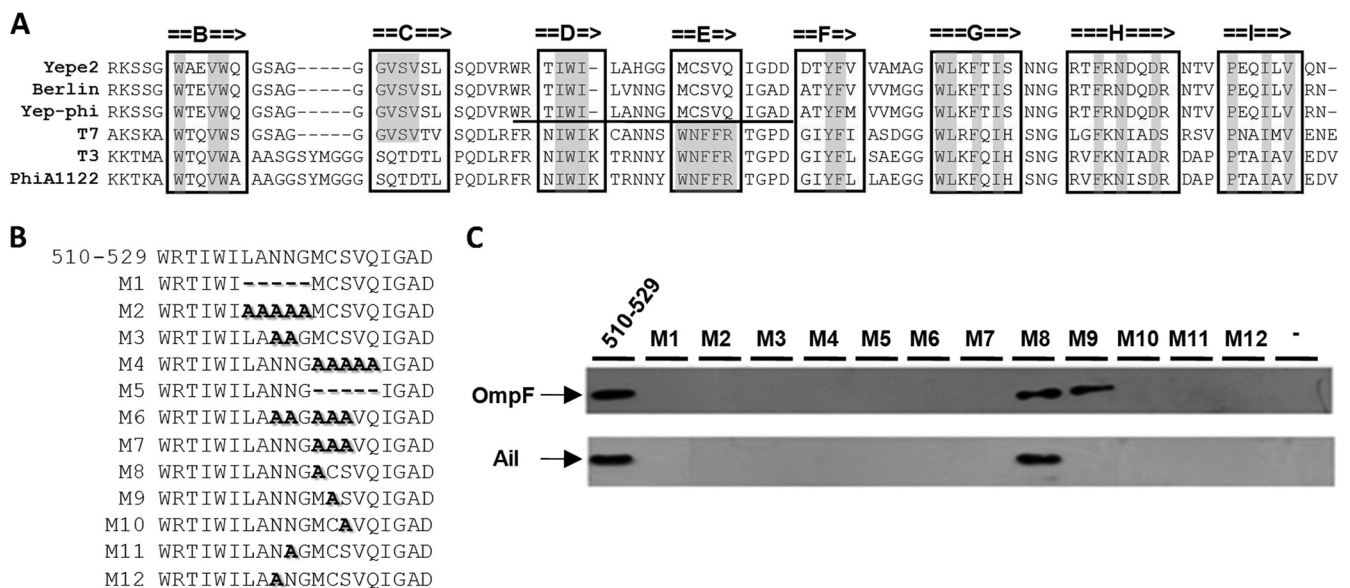Furthermore, our data indicate that residues 510 to 529 of tail

FIG 5 (A) Alignment of the sequence of the tip domain of T7 gp17 with its homologs from *E. coli* phage T3 and *Y. pestis* phages Yep-phi, Berlin, Yepe2, and PhiA1122. Beta-strands are denoted by arrows. The residues of beta-strands that are identical with each other are marked with gray. The domain necessary for the phage Yep-phi and *Y. pestis* interaction is underlined. (B) Locations of deletions and alanine replacements used in this study are indicated by M1 to M12. (C) OmpF and Ail were detected by Western blotting using either rabbit anti-OmpF or anti-Ail polyclonal antibody after affinity chromatography.

fiber protein gp17 are sufficient to allow binding to Ail and OmpF *in vitro*. Residues 518N, 519N, and 523S were determined to be essential for the interaction with OmpF, while residues 518N, 519N, 522C, and 523S were found to be essential for the interaction with Ail.

The results reveal novel phage-host interactions and the infection mechanisms of *Y. pestis*. Three kinds of host factors were specifically identified in this study (LPS, Ail, and OmpF). Although the phage can still lyse the Δ*ail* Δ*ompF* Δ*waaA* strain, it is with significantly decreased infection activity. One possible reason it could still lyse the strain is that there might be additional membrane components cooperatively determining the phage infection efficiency. The method employed in this study for isolating OMP receptors may not have been suitable for isolating proteins of low concentration. Another plausible explanation is that this interaction is presumably followed by a secondary, irreversible attachment of the tail to an unknown receptor (34). Because the irreversible binding triggers the release of naked genome from the virion, followed by the transit of viral DNA to the host cell cytoplasm (20), tail fiber protein gp17 may just keep the phage near the bacterial surface to make productive tail interactions with its receptor more likely (34). Although other factors may also contribute to the adaptation of a phage to a certain host, at least three distinct binding sites are required for the phage to proliferate efficiently on its host. The data presented in this study, together with the available knowledge, allow the proposition of a comprehensive model explaining the events leading to genome ejection from the phage particle.

LPS is required for the structural integrity of numerous OMPs and has significant effects on the cell surface protein composition (10, 16, 45). Close LPS-protein interactions are required for protein support, proper folding, and biological activity (10). For example, a drastic decrease of the concentration of OmpF has been observed in *E. coli* LPS mutants (45). Ail-mediated resistance to human serum depends on the LPS core (39), and changes in the LPS structure may influence the Ail conformation and specific activity (38). However, whether the receptor functions of Ail and OmpF are related to the LPS core remains unknown.

OMPs Ail and OmpF of *Y. pestis* have never been characterized as phage receptors. The current study reports that the host specificity of Yep-phi is mediated, at least in part, through the Ail and OmpF molecules. Affinity chromatography assay showed four OMPs responsible for interacting with the phage tail fiber protein, but the central roles of LamB and OmpA in phage adsorption have yet to be fully described. They are possibly related with the locations of the phage on the cell surface or for a later step of phage DNA injection.

Since *Y. pestis* is classified as a potential biowarfare or bioterror agent, phage therapy may be considered as an approach to counter such a threat (46, 47). A major concern regarding the use of phages in the treatment of infectious diseases still remains the emergence of phage-resistant mutants (23). The mutation of the attachment site is the most frequent route to phage resistance of the host (8). The phage Yep-phi infection has more-complex recognition specificity, requiring at least three host molecules. Various attachment sites can reduce the impact of phage resistance and overcome this problem. Thus, Yep-phi would be highly amenable for use in plague treatments.

Overall, our results represent the first ever demonstration of membrane-bound proteins of *Y. pestis* involved in a T7-related phage adsorption. This work is also the first to identify the residues of the phage tail fiber protein essential for the interaction with OMPs of Gram-negative bacteria. These observations highlight the importance of the tail fiber protein in the evolution and function of various complex phage systems. This information may also contribute to the application of bacteriophages in various settings, such as bacteria typing or phage therapy, through the rational design of mutants binding different receptors or the engineering of artificial, chimeric phage fibers.

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