DTDP-rhamnosyl transferase \( \text{RfbF} \), is a newfound receptor-related regulatory protein for phage \( \text{phiYe-F10} \) specific for \( \text{Yersinia enterocolitica} \) serotype O:3

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Bacteriophages and their hosts are continuously engaged in evolutionary competition. Here we isolated a lytic phage \( \text{phiYe-F10} \) specific for \( \text{Yersinia enterocolitica} \) serotype O:3. We firstly described the phage receptor was regulated by DTDP-rhamnosyl transferase \( \text{RfbF} \), encoded within the \( \text{rfb} \) cluster that was responsible for the biosynthesis of the O antigens. The deletion of DTDP-rhamnosyl transferase \( \text{RfbF} \) of wild type O:3 strain caused failure in \( \text{phiYe-F10} \) adsorption; however, the mutation strain retained agglutination with O:3 antiserum; and complementation of its mutant converted its sensitivity to \( \text{phiYe-F10} \). Therefore, DTDP-rhamnosyl transferase \( \text{RfbF} \) was responsible for the phage infection but did not affect recognition of \( \text{Y. enterocolitica} \) O:3 antiserum. Further, the deletions in the putative O-antigen biosynthesis protein precursor and outer membrane protein had no effect on sensitivity to \( \text{phiYe-F10} \) infection. However, adsorption of phages onto mutant HNF10-\( \Delta \text{O-antigen} \) took longer time than onto the WT, suggesting that deletion of the putative O-antigen biosynthesis protein precursor reduced the infection efficiency.

The primary determinant in the infection of a bacterial host by a bacteriophage is the adsorption of the phage to the host receptor. This receptor recognition is the most reported in phage-host interaction studies\(^1,2\). \( \text{Yersinia enterocolitica} \) is Gram-negative and a globally distributed foodborne human pathogen, belonging to the Enterobacteriaceae \( \text{Yersinia} \) species. There are about 60 different serotypes with variability in the O-antigen. \( \text{Y. enterocolitica} \) clinical isolates from humans predominantly belong to serotypes O:3, O:9, O:8 and O:5, 27 with variability on different continents\(^3,4\); serotypes O:3 and O:9 cause human infections and are most common in Europe, Canada, Japan, China and South Africa; while 1B/O:8 is the primary serotype infecting people in the Americas\(^5,6\). However, at present, serotype O:3 strains are becoming the most frequently detected pathogenic \( \text{Y. enterocolitica} \) all over the world\(^7\)–\(^10\).

To date, the phage phiYeO3-12 and vB_YenP_AP5 display specificity for \( \text{Y. enterocolitica} \) O:3 and phage PY54 exhibits a host range restrict to \( \text{Y. enterocolitica} \) O:5 and O:5, 27 were previously described\(^11\)–\(^15\). Phage viruses have stringent host specificities where the attachment of the virus particle requires specific recognition of phage receptor using a phage receptor binding protein (RBP)\(^16,17\). Several receptors are reported, including flagella/pilus related components\(^18\)–\(^19\), lipopolysaccharides (LPS)\(^20\)–\(^22\), capsular polysaccharides (CPS) and outer membrane proteins (OMP)\(^23\)–\(^24\). Similar to other Gram-negative Enterobacteriaceae bacteria, the structure of the \textit{Yersinia}
enterocolitica LPS has three primary components: lipid A, core oligosaccharide and O-side-chain (O-antigen). LPS acts as an immune stimulatory agent (lipid A) or as a virulence factor (O-antigen)²⁵–²⁷. The lipid A portion is responsible for the endotoxin activity. The O-antigen functions as a barrier against complement-mediated lysis and resists killing of bacteria by microbicidal intracellular granules in polymorpho-nuclear leucocytes. Previously published assays showed that we can detect the serotypes of Y. enterocolitica strains using amplification of O-antigen-encoded genes²⁸. The Yersinia enterocolitica O:3 lipopolysaccharide O-antigen is a homopolymer of 6-deoxy-L-altrose. The genes for the O:3 O-antigen translocation are located within the rfb gene cluster, including 10 open reading frames and eight of the genes, are organized into two operons, rfbABC and rfbDEFGH that are essential for O-antigen synthesis²⁶. A specific detection of Y. enterocolitica serotype O:3 is obtained with fragment of the rfbC gene ²⁹. In this study, we evaluated the lysis ability of the phage phiYe-F10 on the Y. enterocolitica wild strain, a spontaneous rough mutant, three gene deletion strains and a rfbC compensation strain using phage adsorption tests and plaque formation tests. We are first to identify DTDP-rhamnosyl transferase RfbF as the receptor regulator protein, and another protein (putative O-antigen biosynthesis protein precursor) in a Y. enterocolitica O:3 strain is related to phiYe-F10 adsorption.

**Results**

**The host range of phage phiYe-F10.** The 188 Y. enterocolitica strains were (Supplemental data 1): 57 strains serotype O:3, 34 strains serotype O:9, 13 strains serotype O:8, 10 strains serotype O:5, 3 strains serotype O:5, 27, 5 strains of self-agglutinating Y. enterocolitica and 66 other serotypes biotypes 1A Y. enterocolitica (Among the six Y. enterocolitica biotypes, 1A is the most heterogeneous group, including more than 17 different serotypes, some of them are unable to serotyped³⁰). At 25 °C and 37 °C, phiYe-F10 can formed plaques only on serotype O:3 strains, but not on Y. enterocolitica strains other O serogroups, or Y. pseudotuberculosis or Y. pestis strain (Table 1). All of the 57 sensitive strains were O:3 serotype Yersinia enterocolitica carrying the DTDP-rhamnosyl transferase RfbF encoding genes (rfbC), 49 of which were pathogenic Y. enterocolitica (including 39 strains biotype 3, 6 strains biotype 4, and 4 strains of biotype 5); 6 strains were biotype 1A nonpathogenic; and 2 strains were biotype 1A with ail genes (Table 2).

**Morphology.** phiYe-F10 was negatively stained and examined using transmission electron microscopy (Fig. 1A showed with an arrow). The virions showed hexagonal outlines, indicating their icosahedral nature. The head connected with the tail through a short neck which exhibiting T7 symmetry in shape. The phage had a head at approximately 55.0 nm in diameter and a short non-contractile tail of ~11.0 nm in length. Extended tail fibers were not seen. Collectively, these morphological features indicated that this virus belongs to the family Podoviridae.

**General features of the phiYe-F10 genome and comparative genomics.** The phiYe-F10 genome was assembled as a circular molecule when the sequencing was completed. Using PCR, the results of terminal run-off sequencing confirmed that the phiYe-F10 has repeated sequences. The genomes of T7-like phages typically contain direct terminal repeats (DTRs) that are used during genome replication and packaging. For example, Yersinia phage phiYeO3-12 (Genbank No. NC_001271.1) and vB_YenP_AP5 (Genbank No. NC_025451.1) have DTRs of 232 bp and 235 bp, respectively. The lengths of the DTRs in phiYe-F10 (235bp) were in agreement with the reported lengths of T7-like phage members. So we concluded the DNA sequence of the phiYe-F10 consists of a linear double stranded DNA of 39,210 bp, which correlates well with the size of other T7-like phage members. In total, 46 gene products were predicted in the phiYe-F10 genome; functions were assigned to 43 of them based on the similarities of the predicted products to known proteins.
Compared with the other two lytic phages for Y. enterocolitica serotype O:3, the genome of phiYe-F10 was highly similar to the 39,600 bp of Yersinia phage phiYeO3-12 (97%, 623/641) and the 38,646 bp of Yersinia phage vB_Y enP_AP5 (89%, 583/648). Genomic comparisons indicated phages phiYe-F10, phiYeO3-12 and vB_Y enP_AP5 were closely related. A dot plot comparison using the program Gepard was performed to illustrate the genomic similarities among phiYe-F10, phiYeO3-12 and vB_Y enP_AP5. The red dots indicated the corresponding genome regions on the abscissa and the ordinate showed similarity (Fig. 1B-1, B-2). We also performed a graphical comparison of the three genome sequences. Highly related sequences were shown using red shadings. As shown in Fig. 1C, the genome sequence of phiYe-F10 had exactly similar genetic organization and large blocks of homologous synteny when compared to the other two phages. The differences were primarily for the 403 bp and 446 bp deletions (with the region from 26,675 bp to 27,078 bp of Yersinia phage phiYeO3-12 and the 25,710 bp to 26,156 bp of vB_Y enP_AP5 deleted in the genome sequence of phiYe-F10). The missing genes encoded putative 13.5 protein for phage phiYeO3-12; encoded hypothetical protein and portion of internal virion protein A for phage vB_Y enP_AP5. The Fig. 1C data was in concordance with the Fig. 1B-1, B-2 data, strongly indicated that phiYe-F10, phage phiYeO3-12 and vB_Y enP_AP5 were closely associated.

Table 2. The characteristics of the 57 phage sensitive Y. enterocolitica strains of O:3 serotype.

| Serotype | Genotype | Biotype | Sensitive rate |
|----------|----------|---------|---------------|
| O:3      | rfb+, ail+, ystA-, ystB-, yadA+, virF+ | 1A | 0 3 6 4 | 75.44% |
|          | rfb+, ail+, ystA-, ystB-, yadA-, virF- | 2 | 3 3 0 0 | 14.04% |
|          | rfb-, ail-, ystA-, ystB+, yadA-, virF- | 2 | 0 0 0 0 | 3.51% |
| O:3      | rfb-, ail-, ystA-, ystB-, yadA-, virF- | 4 | 0 0 0 0 | 7.02% |

Figure 1. (A) Electron micrograph of phiYe-F10. The phage is negatively stained with 2% potassium phosphotungstate. phiYe-F10 is shown at 135,000× magnification. Scale bar indicates size in nm. (B-1) Dot plot of genome sequences of phiYe-F10 and Yersinia phage phiYeO3-12. (B-2) Dot plot of genome sequences of phiYe-F10 and Yersinia phage vB_Y enP_AP5. (C) Pairwise nucleotide sequence comparison of phiYe-F10, phiYeO3-12 and vB_Y enP_AP5.
were completely lysed at OD $600 \approx 0$. phiYe-F10 adsorbed to the HNF10-Δ

...of the bacterial chromosome, and encoded the dTDP-rhamnosyl transferase RfbF. It directed the O-antigen biosynthesis through synthesizing the O-antigen glycosyltransferases, which transferred the rhamnosyl to growing O-antigen repeats units. As the phage phiYe-F10 specific for...vB_Y enP_AP5. The six families Podoviridae members were classified into the same cluster, the other sequences of the families Myoviridae and Siphoviridae divided from that of the families Podoviridae, and located in different branches (Fig. 2).

Figure 2. The phylogenetic tree of tail fiber proteins. *Strain YeO3-R1, a virulence-plasmid-cured O antigen-negative derivative of Yersinia enterocolitica serotype O:3. The phage receptor of phi R1-37 is in the outer core hexasaccharide of Y. enterocolitica O:3 LPS. UT: untyped; UN: unknown.

For bacteriophages, the primary determinant of the host receptor is the tail fiber protein, so we compared the tail fiber protein of Yersinia phage with those of the families Podoviridae, Myoviridae and Siphoviridae. The alignment showed high homology among phiYe-F10, phiYeO3-12 and vB_Y enP_AP5. The six families Podoviridae members were classified into the same cluster, the other sequences of the families Myoviridae and Siphoviridae divided from that of the families Podoviridae, and located in different branches (Fig. 2).

The adsorption abilities and growth characteristics of the WT strain, its mutants, and compensation strain. Early studies showed the outer membrane protein A (OmpA) was not only a common phage receptor of Enterobacteriaceae32,33, but also a common immune protective antigen beyond the V antigen conserved within Yersinia species34,35. The phage Yep-phi, required not only LPS but also Ail and OmpF for its efficient infection, losing of the ail and ompF products may resulted in defective phage receptors34. In order to identify whether OmpA was involved in the phage receptor of phiYe-F10, we constructed of HNF10-ΔompA knock-out mutants to test the growth characteristic and phage adsorption efficiency. The O-side-chain of Yersinia enterocolitica O:3 is a homopolymer of 6-deoxy-L-altrose35. Our research had suggested that the Yersinia enterocolitica serogroup O:3 were all carried with the fragment of the rfbC gene. The rfc gene clustered in the rfb locus of the bacterial chromosomal, and encoded the dTDP-rhamnosyl transferase RfbF. It directed the O-antigen biosynthesis through synthesizing the O-antigen glycosyltransferases, which transferred the rhamnosyl to growing repeat units. As the phage phiYe-F10 specific for Y. enterocolitica serotype O:3, and the rfc gene was strictly found in sensitive strains, we wanted to identify whether dTDP-rhamnosyl transferase RfbF was involved in the phage infection of phiYe-F10.

The mutants HNF10-ΔrfbF, HNF10-ΔO-antigen and HNF10-ΔompA were verified by PCR (Supplemental data 2). The WT, knock-out mutant strains, the spontaneous rough mutant R-HNF10 (A spontaneous rough derivative of Y. enterocolitica serotype O:3 strain HNF10 carried with virulence-plasmid), and compensation strain HNF10-ΔrfbF/Crfbf were all grown to log phase in LB broth at 25 °C with agitation reaching a similar value of OD$_{600}$ for each group. After infecting with phage phiYe-F10 (at 4.5 h in total growth), every half hour the OD was showed a decrease after one hour (at 5.5 h in total growth) with phage phiYe-F10 infection; and then began lysing (Fig. 3B). The OD$_{600}$ value of the WT and HNF10-ΔompA showed a decrease after one hour (at 5.5 h in total growth) with phage phiYe-F10 infection; and then 2.5 hours later (at 7 h in total growth), both WT and HNF10-ΔompA were completely lysed at OD$_{600}$=0. phiYe-F10 adsorbed to the HNF10-ΔO-antigen and then began lysing (Fig. 3B).

...resistance to phage phiYe-F10. The WT, knock-out mutants strains (HNF10-ΔrfbF, HNF10-ΔO-antigen, HNF10-ΔompA), spontaneous rough mutant R-HNF10, and compensation strain HNF10-ΔrfbF/Crfbf were tested the sensitivity to the phage with the double-layer plaque assay (Fig. 4B). The results showed the knock-out mutant strain HNF10-ΔrfbF and spontaneous rough mutant R-HNF10 were resistant to the phage, producing no plaques and showing a weak positive in the Acriflavine agglutination test; while the WT, HNF10-ΔO-antigen, HNF10-ΔompA, and HNF10-ΔrfbF/Crfbf strains produced plaques and showed negative in the Acriflavine agglutination test (Fig. 4B,C).

Discussion

In natural environments, bacteria and phage are competing and co-existing with each other. In this competition, the bacteria evolve resistance mechanisms against phage infection. These strategies include blocking receptors or altering receptor structures to prevent phage adsorption, preventing phage DNA entry, digesting phage nucleic...
Figure 3. The growth curves of WT, its mutants, and compensation strain. (A) The growth curves of strains without phage infection. (B) The growth curves of strains with phage infection. Approximately $6 \times 10^7$ PFU of phiYe-F10 in 1 ml was mixed with the 10 ml bacterial culture (OD$_{600} \approx 0.1-0.2$), and incubated at 25°C for 9.5 h. Control cultures were grown without phage infection. The OD$_{600}$ of each group was measured every half hour. At the time indicated by arrow, phage phiYe-F10 was added to the culture.

Figure 4. The results of phage adsorption assay, the double-layer plaque assay and Acriflavine agglutination test of WT, its mutants, and compensation strain. (A) Adsorption assay of bacteriophage phiYe-F10 to the test strain. Approximately $6 \times 10^6$ PFU of phiYe-F10 in 100 μl was mixed with 500 μl samples of bacteria (OD$_{600} \approx 1.0$). The adsorption rate of each strain was calculated as $(P_t-P_i)/P_i$. Error bars denote statistical variations. Significance was determined by Dunnett T3 test for comparison between the mutant group and the WT group. *P < 0.05. (B) The double-layer plaque assay. (C) Acriflavine Agglutination test.
acid, inhibiting replication of the phage genome, and cause abortive phage infection. Conversely, bacteriophages are capable of rapid adaptive responses to evolutionary changes in their hosts. As a counter-defensive measure, phages are able to modify their receptor binding proteins, e.g. the tail fiber, to achieve infection and kill the resistant bacterium. For example, Pseudomonas fluorescens SBW25 was found to coevolve with its lytic phage phi2 for more than 300 bacterial generations. This co-evolution is probably due to the continuous modification of the bacterial receptors and phage receptor binding protein

Yersinia enterocolitica is a common foodborne pathogen with O:3 and O:9 as the primary infectious serotypes in most countries. It was reported that, there were two lytic phages which infected Yersinia enterocolitica O:3 had been fully sequenced: phiYeO3-12 (GenBank accession no. AJ251805.1) and vB_YenP_APS (GenBank accession no. KM253764.1). The genome sequences of YeO3-12 and vB_YenP_APS were submitted to the GenBank databases in 1999 by Pajunen, M.I. and colleagues and in 2014 by Leon-Velarde, C.G. and colleagues, respectively. The two phages were all belonged to the family Podoviridae. In this study, the lytic Yersinia phage phiYe-F10, was isolated together with the pathogenic bioserotype 3/O:3 Y. enterocolitica HNF-10 from the same swine rectal swab sample. The whole genome sequence of phage phiYe-F10 showed great similarities to Yersinia phage PhiYeO3-12 and vB_YenP_APS, excepted for minor insertions or deletions among the genome sequences in these phages (Fig. 1C). The genomic comparisons indicated the phiYe-F10, PhiYeO3-12 and vB_YenP_APS were closely genetically related, and most of their DNA sequences appeared to have descended from a single common ancestral phage. The Podoviridae family phage was common in Enterobacteriaceae, which showed the morphologic characteristics of an icosahedral head and a short non-retractable tail when observed under transmission electron microscopy, and the phages were known to have double strands linear genomes with direct terminal repeats.

The Yersinia enterocolitica was isolated together with the pathogenic bioserotype 3/O:3 Y. enterocolitica was genetically related, and most of their DNA sequences appeared to have descended from a single common ancestral phage. The Podoviridae family phage was common in Enterobacteriaceae, which showed the morphologic characteristics of an icosahedral head and a short non-retractable tail when observed under transmission electron microscopy, and the phages were known to have double strands linear genomes with direct terminal repeats.

Currently, the reported Yersinia enterocolitica phage were belonged to different families, phage YeO3-12 and vB_YenP_APS, which specific to infect Y. enterocolitica to the family Podoviridae, however the temperate phage PY54 (GenBank accession no. AJ564013.1) belonged to the family Siphoviridae, which infected Y. enterocolitica of serotype O:5 and O:5, 27; the phage PhiR1-37 (GenBank accession no. AJ972879.2) belonged to the family Myoviridae, which infected strain YeO3-R1 (a virulence-plasmid-cured O antigen-negative derivative of Yersinia enterocolitica serotype O:3). The tail fiber protein sequences alignment revealed that the phages belonged to the family Podoviridae showed a high similarity and were classified into the same cluster (Fig. 2). It has been suggested that they follow a similar DNA ejection mechanism, with tail fibers adsorbed to the host surface LPS. Although the phage vB_YenP_ISA08, PhiR1-37 and PY54 displayed lysis for Y. enterocolitica, the sequences of tail fiber proteins were clustered into different groups, and the hosts serotypes and phage receptors were different from that of phiYe-F10, phiYeO3-12 and vB_YenP_APS (Fig. 2).

Phage phiYe-F10 displayed strict specificity for Y. enterocolitica O:3 at 25 °C and 37 °C. Other serotypes (O:8, O:9, O:5, O:5, 27 et al.) of Y. enterocolitica as well as Y. pseudotuberculosis and Y. pestis were unaffected by the presence of phage phiYe-F10. Previous research indicated that Yersinia enterocolitica phage PhiYeO3-12 and PhiR1-37 used LPS as their receptors. Here we first confirmed that the dTDP-rhamnosyl transferase RfbF, coded by the rfb gene, played a critical role in synthesizing the phage receptor for phiYe-F10. The serotype sensitive Y. enterocolitica of O:3 were all carrying the dTDP-rhamnosyl transferase RfbF encoding gene (rfbc). The rfb gene, which responsible for the biosynthesis of the O side chain of Y. enterocolitica, was chosen for the specific detection target of O:3 serotype. Among the sensitive strains, four strains had different rfb gene with multiple mutation sites compared with the rest of the strains. Although the protein sequence of dTDP-rhamnosyl transferase RfbF changes, the function of the phage receptors was remained and therefore these four strains were still phage sensitive. The rfb gene located within the rfb gene cluster encoded the dTDP-rhamnosyl transferase RfbF, which was involved in the dTDP-L-rhamnose biosynthesis. L-Rhamnose is the receptor on the LPS core for the attachment of O polysaccharides; it is an indispensable component of the lipopolysaccharide synthetic pathway. Y. enterocolitica O:3 O-antigen is a homopolymer of 6-deoxy-L-altrose; the rfb gene regulates the LPS O antigen synthesis pathway. Deletion of dTDP-rhamnosyl transferase RfbF may result in the changes or losses of the O antigen, resulting in the failure of phages to bind and infect the strains. Complementation of dTDP-rhamnosyl transferase RfbF deletion mutant recovered its sensitivity to phiYe-F10. Mutation of dTDP-rhamnosyl transferase RfbF retained agglutination with O:3 antisera, suggested some group modifications of O antigen were responsible for the phage infection but did not affect recognition of the O:3 antisera.

Deletion of the putative O-antigen biosynthesis protein precursor encoding gene may reduced the infection efficiencies; however, the mutant strain was still phiYe-F10 sensitive. When infected with phage phiYe-F10 at a similar OD in logarithmic phase, the WT strain began to lyse after 1 h and reached complete lysis 2.5 hour later; whereas HNF10-ΔO-antigen started lysis at 1.5 h and reached complete lysis 3 hour later. This showed phiYe-F10 adsorbed to HNF10-ΔO-antigen slower than WT; but the time needed from the beginning of lysis to complete lysis was the same for the two strains (Fig. 3B). Consequently, we speculated that the putative O-antigen biosynthesis protein precursor played a role in the first steps of virus-host interaction, the deletion of this protein may affected phage adsorption and phage DNA entry, e.g., blocking the phage tail fiber recognition and binding the receptor, or altering the host receptor structure on the bacterial cell surface to prevent phage adsorption, or preventing phage DNA entry. This needs further investigation.

Many studies showed that OmpA was a common phage receptor of Enterobacteriaceae, but also a common immune protective antigen beyond the V antigen conserved within Yersinia species. Unlike Y. pseudotuberculosis and Y. enterocolitica, Y. pestis had a rough LPS without O antigen where Ail and OmpF were identified to act as the receptors of Yep-phi in addition to the rough lipopolysaccharide of Y. pestis. In our study, the OmpA deletion mutant of Y. enterocolitica did not affect the binding and lysis of phage phiYe-F10, showing OmpA was not the receptor of phiYe-F10.

A gene altered by artificial or natural mutation will lead to the lack or change of the phage receptor; then alter the phage resistance. In Y. pestis, some spontaneous mutations in the core polysaccharide brought about the loss of the LPS core, which resulted in phage resistance. A sequence comparison between the spontaneous resistant
strain (R-HNF10) and the WT strain showed the coding genes of dTDP-rhamnosyl transferase RfbF were completely the same. However, R-HNF10 formed obviously rough colonies on agar; and the Acriflavine agglutination test also showed O-antigen deficiency, which further suggested that the phiYe-F10 resistance mechanism resulted from an O-antigen deficiency. Some of the R-HNF10 resistant strains did not agglutinate with O:3 antiserum and monoclonal antibodies, but the others with serotype not changed. This finding suggested small modifications or other changes of O antigen were responsible for the phage infection (binding) but did not affect recognition of the O:3 antigen. We inferred the epitopes determining serogroup had more and larger distribution than that of phage receptor within the LPS O side-chains.

This study firstly showed the phage receptor of phiYe-F10 was regulated by dTDP-rhamnosyl transferase RfbF from an O-antigen deficiency. Some of the R-HNF10 resistant strains did not agglutinate with O:3 antiserum and monoclonal antibodies, but the others with serotype not changed. This finding suggested small modifications or other changes of O antigen were responsible for the phage infection (binding) but did not affect recognition of the O:3 antigen. We inferred the phage determinations were more and larger distribution than that of phage receptor within the LPS O side-chains.

**Methods**

**Bacteriophage isolation and sensitivity test.** A lytic phage, named phiYe-F10, specific for *Yersinia enterocolitica* was isolated from a swine rectal swab sample. The phage concentration in the swine rectal swab, was estimated using the plaque assay on *Y. enterocolitica* HNF-10 (Bioserotype 3/O:3, virulence plasmid positive). The phage was isolated together with strain HNF-10 from the same sample in a routine prevalence surveillance for *Yersinia* in China.

From these specimens, phiYe-F10 was chosen for detailed study because of its ability to infect *Y. enterocolitica* strains of serotype O:3. The host range of phiYe-F10 was determined using a double-layer plaque assay at 25 °C and at 37 °C, with one *Y. pestis*, 188 *Y. enterocolitica* and 37 *Y. pseudotuberculosis* belonging to different serotypes. Strains used in the bacteriophage sensitivity test were listed in Table 1. Among the 188 different *Y. enterocolitica*, 183 were widely distributed within 17 provinces of China from 1982 to 2014; and 5 reference strains were provided by H. Fukushima at the Shimane Prefectural Institute of Public Health, Matsue, Japan. The strains were collected from routine monitoring of animals, food and patients with diarrhea (Supplemental data 1).

**Electron microscopy.** Filtered phage lysates (about 2 × 10^10 PFU/mL) were pelleted at 25,000 × g for 1 h at 4 °C, using a Beckman high-speed centrifuge with a JA-18.1 fixed-angle rotor (Beckman, Palo Alto, CA, USA). The phage pellet was washed twice in neutral 0.1 M ammonium acetate. The final phage sediment was re-suspended in 150 μL of SM-buffer supplemented with 5 mM CaCl₂. Samples were then deposited onto a carbon-coated Formvar film on copper grids, and stained with 20 μL of 2% potassium phosphotungstate (PT, pH 7.2). Get rid of the dye with filter paper, air dried, and examined under a TECNAI 12 transmission electron microscope (FEI, Hillsboro, OR, USA) at 120 KEV. Images were collected and analyzed using Digital Micrograph™ Software (Gatan, Pleasanton, CA, USA).

**The Genome sequencing of phage DNA, assembly and bioinformatics analysis.** A random "shotgun" library was constructed using a fast nebulization method and the fragments were ligated to the vector pUC118 (TaKaRa Code: 3322, pUC118 HincII/BAP). Cycle sequencing reactions from the plasmid inserts were performed using the ABI PRIS MW Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with a Gene Amp PCR System 9700 (Applied Biosystems). Sequence reactions were analyzed using an ABI PRISMTM 3730XL DNA Analyzer (Applied Biosystems). Sequencing continued until an eight-fold coverage of the sequenced plasmid was attained. Assembly of the sequences was performed using the SeqMan module of the Lasergene software (DNASTAR Inc.). Persisting gaps were closed using primer-walking sequencing of the genomic DNA.

A dot plot comparison and graphical comparison were conducted using BLAST 2.25 and displayed using ACT49. The minimum score cutoff was 100 and the minimum identity cutoff was 50%.

Sequences of tall fiber proteins were collected from NCBI (6 phages for *Y. enterocolitica*, 1 phage for *Y. pestis*, and the *Escherichia coli* phages T7 and T3), the phylogenetic tree was generated using the neighbor-joining method implemented in MEGA6. Bootstrap values representing the percent in 1,000 replicates were shown in the tree.

**Bacteria, plasmids, and growth media.** The *Y. enterocolitica* wild type strain HNF10 was isolated from a swine rectal swab sample from Henan province. R-HNF10 was a spontaneous rough mutant of HNF10. The knock-out mutants HNF10-ΔO-antigen, HNF10-ΔompA, and HNF10-ΔrfbF were constructed in this work. The strains and plasmids used for gene cloning and mutation were listed in Table 3. The serotypes, biotypes, and pathogenicity of these strains were determined as previously described. The E. coli and *Y. enterocolitica* strains were incubated at 37 °C and 28 °C, respectively. Solid and soft agar media contained 2.0% and 0.5% (w/v) agar, respectively. Antibiotics were used at the following concentrations: kanamycin: 50 μg/ml for agar plates and 100 μg/ml for broth; chloramphenicol, 34 μg/ml; cefsulodin, 15 μg/ml; and novobiocin, 2.5 μg/ml.

**Construction of HNF10-ΔrfbF, HNF10-ΔO-antigen and HNF10-ΔompA knock-out mutants.** The deletion mutants were constructed using homologous recombination with the suicide plasmid pDS132. To obtain the knock-out mutants, the corresponding two sets of primers were used to amplify two different fragments respectively. The p1 and p2 primers amplified the upstream region of genes, the P3 and P4 primers...
amplified the downstream region of genes (Table 4). pDS132-O-antigen, pDS132-ompA and pDS132-rfbf were generated using the In-Fusion HD Cloning Kit (Clontech) following the manufacturer’s instructions. The recombinant plasmids were induced into the competent cell S17λpir52, and then mobilized into Y. enterocolitica HNF10 through biparental conjugation. Transconjugants were selected after growth on LB plates containing Yersinia complement (cefsulodin and novobiocin) and chloramphenicol. Bacteria from individual colonies were pooled and allowed to grow in LB without antibiotic overnight at 25 °C. Bacterial cultures were serially diluted in LB without NaCl containing 10% sucrose; the plates were incubated at 25 °C. The recombinants that survived in the 10% sucrose were examined for their antibiotic resistance. The appropriate replacement of the wild-type alleles by the mutants was confirmed using PCR and sequencing.

### Complementation of mutations.

Primers rfbfCF and rfbfCR, incorporating NdeI and SacI restriction sites (Table 4), were used to amplify the ORF of rfbf, including the 904-bp region in the HNF10 genome. The ampli
cion was digested with NdeI and SacI (New England BioLabs) and ligated into the NdeI- and SacI-digested plasmid pSRKKm (D3050; TaKaRa, Japan)53, producing plasmid pSRKKm–rfbf (the rfbf ORF cloned into pSRKKm, KmR); introduced into the competent cell S17λpir; and then mobilized into Y. enterocolitica HNF10-Δrfbf using

### Table 3. Strains and plasmids used for gene cloning and mutation in this study.

| Strains and plasmids | Description or comments | Source or reference |
|----------------------|-------------------------|---------------------|
| **Strains**          |                         |                     |
| Y. enterolitica HNF10 | BioSerotype 3/O:3 with pYY plasmid, isolated in Henan, China from a swine rectal swab | This study |
| HNF10-O-antigen      | Strain HNF10 with a deletion of gene for putative O-antigen biosynthesis protein precursor | This study |
| HNF10-ompA           | Strain HNF10 with a deletion of ompA gene | This study |
| HNF10-rfbc           | Strain HNF10 with a deletion of rfbf gene | This study |
| HNF10-Δrfbf/Crfbf    | rfbf complementation of HNF10-Δrfbf | This study |
| R-HNF10              | Spontaneous rough mutant of HNF10 | This study |
| **Plasmids**         |                         |                     |
| pDS132               | Conditionally replicating vector; R6K origin, mobRK4 transfer origin, sucrose-inducible sacB, CmR | 51 |
| pDS132-O-antigen     | containing a fusion fragment of upstream and downstream of the O-antigen gene | This study |
| pDS132-ompA          | containing a fusion fragment of upstream and downstream of the ompA gene | This study |
| pDS132-rfbc          | containing a fusion fragment of upstream and downstream of the rfbf gene | This study |
| pSRKKm               | Expression vector containing lac promoter and lacIq, KmR | 53 |
| pSRKKm–rfbf          | rfbf cloned into pSRKKm, KmR | This study |

### Table 4. Primers used in this study. Note: Restriction endonuclease sites are underlined.

| Primer | Sequence (5′-3′) |
|--------|-----------------|
| rfbf p1 | AATCAGATCGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| rfbf p2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| rfbf p3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| rfbf p4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |
| ompA p1 | AAAGGATGCTGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| ompA p2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| ompA p3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| ompA p4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |
| Oag P1 | AAAGGATGCTGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| Oag P2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| Oag P3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| Oag P4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |

| Construction of mutants | Sequence (5′-3′) |
|-------------------------|-----------------|
| rfbf p1 | AATCAGATCGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| rfbf p2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| rfbf p3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| rfbf p4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |
| ompA p1 | AAAGGATGCTGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| ompA p2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| ompA p3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| ompA p4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |
| Oag P1 | AAAGGATGCTGATCTCTAGTGAAGGCCGATCCTTTATGGGTAC |
| Oag P2 | GCTAGAAACTCACTAGAAAGATTTAAGAGACCGAAAA |
| Oag P3 | TTTTCGGTCTCTTAAATCTTTCTAGTGAGTTTCTAGC |
| Oag P4 | CCCGGGAGAGCTCGATATCGCAACATCACGGAATCTT |
biparental conjugation. The recombinant clones HNF10-Δrfbf/Crfbf were confirmed using PCR and sequenced to ensure they contained the correct insert sequence.

**Phage adsorption assays.** The WT, R-HNF10, HNF10-Δrfbf, HNF10-ΔO-antigen HNF10-ΔompA knocked-out mutant strains and compensation strain HNF10-Δrfbf/Crfbf were cultured overnight on BHI at 25 °C. Approximately 6 × 10⁶ PFU of phiYe-F10 in 100 μl was mixed with 500 μl samples of bacteria (OD₆₀₀=1.0). The suspension was incubated at room temperature for 5 min and centrifuged at 16,000 × g for 3 min, after which the phage titer remaining in the supernatant was determined as Pt. BHI was used as a non-adsorbing control in each assay, and the phage titer in the control supernatant was set to Pt'. The adsorption rate of each strain was calculated as (Pt-Pt)/Pt × 100%, each strain was repeated three times.

**Agglutination test.** The WT, R-HNF10, knock-out mutant strains and compensation strain were agglutinated using concentrations of Acriflavine. This procedure offered a simple way to distinguish LPS defective bacteria. Bacteria were suspended in 0.2% Acriflavine solution, the LPS defective strains agglutinated, whereas the LPS positive strains showed no agglutination⁴⁹,⁵¹.

**The growth characteristic of WT, mutant strains and compensation strain.** The WT, R-HNF10, HNF10-Δrfbf, HNF10-ΔO-antigen HNF10-ΔompA and HNF10-Δrfbf/Crfbf were grown overnight and 1:100 diluted in fresh LB medium at the zero time point. After 4.5h culture, 1 ml of phiYe-F10 (6 × 10⁶ PFU/ml) was mixed with the bacterial culture (10 ml), and incubated at 25 °C for 9.5 h. Control cultures were grown without phage infection. The OD₆₀₀ of each group was measured every half hour. Each group was repeated three times.

**Ethics statement.** The sample collection and detection protocols were carried out in accordance with relevant guidelines and regulations. All experimental procedures were approved by the Ethics Review Committee [Institutional Review Board (IRB)] of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Signed informed consent was obtained from all study participants.

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Author Contributions

X.W. and H.J. conceived and designed the experiments; J.L., X.L., T.Z., Y.C. and Y.X. performed the experiments; M.S. participated in the sequence alignment; H.H., C.L. and R.D. performed the statistical analysis, J.L., X.L., R.D. and Y.C. wrote the paper. All authors read and approved the final manuscript.
Additional Information

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