*Yersinia enterocolitica* specific infection by bacteriophages TG1 and φR1-RT is dependent on temperature regulated expression of the phage host receptor OmpF

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ABSTRACT

Bacteriophages present huge potential both as a resource for developing novel tools for bacterial diagnostics and for use in phage therapy. This is also valid for bacteriophages specific for *Yersinia enterocolitica*. To increase our knowledge on *Y. enterocolitica*–specific phages we characterized two novel yersiniophages. The genomes of the bacteriophages vB_YenM_TG1 (TG1) and vB_YenM_ϕR1-RT (ϕR1-RT), isolated from pig manure in Canada and from sewage in Finland, consist of linear double-stranded DNA of 162,101 and 168,809 bp respectively. Their genomes encode 262 putative coding sequences and 4 tRNAs genes, and share 91% overall nucleotide identity. Based on phylogenetic analyses of their whole genome sequences and large terminase subunit protein sequences, a genus named *Tg1virus* within the family *Myoviridae* is proposed with TG1 and ϕR1-RT as member species. These bacteriophages exhibit a host range restricted to *Y. enterocolitica*, and display lytic activity against the epidemiologically significant serotypes O:3, O:5,27, and O:9 at and below 25°C. Adsorption analyses of LPS and OmpF mutants demonstrate that these phages use both the LPS inner core heptosyl residues and the outer membrane protein OmpF as phage receptors. Based on RNA-sequencing and quantitative proteomics we also demonstrate the temperature dependent infection is due to strong repression of OmpF at 37°C. In addition, ϕR1-RT was shown to be able to enter into a pseudolysogenic state. All together, this work provides further insight into phage-host cell interactions by highlighting the importance of understanding underlying factors which may affect the abundance of phage host receptors on the cell surface.

IMPORTANCE
Only a small number of bacteriophages infecting *Y. enterocolitica*, the predominant causative agent of yersiniosis, have been previously described. Here, two newly isolated *Y. enterocolitica* phages were studied in detail with the aim of elucidating the host cell receptors required for infection. Our research further expands the repertoire of phages available for consideration as potential antimicrobial agents or as diagnostic tools for this important bacterial pathogen.
INTRODUCTION

Yersinia enterocolitica, a facultative anaerobic, Gram-negative, non-sporulating, short bacillus isolated frequently from soil, water, animals, and foods, is an important zoonotic pathogen leading to human and animal enteric infection (1). The main animal reservoir for Y. enterocolitica is pigs, and pork derived products are thought to be the main source of human infections in addition to drinking of contaminated water and blood-transfusions (1, 2). Symptoms of yersiniosis may include diarrhea, terminal ileitis, mesenteric lymphadenitis, and septicemia (3). Among the species within the genus Yersinia, Y. enterocolitica is highly heterogeneous and is grouped into six phylogroups (4). The widely used bioserotype groups form the basis of the phylogroups such that phylogroup 1 contains the biotype 1A strains, phylogroup 2 the highly pathogenic biotype 1B strains, phylogroup 3 the bioserotype 4/O:3 strains, phylogroup 4 bioserotype 3/O:9 strains, phylogroup 5 bioserotype 2/O:5,27 strains and phylogroup 6 the serotype O:2,3 strains rarely isolated from hares (4–7). Y. enterocolitica is also represented by over 60 serotypes that are determined by the variability of O-antigens present in the outer cell membrane (8, 9). The predominant pathogenic strains associated with yersiniosis belong to bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3, with the last being the most common in Europe, Japan, Canada, and the United States (1, 2). From 2010–2012, 98% of all reported yersiniosis infections worldwide were acquired in Europe, and most (97%) were caused by Y. enterocolitica, with the remainder caused by Y. pseudotuberculosis (10). In 2015, the most commonly reported Y. enterocolitica serotype in the European Union was O:3 (89%), followed by serotypes O:9 (7%), O:5,27 (2%) and O:8 (2%) (10).
Although several bacteriophages infecting *Y. enterocolitica* have been described, few have been studied in detail providing reliable information on morphology, host range, and or receptor specificity. To date, bacteriophages ϕYeO3-12(11–13) and vB_YenP_AP5 (14) with specificity for *Y. enterocolitica* O:3, phage PY54 exhibiting a host range restricted to *Y. enterocolitica* O:5 and O:5,27 (15), *Yersinia* phage ϕR1-37 with a broad host range within the species *Y. enterocolitica* (16, 17) and *Yersinia* phage PY-100 (18) exhibiting a broader host range restricted to the genus *Yersinia*, have been described. These bacteriophages use different parts of the *Y. enterocolitica* lipopolysaccharide (LPS) as receptors (19). Analysis of the host-range combined with genetic and structural data have shown that the receptor for ϕR1-37 is the *Y. enterocolitica* O:3 LPS outer core (OC) hexasaccharide (16). The host receptor for phages ϕYeO3-12 and vB_YenP_AP5 has been determined to be the LPS O-antigen of serotype O:3 consisting of the sugar 6-deoxy-L-altropyranose (12, 14, 20). Given the interest in bacteriophages because of their potential use as therapeutic, diagnostic, and bio-control agents, the aim of this study was to characterize two newly isolated bacteriophages that are active against several epidemiologically significant *Y. enterocolitica* serotypes. In this study, the genome characterization, morphology, host range, host cell receptor specificity, and taxonomic position of the myovirus phages vB_YenM_TG1 (hereafter TG1) and vB_YenM_ϕR1-RT (hereafter ϕR1-RT) are described.

**MATERIALS AND METHODS**

**Bacterial strains, phage isolation, and growth conditions.** Bacterial strains, bacteriophages and plasmids are listed in Table 1. Bacteriophage ϕR1-RT was isolated from the incoming sewage of the Turku (Finland) city sewage treatment
plant, as described for other viruses (19) whereas bacteriophage TG1 was isolated from pig manure collected from a rural farm in Ontario, Canada as described previously for the isolation of *Y. enterocolitica* phages for phagetyping (21). For DNA extraction and morphological studies, ϕR1-RT was propagated on *Y. enterocolitica* strain YeO3-R1 (22) and TG1 on *Y. enterocolitica* strain YeO3-c (23).

**Electron Microscopy.** The preparation of the phage particles for transmission electron microscopy (TEM) was done as described (17, 24). Details are presented in Supplementary Materials and Methods.

**Host Range.** The lytic activity of ϕR1-RT and TG1 was tested on 109 and 160 strains (Table S1), respectively, belonging to 13 *Yersinia* species, as determined by standard spot tests (24). Briefly, 10 µl from a phage suspension containing approximately $10^8$ PFU were spotted in the middle of a lawn of bacteria incubating for 18-24 h. Each strain was tested three times at 25°C and at 37 °C. Bacterial strains were considered sensitive to the phage if the degree of lysis was observed as a complete clearing, clearing throughout but with a faint hazy background, substantial turbidity throughout the cleared zone, or a few individual plaques (24). Bacterial strains were considered resistant if there was no effect of the phage on bacterial growth.

**Genome sequencing and assembly.** Details of the determination of the genomic sequences of phages ϕR1-RT and TG1 as well as the draft genomes of *Y. enterocolitica* strains YeO3-ϕR1-RT-R2, –R7 and –R9 are presented in Supplementary Materials and Methods.
Bioinformatics. Detailed description of the bioinformatics tools used is given in Supplementary Materials and Methods.

Complementation of the *Y. enterocolitica* O:3 OmpF mutant. The full ORF of *ompF* gene plus the upstream promoter region of YeO3-c was cloned as a 2 kb PCR fragment that was amplified with Phusion DNA polymerase using primer pair OmpC-F2 and OmpC-R2 (Table S2) into plasmids pTM100 and pSW25T to obtain plasmids pTM100_OmpF and pSW25T_OmpF, respectively (Table 1). Briefly, the PCR fragments were digested with MfeI and ligated with EcoRI digested, SAP-treated pTM100 or pSW25T. The constructed plasmids were mobilized to the OmpF mutant strain YeO3-c-R1-Cat17 by diparental conjugation as described earlier (25).

Phage adsorption assay. To identify the phage cell host receptors, a variety of *Y. enterocolitica* O:3 mutants (Table 1) were utilized in phage adsorption experiments. Approximately 5x10^3 PFU of phage ϕR1-RT or phage TG1 in 100 μl was mixed with a 400-μl sample of bacteria (*A*<sub>600</sub> ~1.2). The suspension was incubated at RT for 5 min and centrifuged at 16,000 g for 3 min, and the phage titer remaining in the supernatant, *i.e.*, the residual PFU percentage, 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 at least three times.

Total RNA extraction and RNA sequencing. Detailed description of the methods is presented in Supplementary information Materials and Methods. The RNA sequence data has been deposited to Gene Expression Omnibus (accession number GSE66516).
Quantitative proteomics. Detailed description of the methods is presented in Supplementary information Materials and Methods.

Transduction assay. *Y. enterocolitica* O:3 strain YeO3-hfq::Km with hfq gene knocked-out with a kanamycin resistance cassette (Table 1) was used as a donor and transducing particles were produced by infecting this strain with phage φR1-RT using the soft agar overlay method. Following overnight incubation, phages were eluted from the soft agar using SM buffer. The transducing lysates were centrifuged and treated with chloroform to prevent contamination with the donor strain. The titer of the obtained transducing stock was $6.62 \times 10^9$ PFU/mL. The *Y. enterocolitica* strain 6471/76 was used as the recipient. For the transduction of the recipient strain, 10 one mL aliquots of log-phase bacterial cultures containing $10^9$ CFU/mL cells were mixed with 100 μl of $10^{-2}$ diluted transducing phage stock resulting at MOI of 0.006. After 15 min the bacterial cells were centrifuged and washed with LB and centrifuging them down removed the unabsorbed phages. The final cell pellet was resuspended in 100 μl LB, and the cells were allowed to recover during 30 min incubation with vigorous shaking. Subsequently, the bacterial cultures were plated on urea agar plates (0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% KH₂PO₄, 0.00012% phenol red, 2% urea, 1.5% agar) supplemented with kanamycin (200 μg/mL) and incubated for 48h. The kanamycin resistant and urease negative colonies were considered as transduced. The transducing stock was also plated to ensure no contamination with donor strain.

Growth curves. Overnight bacterial cultures were diluted 1:10 in fresh LB medium and 180 μl aliquots were distributed into honeycomb plate wells (Growth Curves Ab Ltd) where they were mixed with 20 μl aliquots of different φR1-RT phage stock
dilutions \((10^0 - 10^{-4})\). A negative control was obtained by mixing 20 µl of phage stock with 180 µl of medium, whereas positive control consisted of 180 µl of bacterial culture and 20 µl of medium. The growth experiments were carried out at 4°C, 10°C, 16°C, 22°C, and 37°C using the Bioscreen C incubator (Growth Curves Ab Ltd) with continuous shaking. The \(OD_{600}\) of the cultures was measured at selected time intervals. The averages were calculated from values obtained for the bacteria grown in 5 parallel wells.

**Phage resistant mutant isolation.** A culture of wild type *Y. enterocolitica* strain 6471/76 was used to flood LB agar plates (LA). After the excess fluid was removed the plates were allowed to dry before two 100 µl aliquots of the \(\phi R1-RT\) stock were pipetted on the lawn of cells. The plates were incubated at 22°C and inspected daily for phage resistant colonies growing within the lysis zones. After three days several colonies appeared and among them three confirmed phage resistant derivatives were isolated. The strains were named YeO3-\(\phi R1-RT\)-R2, YeO3-\(\phi R1-RT\)-R7, and YeO3-\(\phi R1-RT\)-R9.

**CatMu-library screening.** The *CatMu*-transposon insertion library in *Y. enterocolitica* strain YeO3-R1 has been described elsewhere (26, 27). In the present work, a library representing 16,000 independent insertion mutants was screened. The library was grown in LA supplemented with 100 µg/ml chloramphenicol (LA-Clm) until \(OD_{600} = \sim 0.5\). Phage \(\phi R1-RT\) was added to 1 mL of the library culture at MOI \(\sim 10\), fresh LB added to 5 mL and the culture was incubated at 22°C for 2h during which time all phage-sensitive bacteria were expected to be infected and lysed. The surviving bacteria were pelleted by centrifugation, washed twice with 1 mL LB to
remove remaining phages and after resuspending into 100 μl of LB plated on four LA-Clm plates that were incubated at 22°C. The ClmR colonies were re-streaked on LA-Clm plates for further study.

**Arbitrary PCR.** Detailed description of the method is presented in Supplementary information Materials and Methods.

**Cloning, expression and purification of the phage long tail fiber host receptor binding protein.** The phage TG1 distal long tail fiber (LTF) protein Gp37 was co-expressed with phage encoded chaperones Gp57A and Gp38 to synthesize the native form of the putative receptor binding protein (RBP) as described previously for the LTF of phage T4 (28). The Gp37 encoding gene was first cloned into the multiple cloning site (MCS) 1 of pCDF Duet-1 (conferring streptomycin resistance), producing pCDF Duet-1 Gp37. Then, the Gp38 encoding gene was cloned into the MCS 2 of pCDF Duet-1 Gp37, yielding pCDF Duet-1 Gp37-Gp38. Plasmid pET21a(+) conferring ampicillin resistance was used to clone the chaperone Gp57A encoding gene yielding plasmid pET21a(+) Gp57A. The plasmid constructs carry under the control of promoter T7, high level inducible gene expression with a His6 fusion tag at the N-terminus for purification by chelating affinity chromatography (*Fig. S1*). The genes encoding Gp38 and Gp57A however, were expressed without a purification tag. PCR, restriction analysis, and DNA sequencing were used to verify the structure of the plasmids. For expression, *E. coli* BL21 Star™ (DE3) PLYsS cells (Invitrogen) were transformed with pCDF-Duet-1 Gp37 or pCDF-Duet-1 Gp37-Gp38 and the same plasmids were also co-transformed with pET21a(+) Gp57A. Plasmid bearing *E. coli* were grown aerobically at 37°C to an OD_{600} = ~0.6 with shaking at 200 rpm in
250 mL of 2xYT media (16 g/L tryptone, 10 g/L yeast extract, 5.0 g/L NaCl, 0.22 µm filter sterilized, pH 6.5-7.5) supplemented with 50 µg/mL of ampicillin and or 50 µg/mL streptomycin as required. Protein expression was induced by the addition of 1 mM isopropyl-d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) incubating for 24h at 30°C with shaking at 200 rpm. Cells were harvested by centrifugation at 10,000 g for 15 min at 4°C and the pellets were resuspended in 25 mL of buffer A (50 mM sodium phosphate, 300 mM NaCl, 10mM imidazole, pH 8.0) supplemented with a protease inhibitor cocktail (Roche). Cells were disrupted by 10 rounds of 15 s of sonication using a Virsonic Digital 475 ultrasonicator (VirTis, NY, USA) alternating with incubation on ice. Insoluble debris was removed by centrifugation at 18,000 g for 30 min at 4°C and the soluble fraction was filtered through a 0.22 µm pore size filter (EMD Millipore, USA). The protein was purified by immobilized metal ion affinity chromatography using a nickel-nitritotriacetic acid (Ni-NTA) agarose column (Novex, Invitrogen) according to the manufacturer’s protocol. Captured proteins were eluted from the column using buffer B (50 mM sodium phosphate, 300 mM NaCl, 500mM imidazole, pH 8.0) and concentrated using Amicon-Pro centrifuge filters (Millipore) with a 10,000 Da molecular mass exclusion limit incorporating three washes with 10 mM Tris–HCl of pH 8.5. Protein concentration was estimated by measuring sample absorbance at 280 and 260 nm using a Nanodrop 2000 UV-vis Spectrophotometer (Thermo Scientific, USA) and Qubit® Protein Assay Kit using a Qubit® 1.0 fluorometer (Life Technologies) as per the manufacturer instructions. Protein analysis was performed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29) using Mini-Protean®TGX Stain-Free Precast Gels (Bio-Rad Laboratories, USA) and Coomassie blue staining. Precision Plus Protein™ Unstained Standard (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used as a
size marker for the molecular analysis of proteins. Analysis of protein bands and molecular weight (MW) estimates was performed using a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Quantity One® software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Accurate MW determinations and Peptide mass fingerprinting analysis was performed via mass spectrometry (MS) at the Mass Spectrometry Facility, Advanced Analysis Centre of the University of Guelph (Ontario, Canada).

**Cell decoration with bacteriophage host recognition binding proteins.** Confocal laser immunofluorescent microscopy was used to visualize the binding of the phage TG1 LTF protein Gp37 to *Y. enterocolitica* following methodology described by others (30). *Yersinia* strains grown in TSB at 25°C or 37°C for 24 hours were resuspended in wash buffer (50 mM Tris-HCl, pH 7.5) and 10 μl were spotted onto clean glass slides. After air-drying, the cells were fixed in a solution of 5 % gluteraldehyde for 10 min. and blocked with blocking buffer (5% BSA in 50 mM Tris-HCl buffer, pH 7.5) for 10 min. The slides were then incubated for 1 hour in a solution containing of 10 μg/mL of phage TG1 Gp37 (prepared in blocking buffer) followed by washing three times for 5 minutes in wash buffer. The slides were then incubated for 1 hour in anti-His\textsubscript{6} tag (HIS.H8) mouse monoclonal antibody solution prepared in blocking buffer (1:1000 dilution) and washed three times for 5 minutes with wash buffer. In a dark room, the slides were then incubated for 1 hour in goat anti-mouse IgG DyLight 488 polyclonal antibody solution (1:500) prepared in blocking buffer and washed three times for 5 minutes with wash buffer. The slides were air dried prior to analysis. Cells were imaged using an upright Leica DM 6000B confocal laser microscope connected to a Leica TCS SP5 system. Images were
collected digitally using Leica LAS AF Imaging Software and processed using ImageJ (31). To verify the specificity of the fluorescent signal, control samples were immunolabelled as above, with the omission of incubation with the primary antibody. All antibodies were acquired from Pierce Scientific, USA.

**Genome sequences.** The complete genome sequences of *Yersinia* phage vB_YenM_TG1 and vB_YenM_ϕR1-RT were deposited in the NCBI nucleotide database (GenBank) under the accession numbers KP202158 and HE956709, respectively. The RNA sequence data has been deposited to Gene Expression Omnibus (Acc. no GSE66516).

**RESULTS**

**Phage Morphology.** Phages ϕR1-RT and TG1 were negatively stained and examined by TEM. Both phages exhibit a prolate head with apparent icosahedral symmetry and a tubular contractile and rigid tail showing transverse striations (**Fig. 1**). The average dimension for the ϕR1-RT head is 82 ± 4 nm short edge-to-edge, 101 ± 5 nm vertex-to-vertex and the tail including the baseplate is on average 130 ± 7 nm long. The average dimension for the TG1 head is 91 ± 2 nm short edge-to-edge, 115 ± 6 nm vertex-to-vertex and the tail including the baseplate is on average 129 ± 1 nm long. Collectively, these morphological features indicate that these phages belong to the *Myoviridae* family.

**Host specificity.** The host range of phages TG1 and ϕR1-RT were determined by testing their lytic activity on 160 and 109 strains, respectively, belonging to thirteen *Yersinia* species, revealing virulence for *Y. enterocolitica* strains of serotypes O:1,
14 O:2, O:3, O:5, O:6, O:5,27, O:7,8, O:9 and some strains of serotype O:6,30 and O:6,31 while strains from other Y. enterocolitica serotypes and species within the genus Yersinia were resistant to phage infection (Table 2). TG1 and φR1-RT lysed their host when grown at 25°C but not at 37°C. Additionally, TG1 was unable to infect strains belonging to other 20 other genera (Table S3) demonstrating the phages’ host range is restricted to Y. enterocolitica.

**General features of the phage genomes.** The genome of phage φR1-RT is 168,809 bp long with a GC content of 34.5%. The genome encodes 262 ORFs, of which 217 genes are encoded on the reverse strand (as displayed on the genetic map) and 45 genes on the forward strand (Fig. S2), with sizes ranging from 117 bp (product of 38 amino acids) to 3,738 bp (product of 1245 amino acids). The genome of TG1 is smaller than that of φR1-RT at 162,101 bp in length but with a similarly low GC content of 34.6%. TG1 also encodes 262 ORFs of which 223 genes are transcribed on the reverse strand (as displayed on the genetic map) and thirty-nine genes on the forward strand (Fig. S3), with sizes ranging from 114 bp (product of 37 amino acids) to 3,099 bp (product of 1032 amino acids). The GC content of these phages is significantly lower than that associated with the host with a GC content ranging from 47.1 ± 0.2 (32) to 48.5 ± 1.5 % (33). The genomes encode additionally four identical tRNA genes (GlyGGA, TrpTGG, ArgAGA, MetATG) identified using tRNAscan (34) and ARAGORN (35). Constitutively low GC phage genomes are often supplemented with tRNA genes that, once expressed, enhance translation efficiency when infecting high GC content hosts (36). At the DNA level the TG1 genome shows 98% identity with a query coverage of 93%, for an overall DNA sequence identity of 91% with φR1-RT. All ORFs were screened using the BLASTP and PSI-BLAST algorithms (37, 38).
Based on protein homology, putative functions could be assigned to 121 (46%) gene products of phage TG1 and 115 gene products (44%) of phage ϕR1-RT. Most of the identified homologs are conserved among T4-like phages and are either structural, or involved in DNA replication, recombination, repair, or packaging. Thus, the phage T4 gene nomenclature was used to name these genes (Table S4).

**DNA replication, recombination, and repair.** Numerous genes were identified within the phage ϕR1-RT and TG1 genomes that play a direct role in DNA replication, recombination, and repair. Among the genes directly involved in DNA replication are a DNA polymerase, a DNA ligase (Gp30), and three proteins with helicase activity. The closest homologs to the phage TG1 and ϕR1-RT polymerases are found in *Edwardsiella* phage PEi20 [BAQ22701.1] and *Enterobacteria* phage RB69 [NP_861746.1]; all members of the *Myoviridae*. Among the helicases, Gp41 (or Dda) and UvsW homologs are involved in the reorganization of stalled DNA replication forks (39). Other putative proteins identified include homologs to the DNA polymerase sliding clamp loader complex Gp44/Gp62, sliding clamp accessory protein Gp45, single-stranded DNA binding protein Gp32, DNA helicase loader Gp59, and Gp61. In phage T4, the latter is a primase that interacts with helicase Gp41 to form a helicase-primase complex (or primosome). The primosome together with the DNA helicase loader Gp59, unwinds the DNA template and primes DNA synthesis on the discontinuous strand. Among the proteins involved in recombination are type II topoisomerases Gp60 and Gp52, the recombination-related endonuclease pair Gp46/Gp47, the Rec-A like recombination protein UvsX, and a single stranded DNA binding protein, UvsY (40). Lastly, among the proteins involved in repair, a DenV homolog and several RNA ligases were identified. DenV is an N-glycosylase.
UV repair enzyme that excises pyrimidine dimers; the major UV-lesions of DNA, while RNA ligases seal breaks in RNA and may also counteract host defense of cleavage of specific tRNA molecules (41).

**Nucleotide metabolism.** Class I ribonucleotide reductases are responsible for the inter-conversion of ribo- to deoxyribonucleotides and are represented by NrdA-B or NrdE-F which require oxygen for activity, class II containing NrdJ, and the oxygen sensitive class III represented by NrdG-H (42). In TG1 and φR1-RT, genes coding for the aerobic ribonucleotide reductase complex subunits NrdA, NrdB, and NrdH were identified. Additionally, NrdC genes were also located. Other genes identified that are involved in nucleotide metabolism include: thymidylate synthase (Td), thymidine kinase (Tk), dNMP kinase, dCMP deaminase (Cd), dihydrofolate reductase (Fdr), dCTPase-dUTPase, and the exo-deoxyribonuclease *DexA* and endo-deoxyribonuclease *DenA*. A combination of at least some of these genes is required to supplement the intracellular pool of nucleotides for phage DNA and RNA synthesis (41).

**Transcription.** Based on the genome maps presented (Fig. S2 and S3), phage TG1 and φR1-RT present a similar gene arrangement. A search for promoters based on sequence similarity to the host consensus s70 promoter TTGACA(N15-18)TATAAT with a 2 bp mismatch, identified 22 probable host promoters in the phage TG1 genome and 24 probable host promoters in the φR1-RT genome which probably function in early transcription (Tables S5 and S6). Additionally, 15 of the putative host promoters are located in the same relative genomic positions within each phage genome. The genomic layout however, makes it clear that there must be additional
promoters functioning to direct the transition from host to viral metabolism. A search for phage-specific promoters using PHIRE (43) and by analysis of sequences of 100 bp in length upstream of each ORF and submitting them to MEME (44), did not yield additional promoters that could be annotated with confidence. A search for putative rho-independent transcription terminators using ARNold (45, 46) yielded 21 putative terminators in the phage TG1 genome (Table S7) and 24 in the phage ϕR1-RT genome (Table S8). Nevertheless, the presence phage T4 homologs involved in the transcription of late genes: RegA, Gp33, and the sigma factor for late transcription Gp55, suggest that the mechanism for controlling late transcription is similarly complex (41). Likewise, the presence of repressor and translational regulatory protein homologs involved in middle and late transcription including: RegB, DsbA, Alc, MotA, and AsiA, lend further support to this suggestion.

Morphogenesis. The putative structural proteins of TG1 and ϕR1-RT are homologous to existing phage proteins of the T4 supergroup of viruses (Table S4). Among the putative phage structural genes, the phage head is likely composed of the major capsid protein Gp23 and the phage capsid vertex protein Gp24. The prohead precursor and scaffolding proteins Gp68, and Gp67 as well as internal head proteins ipIII and ipII were also identified. Lastly the head portal vertex protein Gp20 that is connected to the neck and through which DNA enters during packaging and exits during infection was also identified. The whiskers and neck are composed of fibrin (wac) and the head completion proteins Gp13 and Gp14. The tail proteins include the tail sheath terminator Gp3, the tail completion protein Gp15, the tail sheath subunit Gp18, and the tail tube subunit Gp19. Proteins that form the baseplate wedge subunits and tail pins that then go on to associate with the central hub to form the viral
baseplate include: Gp5, Gp6, Gp7, Gp8, Gp9, Gp10, Gp11, Gp25, Gp27, Gp28, and Gp53. Among these, Gp5 (ORF150) contains a predicted bacteriophage T4-like lysozyme domain (cd00735) or Phage lysozyme domain (pfam00959), which aids penetration through the peptidoglycan layer during the initial infection process. In phage T4, Gp8 and Gp9 connect the long tail fibers of the virus to the baseplate and trigger tail contraction after viral attachment to a host cell, while Gp11 connects the short tail fiber protein Gp12 (ORF159) to the baseplate (47). The baseplate wedge subunit Gp25, forms a structural component of the outer wedge of the baseplate that has lysozyme activity, evident by the presence of conserved Gene 25-like lysozyme domain (pfam04965). Based on homology and gene synteny the proteins forming the long tail fibers in TG1 and φR1-RT are composed of the tail fiber proximal subunit Gp34, the tail fiber connector or hinge protein Gp35, the proximal tail fiber protein Gp36, and the distal tail fiber protein Gp37 (47). A variety of chaperones or assembly catalysts involved in morphogenesis were also discovered. Head formation chaperones include the capsid vertex assembly chaperone, the prohead assembly proteins Gp21 and Gp22, as well as the head assembly chaperone protein Gp31. Chaperones involved in tail formation include the baseplate hub assembly proteins Gp26 and Gp51. Chaperones for tail fiber assembly include gp57A, gp57B, and Gp38.

**Host cell recognition elements.** In phage T4, phage tail associated receptor-binding proteins (RBPs) Gp37 and Gp12 are necessary for host cell recognition, attachment, and initiation of infection. In the phage TG1 and φR1-RT genomes ORF250 codes for a putative RBP protein of 609 amino acid residues and 503 amino acid residues in length, respectively, sharing 60% overall sequence identity. These proteins also share
40% sequence identity to the distal long tail fiber RBP of *Cronobacter* phage vB_CsaM_GAP161 [YP_006986537.1] and are homologs to the long tail fiber RBP Gp37 of phage T4 [AJC64544.1]. An alignment of these two proteins reveals a high degree of conservation at the N-terminus associated with the proximal tail fiber, as well as at the C-terminus associated with host recognition (Fig. S4). More specifically, the C-terminal 63 amino acids present a 95% sequence identity.

Similarly, in the phage TG1 and φR1-RT genomes, *ORF159* codes for the short tail fiber (STF) protein Gp12, both of 446 amino acid residues in length and which are almost identical to each other, sharing 95% overall sequence identity (Fig. S5). These proteins are homologous to the STF protein Gp12 of phage T4 [NP_049770.1].

**DNA Packaging.** In phage TG1, *ORF164* and *ORF165/ORF167* genes code for the small (TerS) and large (TerL) DNA packaging subunits respectively of a phage terminase protein complex (or holoterminalase) that initiates, drives, and terminates translocation of phage DNA into proheads (48). The homologous genes in phage φR1-RT are represented by *ORF165* (TerS) and *ORF166/ORF168* (TerL). Usually, *terS* and *terL* are arranged side by side but in phage TG1 and φR1-RT two ORFs homologous with *terL* are found. *ORF165* in TG1 and *ORF166* in φR1-RT show sequence similarity to the N-terminus of the phage T4 TerL. Likewise *ORF167* in TG1 and *ORF168* in φR1-RT show sequence similarity to the C-terminus of phage T4 TertL. BLASTX analysis (37, 38) reveals the *terL* gene in both phages is interrupted by a transposase (PHA02552). Additionally, during packaging, the DNA ends are also protected against host RecBCD nuclease action by Gp2, the DNA end protector protein (49); identified in phage TG1 and in φR1-RT as the product of *ORF146*. 
**Homing Endonucleases.** Homing endonuclease genes (HEGs) are not genuine phage DNA, but rather belong to intron associated selfish DNA elements (50) and are commonly found interspersed throughout *Myoviridae* genomes (41). Among the HEGs identified in phage TG1, *ORF148* and *ORF232* exhibit similarity to shortened helix-turn-helix (HN-H) endonucleases, and *ORF9*, *ORF43*, and *ORF66* to GIY-YIG group I intron endonucleases. BLASTX analysis (37, 38) reveals Gp47 (recombination-related endonuclease II) is divided by *ORF66* which contains the HEG. Likewise, the gene coding for UvsX is intersected by *ORF43*, which contains the HEG. *ORF232* also divides the *NrdA* gene. In phage ϕR1-RT five HEGs are also found throughout the genome of which only *ORF148* is homologous to the helix-turn-helix (HN-H) endonuclease that is also located in phage TG1 between Gp4 and Gp53. *ORF20*, *ORF51*, *ORF163*, and *ORF234* exhibit similarity to GIY-YIG group I intron endonucleases, none of which interrupt or intersect other ϕR1-RT genes.

**Lysis.** The final stage of the phage lytic cycle involves the degradation of the bacterial cell wall and release of progeny phages induced by the effect a pore producing protein, the holin, and a peptidoglycan degrading enzyme, the endolysin (51). In TG1, *ORF127* and *ORF122* in ϕR1-RT each encode an obvious endolysin containing a bacteriophage T4-like lysozyme protein domain (pfam00959) and phage-related muramidase (COG3772). Access of the endolysin to the cell wall occurs through the presence of the holin. Holins are small phage encoded proteins characterized by the presence of TMDs, accumulating in the cytoplasmic membrane during infection until suddenly at a specific time, trigger to form lethal lesions resulting in destruction of the cell wall (51, 52). A search for the TG1 and ϕR1-RT holins revealed the putative product of their respective *ORF253* gene contains a predicted t-holin domain.
(pfam11031) with 70% identity to the phage holin of *Enterobacteria* phage CC31 [YP_004010117.1]. The protein sequences are predicted to contain a single TMD spanning aa interval 30-49, as well as a large C-terminal periplasmic domain spanning the aa residue from position 50 to the end terminal amino acid at position 218; a characteristic bitopic topology found in the holin proteins of T4-like phages (53). Moreover, as in phage T4 the putative holin gene is separated from the endolysin gene. An additional search for *Rz/Rz1* genes coding for transmembrane spanins involved in the disruption of the outer membrane of the host was also conducted based on gene arrangement and membrane localization signals (54). The search revealed two candidate genes, ORF225 and ORF224 in phage TG1 and ORF227 and ORF226 in phage φR1-RT, homologous to phage T4 pseT.3 (*Rz*) and pseT.2 (*Rz1*), respectively. As in phage T4, the *Rz/Rz1* genes are adjacent to each other, arranged with overlapping stop and start codons, and additionally no part of the *Rz1* sequence is embedded within the *Rz* coding region (54). In TG1 and φR1-RT, *Rz* possesses a single amino-terminal TMD, and *Rz1* encodes an outer membrane lipoprotein based on the presence of a signal peptidase II (SPII) cleavage site located between amino acid residues 16 and 17 as predicted by LipoP (54, 55). Lastly, the presence of phage T4 homologs to *rI* lysis inhibition regulator membrane protein and *rIII* lysis inhibitor accessory protein in TG1 and φR1-RT suggest the potential for lysis inhibition (LIN) following superinfection (56, 57).

**Phylogeny of TG1.** It is interesting to note that very similar bacteriophages with an overall DNA sequence identity of 91% were isolated from such different locations and sources, as phages TG1 and φR1-RT were isolated in Canada from pig manure, and in Finland from raw sewage, respectively. Moreover, less than 34% overall DNA
similarity exists with their closest neighbours within the *Myoviridae* (Table S9). The relatedness of these two phages was further explored using progressiveMauve (Fig. 2) (58, 59); CoreGenes (60, 61) which the Bacterial and Archaeal Virus Subcommittee of the International Committee on Taxonomy of Viruses (ICTV) has extensively used to compare the proteomes of viruses; and by phylogenetic analysis of their whole genome sequences (Fig. S6) and their large terminase subunit protein sequences (Fig. 3). It is evident from phylogenetic analyses that TG1 and ϕR1-RT form a distinct taxonomic clade among their closest neighbours. Based on these observations and using a 95% DNA sequence identity as the criterion for demarcation for a species, a new genus named *Tg1virus* with phages TG1 and ϕR1-RT as member species was proposed to the ICTV (approved in 2016 and pending ratification).

**Growth curves.** In order to study the efficiency of phage infection at different temperatures bacterial growth after phage infection with ϕR1-RT was measured. Host bacterial strain was grown at selected temperatures with addition of different phage stock dilutions. Bacterial growth was followed for 3 d at 4°C, 2 d at 10°C and 16°C and 1 d at 22°C and 37°C. Lysis of the bacterial cultures was observed at 4°C, 10°C, 16°C and 22°C, whereas at 37°C the bacteria were not significantly affected even with the highest initial phage concentrations (Fig. 4, panels A). The onset time of lysis depended on the temperature and initial phage titer. At 4°C the bacterial culture started to lyse after 56-60 h, at 10°C already at 16 h with highest phage titer and at 24-28 h with the lowest phage titer. The corresponding times for 16°C and 22°C were 6 and 12 h. While the lysis at 10°C and 16°C was complete, at 22°C strong regrowth after the initial lysis took place. At 4°C the 3 d incubation time was not long enough to follow the lysis to completion. Under all tested conditions negative (medium only)
controls showed no increase in the absorbance, whereas the positive (bacteria only) controls presented the normal bacterial growth pattern.

Transduction. To study the transducing potential of ϕR1-RT, transduction of the KmR and urease-negative phenotype of strain YeO3-hfq::Km to the KmS and urease-positive wild type strain 64741/76 was assayed. Repression of urease activity is one of the phenotypes of the hfq mutant (62) and could be used to confirm the transduction of the hfq::Km allele. The transduction assays were performed in 10 parallel tubes using a MOI of 0.006. A total of 6.6×10^8 PFU from the transducing lysate resulted in a total of 3 KmR urease-negative colonies that were confirmed by PCR. From this the calculated transduction frequency in the experiment was 4.5×10^-7 transductants per PFU.

Identification of the phage receptors – pseudolysogeny. As the LPS and protein profiles of the phage resistant mutants YeO3-ϕR1-RT-2, YeO3-ϕR1-RT-7, and YeO3-ϕR1-RT-9 did not differ from those of the wild type bacteria (data not shown) the genomic DNA of the mutant and the wild type strains were sequenced. The de novo assembly results showed that the total scaffold sizes of the assembled genomes of the three mutants were ~165-173 kb larger than that of the wild type parental strain (Table S10). This suggested that the mutants carried extra DNA and the size matched very close to the size of phage ϕR1-RT genome (168,809 bp). This immediately raised the possibility that the phage had lysogenized the host and would reside as a prophage. In all three draft genomes the phage genome sequence was indeed identified and in all it formed the scaffold 4.1 with almost identical sizes (Table S10). Significantly, in all three cases the scaffold sequences were 100% identical to phage
ϕR1-RT sequence without any flanking host sequences, suggesting that the phage genome resided in these bacteria as an autonomous replicating unit in a state known as pseudolysogeny. Such state has been described for T4-like phages (63).

Identification of the phage receptors – transposon insertion library screening. As selection of spontaneous phage resistant mutants seemed to favor pseudolysogeny we decided to use a different approach. A CatMu-transposon library of strain YeO3-R1 (26) was exposed the ϕR1-RT for 2 hr and the surviving phage-resistant mutants were grown on LA-Clm plates. The recovered colonies were tested to be true ϕR1-RT resistant mutants. In order to exclude pseudolysogens, the clones were screened with ϕR1-RT specific PCR, and negative ones were further analysed by CatMu-specific arbitrary PCR to identify the CatMu insertion site (26). For four of the candidates the transposon insertion site was identified as gene Y11_04441 of the Y. enterocolitica O:3 strain Y11 genome (NC_017564.1). In strain Y11 genome the gene was annotated to encode for the outer membrane porin OmpC, however, in all other Y. enterocolitica genomic sequences as OmpF, therefore we opted to use OmpF. To confirm that OmpF is the ϕR1-RT receptor, one of the mutants YeO3-R1-Cat17 was complemented with the wild type ompF gene either in trans with plasmid pTM100_OmpF or in cis by suicide plasmid pSW25T_OmpF. Both of these approaches resulted in regaining the phage sensitivity thus confirming that OmpF serves as ϕR1-RT receptor.

The LPS inner core heptose region functions as a receptor. Adsorption experiments were carried out to study the ability of ϕR1-RT and TG1 to interact with Y. enterocolitica O:3 derivatives differing mainly in their LPS composition (Fig. 5). A
short 5 min adsorption time was used as it produced highest resolution between the
strains. A general observation was that TG1 adsorbed faster than φR1-RT. Both
phages showed negligible adsorption to YeO3-c-R1-Cat17, the ompF mutant strain
and adsorbed well to both ompF-complemented strains. Both phages showed reduced
but clear adsorption to the pseudolysogen, indicating changes in abundance or
exposure of the phage receptor(s). Finally, the adsorption to the inner core mutants
decreased with the truncation of the core oligosaccharide suggesting that the inner
core heptoses are part of the secondary receptor (Fig. 5).

Temperature-dependence of ompF expression. We then wondered whether the
temperature-dependent sensitivity of Y. enterocolitica O:3 could be due to ompF
regulation. The expression of ompF under different growth temperatures was analysed
from RNA-sequencing and quantitative proteomics (LC-MS/MS) data. The
transcriptomic data showed an inverse correlation between the expression of ompF
and the temperature of incubation (Fig. 4, panels B). Consistently, the quantitative
proteomics demonstrated much higher abundance of the OmpF protein in the 22°C
sample when compared to the 37°C sample, where the abundance barely exceeded the
threshold of identification (Fig. 4, panels B).

In vitro expression of the long tail fiber protein Gp37 of phage TG1. In this study,
co-expression with the phage encoded chaperones Gp38 (required for
oligomerization) and Gp57A, which is also thought to participate in assembly (64, 65)
was utilized in an attempt to synthesize the native form of distal long tail fiber protein
of phage TG1 as previously described for the production of Gp37 from phage T4 (28).
SDS-PAGE demonstrated that an oligomer of approximately 210 kDa was obtained
when Gp37 was co-expressed with Gp38 in a bicistronic plasmid (pCDF-Duet-1 Gp37-Gp38) or when this same plasmid was co-expressed with Gp57A (Fig. S7, lanes 3 and 5). Under reduced conditions Gp37 appears as a monomer of approximately 70 kDa in size (Fig. S7, lanes 4 and 6). This estimate is close to the predicted molecular mass of the recombinant phage TG1 Gp37 determined via MS at approximately 68.050 kDa. Peptide mass fingerprinting confirmed the identity of the protein (Fig. S8). Based on the protein expression results obtained, it appears that in phage TG1 only the Gp38 chaperone is essential and the general chaperone Gp57A is not required for *in-vitro* protein folding of Gp37 as has been reported for phage T4 (28). The formation of higher molecular weight oligomers of phage TG1 Gp37 is consistent with previous reports that describe RBPs of phages present as homotrimers in solution migrating in the SDS-PAGE with a mobility that corresponds to that of oligomeric forms (28, 66–68).

**Confirmation of host binding specificity.** Host binding specificity was then tested through immunolabeling of bacterial cells with phage TG1 LTF protein Gp37 followed by detection with anti-His$_6$ antibodies and DyLight 488 conjugated secondary antibodies. Consistent with the temperature dependent infection of phage TG1, the application of the LTF protein Gp37 to *Y. enterocolitica* cells showed decoration of the surface of *Y. enterocolitica* O:3, O:5,27, and O:9 cells when these were grown at 25°C but not at 37°C (Fig. 6). Notably, binding was more apparent near the apex of the cells which is also reported to occur in other phages such as λ, T4, T7, KVP40 and φA1122, preferentially infecting cells at the poles (69).

**DISCUSSION**
Among bacteriophages, the C-terminus of RBPs involved in ligand interactions usually exhibits considerable sequence divergence, thus providing diversity in host specificity. In the case of ϕR1-RT and TG1, the high sequence identity at the C-terminus of their long tail fiber and short tail fiber proteins may account for the striking similarity in virulence of these two phages for *Y. enterocolitica*. Notably, phage ϕR1-RT shows virulence to strains of the same serotypes as phage TG1. Based on adsorption experiments, the outer membrane protein OmpF and the inner core heptosyl residues of the LPS serve as phage receptors for phage TG1 and ϕR1-RT. It is worth noting however, that the *E. coli* strain DH10B/pTM100_OmpF was not sensitive to ϕR1-RT. We reasoned that this could be due to poor expression of *Y. enterocolitica* OmpF in *E. coli* or more likely that the LPS inner core, known to be used by T4-like phages as the secondary receptor (76, 77) was not compatible. The inner core structures of *E. coli* and *Y. enterocolitica* differ substantially potentially explaining this result.

Multiple lines of evidence suggest OmpF is the primary host range determinant for these two bacteriophages. First, a multiple alignment of OmpF amino acid sequences of *Y. enterocolitica* (from a BLASTP search of sequence databases using the O:3 OmpF sequence as query) suggest the restricted host range of these phages among *Y. enterocolitica* serotypes could be due to OmpF. The alignment provided a distribution of conserved amino acid residues and the presence of regions with high and low homologies, which coincide with eight transmembrane domains and eight “external” loops, respectively of the topology of the OmpF porin from *E. coli* (70, 71). The search and alignment of the sequences (Fig. S9) revealed that the OmpF sequences of the ϕR1-RT sensitive serotypes are 100% identical. The most dramatic differences
between the serotypes map to loop 4. In the alignment most close to the O:3 sequence is the serotype O:7,8,19 OmpF that is 96% identical to O:3 and may still be sensitive to φR1-RT; in it the loop 4 sequence differs least, while in others differences are bigger and also accumulate in other loops, mainly in loops 5, 6, and 7 (Fig. S9). The porin loops are plausible binding sites for bacteriophages as demonstrated by the interaction of *E. coli* OmpF and K20 phages which bind to the L5, L6, and L7 external loops (72–74). Thus it is likely that the loop 4 sequence is targeted by the φR1-RT or TG1 receptor binding proteins, however, experimental evidence is necessary to confirm this. Secondly, RNA-sequencing and quantitative proteomics data, the analysis of growth curves of *Y. enterocolitica* infected with φR1-RT at various temperatures (4°C to 37°C), as well as phage host range analysis results conducted at 25°C and 37°C clearly indicate that the failure of φR1-RT and TG1 to infect *Y. enterocolitica* O:3 at 37°C is due to the strong repression of the *ompF* gene. The temperature dependent expression of OmpF results also agree with a previous study, where two-dimensional gel electrophoresis of whole-cell proteins of *Y. enterocolitica* cultured at 25°C and 37°C suggested that OmpF is downregulated when the bacteria were cultured at 37°C (75). Consistent with this observation, the application of immunolabelled phage TG1 receptor binding protein Gp37 to *Y. enterocolitica* cells showed decoration of the surface of *Y. enterocolitica* O:3, O:5,27, and O:9 cells when these were cultured at 25°C but not at 37°C. The decoration of the cell surface agrees with a high level expression of this major outer membrane protein class depending on the bacterial species and the environmental conditions, which can reach about $10^4$–$10^6$ copies per cell (72). It is reasonable to suggest then that the phage TG1 distal long tail fiber protein Gp37 (and by extension, its homolog in φR1-RT) is specifically involved in binding to OmpF while presumably, the short tail fiber
protein Gp12 binds to the inner core of LPS, as is reported to occur in other T even
phages as a secondary receptor (76, 77).

The in vitro temperature dependent infection of these two highly related phages,
questions their potential use as biocontrol or therapeutic agents as has been suggested
for the temperate Yersinia phage PY100 (18, 78). On the other hand, it is not known
whether the ompF gene is expressed in vivo justifying further studies towards finding
that out. However, due to their marked specificity for the epidemiological relevant Y.
enterocolitica serotypes O:3, O:5,27, and O:9, these phages may prove useful for
diagnostic purposes. In addition, the successful synthesis of the long tail fiber of
phage TG1 opens up the possibility of its use as a probe as well as for the production
of suitable amounts of protein for X-ray crystallography to elucidate its atomic
structure or co-crystallization with its receptor OmpF to shed light on specific host
cell receptor-virus interactions.

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**Escherichia coli**

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**Bacteriophages**

TG1 | This study
φR1-RT | This study
Table 2. Lytic activities of phages TG1 and φR1-RT. The sensitivity was tested on 160 Yersinia species strains (Table S1) at 25°C.

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<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
<td>I [2], O:1b [2], O:3 [2]</td>
</tr>
<tr>
<td><strong>Y. rohdei</strong></td>
<td></td>
<td></td>
<td>UT[2]</td>
</tr>
<tr>
<td><strong>Y. ruckeri</strong></td>
<td></td>
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<td>NT[1], UT[5]</td>
</tr>
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</table>

\(^a\)The number of strains studied is given in brackets. Phage φR1-RT sensitivity was tested only with the 109 UH-source strains (Table S1).

\(^b\)NT, non-typeable and either cross-reacting or not agglutinating with Y. enterocolitica O:3, O:5, O:8 or O:9 antisera. UT, untyped.
FIGURE LEGENDS

Figure 1. Bacteriophage φR1-RT and TG1 morphology by electron microscopy. Panel A. φR1-RT virions at 39,440x magnification. The virion head and tail are indicated, as well as long tail fibers (LTF) and a baseplate with protruding tail pins (B). Scale bar, 100 nm. Panel B. A φR1-RT virion at 84,320x magnification. A baseplate with protruding tail pins (B), and a neck and collar with neck fibers (A) can be observed. Scale bar, 50 nm. Panel C. A φR1-RT virion at 108,800x magnification. Suggested long tail fibers (LTF) can be seen bent up towards the head along the tail sheat, as described for bacteriophage T4 (84). Scale bar, 50 nm. Panel D. Bacteriophage TG1 virion shown at 150,000x magnification. A neck and collar with neck fibers (A), a baseplate with protruding tail pins (B), and an extended long tail fiber (LTF) can be observed. Scale bar indicates size in nm.

Figure 2. ProgressiveMauve alignment of phage TG1 and φR1-RT. The genome of φR1-RT [HE956709] is indicated on the top and that of TG1 [KP202158] is shown in the bottom of the figure. The degree of sequence similarity between regions is given by a similarity plot within the coloured blocks with the height of the plot proportional to the average nucleotide identity. Below these are illustrated the phage genes as outlined boxes on the plus (above horizontal) and minus (below horizontal) strands.

Figure 3. Phylogenetic analysis of the large terminase subunit protein sequences of phages TG1, φR1-RT and related bacteriophages. The phylogenetic analysis was constructed using “one click” at phylogeny.fr using MUSCLE for multiple alignment and PhyML for phylogeny (85).

Figure 4. Phage φR1-RT does not propagate at 37°C. Panel A. The growth curves of Y. enterocolitica infected with φR1-RT. Bacteria were cultured with different concentrations of phage particles in LB at 4°C, 10°C, 16°C, 22°C, and 37°C. Each graph represents the average of five replicates. Note the different scales used for the X-axis at different temperatures. Panel B. Analysis of the ompF gene expression (left) and protein abundance (right) at different temperatures. The mean expression levels of the ompF gene were obtained from RNA-sequencing analysis. The production levels of the OmpF protein was obtained from normalized mean spectral values for the proteins detected by the LC-MS/MS analysis. Error bars represent the calculated standard deviation.

Figure 5. Phages φR1-RT and TG1 use OmpF and LPS inner core heptose region of Y. enterocolitica O:3 as receptors. Panel A. Adsorption experiments were performed with different LPS and ompF mutants, with the complemented strains, and with the pseudolysogen. All strains are OmpF positive with the exception of YeO3-c-R1-Cat17. The TG1 and φR1-RT adsorptions to the bacteria at 5 min are shown as residual PFU percentages. Error bars indicate standard deviations. The no bacteria control (LB) and strains used for adsorptions are indicated below the columns. The LPS chemotypes of the strains are indicated on top of the columns. Panel B. The schematic structures of the Y. enterocolitica O:3 LPS molecules of different chemotypes (86). Please note that Y. enterocolitica O:3 carries simultaneously the S and Ra type LPS molecules. This is indicated in panel A by a plus sign. O-ag, O-antigen or O polysaccharide; OC, outer core hexasaccharide; IC, inner core; LA, lipid A.

Figure 6. Confocal immunofluorescence microscopy images of Y. enterocolitica cells after incubation with LTF protein Gp37 derived from phage TG1. Gp37 decorates the cell surface of Y. enterocolitica strain K14 of serotype O:9 (a), Y. enterocolitica strain gc815-73 of serotype O:5,27 (c), and Y. enterocolitica strain 6471/76 of serotype O:3 (e) grown at 25°C, whereas Y. enterocolitica strain 8081 of serotype O:8 (g) does not show cell decoration with Gp37. Similar
images to that presented in g were observed when the same strains were grown at 37°C.
Differential interference contrast microscopy images of a, c, e and g, are shown in b, d, f and h,
respectively. Scale bar represent size in µm.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Plasmid constructs for chaperone-assisted expression of the distal long tail fiber
protein Gp37 of phage TG1. The location of the origins of replication, antibiotic resistance genes
(Sm R, streptomycin resistance; Amp R, ampicillin resistance), relevant promoters (T7 lac
promoter), Lac I repressor, Multiple cloning sites (yellow) and sequences coding phage Gp37
(red), and chaperones Gp38 and Gp57A (green) are presented.

Figure S2. Map of the phage ϕR1-RT genome [HE956709]. The genes are shown by different-
colored arrows. The arrow direction indicates the coding direction of the genes. The genes
encoding putative proteins with an assigned function are shown in black (see also Table S3). The
locations of tRNA encoding genes are shown in blue. Hypothetical proteins with an unknown
function are depicted in yellow. Homing endonuclease genes are shown in orange. Putative host
promoters are shown as pink triangles above the sequence, and putative rho independent
terminators are shown as green triangles below the sequence.

Figure S3. Map of the phage TG1 genome [KP202158]. The genes are shown by different-
colored arrows. The arrow direction indicates the coding direction of the genes. The genes
encoding putative proteins with an assigned function are shown in black (see also Table S2). The
locations of tRNA encoding genes are shown in blue. Hypothetical proteins with an unknown
function are depicted in yellow. Homing endonuclease genes are shown in orange. Putative host
promoters are shown as pink triangles above the sequence, and putative rho independent
terminators are shown as green triangles below the sequence.

Figure S4. Multiple sequence alignment of the long tail fiber (Gp37) sequences of phage TG1
and ϕR1-RT. Multiple sequence alignment was performed using Clustal W via Geneious R9
software version 9.0.2. (Biomatters Ltd). Positions which have a single, fully conserved aa
residue (100% similarity) are highlighted in black; aa present in 2 of the sequences are
highlighted in grey. The homologous phage T4 Gp37 sequence was included in the alignment or
comparison.

Figure S5. Multiple sequence alignment of the short tail fiber (Gp12) protein sequences of
phage TG1 and ϕR1-RT. Multiple sequence alignment was performed using Clustal W via Geneious R9
software version 9.0.2. (Biomatters Ltd). Positions which have a single, fully conserved aa
residue (100% similarity) are highlighted in black; aa present in 2 of the sequences are
highlighted in grey. The homologous phage T4 Gp12 sequence was included in the sequence
alignment for comparison.

Figure S6. Protein mass fingerprinting of phage TG1 Gp37. The amino acid sequence of phage
TG1 Gp37 is shown. Peptide fragments from the analysis of gel slices corresponding to the
reduced form of the protein and identified via protein mass fingerprinting are underlined and
shown in bold.

Figure S7. Whole genome phylogeny of phages TG1, ϕR1-RT and related bacteriophages. The
phylogenetic tree was generated using using “one click” at phylogeny.fr using MUSCLE for
multiple alignment and PhyML for phylogeny (85).
Figure S8. Expression of N-terminal His6 tagged phage TG1 Gp37. 4-15% Tris-HCl SDS-PAGE run at 4°C, 100V. Lane 1, pCDF-Duet-1 Gp37 (unheated sample); Lane 2, pCDF-Duet-1 Gp37 under reduced conditions (sample heated at 100°C for 10 min in the presence of SDS and β-mercaptoethanol); Lane 3, pCDF-Duet-1 Gp37-Gp38 (unheated sample); Lane 4, pCDF-Duet-1 Gp37-Gp38 reduced conditions; Lane 5, pCDF-Duet-1 Gp37-Gp38 co-expressed with pET21a(+) Gp57A (unheated sample); Lane 6 pCDF-Duet-1 Gp37-Gp38 co-expressed with pET21a(+) Gp57A reduced conditions; Lane 7, pCDF-Duet-1 Gp37 co-expressed with pET21a(+) Gp57A (unheated sample); Lane 8, pCDF-Duet-1 Ggp37 co-expressed with pET21a(+) Gp57A reduced conditions; Lane M, molecular weight markers.

Figure S9. CLUSTAL W multiple sequence alignment of the OmpF proteins of Y. enterocolitica and related species. The external loops, indicated by brown highlighting and box, were identified based on OmpF alignment of YeO3 OmpF with that of E. coli, shown at the bottom. The N-terminal signal-peptide is indicated by blue highlighting and box. Use the zoom-in option to see details of the alignment.

SUPPLEMENTARY TABLES

Table S1. List of Yersinia strains used in phage host range experiments
Table S2. Primers used in this work
Table S3. Bacterial strains used to examine the cross infectivity of phage TG1
Table S4. Annotations of bacteriophage TG1 and φR1-RT genes
Table S5. Putative Phage TG1 promoters
Table S6. Putative Phage φR1-RT promoters
Table S7. Predicted terminator sequences of phage TG1
Table S8. Predicted terminator sequences of phage φR1-RT
Table S9. TG1 genome BLASTN analysis
Table S10. Whole genome sequencing statistics after de novo assembly of φR1-RT resistant mutants.
Figure 1.
Figure 2.
Figure 3
Figure 4
Figure 5
Figure 6