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Broad-Host-Range *Yersinia* Phage PY100: Genome Sequence, Proteome Analysis of Virions, and DNA Packaging Strategy▽

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PY100 is a lytic bacteriophage with a broad host range within the genus *Yersinia*. The phage forms plaques on strains of the three human pathogenic species *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* at 37°C. PY100 was isolated from farm manure and intended to be used in phage therapy trials. PY100 has an icosahedral capsid containing double-stranded DNA and a contractile tail. The genome consists of 50,291 bp and is predicted to contain 93 open reading frames (ORFs). PY100 gene products were found to be homologous to the capsid proteins and proteins involved in DNA metabolism of the enterobacterial phage T1; PY100 tail proteins possess homologies to putative tail proteins of phage AaΦ23 of *Actinobacillus actinomycetemcomitans*. In a proteome analysis of virion particles, 15 proteins of the head and tail structures were identified by mass spectrometry. The putative gene product of ORF2 of PY100 shows significant homology to the gene *3* product (small terminase subunit) of *Salmonella* phage P22 that is involved in packaging of the concatemeric phage DNA. The packaging mechanism of PY100 was analyzed by hybridization and sequence analysis of DNA isolated from virion particles. Newly replicated PY100 DNA is cut initially at a *pac* recognition site, which is located in the coding region of ORF2.

Bacteriophages were discovered independently by F. Twort in 1915 and F. d'Herelle in 1917. In the first decades after their discovery, phages were used to combat bacterial infections, first in animals and later in humans (46, 47). The use of phages for the treatment of bacterial infections was abandoned in Western countries with the advent of antibiotics, largely since many of the results of early phage therapies had been ambiguous. In the 1940s in the United States scientists began using phages in basic genetic studies, and the findings and results of those experiments formed the basis of molecular biology (48).

In the genus *Yersinia*, phages have been used for typing, and phage sets have been worked out for typing *Yersinia enterocolitica* (6, 24). The genomes of two lytic yersiniophages have been determined; both phages show a close relationship to the *Escherichia coli* phages T3 and T7 and possess short noncontractile tails. Phage ΦYeO3-12 is specific for *Y. enterocolitica* serotype O3 (31), and phage ΦA1122 has been used for typing of *Y. pestis* (17). Their genome sizes are 37,555 and 39,600 bp, respectively. Recently, the lytic yersiniophage ΦR1-37 was described; this phage has a broader host range within *Y. enterocolitica* and possesses a contractile tail, however, the genome size of ΦR1-37 is estimated to be 270 kb (25). In our laboratory the temperate yersiniophage PY54 with a genome size of 46,339 bp is studied because of its replication modus as a linear prophage (20, 21). The host range of PY54 is restricted to *Y. enterocolitica* strains serotypes O5 and O5,27.

Reports on phage therapy experiments with yersiniophages are rare in the literature; however, one remarkable historic report is from the experiments of d'Herelle, who reported the successful treatment of four plague patients with lytic phages (13, 4). The renewed interest in phage therapy experiments due to the increase in antibiotic resistance of several pathogenic bacteria prompted us to search for *Yersinia* phages that lyse their hosts at 37°C. The aim of these experiments was to study the possibility to reduce or eradicate enteropathogenic *Y. enterocolitica* in infected pigs, which are the main reservoir for human food-borne infections (16). Applications of phages against bacterial pathogens in the food production chain are intended to reduce zoonotic bacteria in domestic animals or to use them as biocontrol agents for food preservation (18, 22).

We isolated the phage PY100 from the manure of a pig farm in Germany. PY100 was found by its ability to form large clear plaques on susceptible *Y. enterocolitica* strains at 37°C. We investigated, in an animal model for enteropathogenic *Y. enterocolitica*, whether PY100 or the yersiniophage ΦYeO3-12 (30) was able to inhibit colonization of the guts of mice by strains of *Y. enterocolitica* biotype 4, serotype O3 (43), which causes most of the human cases of yersiniosis in Europe.

Our studies of the PY100 biology revealed a very broad host range of the phage in the genus *Yersinia*, which included also the two other human pathogenic species *Y. pseudotuberculosis* and *Y. pestis*. In all cases, PY100 formed large plaques at 37°C on the bacterial lawns and may be of interest for treating infections with these pathogens. Especially, since *Y. pestis* is classified as a potential biowarfare or bioterror agent, phage

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therapy may be considered as an approach to counter such a threat (4, 17). We report here on the host range, burst size, genome sequence, proteomic characteristics, and packaging mechanism of this phage.

MATERIALS AND METHODS

Bacterial strains. *Yersinia* strains and other enterobacterial strains were obtained from the Robert Koch Institute collection (27, 44) and the Institute Pasteur, Paris, France. *Y. pestis* strains KIM (12) and EV76 (38) were tested in the biosafety level III laboratory of the Robert Koch Institute. Other investigated strains used were *Y. enterocolitica* 8081 (serotype O:8) (49), *Y. enterocolitica* 6471/76 (serotype O:3) (42), and *Y. pseudotuberculosis* YPIII (7).

Isolation of PY100, propagation, and plaque assay. Manure was centrifuged twice at 10,000 × *g*, and the supernatant was passed through a 0.45-μm-pore-size filter. Serial dilutions of the supernatant were made in SM buffer (5.8 g of NaCl per liter, 2.0 g of $MgSO_4 \cdot 7H_2O$ per liter, 50 mM Tris-HCl [pH 7.5]) (39). Portions (20 μl) of the dilutions were spotted onto a lawn of the indicator strain *Y. enterocolitica* 13169 (44). PY100 was purified by repeated single plaque isolation.

To determine the titer of PY100 preparations, 0.1-ml portions of the phage dilutions were mixed with 0.1 ml of the overnight culture of strain 13169, followed by incubation for 15 min. After the addition of 3 ml of 48°C warm soft agar medium (Luria-Bertani [LB] medium with 10 mM $CaCl_2$, 10 mM $MgSO_4$, and 0.6% agar), the mixture was poured on LB solid medium and incubated overnight at 37°C.

The host range of PY100 was determined by spotting 20 μl of phage suspensions containing approximately 10^8, 10^6, or 10^3 PFU ml^{-1} (determined on strain 13169) on lawns of test bacteria, followed by incubation overnight at 37°C. The overlays were prepared with 0.1 ml overnight cultures grown in LB broth that were mixed with 3 ml of LB soft agar (see above). Each strain was tested three times, except for *Y. pestis* strains that were tested only twice. One-step growth curves were carried out with mid-exponential-phase cultures of strain 13169 with 5×10^6 PFU of PY100. Samples were taken in intervals of 5 min.

Electron microscopy. Phages were isolated in CsCl step gradients as described earlier (45). Transmission electron microscopy was performed with a Philips 400 electron microscope. Negative staining of phage preparations were performed with 1% uranyl acetate or 1% phosphotungstic acid (pH 6.8) (44).

Cloning and nucleotide sequence determination. A random or "shotgun" library containing 1.4-kb DNA fragments was constructed using a previously described fast nebulization method (35). The vector pCR4Blunt-TOPO (Invitrogen) was used according to the manufacturer's recommendations. Plasmid DNA was prepared by using the QIAprep 96 Turbo BioRobot kit (Qiagen) on a BioRobot 8000 (Qiagen). Cycle sequencing reactions from plasmid inserts were performed by using an ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems) on a GeneAmp PCR System 9700 (Applied Biosystems). Sequence reactions were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequencing was continued until an eightfold coverage of sequence data was reached. Assembly of sequences was performed by using the SeqMan module of the Lasergene software (DNASTAR, Inc., Madison, WI). Persisting gaps were closed by primer walking on genomic DNA.

Open reading frame (ORF) prediction and initial analysis was performed with GLIMMER (HUSAR package at http://genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/). In addition, the Find ORF feature of SeqEdit and Coding Prediction-Borodovsky method of GeneQuest (DNASTAR) was used to visually scan the sequence for potential genes (cutoff, 90 bp). Putative translated proteins were scanned for homologues using BLASTP and Psi-BLAST (2, 3) at http://www.ncbi.nlm.nih.gov. A tRNA search was performed with tRNAscan (http://www.genetics.wustl.edu/eddy/tRNAscan-SE) (28). A terminator search was performed with the tool TERMINATOR search (HUSAR package). Further analyses were carried out with the software package of MacVector (version 7.0; Oxford Molecular Group).

Nucleotide sequence accession number. The nucleotide sequence of the PY100 genome was submitted to EMBL data library under accession number AM076770.

SDS-PAGE and mass spectrometric analysis of PY100 proteins. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system as described by Laemmli (26) was used. Samples were suspended in loading buffer (Bio-Rad, Munich, Germany), boiled for 10 min, and electrophoresed on a 12% (wt/vol) polyacrylamide gel at 20 mA at 8°C. Proteins were visualized by using Coomassie brilliant blue R-250 (Bio-Rad, Munich, Germany) staining. For further analysis the bands of interest were excised, and in-gel digestion was performed according to a standard procedure. For peptide sequence determination, liquid chromatography-mass spectrometry (LC-MS) measurements were performed using a Qstar XL hybrid mass spectrometer (Applied Biosystems, Foster City, CA) coupled to an Ultimate Nano-HPLC system (LC Packings, Amsterdam, The Netherlands) by using a nano-electrospray source. Tandem MS spectra were acquired during high-pressure liquid chromatography run using the data-dependent acquisition abilities of the Analyst software package (Applied Biosystems). Peptide sequences were assigned manually using Bioanalyst software (Applied Biosystems), and peptide sequences were aligned to the phage genome using MacVector (Accelrys, Cambridge, United Kingdom).

Analysis of *pac* fragments. Virion DNA was digested with BglII and run on an 0.8% agarose gel. The *pac* fragment was cut out and digested with DraI. The resulting DNA fragments were ligated with pLitmus38 which had been digested with EcoRV to have blunt ends. The ligation mixture was introduced into *E. coli* DH5α, and AmpR transformants were selected. Plasmid DNA was prepared and hybridized to a PCR probe (345 bp) generated with the primers PY100-5 (5′-CAAGTAGCGATGGTTCTATGAGTCC-3′) and PY100-6 (5′-CTTGCTTTGCGTCTTCCAGTG-3′). The probe was labeled with fluorescein, and hybridization was carried out according to standard procedures (39). Hybridizing plasmids were sequenced.

TABLE 1. Host range of PY100[a]

Organism	Source	Strain properties	No. of strains tested	No. of PY100-sensitive strains
Y. enterocolitica	RKI, IP	Serogroup O:3	10	9
Y. enterocolitica	RKI, IP	Serogroup O:5,27	5	5
Y. enterocolitica	RKI, IP	Serogroup O:9	9	8
Y. enterocolitica	RKI, IP	Serogroup O:8	6	4
Y. enterocolitica	RKI, IP	Biogroup 1A, various serogroups, NT	20	15
Y. pseudotuberculosis	RKI, IP	Serogroups I to VII	10	10
Y. pestis		KIM, EV76	2	2
Y. intermedia	RKI, IP	Various serogroups, NT	10	6
Y. kristensenii	RKI, IP	Various serogroups, NT	10	6
Y. frederiksenii	RKI, IP	Various serogroups, NT	10	4
Y. mollaretii	RKI, IP	Various serogroups, NT	10	3
Y. rohdei	RKI		1	0

[a] Tests were performed by spotting 20 μl of phage suspensions containing approximately 10^8, 10^6, or 10^3 PFU/ml on lawns of test bacteria (100 μl of overnight culture mixed with 3 ml of LB soft agar). Plaques were determined after overnight incubation at 37°C. Abbreviations: RKI, strain collection of the Robert Koch Institute; IP, strains from the Institute Pasteur, Paris, France. NT, strains not typeable with standard sera.

RESULTS AND DISCUSSION

Isolation and host range of PY100. PY100 was isolated from pig manure collected on a farm in Germany by single plaque purification on *Y. enterocolitica* 13169 (43). Since the phage formed clear large plaques at 37°C, it was chosen for further experiments. We found that the incubation temperature during the development of the bacterial lawn influenced the number of plaques significantly. Plaque titers of the same PY100 preparation were lower in bacterial lawns after incubation at 30°C or 20°C compared to 37°C, suggesting that phage infections are more efficient at 37°C.

To determine the host range of the phage a number of bacterial strains were tested (Table 1). The host range of the phage was very broad within the genus *Yersinia* and included strains from the three human pathogenic species *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. Among the susceptible *Yersinia* strains are *Y. enterocolitica* 8081 (serotype O:8), *Y. enterocolitica* 6471/76 (serotype O:3), *Y. pseudotuberculosis*

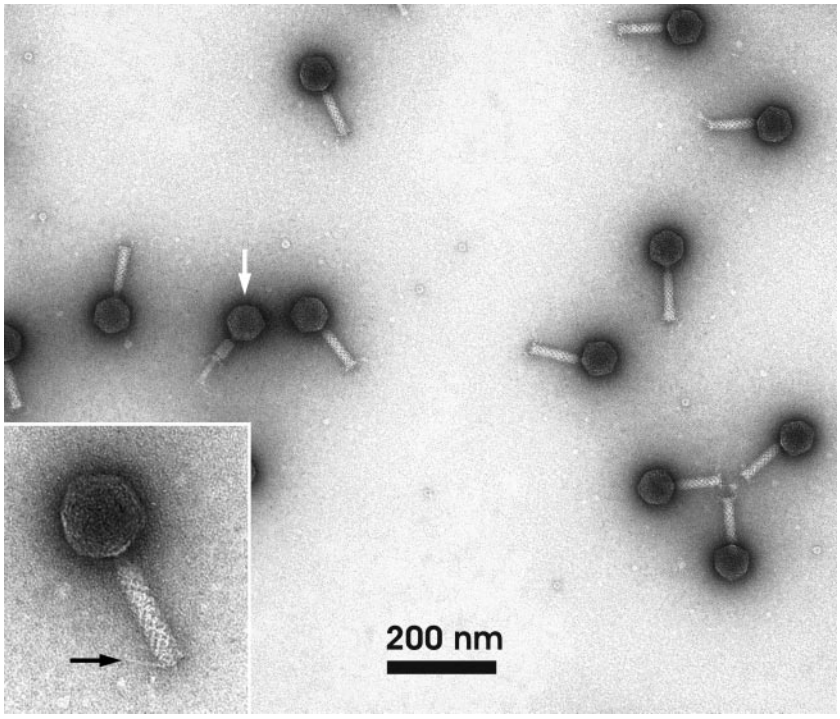

FIG. 1. Electron micrograph of CsCl-purified phage PY100 particles. The white arrow indicates phage with a contracted tail; the black arrow (inset) indicates tail fiber.

YPIII, and *Y. pestis* KIM. In addition, we tested the phage on other species belonging to the *Enterobacteriaceae*: *E. coli* (12 strains) *Shigella* sp. (9 strains), *Salmonella enterica* (10 strains), *Proteus* sp. (3 strains), *Enterobacter cloacae* (1 strain), *Citrobacter freundii* (2 strains), and *Serratia marcescens* (1 strain). We did not detect any plaque formation under the described conditions, suggesting that the host range of the phage is restricted to *Yersinia* strains.

Morphology of phage. Electron micrographs revealed that PY100 virions possess an icosahedral capsid and a contractile tail with fibers (Fig. 1). The diameter of the capsid is approximately 70 nm, and the length of the tail approximately 80 nm. The capsid contains double-stranded DNA with a length of 50,291 bp. Based on these properties the phage belongs to the family of *Myoviridae* (1).

Propagation of PY100 and burst size. Occasionally, plaques of PY100 on bacterial lawns appeared to be turbid. Therefore, we isolated bacteria from these plaques and investigated whether the bacteria might have become lysogenic. After repeated streaking on agar plates, single colonies were picked and grown in liquid culture. Phage induction was carried out by mitomycin C addition in the early logarithmic phase. However, we never succeeded in isolating viable phages from such liquid cultures, and bacteria from these cultures tested for phage susceptibility always allowed plaque formation. Thus, we conclude that PY100 does not integrate into the chromosome and replicates only via the lytic cycle.

To estimate the burst size of the phage, single growth experiments were carried out with strain *Y. enterocolitica* 13169 (30). The lytic cycle lasts approximately 30 min, and the burst size deduced from these experiments was estimated to be about 120 phages per infected cell at 37°C (data not shown).

Genome analysis of PY100. The complete nucleotide sequence of the PY100 genome (GenBank accession AM076770) was determined by sequencing random DNA clones of virion DNA fragments as described in Materials and Methods. The sequence was assembled into a single sequence of 50,291 bp in length, which is predicted to code for 93 genes (Fig. 2). To define the left end of the circular permutated genome, the order of the genes was arranged commencing with the small terminase gene. This arrangement facilitates the comparison with genomic maps of other phages with the gene order "terminase - head - tail" (37). A description of annotated genes is summarized in Table 2; no tRNA genes were found in the PY100 genome.

The left side of the genome (nucleotides 1 to 20,000) contains mostly smaller ORFs. Homology to known proteins was found only for a few gene products. ORF2 encodes a putative protein with significant similarity to the P22 gene *3* product (small terminase) of *Salmonella* phage P22 (33, 50). The putative gene product of ORF18, located on the minus strand, shows similarity to the large terminase subunits of a number of phages. The arrangement of ORF2 and ORF18 is unusual since most terminase genes are adjacent and located on the same DNA strand (11). In PY100 both putative terminase genes are followed by a transcription terminator. A number of small ORFs, whose putative gene products have no similarity to known proteins, are located between ORF2 and ORF18. The next ORFs coding for gene products with significant homology to known proteins are ORF34 and ORF35 on the

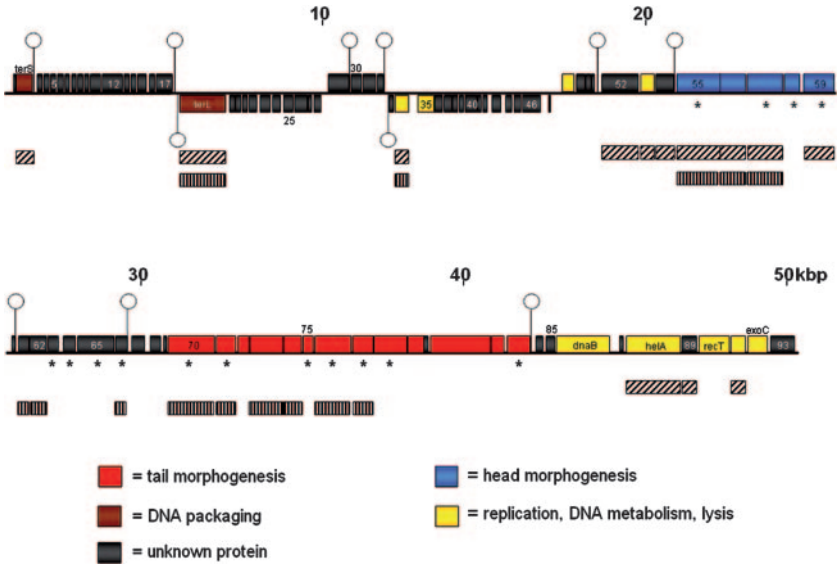

FIG. 2. Genetic map of the PY100 genome. Colored boxes above the black line represent ORFs of the positive strand; colored boxes below the black line represent ORFs of the negative strand. Colors indicate functional assignments. Rho-independent terminators are indicated by hairpins (above the sequence, terminators on the positive strand; below the sequence, terminators on the negative strand). Asterisks indicates ORFs whose gene products were detected by MS. Filled boxes with diagonal and vertical lines indicate regions of homology to the genome of phage T1 and phage AaΦ23, respectively.

minus strand. Their products are similar to DNA methyltransferases and single-stranded DNA-binding proteins, respectively. The putative gene product of ORF48 may be an endolysin. Although few functional assignments could be made for genes of the left side of the phage genome, the arrangement of rho-independent terminators (indicated by hairpins, see Fig. 2) support the ORF analysis. Table 3 displays the sequences of potential terminators.

The putative gene products of ORF52, ORF53, and ORF54 display similarity to the three enterobacterial phage T1 proteins T1p16, T1p64, and T1p62, respectively. Protein T1p64 may be a polynucleotide kinase or phosphatase (37), while for the protein homologs to the ORF52 and ORF54 gene products no functions are known.

ORF55 to ORF83, spanning the region from nucleotides 20900 to 42300, encode the structural proteins of the virion particle and are also discussed with the results of the proteomic investigation (see below). ORF55 may encode a portal protein and gene products of ORF56, and ORF57 and ORF59 are related to different head proteins of other phages. Protein homologs to the ORF61, ORF62, and ORF66 gene products exist in phage AaΦ23 of *Actinobacillus actinomycetemcomitans*, although no function of these gene products is known (36). ORF70 is related to phage tail tape measure proteins, and ORF74, ORF78, ORF79, ORF81, ORF82, and ORF83 encode proteins related to different tail proteins, such as baseplate proteins, tail fiber proteins, and the tail fiber assembly proteins of other phages (Table 2).

The right side of the genome from ORF84 to ORF93 contains some genes that encode proteins that are known to play a role in DNA metabolism, including synthesis, degradation, and recombination. ORF86 encodes a protein with homology to the replicative helicase RepA of gram-negative plasmids belonging to the DnaB-like helicase family. These proteins unwind double-stranded DNA into single-stranded intermediates fueled by nucleoside triphosphate hydrolysis (29). The putative protein encoded by ORF88 shows high homology to the T1p22 helicase, another protein involved in DNA unwinding and synthesis (37). Interestingly, the putative gene product of ORF89 is similar to the putative T1p21 protein, for which no function is known thus far. ORF90 encodes a DNA recombinase protein, and ORF91 encodes an endonuclease related to the T1p63 gene product of phage T1. The latter protein is a zinc-dependent HNH homing endonuclease, whose function in phages is unknown. It has been proposed that these endonuclease genes in phage genomes can be considered as analogous to insertion or transposon elements in bacterial genomes (37). ORF92 encodes an exonuclease that may be involved in host DNA degradation.

Proteomic analysis of virion particles. The bioinformatic analysis of the PY100 genome suggested that the region from ORF55 to ORF83 harbors most of the genes encoding the proteins building the mature virion particle. From Coomassie blue-stained two-dimensional gels, one protein encoded by ORF59 could be identified, which was isolated from several spots, indicating that it might be modified posttranslationally (data not shown). We therefore decided to analyze one-dimensional SDS gels, to cut out all visible bands and perform an MS analysis after trypsin digestion. The LC-MS analysis of eight protein bands visualized by Coomassie staining (Fig. 3, bands A to H) led to the identification of 15 structural proteins of the phage PY100. The sequence coverage and the number of identified peptides are given in Table 4. We were able to identify up to three proteins in one band by using the LC-MS system with integrated tandem MS capabilities. In some cases, peptides confirming the N terminus of the PY100 proteins were found (see Table 4).

Based on homology to known phage proteins, the detected PY100 proteins of ORF55, ORF57, ORF58, and ORF59 form

TABLE 2. Selected genes of phage PY100[a]

ORF	Gene	Start-stop	Protein size (aa)[b]	Potential function (gene)	Significant matches (source)
2	ORF2	491–1045	184	Terminase small subunit (*terS*)	Terminase small subunit (enterobacterium phage P22)
18	ORF18	6909–5440	489	Terminase large subunit (*terL*)	Putative terminase large subunit TerL (bacteriophage AaΦ23)
34	ORF34	12756–12109	215	Dam-methylase (*dam*)	Phage DNA methyltransferase (*Sodalis glossinidius* strain) (enterobacterium phage T1)
35	ORF35	13367–12804	187	Single-stranded DNA binding (*ssb*)	Single-stranded DNA-binding protein (*Vibrio* sp. strain MED222)
48	ORF48	17422–17838	127	Endolysine	Bacteriophage P7-related protein (*Yersinia pseudotuberculosis* IP 32953)
52	ORF52	18620–19810	396		Hypothetical protein T1p16 (enterobacterium phage T1)
53	ORF53	19854–20312	152	Polynucleotide kinase/phosphatase	Putative polynucleotide kinase/phosphatase (enterobacterium phage T1)
54	ORF54	20235–20912	195		Hypothetical protein T1p62 (enterobacterium phage)
55	**ORF55**	**20979–22340**	**453**	**Portal protein**	**Hypothetical protein (XF1571 *Xylella fastidiosa* 9a5c) (T1p52 enterobacterium phage T1)**
56	ORF56	22300–23133	277	Head protein	Putative bacteriophage protein (*Acinetobacter* sp. strain ADP1) (T1p51 enterobacterium phage T1)
57	**ORF57**	**23145–24305**	**386**	**Head protein**	**Hypothetical protein (lin1728 *Listeria innocua* Clip11262) (T1p50 enterobacterium phage T1)**
58	**ORF58**	**24308–24796**	**162**		**Hypothetical protein lin1727 (*Listeria innocua* Clip11262)**
59	**ORF59**	**24886–25893**	**335**	**Head protein**	**Hypothetical protein (CGSHi22121_00927) (*Haemophilus influenzae* 22.1-21) (T1p47 enterobacterium phage T1)**
63	**ORF63**	**27035–27403**	**122**		
64	**ORF64**	**27400–27930**	**176**		
65	**ORF65**	**27975–29114**	**379**		**Bacteriophage Felix 01**
66	**ORF66**	**29118–29546**	**142**		**Hypothetical protein PD1187 (*Xylella fastidiosa* Temecula1)**
70	**ORF70**	**30763–32271**	**502**	**Tail tape measure protein**	**Phage-related protein (*Xylella fastidiosa* Ann-1)**
71	**ORF71**	**32271–32918**	**215**		**Putative bacteriophage protein (*Ralstonia eutropha* JMP134)**
73	ORF73	33313–34380	355		Phage-related protein (*Xylella fastidiosa* Dixon)
74	ORF74	34358–34951	197	Baseplate protein	Conserved hypothetical protein (*Xylella fastidiosa* Dixon)
75	**ORF75**	**34948–35304**	**118**		**Hypothetical protein CGSHi22121_00997 (*Haemophilus influenzae* 22.1-21)**
76	**ORF76**	**35315–36490**	**391**		**COG3299: uncharacterized homolog of phage Mu protein gp47 (*Magnetospirillum magnetotacticum* MS-1)**
77	**ORF77**	**36493–37158**	**221**		**Hypothetical protein plu2875 (*Photorhabdus luminescens* subsp. *laumondii* TTO1)**
78	**ORF78**	**37151–38206**	**351**	**Tail fiber protein**	**Hypothetical protein (Ecol5_01002866 *Escherichia coli* 53638)**
79	ORF79	38179–38736	185	Tail fiber assembly protein	Putative bacteriophage protein (*Yersinia pseudotuberculosis* IP 32953)
81	ORF81	38887–40809	640	Tail fiber protein	Tail fiber protein (*Yersinia* phage PY54)
82	ORF82	40794–41225	143	Tail fiber assembly protein	Tail fiber assembly protein (*Yersinia* phage PY54)
83	**ORF83**	**41268–42035**	**255**	**Tail protein**	**gp37 long tail fiber, distal subunit (enterobacterium phage T4)**
86	ORF86	42787–44475	562	Replicative DNA helicase (*dnaB*)	Hypothetical protein PBPRB1439 (*Photobacterium profundum* SS9)
88	ORF88	44964–46682	572	NTP-dependent helicase (*helA*)	Putative ATP-dependent helicase (enterobacterium phage T1)
89	ORF89	46682–47185	167		Hypothetical protein T1p21 (enterobacterium phage T1)
90	ORF90	47215–48213	332	DNA recombination protein (*recT*)	Phage-related DNA recombination protein (*Bordetella bronchiseptica* RB50)
91	ORF91	48214–48702	162	HNH endonuclease (*endC*)	Bacteriophage Felix 01
92	ORF92	48702–49400	232	Exonuclease	Exonuclease (*Escherichia coli* O157:H7 strain Sakai)

[a] ORFs whose gene products were detected by MS are indicated in boldface type.
[b] aa, amino acids.

the capsid. The head protein encoded by ORF59 is the highly abundant protein seen in band E (Fig. 3). The proteomic approach proves that some ORFs are indeed protein coding sequences, although the proteins have no functionally assigned homologs in the database, e.g., proteins of ORF63, ORF64, and ORF65 (see Table 3). A homolog of the ORF66 protein with unknown function is encoded in the genome of phage AaΦ23. The ORFs downstream (ORF70 to ORF83) encode the tail proteins with its various structures, such as tail length tape measure (ORF70 protein), fibers, base plate, etc. (see

TABLE 3. Potential rho-independent terminators in the PY100 genome

Position	Direction	ORF no.[a]	Sequence[b]
1055	+	2	AAAGCCCTTGACATTGTTCAGGGGCTTTTCTATTATATGCGC
5407	+	17	GCCGGATCGGGTTTCCCTTTTCCGGCTTTTCTTTTATCCCCAC
5433	−	18	AGCCGGAAAAGGGAAACCCGATCCGGCTCTTTTTTTTGTT
10843	+	29	AAGCGCCCTTAATTGGGCGCTTTTACTATCAACTT
11922	+	32	AAGGGGCCATATGGCCCCTTTCTTTTGTCTATTCAACTG
11941	−	33	AAGGGGCCATATGGCCCCTTAATCGTTTTTAGCGCTG
18502	+	51	TGCCCCGCTCCGGCGGGGGTATTTTTTTTATCTGAAA
20929	+	54	CCCGTAAGCTATAATAGTTTACGGGCTTATTTATTTTTCGAG
26094	+	60	GGGGAGAGGCTACGGCCTTTCCCCTTTTTTATATGGAGGC
29554	+	66	AAAAGGGGTTGACTTCGGTCAATCCCTTTTCTATTATGCGC
42038	+	83	AAAGGGGCTTCGGCCCCTTTATTTTTTTATAAA

[a] That is, the preceding ORF.
[b] Underlining indicates dyad symmetries that potentially yield stable hairpin structures in the RNA transcript.

Table 2). In most cases, the observed molecular masses correspond well to the theoretical molecular size deduced from the corresponding ORF. However, ORF57 would encode a protein of 366 amino acids with a size of 43.3 kDa, while peptides of the ORF57 gene product were found in a protein band with an observed mass of 19 kDa. Since the detected peptides are from the N-terminal part of the protein (amino acid residues 21 to 28, 36 to 44, and 161 to 172), no obvious explanation for this observation can be given. It is possible that the C-terminal part of the protein is proteolytically cut off during assembly of the phage particle, as is described for some phage proteins, e.g., gp5 protein of phage T4 (52).

Relationship of PY100 to other bacteriophages and prophage sequences. BLASTN searches with the PY100 sequence against the database (nucleotide collection [nr/nt]; http://www.ncbi.nlm.nih.gov/BLAST/) yielded only short sequences (<50 bp) of similarity. All functional assignments were made on the basis of translated ORFs against the database (BLASTX). Thus, the PY100 genome harbors several gene products with homology to proteins of the enterobacterial phage T1 (37). Gene products of ORFs showing homology to the T1 proteins are the capsid proteins and proteins involved in replication and DNA packaging (Fig. 2). Phage T1 is a *Siphoviridae*, a phage with a flexible, noncontractile tail, whereas PY100 has a contractile tail. Another phage possessing several putative gene products with homology to the PY100 is the temperate bacteriophage AaΦ23 of *A. actinomycetemcomitans*, which has a contractile tail like PY100. The homologous gene products of phage AaΦ23 are putative tail and head proteins (36).

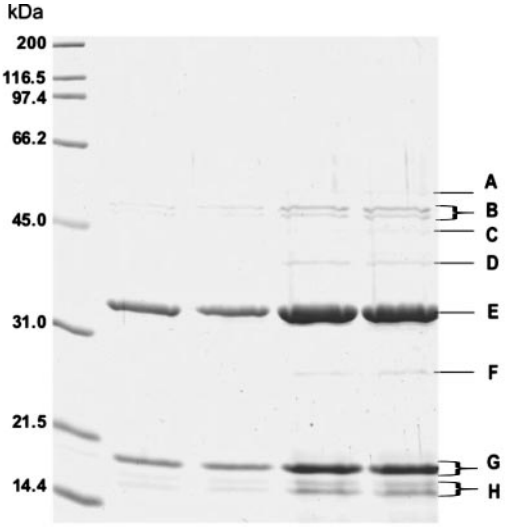

FIG. 3. SDS-PAGE analysis of proteins from PY100 virions (different protein concentrations were loaded from left to right). Capital letters indicate protein bands excised for MS analyses.

The BLASTX search of the PY100 ORFs revealed some genomic regions in other gram-negative bacteria that encode putative gene products with significant homology to the PY100 structural head and tail proteins. These genomic regions are prophage remnants, for example, two genomic regions in the *S. enterica* strain CT18. Thirteen putative gene products of the region from STY1058 to STY1073 and eleven gene products of the region from STY2038 to STY2015 share homology with some of the proteins from ORF55 to ORF77 of PY100 and have a similar gene order (32). Other putative gene products of prophage regions with homologies to the head and tail PY100 proteins are found in *Xylella fastidiosa* strain 9a5c (region from genes XF1571 to XF1595 and region from genes XF1676 to XF1706, accession no. NC_002488 [41]). In *Photorhabdus luminescens* subsp. *laumondii* TTO1, several genes of phage origin encode putative proteins with homology to PY100 proteins (accession no. NC_005126 [14]).

Packaging mechanism of PY100. In an attempt to elucidate whether *cos* sites were present in the PY100 genome, DNA of the phage was ligated and digestions with selected restriction enzymes were carried out. When the restriction patterns of unligated and ligated phage DNA obtained with the same enzyme were compared, no fragments arising from ligation of the *cos* sites were detectable.

The analysis of the genomic sequence indicated a gene product (encoded by ORF2) with significant similarity to the product of gene *3* of the *Salmonella* phage P22 (33, 50). This protein is part of the terminase complex of phage P22 and initiates the packaging of phage DNA by cutting the concatemeric P22 DNA close to or at the *pac* site. The *pac* site is 22 bp long with a consensus sequence of 12 bp (AAGATTTATCTG) and lies within the coding region of the P22 gene *3*. After the initial endonucleolytic cut, packaging of DNA proceeds in one direction and sequentially DNA from the concatemer is packaged by a headful mechanism (9, 51). As a consequence of the packaging process starting at a *pac* site, a restriction fragment

TABLE 4. PY100 virion proteins detected by MS

Band[a]	Observed avg mass (kDa)	ORF	Theoretical avg mass (kDa)	Amino acids	No. of peptides found	Sequence coverage (%)	Putative function[b]	N-terminal peptide[c]
A	51	70	53.9	502	7	15.1	Tail-length tape measure protein	
B	48	55	50.0	453	7	16.6	Portal protein	+
	48/40	76	42.7	391	4	13.0	–	
C	40	65	42.6	379	3	5.3	–	
D	38	78	37.7	351	6	21.1	Tail fiber	
E	35	59	37.2	335	15	30.7	Head protein	
	35	83	27.7	255	7	19.2	Tail fiber	
F	28	77	24.8	221	7	23.9	–	+
	28	71	23.7	215	4	19.0	–	+
G	19	57	42.6	386	3	7.4	Head protein	
	19	64	19.8	176	8	44.3	–	
	19	58	17.2	162	8	38.8	–	+
H	16	66	15.2	142	7	32.4	–	
	16	63	14.2	122	3	22.1	–	
	16	75	13.1	118	1	9.3	–	

[a] See Fig. 3.
[b] –, The annotation was "phage related, unknown function."
[c] +, Peptides were found corresponding to the deduced N terminus.

with reduced abundance is found in agarose gels after digestion of virion DNA ("*pac* fragment") with suitable restriction enzymes (23). The *pac* fragment is also visualized by hybridization when using a hybridization probe consisting of sequences downstream from the *pac* site.

In an experiment analogous to that used to examine P22, we performed restriction enzyme analysis of the PY100 virion DNA and hybridization studies. Analysis of the restriction patterns of the PY100 DNA revealed the presence of the expected restriction fragments plus an additional fragment with a lower intensity. Since the *pac* site of P22 is located within the coding region of gene *3*, we deduced primers for a 348-bp hybridization probe from the PY100 sequence (primers PY100-5 and PY100-6) spanning the 3′ region of ORF2 and its downstream region (see Fig. 5). Hybridization studies with a labeled PCR probe revealed two positive bands in each digest (Fig. 4). One of these hybridizing bands corresponded to the band with lower abundance and results from initiation of DNA packaging at the *pac* site ("*pac* fragment"). The second band was the expected restriction fragment derived from the genomic sequence containing the probe sequence (Fig. 5).

To determine the position of the *pac* site in the PY100 sequence more precisely, an alignment of the PY100 ORF2 DNA sequence and the gene *3* sequence of P22 was performed (data not shown). The overall similarity of the two coding regions is 39% (four gaps introduced). A nucleotide sequence identical to the *pac* consensus sequence of P22 (51) is not present in the coding region of ORF2. Allowing mismatches, two sequences can be found in which 8 of 12 bp are identical to the *pac* consensus sequence of P22. One of these sequences (AAttTTTAcCaG) is at a similar position in the coding sequence of PY100 ORF2 (approximately the middle of the coding region, positions 821 to 833 of the genomic sequence) as the *pac* site in gene *3* of P22 (position 269; length of coding sequence, 489 bp) and may serve as a recognition site for the terminase complex (Fig. 5).

Since another short nucleotide sequence has also been proposed to function as a secondary *pac* site for the P22 gp3

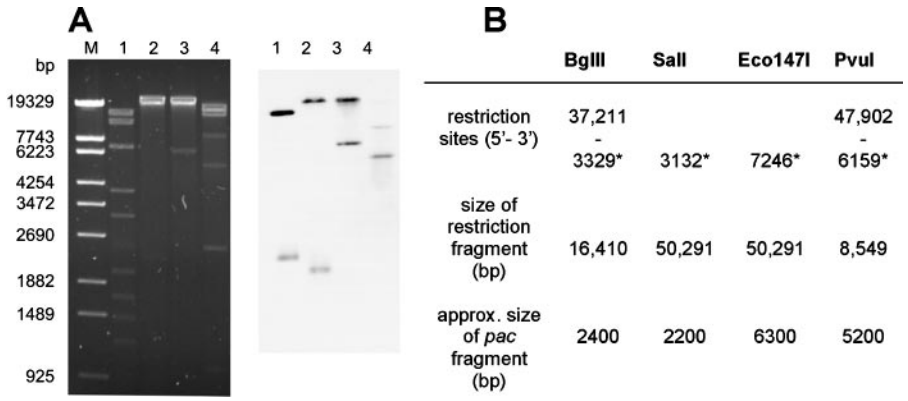

FIG. 4. (A) Detection of *pac* fragment. The left panel shows a 0.8% agarose gel of restriction of PY100 genomic DNA with BglII (lane 1), SalI (lane 2), Eco147I (lane 3), and PvuI (lane 4). Lane M contains lambda DNA digested with Eco130I. The right panel shows a Southern blot with PCR probe generated with the primers PY100-5 and PY100-6 (see Fig. 5). (B) Sizes of restriction fragments containing ORF2 and *pac* fragments (in base pairs). The positions of restriction sites are given according to PY100 numbering. Asterisks indicate sites at the 3′ end of *pac* fragments. Enzymes SalI and Eco147I have only one recognition site in the PY100 genome.

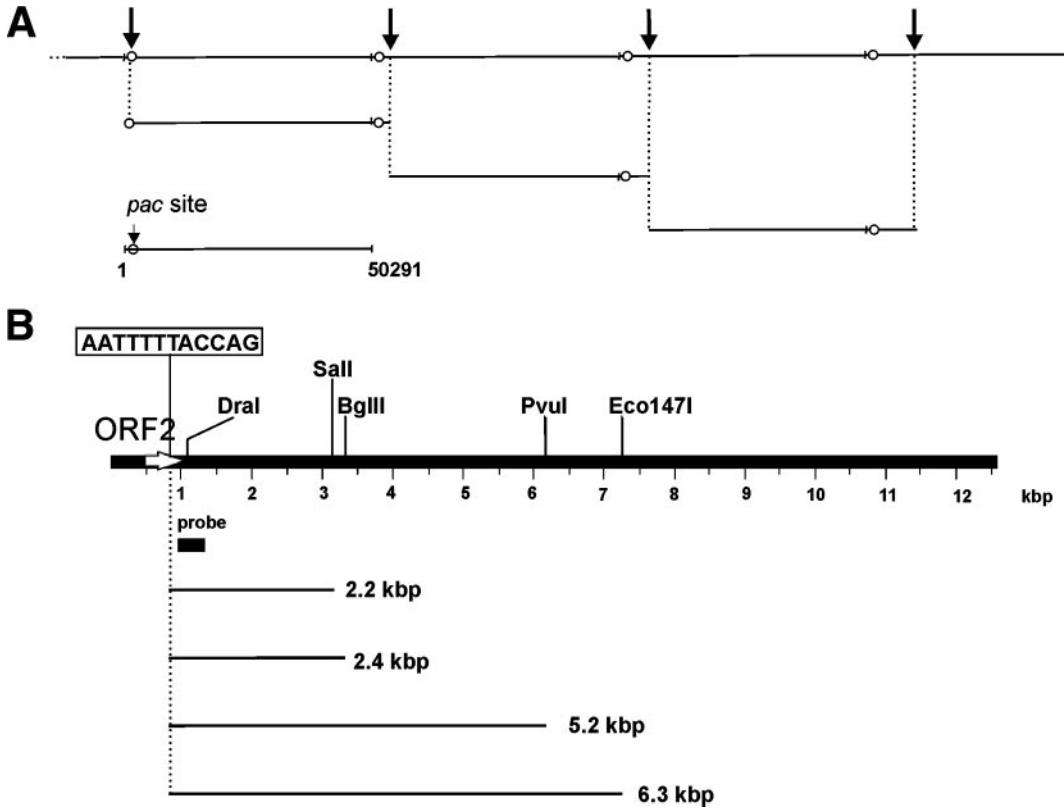

FIG. 5. Generation of *pac* fragments. (A) Cutting of concatemeric DNA starting from the *pac* site (○), with sequential cuts according to the headful mechanism. (B) Partial genomic map of PY100 showing the position of the putative *pac* site (boxed nucleotides) and selected restriction sites of enzymes generating the *pac* fragments (see also Fig. 4). The black box indicates the PCR probe (primers PY100-5 and PY100-6) that was used for hybridization.

protein (10, 34), we performed an additional search. The putative secondary *pac* site of P22 was assumed to consist of nine nucleotides and is located 110 bp downstream of the consensus *pac* site in the gene *3* coding sequence. We also found a similar sequence (seven out of nine identical) in the corresponding region of ORF2 of PY100 (160 bp downstream of the *pac* site; data not shown). The functionality of this secondary *pac* site, however, was questioned later (51).

In P22 the *pac* site serves as a packaging recognition signal for the terminase complex. The initial cleavage of the concatemeric DNA, however, takes place in six "*end*" regions that span 120 bp within the coding region of gene *3*. The *end* regions are separated by approximately 20 bp or multiples of 20 bp, and individual cuts might be spread over up to 3 to 5 bp (9). The *pac* consensus recognition site lies between *end* regions 3 and 4.

To determine the initial cleavage site of the terminase reaction in the PY100 genome, the *pac* fragment obtained after BglII digestion of virion DNA was cut out from agarose gels (Fig. 6A). After digestion with DraI and hybridization with the PCR probe obtained with the primers PY100-5 and PY100-6, the hybridization signal indicated a variable length of the *pac* fragment of PY100 (Fig. 6B). The fragment was cloned into a standard vector, and a number of recombinant plasmids were sequenced. In total, the molecular ends formed at the packaging initiation site of 154 *pac* fragments were determined. Figure 6C shows the distribution of the cleavage sites along the genomic sequence of PY100. All sites are within the coding region of ORF2 (small terminase homolog), and most of the cuts (87%) lie within 140 bp (from positions 740 to 880). The distribution of the cleavage sites does not show any obvious clustering, nor does the analysis of the nucleotides at the sites reveal any significant structures. The putative *pac* site at positions 821 to 833 lies in the middle of the cleavage sites region, as is also the case for the *pac* site of P22 (5, 10).

PY100 for genetic studies of *Yersinia*? The similarity of the packaging mechanism of PY100 and P22 suggests that PY100 might be able to perform transduction in *Yersinia*. Bacteriophage P22 was the first phage shown to be able to perform generalized transduction (53). During the assembly of progeny particles host DNA is "incorrectly" recognized for packaging by the P22 gene *3* protein in ca. 2% (15). The particles thus formed can inject a contiguous fragment of host DNA into a susceptible bacterium. For this reason, P22 can move genes from one bacterium to another and is used in genetic studies of *S. enterica* serovar Typhimurium Given its wide host range, PY100 could be a useful genetic tool for *Yersinia*.

If PY100 should be used for genetic experiments, a high plating efficiency resulting in comparable titers on different recipient strains would be preferable. It is known that phage particles growing well and efficiently on one strain of bacteria are often unable to do so in other strains of the same bacterial

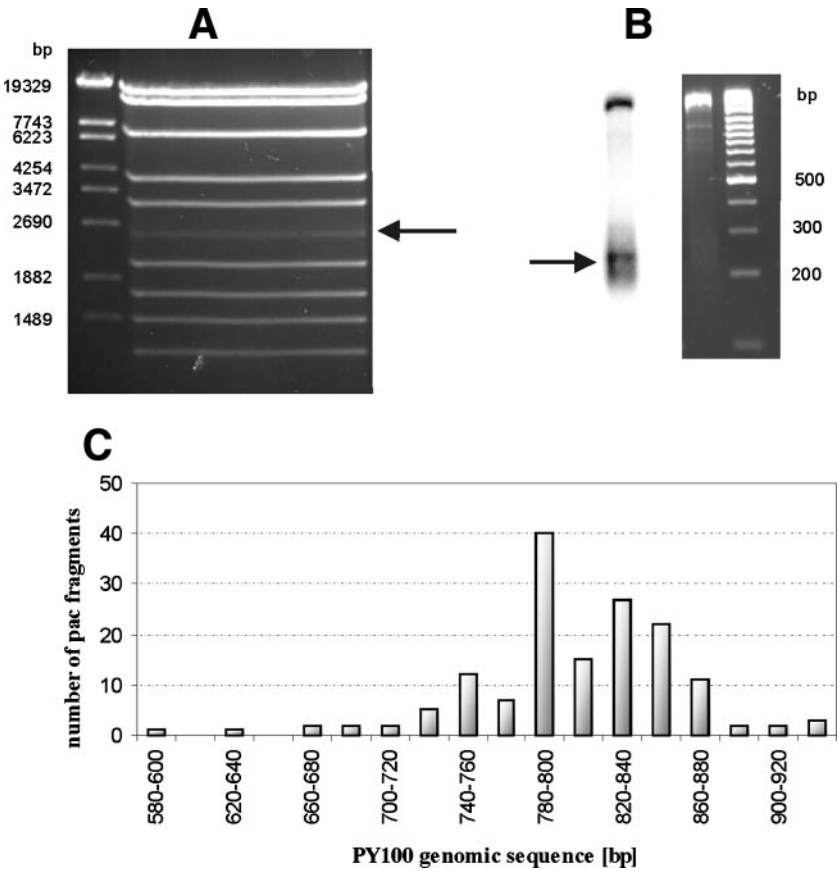

FIG. 6. Determination of the position of *end* sites. (A) BglII digest of PY100 DNA. The *pac* fragment was excised (arrow). (B) Digest of the BglII *pac* fragment with DraI and a Southern blot with probe (see Fig. 5B). The arrow indicates fragments with *end* sites. (C) Distribution of initial cuts within the coding region of ORF2 (positions are according to PY100 sequence numbering).

species due to restriction modification systems. We investigated the plating efficiency of PY100 preparations on 11 *Y. enterocolitica* strains belonging to the four most important pathogenic serotypes (O3, O5,27, O8, and O9; Table 5). From each strain phage lysates were prepared and tested on the donor and on all other strains, yielding 121 (11 × 11) phage titer experiments. We observed no restriction of phage growth between the O3 and O5,27 strains. One O8 strain (ATCC 9610) and two O9 strains restricted phage growth to some extent; however, in total only in 4 cases (out of 121) did lysates

TABLE 5. Efficiency of plaque formation of PY100 in *Y. enterocolitica*[a]

Recipient strain	Efficiency of plaque formation of PY100 phage lysate (host strain) for serotype:										
	O3, strain:			O5,27, strain:			O9, strain:		O8, strain:		
	2*	16*	738/84*	83/88	44	97	9610*	8081	8	36	38
2*	100%	+	+	+	+	+	+	+	+	+	+
16*	+	100%	+	+	+	+	+	+	+	+	+
738/84*	+	+	100%	+	+	+	+	+	+	+	+
83/88*	+	+	+	100%	+	+	+	+	+	+	+
44*	+	+	+	+	100%	+	+	+	+	+	+
97*	+	+	+	+	+/–	100%	+	+	+	+	+
9610†	Low	–	Low	Low	–	+/–	100%	Low	+/–	+/−	+/–
8081	+	+	+	+	+	+	+	100%	+	+	+
8*	+	+	+	+	+/–	+	+	+	100%	+	+/–
36*	+/–	Low	+	+/–	–	+/–	+	+/–	+/–	100%	+/–
38*	+/–	Low	+	+/–	–	+/–	+	+	+/–	+/–	100%

[a] PY100 preparations derived from 11 host strains belonging to the main pathogenic serotypes O3, O5,27, O8, and O9 were prepared. Efficiency was determined as follows. The PFU titer of lysate titered on the same strain was set to 100% (100% values). All PY100 titers on host strains were greater than 10^7 PFU ml^{-1}; only PY100 preparations derived from strains 36 and 38 were 5×10^5 and 3×10^5, respectively. Key: +, approximately the same PFU on the recipient strain as on the host strain; +/−, 1 to 10% PFU on the recipient strain compared to the PFU on the host strain; Low, <1% PFU on the recipient strain compared to the PFU on the host strain; −, no PFU. *, *Y. enterocolitica* RKI strain collection; †, *Y. enterocolitica* ATCC 9610.

not result in plaque formation. These experiments indicate that PY100 has a high plating efficiency, which is a prerequisite for its use as genetic tool.

Conclusions. PY100 is a lytic phage with a broad host range in *Yersinia.* The genome sequence revealed that several gene products possess homologies to proteins of the lambdoid phages T1 and aaphi23. According to the modular theory of phage evolution, phages are built from interchangeable units that can be multigenic and fulfill homologous functions but may lack any sequence similarity (8). From this point of view, PY100 possesses some modules that are known from other phages, especially the units that encode the structural proteins of the head and tail. Some of these structural proteins were also identified in a proteomic analysis.

However, surprisingly, a significant portion of ORFs located on the left arm of the PY100 genome encode gene products for which at present no functional assignment can be made. ORFs encoding known integrases and repressor/antirepressor proteins were not identified, indicating that PY100 does not lysogenize its hosts and confirming the experimental observations. This is a good precondition if one considers antimicrobial strategies such as phage therapy using PY100. The ability of PY100 to lyse susceptible strains at 37°C is remarkable, since most described yersiniophages are propagated at temperatures below 30°C (6, 24, 25, 30).

We applied PY100 phage preparations by the oral route in a mouse model with enteropathogenic *Y. enterocolitica* O3. These trials, however, did not yield satisfying results since it was not possible to prevent the colonization of the gut by *Y. enterocolitica* (43). Nevertheless, PY100 may still be an interesting tool for antimicrobial strategies, since the efficacy of phage treatments depends heavily on the route (oral, intramuscular, aerosol inhalation, etc.) and/or the frequency of the phage application (4). Optimal conditions for PY100 applications may have yet to be found. Antimicrobial strategies using lytic phages against *Y. pestis* is still in consideration, especially if antibiotic-resistant strains of this human pathogen would be used as a bioterror agent.

The genome sequence analysis suggested that PY100 uses a headful packaging strategy starting at a *pac* recognition site in the concatemeric DNA formed by rolling-circle replication. This mechanism could be confirmed and a clustering of cuts around a putative *pac* site within ORF2 was found. Based on the similarity to the packaging strategy of *Salmonella* phage P22, it seems possible that PY100 can be used for transduction. Lytic *Yersinia* phages from environmental sources have been shown to perform generalized transduction of small plasmids (19). Plasmid transduction with phage P22 was also reported either by homologous recombination between phage DNA and plasmid DNA that harbored P22 sequences (cointegrate formation), or when a *pac*-like sequence was present on a plasmid that can be packaged in a headful mechanism from concatemeric DNA (40). It seems feasible to develop PY100 phage derivatives that can be used for transduction.

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