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PY54, a linear plasmid prophage of *Yersinia enterocolitica* with covalently closed ends

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Summary

PY54 is a temperate phage isolated from *Yersinia enterocolitica*. Lysogenic *Yersinia* strains harbour the PY54 prophage as a plasmid (pY54). The plasmid has the same size (46 kb) as the PY54 genome isolated from phage particles. By electron microscopy, restriction analysis and DNA sequencing, it was demonstrated that the phage and the plasmid DNAs are linear, circularly permuted molecules. Unusually for phages of Gram-negative bacteria, the phage genome has 3′-protruding ends. The linear plasmid pY54 has covalently closed ends forming telomere-like hairpins. The equivalent DNA sequence of the phage genome is a 42 bp perfect palindrome. Downstream from the palindrome, an open reading frame (ORF) was identified that revealed strong DNA homology to the *telN* gene of *Escherichia coli* phage N15 encoding a protelomerase. Similar to PY54, the N15 prophage is a linear plasmid with telomeres. The N15 protelomerase has cleaving/joining activity generating the telomeres by processing a 56 bp palindrome (telomere resolution site *telRL*). To study the activity of the PY54 protein, the *telN*-like gene was cloned and expressed in *E. coli*. A 77 kDa protein was obtained and partially purified. The protein was found to process recombinant plasmids containing the 42 bp palindrome. Telomere resolution of plasmids under *in vivo* conditions was also investigated in *Yersinia* infected with PY54. Processing required a plasmid containing the palindrome as well as adjacent DNA sequences from the phage including an additional inverted repeat. Regions on the phage genome important for plasmid maintenance were defined by the construction of linear and circular miniplasmid derivatives of pY54, of which the smallest miniplasmid comprises a 4.5 kb DNA fragment of the plasmid prophage.

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Introduction

The species *Yersinia enterocolitica* belongs to the family *Enterobacteriaceae* and contains about 70 serogroups, some of which are enteropathogenic for humans. In Europe, *Yersinia* infections are mainly caused by strains belonging to the serogroups O:3, O:5,27 and O:9, whereas O:8 is the predominant serogroup in the United States (Kapperud, 1991; Bottone, 1997). The bacteria are predominantly transmitted by raw meat and meat products (Tauxe *et al*., 1987). Pigs are known to be an important reservoir for pathogenic *Yersinia* strains. In contrast to the serogroups mentioned above, strains belonging to the serogroup 5/biogroup 1A are considered to be non-pathogenic. Non-pathogenic *Yersinia* strains can be found readily in the environment (Marranzano *et al*., 1993). However, there are some reports that describe the isolation of biogroup 1A strains from clinical samples (Ratnam *et al*., 1982; Mingrone *et al*., 1987; Bissett *et al*., 1990). Therefore, a pathogenic potential for these strains has been suggested (Morris *et al*., 1991; Burnens *et al*., 1996; Sulakvelidze *et al*., 1996). As biogroup 1A strains sometimes contain plasmids that are partially homologous to the virulence plasmid harboured by pathogenic strains (Hoffmann *et al*., 1998), it is likely that gene transfer occurs in *Yersinia*, enabling environmental strains to cause infections. This possibility, along with the fact that the virulence plasmid is known to be non-conjugative, prompted us to isolate and characterize temperate *Yersinia* phages in order to unravel whether gene exchange by transduction occurs between pathogenic and non-pathogenic strains (Popp *et al*., 2000). These studies demonstrated that a number of *Yersinia* strains harboured temperate phages, for which no indicator strain could be found. Furthermore, most of the propagatable phages had a narrow host range and were only active on strains belonging to the same serogroup from which they were isolated. One temperate phage specific for O:3 strains as well as phage mixtures isolated from sewage, which mainly contained virulent phages, were able to transduce small *Yersinia* plasmids, but not the 70 kb virulence plasmid pYV (Hertwig *et al*., 1999). From these results, it can be concluded that phage-mediated transfer of the virulence plasmid from pathogenic

to non-pathogenic *Yersinia* strains is rather unlikely. However, it should be taken into account that, as yet, only a few *Yersinia* phages have been characterized in detail. Recently, one O:3-specific virulent phage was sequenced and shown to be closely related to the *Escherichia coli* phages T3 and T7 (Pajunen *et al.*, 2001; 2002).

Here, we concentrate on the characterization of the temperate *Yersinia* phage PY54, which infects pathogenic and non-pathogenic *Y. enterocolitica* strains (Popp *et al.*, 2000). The phage has a lambda-like morphology, belongs to the family *Siphoviridae* and has a genome composed of double-stranded DNA ≈ 46 kb in size. PY54 was indistinguishable from the temperate *Yersinia* phage PY30 and showed only weak homologies to other temperate phages (Popp *et al.*, 2000). This study shows that the PY54 genome contains 3′-protruding ends and that the PY54 prophage is a linear plasmid with terminal hairpins. These telomere-like structures are generated by a phage-encoded protelomerase. The characterization of phage PY54 demonstrated that it has properties similar to the *E. coli* phage N15 (Ravin and Shulga, 1970), which was isolated in 1964 and, up to now, was unique in that the prophage is a linear plasmid with covalently closed ends.

Results

Lysogenic Yersinia strains harbour the PY54 prophage as plasmid

Phage PY54 was isolated from the non-pathogenic *Y. enterocolitica* O:5 strain 29854 by induction with mitomycin C (Popp *et al.*, 2000). As strain 29854 also contains large plasmids, we investigated whether any homology exists between the plasmids and the phage. DNA hybridization experiments revealed strong regions of homology between PY54 and one of the plasmids (data not shown). Moreover, the restriction patterns of the plasmid and the phage were very similar (Fig. 1A). For both molecules, an identical size of 46 kb was determined. To find out whether the plasmid is equivalent to the PY54 prophage, other *Yersinia* strains were lysogenized with the phage and analysed for plasmid content. All lysogenized *Yersinia* strains contained a low-copy-number plasmid that was indistinguishable from the plasmid of strain 29854. This finding indicates that, in all infected *Yersinia* strains, PY54 replicated as a plasmid. The plasmid was named pY54 to distinguish it from PY54 phage particles. To determine whether the phage genome can also integrate into the chromosome of bacteria, we analysed restriction patterns of total DNAs from lysogenic and non-lysogenic *Yersinia* strains by pulsed field gel electrophoresis (PFGE) and hybridized the digested DNAs with the labelled phage genome. This study provided no indication of a chromosomal insertion of the prophage DNA (data not shown).

The PY54 phage genome and plasmid prophage are linear, circularly permuted molecules

Restriction patterns of the phage genome and plasmid were analysed in detail. For this purpose, the restriction endonuclease *Dra*I was used, which cuts the DNAs into at least 20 fragments. The *Dra*I restriction patterns of the phage and plasmid turned out to be nearly identical with only two different restriction fragments. The phage contained a 1 kb *Dra*I fragment, whereas a fragment of only 850 bp was detected in the plasmid. In addition, a 700 bp fragment was observed in the plasmid digests that was frequently absent in the digested phage DNA (Fig. 1A and

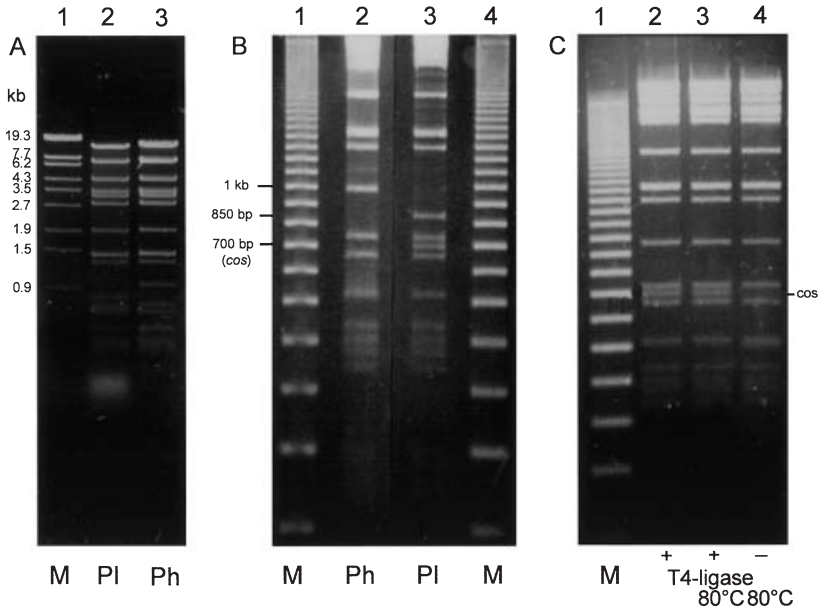

Fig. 1. *Dra*I restriction analysis of the PY54 phage and plasmid DNAs.
A. 0.8% agarose gel. Lane 1, M, marker (λ *Eco*130I); lane 2, Pl, plasmid DNA; lane 3, Ph, phage DNA.
B. 2% agarose gel. Lanes 1 and 4, marker (100 bp ladder); lane 2, phage DNA; lane 3, plasmid DNA.
C. Detection of the *Dra*I fragment containing the cohesive ends. Lane 1, marker (100 bp ladder); lane 2, T4 ligase-treated phage DNA; lane 3, same DNA as in lane 2, but heated for 10 min at 80°C before being loaded onto the gel; lane 4, phage DNA that was heated, but not treated with T4 ligase.

B). Some *Dra*I digests of the phage DNA, however, contained the 700 bp fragment, which appeared as a weak band. To determine whether this fragment contains cohesive ends, digested phage DNA was heated for 10 min at 80°C and immediately chilled on ice before loading onto an agarose gel. The treatment revealed that the 700 bp *Dra*I fragment of the phage disappeared completely by heating, whereas the corresponding *Dra*I fragment of the plasmid was stable. By incubation with T4 ligase before digestion, the fragment of the phage became robust and stable too (Fig. 1C), suggesting that the 700 bp *Dra*I fragment contains cohesive ends that are only present in the phage DNA.

Hybridization experiments revealed strong homology between the 1 kb *Dra*I fragment of the phage and the 850 bp fragment of the plasmid. Additionally, the 1 kb fragment hybridized to a very small *Dra*I fragment of the plasmid (data not shown). This pointed to the possibility that the plasmid is also a linear molecule, and that the aforementioned *Dra*I fragments of the plasmid are equivalent to the 1 kb fragment of the phage and are located at the ends of the plasmid. Through further restriction and mapping analysis, it was confirmed that the phage and plasmid DNAs are indeed linear circularly permuted molecules. The end sequences of both molecules were analysed in detail (see below). The results of this restriction analysis were confirmed by electron microscopy. The phage DNA exhibited linear and circular molecules, whereas in the preparation of the plasmid, exclusively linear molecules were visible (Fig. 2A and B). As differences between the phage genome and the plasmid were only detected at the ends of the molecules, a common restriction map was constructed (Fig. 3). The map shows that the phage genome and plasmid prophage are nearly 50% permuted so that the *cos* site of the phage is located approximately in the middle of the plasmid. Also mapped was the approximate position of a partial PY54 genome that we found in DNA preparations of phage lysates not purified by caesium chloride step gradients. In caesium chloride step gradients, phage particles containing the complete or partial genome were separated. On agarose gels, the partial genome was visible as a fuzzy DNA band that migrated close to the band of the complete phage genome. By restriction analysis and DNA hybridization, it was demonstrated that the partial genome has a size between 12 and 14 kb, with the left end mapping to the position of the cohesive ends (data not shown). We suspect that the partial genome is generated during the replication and packaging of the phage DNA and are currently investigating this subject in detail.

Phage PY54 DNA has 3′-protruding ends

The *cos* region of phage PY54 was analysed by cloning and sequencing of the 700 bp *Dra*I fragment of pY54 harbouring the *cos* site. The DNA sequence (AJ348843)

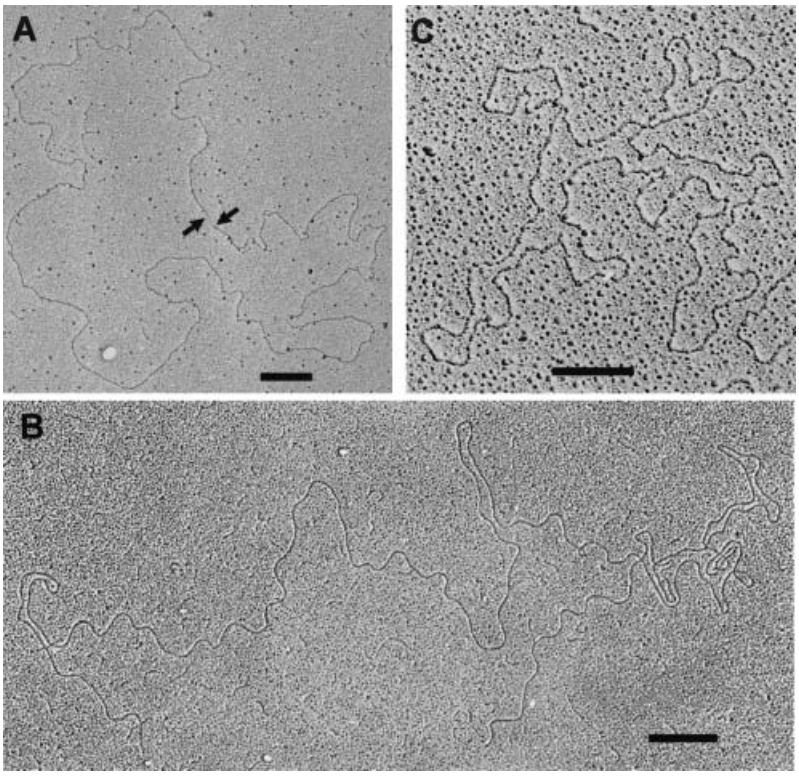

Fig. 2. Electron micrographs of PY54 phage and plasmid DNAs.
A. Phage DNA. The arrows indicate the positions of the cohesive ends.
B. Plasmid prophage DNA.
C. Denatured DNA of the miniplasmid pSH95 (16.5 kb). Seventeen molecules were measured [size 32.73 kilonucleotides (knt), SD = 1.44 knt = 4.4%]. Bars indicate 1 kb (A and B) and 1 knt (C) respectively.

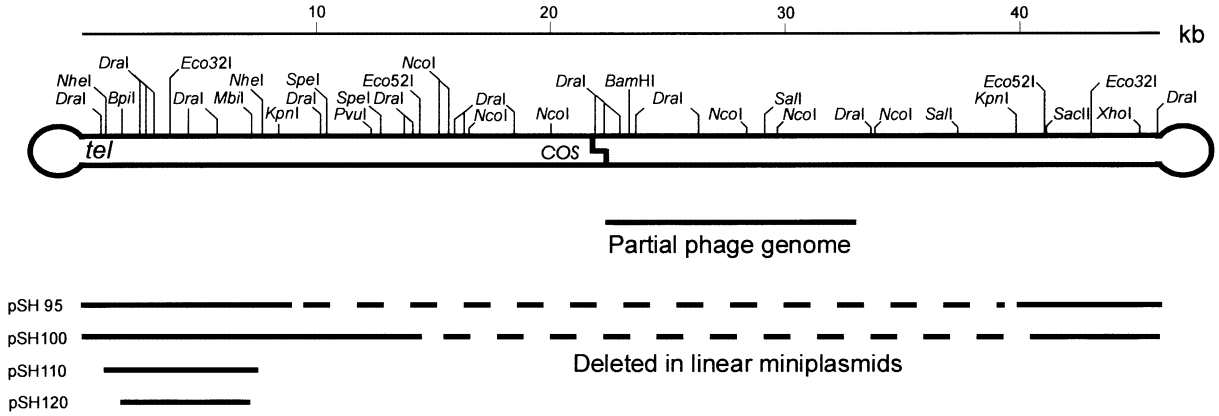

Fig. 3. Physical map of PY54. The positions of the *tel* gene, *cos* site, partial phage genome and miniplasmid derivatives are indicated. See text for details.

of the *cos* region is shown in Fig. 4. To find out whether any differences exist between this plasmid sequence and the corresponding sequence of the phage DNA, the *cos* region of the phage was amplified by polymerase chain reaction (PCR) using the primers Cos-F and Cos-R (Table 1) deduced from the plasmid sequence. Additionally, T4 ligase-treated phage DNA was sequenced using these primers. We could not detect any discrepancy between the *cos* region of the plasmid and the phage. To define the protruding end sequences of the phage genome, the DNA was heated (10 min for 60°C, 70°C or 80°C), chilled on ice and treated with DNA polymerase I (large fragment). It was expected that 5′-protruding ends would be filled in by the polymerase activity of the large fragment yielding blunt ends. In the case of 3′-protruding ends, the exonuclease activity of the enzyme would trim the single strands and also make blunt ends. The end sequences of the PY54 phage genome were determined using the *cos* primers. A comparison of the DNA sequences of the ends with the sequence of the total *cos* region revealed a gap of 10 bp in the phage DNA treated with the large fragment of DNA polymerase I (Fig. 4).

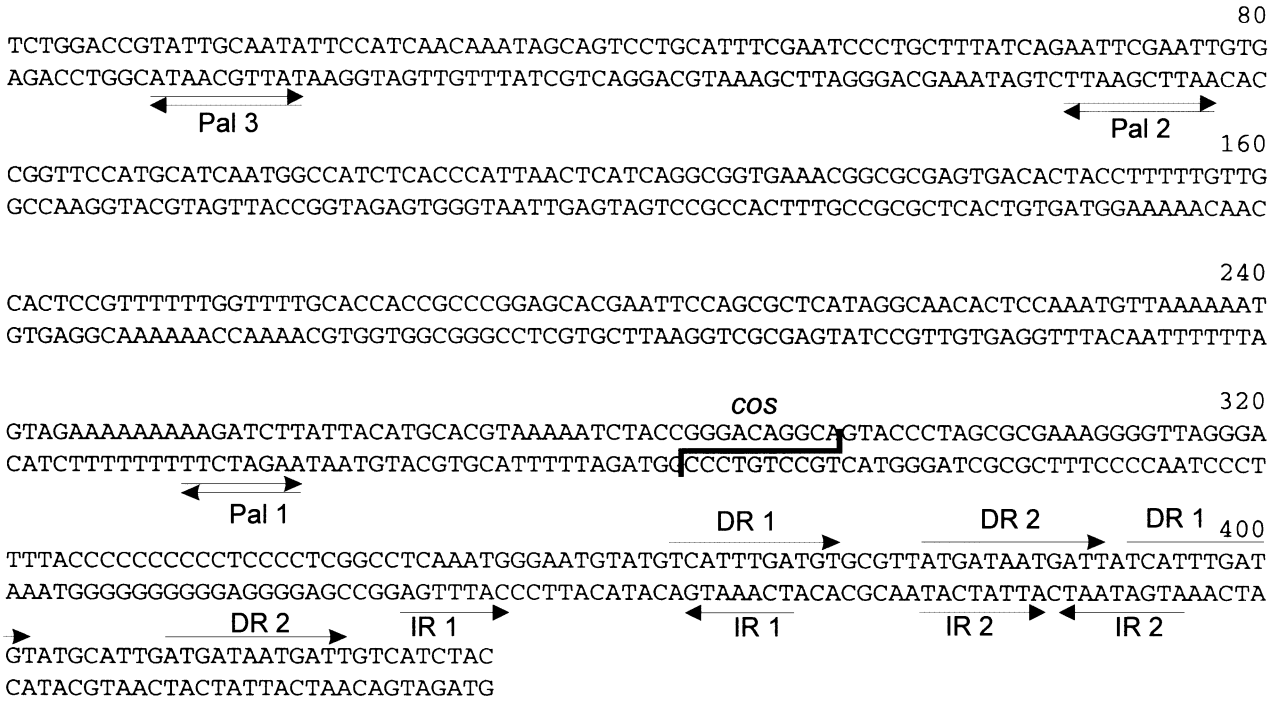

Fig. 4. Nucleotide sequence of the *cos* region (AJ348843). Only the relevant section is shown. Arrows indicate direct (DR) and inverted (IR) repeats. Palindromes are abbreviated as Pal. Positions of the cleavage sites creating the cohesive ends are indicated by vertical bars.

Table 1. Nucleotide sequences of primers used in this study.

Primer	Nucleotide sequence	Target sequence
Cos-F	5′-GCTTCTAACACGGCTTGTTGG-3′	*Cos* region
Cos-R	5′-GTAGGCCACCCTTCAAATAGG-3′	*Cos* region
Tel-Long	5′-CAATCAAATAAAGCCAATTAAAGTAACCCATACAATCATATAAATGCA-CATTGAAATCTATTCACACATTG-3′	Right plasmid end
Pal-850	5′-CGTAACCCATTCCAACACACAAATTAAAGTAACCCAATCAACCACC-3′	Left plasmid end
Tel-F1-2	5′-GCGGATCCGTGATAGTGTGAATGGGTTAC-3′	Protelomerase gene
Tel-R1-2	5′-GCAGGATGAGAAAGCTTTTGATGACATTCGGTTCCCTG-3′	Protelomerase gene
8del-F	5′-GCTAACTCTAGCAACAAGC-3′	42 bp palindrome flanking region
8del-R	5′-AGTATGCTGAAATAGGTGAC-3′	42 bp palindrome
Tel-1	5′-CCGAGTTTTGTCACCCTGCG-3′	42 bp palindrome flanking region
PalIRF	5′-AATGGATCCAATTAAAGTAACCCATACAATCAT	42 bp palindrome flanking region
PalIRR	5′-GGCAAGCTTAATTAAAGTAACCCAATCAACCAC	42 bp palindrome flanking region

These data demonstrated that the phage genome contains 3′-protruding ends of 10 nucleotides (5′-GGGACAGGCA-3′ and 5′-TGCCTGTCCC-3′), which were removed by the 3′ to 5′ proofreading exonucleolytic activity of the DNA polymerase I. The location of the *cos* site on the 700 bp *Dra*I fragment indicates that the fragment can be resolved by heating into two fragments of about 500 bp and 200 bp. These fragments could not be detected easily on agarose gels because other *Dra*I restriction fragments similar in size were present in the digests.

The analysis of the *cos* region revealed a number of direct repeats, inverted repeats and palindromes located on both sides of the *cos* site (Fig. 4). In order to elucidate whether the *cos* region allows *in vivo* packaging and subsequent transduction of a plasmid by PY54, we cloned several restriction fragments with or without the *cos* site in *E. coli* using the vector pIV2 that contains a kanamycin resistance gene. The plasmids pSH35 and pSH36 harboured the 700 bp *Dra*I fragment and a larger 3.4 kb *Bam*HI–*Nco*I fragment containing the *cos* region respectively (Fig. 3). Plasmid pSH37 was used as a control plasmid containing a 1 kb *Eco*RI restriction fragment of the phage located adjacent to the *cos* site. The plasmids were introduced into the *Y. enterocolitica* strains 29807/6 and 83/88 by transformation. After lysogenization by PY54, the strains were induced with mitomycin C, and the lysates were used for transduction of the plasmids into the strains 29807/6 and 83/88 and into their lysogenic derivatives harbouring PY54. We determined nearly the same transduction frequencies (10^{-4}–$10^{-5)}$ for all donor strains. The lysogenic recipient strains yielded transduction frequencies approximately one order of magnitude higher than the non-lysogenic strains. The vector pIV2 was not transduced by PY54. We also studied encapsidation of plasmids during the lytic cycle, but the determined transduction frequencies were even lower than those obtained by induction of lysogenic strains (data not shown). Thus, the presence of the PY54 *cos* region on the plasmids pSH35 and pSH36 did not result in a more efficient transduction than a restriction fragment of the phage without the *cos* site.

The ends of plasmid pY54 are covalently closed hairpins

In order to characterize the ends of the plasmid, attempts were made to clone the terminal 850 bp and 150 bp *Dra*I fragments of pY54. These proved unsuccessful, so the homologous 1 kb *Dra*I fragment of the phage genome was cloned in *E. coli* using the vector pLitmus28. The sequence analysis of the insert of plasmid pSH8 containing the 1 kb *Dra*I fragment exhibited part of an open reading frame (ORF) and a perfect 42 bp palindrome upstream from the ORF. The ORF showed significant homology to the gene *telN* (AF064539) of *E. coli* phage N15, which encodes the protelomerase TelN (data not shown). This enzyme converts the circular N15 phage genome into the linear plasmid prophage (Deneke *et al*., 2000). To elucidate whether phage PY54 contains a complete protelomerase-like gene, a 2.2 kb *Hpa*I fragment overlapping with the insert of pSH8 was cloned and sequenced (pSH82). With this, the complete sequence of the ORF was determined (AJ348844). The alignment with the *telN* gene of phage N15 revealed that the genes are closely related and have nearly the same size. The protelomerase gene of phage PY54 encodes a protein of about 72 kDa with an overall similarity of about 60% to TelN (Fig. 5). Two possible start codons for methionine were detected, one of them downstream from a putative ribosome binding site and possible promoter sequences (Fig. 6A). The 42 bp palindrome that we found 152 bp upstream from the putative start codon of the protelomerase gene revealed partial homology to the 56 bp palindrome (*telRL*), which is located at nearly the same distance from the *telN* gene of N15. Ten nucleotides (5′-TACGCGCGTA-3′) in the middle of the palindromes are identical. The 56 bp palindrome of N15 is the substrate for TelN that has cleaving–joining activity and generates covalently closed hairpin structures (telomeres), the ends of the N15 plasmid prophage. The 42 bp palindrome of

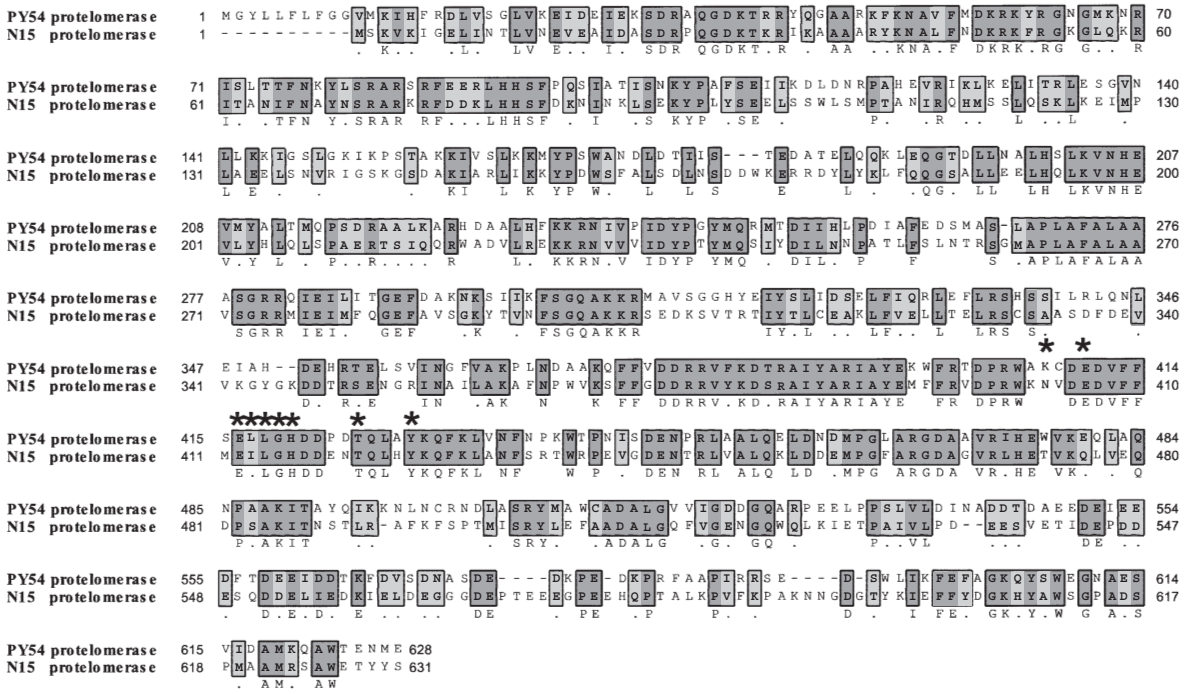

Fig. 5. Alignment of the PY54 and N15 protelomerases. Asterisks indicate amino acids belonging to a motif (bxxaxxxxxahLGHxxxxTxxxY; b, basic residue; a, acidic residue; h, hydrophobic residue) that was suggested to be part of the active centre of the N15 protelomerase (Deneke *et al*., 2000).

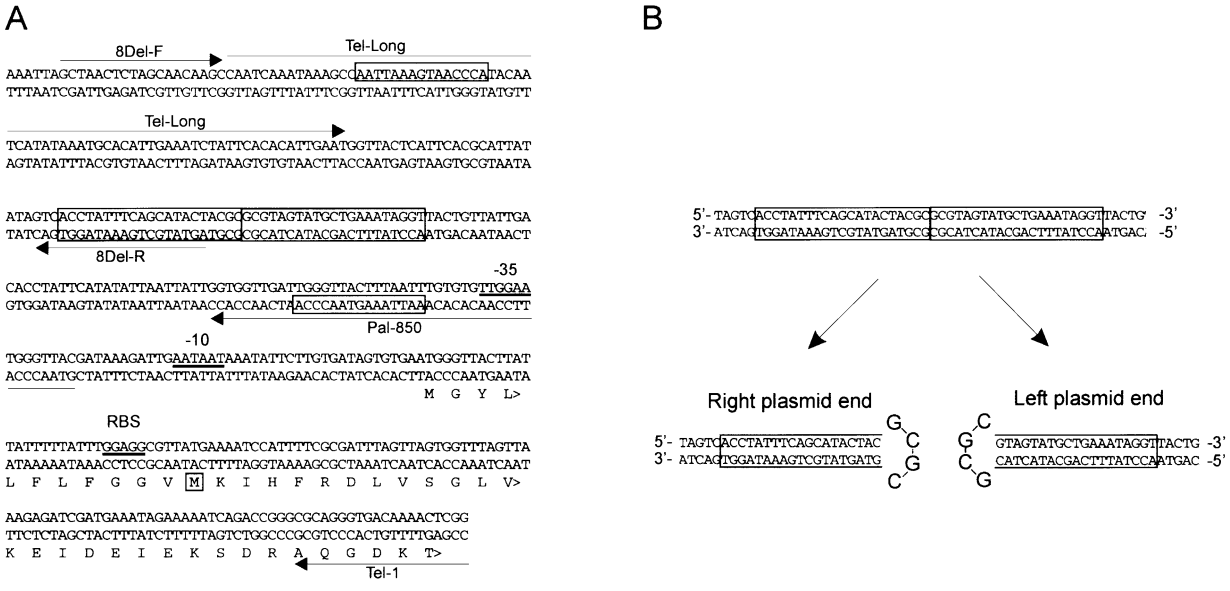

Fig. 6. Determination of the plasmid pY54 end sequences.
A. Nucleotide sequence of the phage PY54 DNA region upstream from the *tel* gene (AJ348844). The 42 bp palindrome, its flanking inverted repeat and the putative start codon are boxed. The possible ribosome binding site (RBS) and promoter sequences are underlined. Arrows indicate primers used for PCR and sequencing.
B. DNA sequences of the right and left plasmid ends.

PY54 is located on a 1 kb *Dra*I fragment of the phage DNA. Cleavage of the palindrome would yield two *Dra*I fragments of about 850 bp and 150 bp, which corresponds to the sizes of the terminal *Dra*I fragments of plasmid pY54.

The covalently closed ends of plasmid pY54 were detected in digests of the plasmid analysed on non-denaturing and alkaline agarose gels. To simplify the analysis, this study was also performed with the linear miniplasmid derivative pSH100 (see below), which yields a smaller number of fragments. The miniplasmid pSH100 has a size of about 21 kb and comprises the left and right *Eco*52I fragments of pY54 (Fig. 3). The comparison of the *Dra*I restriction patterns of the plasmids pY54 and pSH100 demonstrated that the left terminal 850 bp fragment was missing under denaturing conditions. Instead, a 1.7 kb fragment appeared on alkaline agarose gels. In contrast, the other *Dra*I fragments of the digests exhibited identical sizes under non-denaturing and denaturing conditions (Fig. 7A and B). This finding indicated that the left terminal *Dra*I fragment of the plasmids has twice the size under denaturing conditions. As the gel electrophoretic analysis of the right terminal 150 bp *Dra*I fragment did not give satisfactory results, we also examined the *Eco*32I digest of the plasmid pSH100. The terminal *Eco*32I fragments of pSH100 are 4.7 kb and 3.3 kb in size. Only these fragments were shifted under denaturing conditions and displayed a size of 9.4 kb and 6.6 kb (Fig. 7C and D). The same results were obtained when the digested plasmids were incubated with proteinase K, suggesting that the ends of the plasmids are not protected by protein, but contain an additional covalent linkage that might be part of a hairpin-like structure.

The hairpin structure was verified by the determination of the plasmid end sequences. For this procedure, the terminal 850 bp *Dra*I fragment (left end) and the terminal 1.1 kb *Xho*I fragment (right end) were isolated. The primers used for sequencing had a length of 71 bp (Tel-Long) and 46 bp (Pal-850) and matched about 30 bp upstream and downstream from the palindrome respectively (Table 1 and Fig. 6A). The sequence analysis revealed that both plasmid end fragments comprise half the palindrome and create terminal loops (Fig. 6B). Each double-stranded terminal fragment could be sequenced as a continuous single-stranded hairpin. In contrast to the N15 telomeres, the PY54 hairpins consist of identical sequences because the 42 bp palindrome is perfect without any mismatches.

The 42 bp palindrome is the target for the PY54 protelomerase

In order to elucidate whether the PY54 protelomerase is

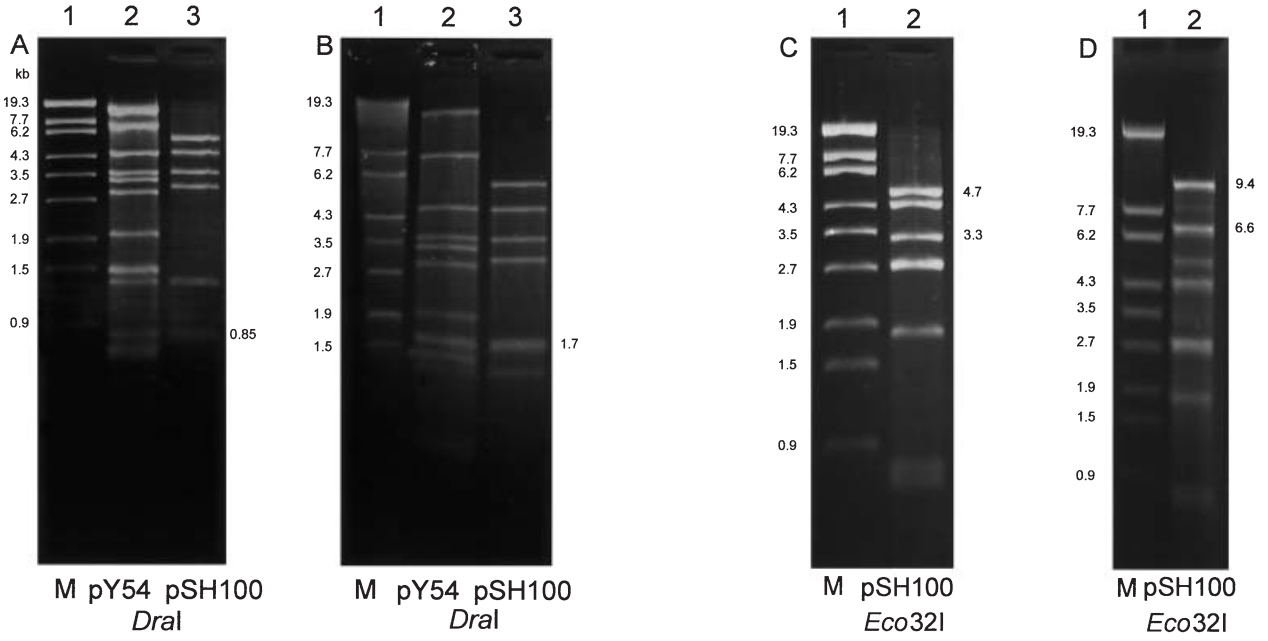

Fig. 7. Detection of the covalently closed end fragments of the plasmids pY54 and pSH100 (linear miniplasmid derivative, Fig. 3). M, marker (λ *Eco*130I).
A. *Dra*I restriction patterns on a non-denaturing agarose gel.
B. *Dra*I restriction patterns on an alkaline agarose gel.
C. *Eco*32I restriction pattern of pSH100 on a non-denaturing agarose gel.
D. *Eco*32I restriction pattern of pSH100 on an alkaline agarose gel. Restriction fragments containing the covalently closed ends of the plasmids are indicated.

involved in the generation of the terminal hairpins of the plasmid prophage, the activity of the enzyme was investigated in detail. The *telN*-like gene was amplified by PCR, cloned and expressed in *E. coli* K-12 using the inducible vector pMS119EH, creating pSH101. The primers Tel-F1-2 and Tel-R1-2 (Table 1), which were designed for the PCR, encompassed the whole ORF including the putative ribosome binding site. After cloning and sequencing, gene expression was induced with IPTG, and whole-cell extracts were analysed by SDS-PAGE. Upon induction, a protein of about 77 kDa was detected in bacteria containing plasmid pSH101 which was absent under non-induced conditions (Fig. 8A). The size of 77 kDa is close to the expected size of 72 kDa calculated from the amino acid sequence of the protein.

Enzyme preparations suitable for an *in vitro* assay were obtained by partial purification of the protelomerase (data not shown). The purification procedure was performed as described previously for TelN (Deneke *et al*., 2000). The substrates for the activity assay comprised PY54 DNA fragments cloned in pBR329 that carried the complete or partial 42 bp palindrome (Fig. 8B). After incubation with the enzyme preparation, the recombinant plasmids were analysed on 0.8% agarose gels. The data demonstrated that the plasmids pJD107 and pSH104, which harboured the 42 bp palindrome and a 407 bp DNA fragment including the palindrome, respectively, were processed by the PY54 protelomerase. In contrast, plasmid pSH104-1, containing only part of the palindrome on a 137 bp fragment, was not processed (Fig. 8C). The results indicate that the 42 bp palindrome is the target for the PY54 protelomerase under *in vitro* conditions.

We suspect that, under *in vivo* conditions, the protelomerase is active after the phage DNA has entered the bacterial cell and circularized by annealing of the cohesive ends. The subsequent processing of the circle at the target sequence, which is located opposite to the *cos* region, would convert the circular phage DNA to the linear plasmid. We therefore studied whether the processing of the substrates used in the *in vitro* assay can also be observed in infected bacteria. The recombinant plasmids were introduced into the non-pathogenic *Y. enterocolitica* O:5 strain 29807/6 and the pathogenic O:5,27 strain 83/88. Both strains are susceptible to PY54 and were lysogenized with a phage mutant (C) containing a kanamycin resistance gene (S. Hertwig, I. Klein, V. Schmidt, S. Beck, J. A. Hammerl and B. Appel, unpublished). The lysogenized bacteria were plated on agar containing kanamycin. Lysogens were isolated and plated on agar containing chloramphenicol. Strains harbouring plasmid pSH104 became sensitive to chloramphenicol upon infection with PY54. The analysis of the plasmid content confirmed that both *Yersinia* strains had lost pSH104. In contrast, strains containing pBR329, pSH104-1 or pJD107 retained the plasmids upon infection. This result was a little surprising, as pSH104 and pJD107 were both efficient substrates for the protelomerase under *in vitro* conditions. The detailed analysis of the upstream region of the PY54 *telN*-like gene revealed an additional 15 bp inverted repeat flanking the 42 bp palindrome (Fig. 6). To elucidate whether this repetitive sequence might affect the processing of the palindrome under *in vivo* conditions, we cloned the 188 bp DNA fragment containing the palindrome plus the flanking inverted repeat using pBR329 (see *Experimental procedures*). Upon introduction of the resulting plasmid pSH105 into *Yersinia* and subsequent lysogenization by PY54, this plasmid was lost. To examine whether the loss of plasmid pSH105 was initiated by telomere resolution, the lysogenized *Yersinia* strain 29807/6 was analysed after different times of infection. A significant processing of plasmid pSH105 was already visible after 10 min of infection. After 6 h, most of the supercoiled plasmid DNA was converted to the linear form (Fig. 9, lane 17), whereas no conversion was detected in the non-infected strain (Fig. 9, lanes 12–14). Moreover, the amount of total plasmid DNA isolated from the infected strain was lower than that of the non-infected strain. The difference in plasmid content was not caused by lysis of the infected strain because we used a phage mutant unable to lyse its host (S. Hertwig, I. Klein, V. Schmidt, S. Beck, J. A. Hammerl and B. Appel, unpublished), and both strains showed nearly identical growth characteristics. Hence, it is more probable that the divergent plasmid yields reflect the loss of pSH105 caused by linearization. It is very likely that the processed plasmid cannot be replicated or is unstable, which would consequently result in plasmid loss. We also analysed plasmid pJD107 in *Yersinia*. In contrast to pSH105, we could not observe any processing of pJD107 (Fig. 9, lanes 9–11). The *in vivo* results suggest that the loss of plasmid pSH105 containing the palindrome on a 188 bp DNA fragment of PY54 was initiated by processing. In the *in vitro* assays, plasmid pSH105 was not processed more efficiently by the purified protelomerase than by pJD107 (data not shown). Taking the *in vitro* and *in vivo* data together, we assume that the 42 bp palindrome is the target for the PY54 protelomerase, but that the flanking inverted repeat is necessary for efficient processing under *in vivo* conditions.

The DNA region essential for plasmid maintenance maps on a 4.5 kb fragment

The region of the PY54 genome important for plasmid maintenance was identified by the construction of linear and circular miniplasmid derivatives of pY54. In a first experiment, we examined whether pY54 is capable of

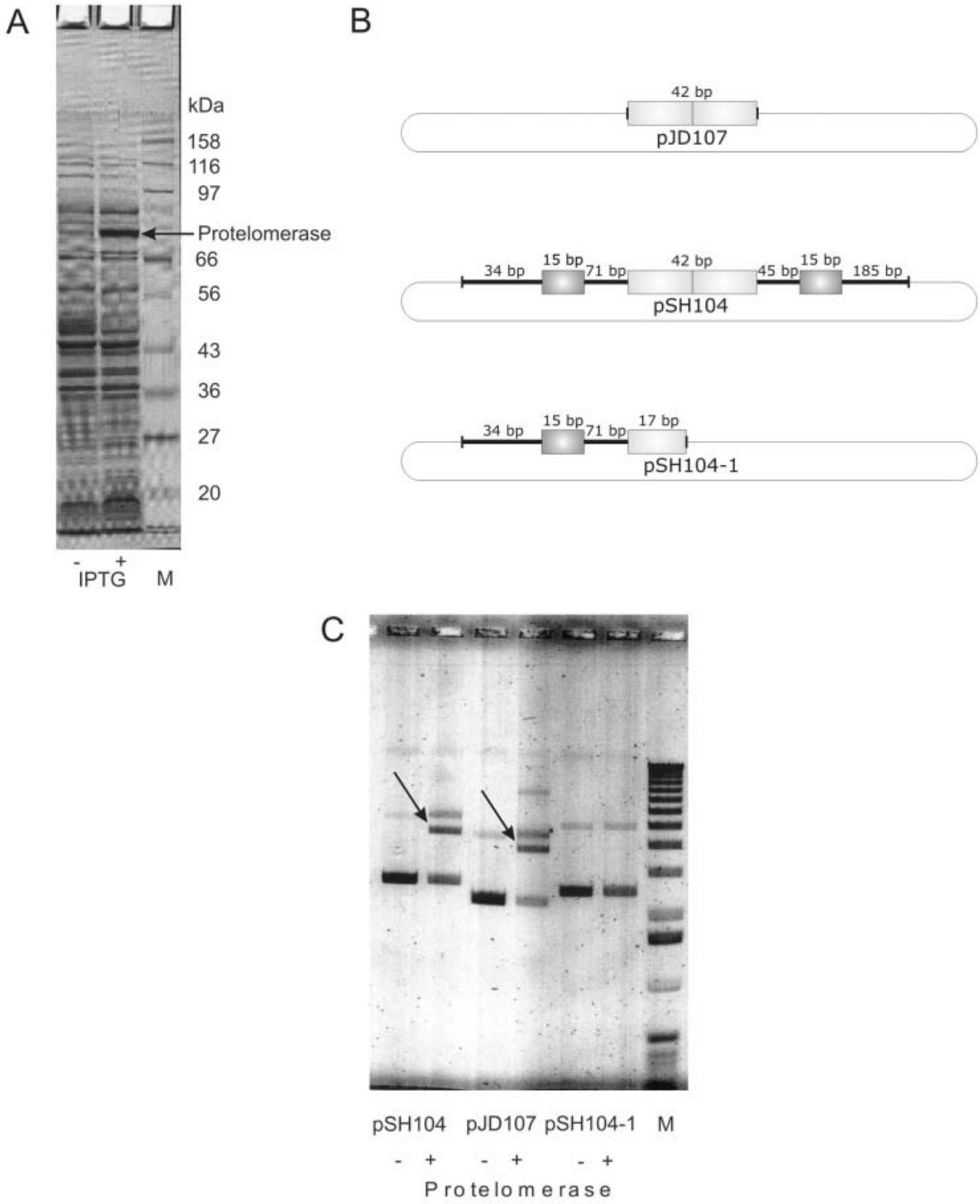

Fig. 8. Overexpression of the PY54 *tel* gene and activity of the protelomerase.
A. Polyacrylamide gel electrophoresis of the protelomerase of IPTG-induced SCS1 (pSH101) cells. M, marker (New England Biolabs).
B. Scheme of the inserts of the plasmids pJD107, pSH104 and pSH104-1 used as substrates for the PY54 protelomerase. Plasmid pJD107 contains the 42 bp palindrome. Plasmid pSH104 contains a 407 bp DNA fragment of the phage DNA including the complete 42 bp palindrome and a flanking 15 bp inverted repeat. Plasmid pSH104-1 contains a 137 bp DNA fragment of the phage DNA including part of the palindrome.
C. Activity assay for the partially purified protelomerase. M, marker (1 kb ladder). Arrows indicate the position of linearized plasmids.

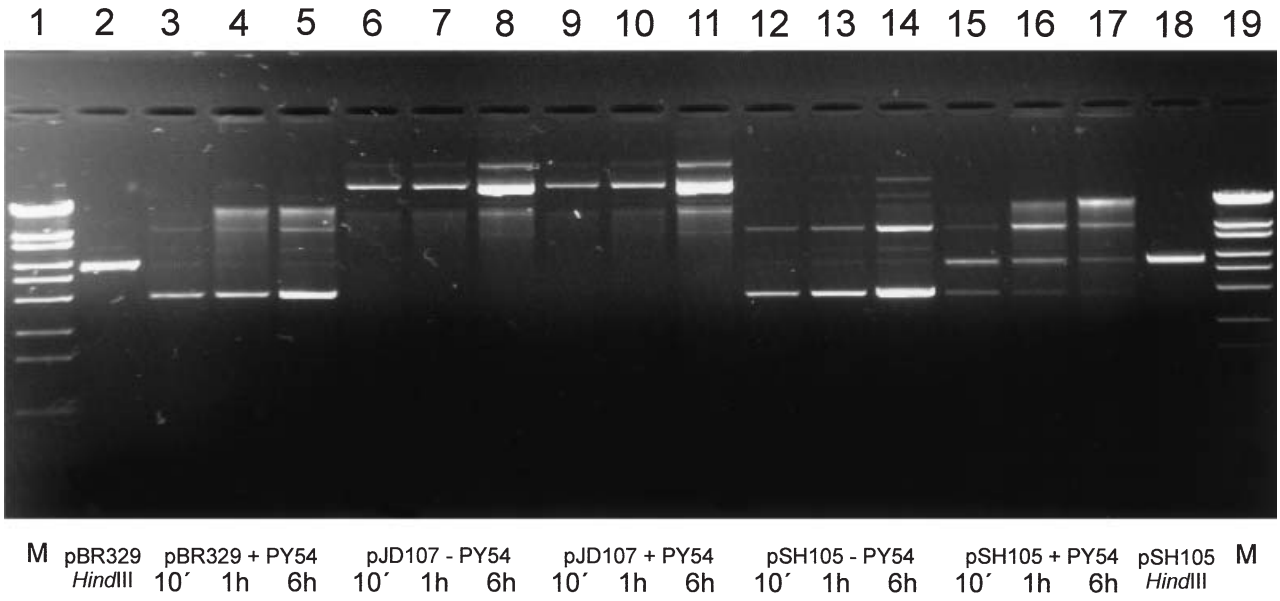

Fig. 9. *In vivo* linearization of plasmid pSH105 by phage PY54. Lanes 1 and 19, marker (λ *Eco*130I); lane 2, pBR329 digested with *Hin*dIII; lanes 3–5, pBR329 isolated from an infected *Yersinia* strain; lanes 6–8, pJD107 isolated from a non-infected *Yersinia* strain; lanes 9–11, pJD107 isolated from an infected *Yersinia* strain; lanes 12–14, pSH105 isolated from a non-infected *Yersinia* strain; lanes 15–17, pSH105 isolated from an infected *Yersinia* strain; lane 18, pSH105 digested with *Hin*dIII. Plasmids were analysed after 10 min, 1 h or 6 h of infection.

replicating in *E. coli* DH5α. For this purpose, the bacteria were transformed with circular phage DNA (mutant I; S. Hertwig, I. Klein, V. Schmidt, S. Beck, J. A. Hammerl and B. Appel, unpublished) obtained by treatment with T4 ligase before transformation. After 2 days of incubation, kanamycin-resistant colonies appeared on agar plates, which were found to harbour the linear plasmid prophage. This proved that, upon transformation of *E. coli*, the circular phage DNA was converted to the linear plasmid. However, we were unable to induce PY54 phages in *E. coli* DH5α. To isolate smaller derivatives of pY54, the phage DNA was digested with various restriction endonucleases, religated and used for transformation of *E. coli.* The first isolated miniplasmid pSH100 has a size of 21 kb (including the marker gene) and was isolated after digesting the phage DNA with *Eco*52I. A similar miniplasmid of 16.5 kb (pSH95) was isolated by cleaving the phage DNA with *Kpn*I (Fig. 3). The miniplasmids pSH95 and pSH100 were propagated in bacteria as linear molecules, similar to pY54. The existence of covalently closed end fragments could be demonstrated on alkaline agarose gels (Fig. 7). Furthermore, the analysis of the linear miniplasmids by electron microscopy revealed single-stranded circular DNA with exactly twice the length under denaturing conditions (Fig. 2C). The size of pSH100 was reduced further by digestion with *Nhe*I yielding a circular plasmid of 8 kb retaining replicative competence. In this plasmid, termed pSH110, the 42 bp palindrome and 5′ portion of the protelomerase gene are lacking. The smallest replicative miniplasmid that we could isolate so far was obtained by digestion of plasmid pSH110 with the restriction enzymes *Bpi*I and *Mbi*I. The resulting plasmid pSH120 has a size of 6 kb, of which about 1.5 kb originate from the transposon vector pUTKm. Figure 3 shows the positions of all isolated miniplasmids.

Discussion

Despite the fact that, at the lysogenic stage, the genomes of temperate bacteriophages are generally integrated into the bacterial chromosome, there are some prophages that replicate as plasmids, e.g. the *E. coli* phages P1 (Ikeda and Tomizawa, 1968), P4 (Briani *et al*., 2001) and N15 (Ravin and Shulga, 1970), phage cp32 from *Borrelia burgdorferi* (Eggers and Samuels, 1999) and the *Leptospira biflexa* phage LE1 (Saint Girons *et al*., 2000). Unlike the other aforementioned phages, the N15 prophage is unique in that it is a linear plasmid with covalently closed ends (Rybchin and Svarchevsky, 1999; Ravin *et al*., 2000). Linear plasmids have mostly been found in actinomycetes, particularly in the genus *Streptomyces* (Hirochika *et al*., 1984; Kinashi *et al*., 1991; Kalkus *et al*., 1998; Polo *et al*., 1998). However, there are also a number of reports describing linear plasmids in *Borrelia* (Casjens *et al*., 2000), *Mycobacterium* (Picardeau and Vincent, 1998) and even in a fungus (Katsura *et al*., 2001). Common features of linear plasmids of actinomycetes and mycobacteria are terminal inverted repeats and terminal

proteins bound to the 5′ ends, also described for the genomes of *Pseudomonas* phage PRD1 (Savilahti and Bamford, 1993) and *Bacillus subtilis* phage ϕ29 (reviewed by Salas, 1991). In contrast, linear plasmids of *Borrelia* have covalently closed terminal hairpin telomeres (Hinnebusch and Barbour, 1991; Chaconas *et al*., 2001). Similar structures have been found in phage N15, the first linear plasmid prophage in prokaryotes and, up to now, the only known linear plasmid in *E. coli* (Malinin *et al*., 1992).

The characterization of the *Yersinia* phage PY54 in this study revealed some quite interesting similarities to and differences from N15. Both phages have a lambda-like morphology and a genome of about 46 kb composed of double-stranded DNA. However, N15 was reported to be closely related to λ (Ravin and Shulga, 1970), whereas the PY54 DNA showed no cross-hybridization with λ (Popp *et al*., 2000), suggesting that these phages are not cognate. The different relationship to λ was also confirmed by the comparison of the cohesive ends of the phage DNAs. The mature genomes of N15 and λ have 5′-protruding cohesive ends, of which 10 out of 12 nucleotides are identical (Ravin *et al*., 1998). The mature phage PY54 genome has 3′-protruding cohesive ends. This is rather unusual for phages of Gram-negative bacteria, which mostly possess 5′ overhanging ends. In the vicinity of the *cos* site of phage PY54, a number of repeated sequences were detected. Inverted repeats are frequently found close to the *cos* sites of phages (Kinner *et al*., 1994). They may serve as recognition sequences for DNA-binding proteins, e.g. terminases. Our transduction studies could not demonstrate that the 700 bp *Dra*I fragment of PY54 containing the *cos* site is an efficient DNA packaging signal. The same is true for a larger PY54 restriction fragment enclosing this *Dra*I fragment. The transduction frequencies determined for the recombinant plasmids were not significantly higher than those obtained with another cloned restriction fragment of the phage. Hence, transduction was apparently not achieved by *cos*-dependent encapsidation, but by rare recombination between the phage and the plasmids, as already found with phage P22 (Schmidt and Schmieger, 1984). Using lysogenic recipient strains, higher transduction frequencies were observed, probably by the protection of these strains from lysis by the prophage repressor. Interestingly, 700 bp restriction fragments of the phages RP3 and D3 containing the *cos* sites were sufficient for high transduction frequencies of cosmid derivatives (Kinner *et al*., 1994; Sharp *et al*., 1996). Similar to PY54, the *cos* regions of these phages harbour repetitive DNA sequences. However, the determined transduction frequencies were several orders of magnitude higher than those of PY54. It is conceivable that the recombinant plasmids that we have used for transduction studies do not have the optimal size for encapsidation. For phage λ and the actinophage ϕC31, it has been shown that the DNA to be encapsidated into the phage head must have a minimal length of around 80% of the genome (Harris *et al*., 1983).

Despite the differences, the prophages PY54 and N15 share the uncommon property of replicating as linear plasmids. Moreover, analysis of the DNA regions of the phages that account for this trait revealed striking similarities. Both phages have a genome ≈ 50% circularly permuted to the respective plasmid. This means that the junction of the cohesive ends of the phages and the junction of the plasmid ends are located nearly in the middle of the linear plasmid map and phage map respectively. The *telN* gene of N15 and the homologous gene of PY54 are located at the left ends of the plasmids. In addition, the palindromes located upstream from the protelomerase genes exhibit partial homology and are located at nearly the same distance (PY54, 152 bp; N15, 164 bp) from the putative start codons of the *tel* genes. This coincidence was verified by comparison of the plasmid end structures. Our results demonstrate that the plasmid pY54 contains ends that are covalently closed. Furthermore, the obtained sequencing data indicate that the 42 bp palindrome on the phage genome is essential for the conversion of the phage DNA to the linear plasmid. The conversion results in two identical terminal hairpins, each composed of half the palindrome. From our data, it can be expected that the mechanism by which the conversion occurs is through cleavage of the palindrome followed by joining of the cleaved DNA strands, as already demonstrated for phage N15 (Deneke *et al*., 2000). Most likely, this can be achieved by staggered nicking at the ends of the palindrome, generating overhanging DNA strands that are self-complementary and able to snap back (Rybchin and Svarchevsky, 1999). In PY54, this process might be favoured by a 10 bp alternating Pyr/Pur sequence in the centre of the palindrome, which could be a potential region of Z-DNA structure. The same sequence is present in the middle of the N15 palindrome and harbours a central $(CG)_3$ essential for processing (Deneke *et al*., 2002). The exact cleavage sites on the PY54 and N15 palindromes have still to be elucidated. The assay performed with the partially purified PY54 protelomerase demonstrated that, under *in vitro* conditions, the 42 bp palindrome functions as a substrate for the enzyme. However, the *in vivo* assay revealed that, upon infection, only plasmid pSH105 containing the palindrome plus adjacent DNA sequences of the phage genome was processed and then lost. It is very likely that the processing of this plasmid was caused by the protelomerase, which points to the possibility that the PY54 palindrome is the target for the protelomerase, but that the maximum activity of the enzyme under *in vivo* conditions requires a substrate that comprises flanking DNA sequences. These additional sequences might be important for the efficient binding of

the enzyme. In N15, the 56 bp palindrome is the target for TelN under *in vitro* conditions. It has been demonstrated that two TelN molecules bind to *telRL*, but that additional sequences of the *tos* (telomerase occupancy site) stabilize the TelN–target complexes (Deneke *et al*., 2002). *Tos* is composed of a series of inverted repeats centred on the 56 bp palindrome *telRL*. In contrast to N15, the palindromic sequence of PY54 is flanked by only one inverted repeat. Another discrepancy between the phages is that the 42 bp palindrome of PY54 is perfect, whereas the 56 bp sequence of N15 has two mismatches. These differences might be significant for the substrate specificities of the protelomerases. However, the mechanisms of PY54 and N15 target sequence processing by the protelomerases of the phages are apparently similar. The N15 protelomerase contains a sequence motif at the C-terminus thought to belong to the active centre of the enzyme (Deneke *et al*., 2000). This motif can also be identified in several integrases and was found in this study to be present at the C-terminus of the PY54 protelomerase (Fig. 5).

By transposon mutagenesis, we have isolated a number of phage mutants containing a kanamycin resistance gene (S. Hertwig, I. Klein, V. Schmidt, S. Beck, J.A. Hammerl and B. Appel, unpublished). One of the non-defective mutants was used for the isolation of miniplasmid derivatives of pY54. The plasmid prophage and the miniplasmid derivatives are able to replicate in *E. coli* DH5α, but we could not isolate any phages upon induction with mitomycin C. These data suggest that *Yersinia*-specific factors are necessary for a phage release. In λ, a cellular factor (FIS) is a component of the site-specific recombination pathway that stimulates excision of the prophage significantly (Thompson *et al*., 1987). The miniplasmids pSH95 and pSH100 contain the complete *tel* gene. By electron microscopy, comparison of restriction patterns and DNA sequencing, it became evident that these miniplasmids are linear molecules with terminal hairpins, like the whole plasmid prophage. It is very likely that the PY54 protelomerase is essential for the replication of the linear plasmids. Ravin *et al*. (2001) demonstrated that the N15 protelomerase is necessary for replication of the linear prophage. TelN is an end-resolving enzyme responsible for processing of replicative intermediates. These authors also showed that TelN and *telRL* are sufficient to convert some circular plasmids (mini-F, mini-P1) into linear plasmids with covalently closed ends.

In order to confine the DNA region essential for PY54 plasmid replication, the size of plasmid pSH100 was reduced by restriction and ligation steps. By this procedure, circular miniplasmids were isolated in which the *tel* gene and the palindromic sequence were deleted. The smallest miniplasmid comprises 4.5 kb of the phage genome. Interestingly, similar-sized miniplasmids have been derived from the N15 plasmid (Rybchin and Svarchevsky, 1999). As in PY54, the smallest linear miniplasmid of N15 contains the *telN* gene and the terminal hairpins. In addition, the partition locus *par* is essential for the maintenance of the linear N15 plasmids (Sankova *et al*., 1992; Ravin and Lane, 1999). The minimal circular N15 plasmid has a size of 5.2 kb and mainly consists of one ORF (*repA*) encoding a replication protein. The comparison of the published map of the N15 plasmid (Rybchin and Svarchevsky, 1999) with the map of pY54 including the positions of the miniplasmids of both phages displays striking resemblances. Analysis of the PY54 DNA sequence confirmed that the miniplasmids of both phages are composed of similar sets of genes (S. Hertwig, I. Klein, V. Schmidt, S. Beck, J.A. Hammerl and B. Appel, unpublished). The analysis showed that both phages contain related gene clusters comprising DNA sequences necessary for plasmid replication and maintenance, whereas other genes important for phage propagation are not related. It can be suggested that, from an evolutionary point of view, the DNA region responsible for the unusual property of linear plasmid prophage replication was acquired by gene exchange between otherwise unrelated phages or by independent recombination events between the phage genomes and plasmids. In conclusion, this study demonstrates that linear plasmid prophages are not limited to *E. coli.* The fact that, in contrast to N15, phage PY54 is not related to phage λ indicates that these phages do not share a common ancestor.

Experimental procedures

Bacterial strains, growth conditions and plasmids

Escherichia coli DH5α [F^- ϕ80 d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^- m_K^+) *pho*A *sup*E44 l^- *thi*1 *gyr*A96 *rel*A1; Woodcock *et al*., 1989] was used as the plasmid host for most of the cloning procedures. Phage PY54 was propagated on the *Y. enterocolitica* strains 29807/6 (serogroup O:5, biogroup 1A, plasmid-cured derivative of 29807; Strauch *et al*., 2000) and 83/88 (serogroup O:5,27, biogroup 2; strain collection of the Robert-Koch-Institut). Strains were grown in Luria–Bertani broth (LB). The *E. coli* and *Y. enterocolitica* strains were incubated at 37°C and 28°C respectively. Solid and soft agar media contained 1.8% and 0.7% (w/v) agar respectively. When required, ampicillin and neomycin were supplemented at 100 μg ml^{-1} and chloramphenicol and tetracycline at 12.5 μg ml^{-1}. Vectors used for molecular cloning were pUC18 (Ap^r; Norrander *et al*., 1983), pLitmus28 (Ap^r; New England Biolabs), pBR329 (Ap^r, Cm^r, Tc^r; Covarrubias and Bolivar, 1982) and pMS119EH (Ap^r; Strack *et al*., 1992).

Isolation of phage DNA and plasmids

PY54 was propagated by the soft agar overlay method

(Sambrook and Russell, 2001). Phages were purified by caesium chloride step gradients, and phage DNA was isolated as described previously (Hertwig *et al*., 1999). Plasmids were isolated from *E. coli* and *Y. enterocolitica* by the alkaline lysis procedure (Birnboim and Doly, 1979). For the extraction of the low-copy-number plasmid pY54 and its miniplasmid derivatives, 10 ml or 500 ml cultures were used.

Electron microscopy

Double-stranded DNA samples were prepared by the microdiffusion technique according to the method of Lang and Mitani (1970). To demonstrate the covalently closed ends of the linear plasmid prophage DNA by electron microscopy, the DNA was denatured and spread on a water surface in the presence of cytochrome *c* as described previously (Deneke *et al*., 2000). Samples were examined using a Philips CM 100 electron microscope.

DNA manipulations and sequence analysis

DNA manipulations (restriction digestions, ligations, generation of blunt-ended DNA) and transformations were performed using standard methods (Sambrook and Russell, 2001). Restriction and DNA-modifying enzymes were obtained from New England Biolabs or MBI Fermentas. DNA electrophoresis was carried out in agarose gels with TBE buffer (Sambrook and Russell, 2001). Under denaturing conditions, gels were run in 50 mM NaOH–1 mM EDTA (Fürste *et al*., 1989). Hybridization experiments were carried out overnight at 65°C in 2× SSC buffer (Sambrook and Russell, 2001) containing 5% (w/v) dextran sulphate, 0.1% (w/v) SDS and 0.5% (w/v) blocking reagent. For the labelling of DNA probes and the detection of hybridization signals by chemoluminescence, the Renaissance kit (DuPont NEN) was used. Oligonucleotides used for PCR and sequencing are listed in Table 1 and were purchased from Pharmacia Biotech and Metabion. DNA sequencing was conducted using the ABI *Taq* dye deoxy cycle sequencing kit and an ABI 373 DNA sequencer (PE Applied Biosystems). For the sequencing of the hairpin telomeres, plasmid fragments were incubated at 98°C for 5 min before adding the sequencing mix. Then, the DNA was denatured at 98°C for 5 s, annealed at 70–75°C for 5 s and extended at 60–65°C for 3 min.

Construction of plasmids

The *cos* region of the phage was cloned by inserting the 700 bp *Dra*I fragment of pY54 into the *Eco*32I site of pLitmus28 (pSH34). The insert of pSH34 was hybridized to several phage digests confirming that it contained the *cos* region. For the construction of pSH35 and pSH37, the 700 bp *Dra*I fragment and a 1 kb *Eco*RI fragment of PY54 were inserted into the *Eco*32I site and *Eco*RI site of pIV2 respectively (Strauch *et al*., 2000). Plasmid pSH36 was constructed by cloning the 3.4 kb *Bam*HI–*Nco*I restriction fragment into the corresponding sites of pLitmus28, digesting the recombinant plasmid with *Bam*HI and *Sac*I and inserting the fragment into the *Bam*HI–*Sac*I sites of pIV2. To determine the sequence of the terminal 850 bp and 150 bp *Dra*I fragments of pY54, including part of the protelomerase gene, the homologous 1 kb *Dra*I fragment of phage PY54 was inserted into the *Eco*32I site of pLitmus28, resulting in plasmid pSH8. The remaining sequence of the PY54 protelomerase gene was obtained by cloning a 2.2 kb *Hpa*I fragment in pUC18 (pSH82) that overlapped with the *Dra*I fragment. To clone the complete protelomerase gene in the inducible vector pMS119EH, the gene was amplified by PCR using the primers Tel-F1-2 and Tel-R1-2, which contained restriction sites for *Bam*HI and *Hin*dIII respectively (Table 1). The PCR product was inserted into the corresponding sites of the vector yielding plasmid pSH101. By sequencing, it was verified that the PCR product contained no mismatches. The substrates for the protelomerase were produced by cloning of PY54 DNA fragments in pBR329. For the construction of plasmid pJD107, both DNA strands of the 42 bp palindrome were synthesized. A *Bam*HI site was introduced at the 5′ end and a *Hin*dIII site at the 3′ end of the sequence. The oligonucleotides were annealed and inserted into the corresponding sites of pBR329. Plasmid pSH104 contained a 407 bp fragment including the palindrome that was obtained by PCR using the primers 8delF and Tel1 (Table 1, Fig. 6A). For the construction of the plasmid pSH104-1, the primers 8delF and 8delR (Table 1) were used, which yielded a PCR product of 137 bp containing part of the palindrome (Fig. 6A). These PCR products were inserted into the *Eco*32I site of pBR329. Plasmid pSH105 contained a 188 bp fragment including the palindrome and the flanking inverted repeat (Fig. 6A). The insert of this plasmid was obtained by PCR using the primers PalIRF and PalIRR (Table 1), in which a *Bam*HI site and a *Hin*dIII site was introduced at the 5′ end respectively. The digested PCR product was inserted into the corresponding sites of pBR329. All sequences of the constructs were verified.

Partial purification of the PY54 protelomerase and activity assays

The purification of the protelomerase and the *in vitro* assay were carried out as described for the N15 protelomerase (Deneke *et al*., 2000). Proteins were analysed by SDS-PAGE according to the method of Laemmli (1970). For the *in vivo* assay, the plasmids pBR329, pJD107, pSH104, pSH104-1 and pSH105 were introduced into the *Y. enterocolitica* strains 29807/6 and 83/88. The strains were infected with the phage mutant C, which had lost the lytic activity (S. Hertwig, I. Klein, V. Schmidt, S. Beck, J.A. Hammerl and B. Appel, unpublished). Lysogenic bacteria were selected on LB agar containing kanamycin and were then plated on agar containing chloramphenicol. In addition, the plasmid content of the infected bacteria was examined. To study the kinetics of the *in vivo* linearization of the plasmids, strains containing the plasmids pBR329, pJD107 or pSH105 were grown in LB broth containing chloramphenicol to an optical density (588 nm) of about 0.5. The strains were infected with phage mutant C at a multiplicity of infection of ≈ 1. At different time points (10 min, 1 h, 6 h) after infection, plasmids were isolated and analysed on 0.8% agarose gels.

Accession numbers

The nucleotide sequences of the PY54 *cos* region and pro-

telomerase gene have been submitted to the EMBL databank under the accession numbers AJ348843 and AJ348844 respectively.

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