Virology 407 (2010) 43–52

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Insights into the infective properties of YpfΦ, the *Yersinia pestis* filamentous phage

Iman Chouikha, Lucie Charrier [1], Sofia Filali, Anne Derbise, Elisabeth Carniel *

Yersinia Research Unit, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris cedex 15, France

ARTICLE INFO

Article history:
Received 3 May 2010
Returned to author for revision 17 June 2010
Accepted 30 July 2010
Available online 21 August 2010

Keywords:
Plague
Y. enterocolitica
Y. pseudotuberculosis
Transduction
Horizontal transfer
dif
CTXΦ
Antiqua
Medievalis
Orientalis

ABSTRACT

YpfΦ is a filamentous phage that infected *Yersinia pestis*, the plague bacillus, during its emergence. Using an experimental transduction approach, we show here that this phage has the capacity to infect with variable efficiencies, all three pathogenic *Yersinia* species as well as *Escherichia coli*. Like other *Inovirus* phages, its genetic organization comprises three functional modules necessary for the production of infectious virions. Upon infection, YpfΦ integrates into the chromosomal *dif* site, but extrachromosomal forms are also frequently observed. Several pieces of evidence suggest that the absence of chromosomal YpfΦ in natural non-Orientalis *Y. pestis* isolates results from a higher chromosomal excision rate rather than from a defective integration machinery. A resident YpfΦ confers some protection against a superinfection. In contrast to other filamentous phages, the incoming YpfΦ genome inserts itself between two copies of the resident prophage. This analysis thus unravels infective properties specific to YpfΦ.

Introduction

Filamentous bacteriophages of the genus *Inovirus* (*Inoviridae* family) infect almost exclusively Gram negative bacteria (Deng et al., 1999; Jacobson, 1972). Some of these filamentous phages are associated with bacterial virulence. The best studied is CTXΦ, that carries the *ctxAB* genes coding for cholera toxin, the most important virulence factor of *Vibrio cholerae* (Waldor and Mekalanos, 1996). This phage is integrated into the *V. cholerae* chromosome and is able to infect non-pathogenic environmental *Vibrio* strains, to form toxigenic lysogens (Davis and Waldor, 2003; Waldor and Mekalanos, 1996). Another filamentous phage of this genus (CUS-1) has been associated with the virulence of extra-intestinal *Escherichia coli* K1 strains (Gonzalez et al., 2002). A new *Inovirus* member, YpfΦ (for *Yersinia pestis* filamentous phage), has been recently described and characterized (Derbise et al., 2007). This filamentous phage, which shares almost the same backbone as CUS-1 (Gonzalez et al., 2002), infected the plague bacillus after it evolved from its ancestor, conferring on it an increased fitness during the infectious process, by an as yet unidentified mechanism.

The genome of *Inovirus* phages is organized in three putative functional domains (Model and Russel, 1988): (i) the replication module which contains the genes encoding the rolling-circle machinery of DNA replication and single-stranded DNA-binding proteins, (ii) the structural module which encodes the major and minor coat proteins, and the host recognition and adsorption protein, and (iii) the assembly/secretion module which is responsible for the extrusion of the phage particles (Marvin, 1998; Model and Russel, 1988). The 8.7 kb YpfΦ genome has a genetic organization which is similar to that of other *Inovirus* phages (Fig. 1). This genome encodes a functional phage since YpfΦ particles approximately 8 nm wide and 1,200 nm long that contain the (+) ssDNA of the circular phage genome are synthesized and secreted into the supernatant. Furthermore, these particles were shown to be infectious for a YpfΦ-negative *Y. pestis* isolate (Derbise et al., 2007). The YpfΦ prophage also carries at its extremities coding sequences (CDS) of unknown functions (Fig. 1), one of which (YPO2273) is similar to the transcriptional repressors RstR of CTXΦ (Davis et al., 2002; Waldor et al., 1997), and Vpf122 of Vf12 and Vf33 (Chang et al., 1998). The other flanking CDS have no similarities with known proteins.

The YpfΦ natural bacterial host, *Y. pestis*, is a Gram-negative bacterium that belongs to the genus *Yersinia* and to the family *Enterobacteriaceae*. This genus comprises three species that are pathogenic for humans and animals: *Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. While *Y. enterocolitica* and *Y. pseudotuberculosis* behave like true enteropathogens, i.e. they are transmitted by the fecal-oral route and cause intestinal symptoms of moderate intensity (Brubaker, 1991), *Y. pestis* is the etiologic agent of plague, a highly severe and often fatal disease transmitted by fleas. A phylogenetic analysis of the three pathogenic *Yersinia* species demonstrated that *Y. pseudotuberculosis* and *Y. enterocolitica* diverged from a common ancestor between 0.4

* Corresponding author. Fax: +33 1 45 68 89 54.
E-mail address: elisabeth.carniel@pasteur.fr (E. Carniel).
[1] Present address: Plague Unit and Plague central laboratory, Institut Pasteur of Madagascar, B.P. 1274 Ambatofotsikely, 101 Antananarivo, Madagascar.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.07.048

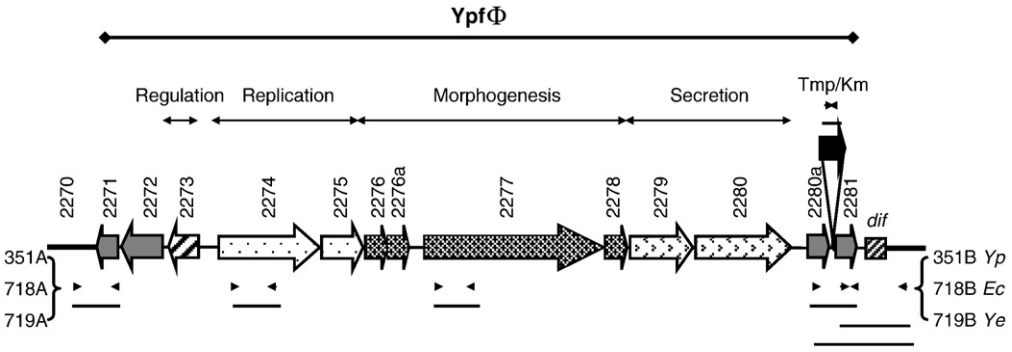

Fig. 1. Genetic organization of the YpfΦ::Tmp phage. The ORFs and directions of transcription are indicated by large arrows, with their YPO number according to the CO92 annotation. Small arrows below the genetic map indicate the location of the primers used to PCR amplify portions of the phage or regions overlapping the phage borders. Horizontal bars below the genetic map indicate the amplified regions. Because of a heterogeneity in the flanking sequences, different sets of primers were used to amplify the chromosomal regions flanking YpfΦ::Tmp in *Y. pseudotuberculosis*/*Y. pestis* (*Yp*) *E. coli* (*Ec*) and *Y. enterocolitica* (*Ye*).

and 1.9 million years ago and that the plague bacillus is a clone of *Y. pseudotuberculosis* that emerged over the last 20,000 years (Achtman et al., 1999). The transformation of *Y. pseudotuberculosis* into *Y. pestis* was accompanied by a massive loss of functions and by the horizontal acquisition of a few genetic elements (Chain et al., 2004; Hinchliffe et al., 2003), one of which being the filamentous phage YpfΦ. After its divergence from its *Y. pseudotuberculosis* ancestor, the plague bacillus split into two main branches: branch 1 which formed the two sub-branches 1.ORI (biovar Orientalis) and 1.ANT (biovar Antiqua); and branch 2 which divided into sub-branches 2.MED (biovar Medievalis) and 2.ANT (another subset of biovar Antiqua) (Achtman et al., 2004). YpfΦ is essentially found as a prophage stably integrated into the bacterial chromosome in 1.ORI strains, while in the other sub-branches it is present almost exclusively as extrachromosomal and highly unstable replicons (Derbise et al., 2007; Li et al., 2008). This difference in sub-cellular location is not due to different phage properties since the entire phage nucleotide (nt) sequence is 100% identical in different *Y. pestis* sub-branches (Derbise et al., 2007). As with other integrating filamentous phages, the YpfΦ genome in 1.ORI strains forms tandem repeats integrated into the chromosomal *dif* site (a 28 bp sequence used by the XerCD recombinases to resolve chromosome dimers during bacterial division) (Blakely et al., 1993; Lesterlin et al., 2004).

Since the *Y. pestis* filamentous phage has been identified recently, little is known about its physiology. The aim of this study was to shed some lights on the physiological and infective properties of this phage.

Results and discussion

Infectivity of YpfΦ for various Y. pestis isolates

YpfΦ was previously shown to be able to infect a *Y. pestis* isolate that does not harbor the phage (Derbise et al., 2007). To determine whether this infectious capacity can be extended to all *Y. pestis* isolates and whether the efficiency of infection is similar for all strains, the previously constructed YpfΦ::Tmp phage, tagged with a trimethoprim resistance cassette (Derbise et al., 2007), was used to infect a panel of 17 *Y. pestis* isolates (Table 1). To avoid any possible bias due to cross-immunity mechanisms, these strains were selected after screening by PCR for the absence of YpfΦ-specific sequences, using primer pairs amplifying internal portions of several phage ORFs (YPO2271, YPO2274, YPO2277 and YPO2281, Fig. 1). In addition, since all 1.ORI *Y. pestis* contain a stable chromosomally-integrated prophage, a previously constructed CO92ΔYpfΦ mutant (Derbise et al., 2007) in which the entire phage genome has been replaced by a kanamycin resistance cassette was also used as a recipient.

The ability of the tagged YpfΦ::Tmp phage to infect these various strains was then investigated. The efficacy of transduction, calculated as an infectivity rate (IR, see Materials and methods), was divided into four categories: 0 (no transductant detected, detection limit of 2×10^{-9}), I ($10^{-7}\geq IR>10^{-9}$), II ($10^{-4}\geq IR>10^{-7}$) and III ($IR>10^{-4}$). The transduction experiments were repeated three times independently for each recipient strain. As shown in Table 1, YpfΦ::Tmp had the capacity to infect almost all *Y. pestis* strains (16 out of 17). Since the *Y. pestis* genome is naturally prone to frequent *in vitro* recombinations (Guiyoule et al., 1994), it is possible that the only refractory strain (IP542) underwent rearrangements that led to the artificial loss of functions necessary for YpfΦ infection.

The efficiency of YpfΦ::Tmp transduction varied depending on the isolates. Remarkably, the phage was the most infectious for the 1.ANT group of strains (IR in category III for 4/6 isolates). This variability in infectivity between different sub-branches was unexpected because *Y. pestis* is a highly monomorphic species (Achtman et al., 1999) and a comparison of three *Y. pestis* genomes belonging to the three main phylogenetic branches (0, 1 and 2) identified only 76 synonymous single nucleotide polymorphisms in 3,250 orthologous sequences (Achtman et al., 2004). Therefore, subtle bacterial genetic differences might cause the variations in the efficiency of YpfΦ to infect *Y. pestis*. It should be noted however that all 1.ANT strains were isolated in Kenya, so the possibility that the strains from this specific geographical area display a peculiar feature that renders them highly susceptible to a YpfΦ infection cannot be excluded. Curiously, although the 1.ORI sub-branch is the only one to stably harbor the phage, YpfΦ::Tmp displayed a low IR for the Orientalis strain CO92ΔYpfΦ (Table 1). One possible explanation would be that the replacement of the endogenous prophage by a Km cassette had generated a conformational change in the integration site that impairs the efficiency of insertion. Another explanation would be that, after acquiring a stable form of the phage, some mutations deleterious for subsequent YpfΦ infections occurred in this *Y. pestis* sub-branch.

Protection conferred by the presence of an endogenous filamentous phage

The presence of a resident phage has been shown in several instances to partially protect the host bacterium from a subsequent infection by the same phage (Davis et al., 1999; Kimsey and Waldor, 1998). Whether an endogenous YpfΦ could confer some immunity was studied by comparing the susceptibility of three naturally YpfΦ-positive *Y. pestis* strains belonging to biovar Orientalis (CO92), Antiqua (IP550-HC1) and Medievalis (IP1865-12), to that of their YpfΦ-negative derivatives. These derivatives were either the CO92ΔYpfΦ strain artificially deleted of the prophage genome, or spontaneous phage-negative colonies (IP1865-1 and IP550-HC2), easily obtained because of the high instability of the phage in these biovars. Upon infection with YpfΦ::Tmp, Tmp^R colonies were recovered from all strains tested and the presence of YpfΦ::Tmp was confirmed by PCR, thus indicating that an endogenous YpfΦ phage does not fully protect *Y. pestis* against a superinfection by the

Table 1
Susceptibility of *Yersinia* and *E. coli* strains to an infection with YpfΦ::Tmp.

Strains	Biovar[a]/Biotype	Serotype	Infectivity rate[b]	Infectivity rate category
Y. pestis				
CO92ΔYpfΦ	1.ORI	NA	$6.9\ (\pm 2.5)\times 10^{-8}$	I
IP550-HC2	1.ANT	NA	$6.9\ (\pm 3.6)\times 10^{-3}$	III
IP551	1.ANT	NA	$6.6\ (\pm 2.7)\times 10^{-2}$	III
IP554	1.ANT	NA	$2.8\ (\pm 1.5)\times 10^{-1}$	III
IP542	1.ANT	NA	0	0
IP545	1.ANT	NA	$4.5\ (\pm 1.7)\times 10^{-2}$	III
IP547	1.ANT	NA	$1.3\ (\pm 1.3)\times 10^{-8}$	I
IP546	2.ANT	NA	$1.3\ (\pm 0.3)\times 10^{-8}$	I
IP611	2.ANT	NA	$2.2\ (\pm 1.5)\times 10^{-8}$	I
IP541	2.ANT	NA	$3.8\ (\pm 2.5)\times 10^{-8}$	I
IP1865-1	2.MED	NA	$1.5\ (\pm 0{,}5)\times 10^{-3}$	III
KIM6	2.MED	NA	$1.8\ (\pm 1.4)\times 10^{-8}$	I
IP556	2.MED	NA	$3.2\ (\pm 3.1)\times 10^{-9}$	I
IP617	2.MED	NA	$1.6\ (\pm 1.0)\times 10^{-8}$	I
IP669	2.MED	NA	$4.4\ (\pm 2.3)\times 10^{-8}$	I
IP670	2.MED	NA	$2.4\ (\pm 2.3)\times 10^{-8}$	I
IP616	2.MED	NA	$1.9\ (\pm 0.8)\times 10^{-8}$	I
Y. enterocolitica				
IP29175	2	O:9	$4.1\ (\pm 1.8)\times 10^{-9}$	I
IP28938	2	O:9	$6.2\ (\pm 3.3)\times 10^{-8}$	I
IP28193	2	O:9	$2.0\ (\pm 1.2)\times 10^{-9}$	I
IP28109	2	O:9	$1.1\ (\pm 0.7)\times 10^{-1}$	III
IP28302	2	O:5-27	$2.8\ (\pm 1.5)\times 10^{-8}$	I
IP29127	3	O:5-27	$2.3\ (\pm 0.8)\times 10^{-1}$	III
IP29435	1A	O:37	$1.5\ (\pm 0.6)\times 10^{-6}$	II
IP29433	1A	NAg	$2.4\ (\pm 1.4)\times 10^{-7}$	II
IP29431	1A	O:6,30-6,31	$3.6\ (\pm 3.5)\times 10^{-9}$	I
IP29430	1A	O:7,8-8-8,19	0	0
IP29419	1A	O:7,8-8-8,19	0	0
IP17451	1B	O:8	0	0
IP1105	1B	O:8	0	0
IP22596	1B	O:8-8,19	0	0
IP24766	1B	O:7,8-8-8,19	0	0
IP24502	1B	O:20	0	0
Ye 8081	1B	O:8	0	0
Y. pseudotuberculosis				
IP32953	NA	I	$4.4\ (\pm 2.2)\times 10^{-8}$	I
IP30642	NA	I	0	0
IP33306	NA	II	0	0
IP32544	NA	III	0	0
IP31830	NA	IV	0	0
IP32463	NA	V	$1.4\ (\pm 1.3)\times 10^{-9}$	I
E. coli				
Top10[c]	NA	NA	$1.2\ (\pm 0.7)\times 10^{-8}$	I
MG1655	NA	OR:H48:K- F-	0	0
536	NA	O6:K15:H31	0	0
Nissle 1917	NA	O6:K5:H1	0	0
ECOR 31	NA	O79:H43	$1.4\ (\pm 0.7)\times 10^{-7}$	II

Nag: Non-agglutinable, NA: Not Applicable.
[a] : according to Achtman et al. (1999).
[b] : mean and standard deviation (in parentheses) of 3 independent experiments.
[c] : F⁻ strain.

same phage. In the three strains, the presence of a resident phage was associated with a lower infectivity rate, and this difference was statistically significant for two of them (CO92 and IP550, Fig. 2). Therefore, an endogenous YpfΦ confers some level of protection against a superinfection by the same phage.

In *V. cholerae*, immunity against an infection by a new CTXΦ virion has been attributed to the phage-borne *rstR* gene that codes for a repressor (Davis et al., 1999). Based on blast results and position within the YpfΦ genome (Fig. 1), YPO2273 is predicted to be the *rstR* homolog. The possibility that YPO2273 may be responsible for the protection observed was investigated by deleting this gene from the prophage genome and comparing the transduction efficacy of YpfΦ:: Tmp in the CO92 parental strain and the CO92(YpfΦΔ73) derivatives. In the absence of YPO2273, the efficacy of transduction of CO92 (YpfΦΔ73) (6.1×10^{-9}) remained as low as that of the parental strain, thus indicating that the protection conferred by the presence of a resident phage is not mediated by the *rstR* homolog in YpfΦ.

Involvement of the three putative functional modules in YpfΦ infectivity

The core region of YpfΦ has a genetic organization reminiscent of that of other filamentous phages and is thus predicted to comprise three functional domains involved in phage replication, morphogenesis and secretion (Fig. 1). The morphogenesis and secretion modules are directly involved in the production of phage particles. To determine whether the two corresponding regions of YpfΦ are also involved in the production of infectious virions, one gene in each of these two putative modules (YPO2277 and YPO2279) was deleted from the *Y. pestis* CO92 chromosome by allelic exchange with a non-polar kanamycin resistance cassette. RT-PCR were performed to ensure that the inserted cassette did not impair the expression of the downstream genes. The YPO2279 putative product is homologous to pI, a protein that interacts with the phage DNA packaging signal and is essential for phage assembly (Haigh and Webster, 1999; Rapoza and Webster, 1995; Russel, 1995). The YPO2277 putative product is

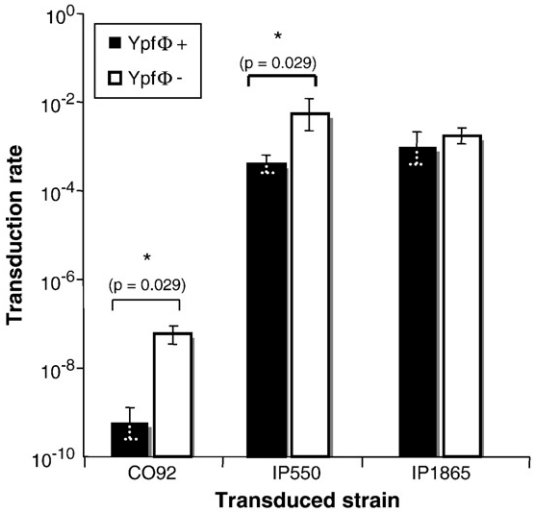

Fig. 2. Susceptibility to a YpfΦ::Tmp infection of YpfΦ-positive and -negative *Y. pestis* strains. Standard deviations of three independent experiments are represented by vertical bars. Statistical analysis of data was determined with the Student's *t*-test. A star indicates a statistically significant difference with the *p* value inside parentheses.

homologous to pIII, a protein necessary for the assembly and release of phage particles (Crissman and Smith, 1984; Rakonjac and Model, 1998). This protein, localized on the tip of the virion, also mediates the initial step of Ff phage infection by binding to the F pilus (Deng et al., 1999; Jacobson, 1972). The control strain CO92(YpfΦ::Km) was generated by inserting a kanamycin cassette in the intergenic region between YPO2280a and YPO2281 in the phage genome (Fig. 1). Phage preparations from these two mutants were used to infect the phage-negative *Y. pestis* strain IP550-HC2. While phage preparations from the control strain were able to infect the *Y. pestis* recipient, those from the CO92(YpfΦΔ77) and CO92(YpfΦΔ79) deletants were unable to do so (Table 2), confirming that, as predicted from genetic similarities, the two domains need to be functional to produce infectious YpfΦ virions.

The third module is also involved, although indirectly, in the generation of phage particles since it promotes replication of the phage genome. One gene in this module (YPO2274, Fig. 1) was deleted from the YpfΦ prophage genome by allelic exchange. The YPO2274 product is homologous to the pII protein of the *E. coli* Ff phages (Dotto et al., 1981; Meyer and Geider, 1979a; Meyer and Geider, 1979b) and to a similar protein encoded by the ΦLf phage of *Xanthomonas campestris* (Lin et al., 1996; Wen and Tseng, 1994). These proteins are involved in the replication of the phage genome. Phage preparations from the CO92(YpfΦΔ74) mutant were unable to infect the *Y. pestis* recipient strain (Table 2), indicating that this gene is also necessary for the generation of functional YpfΦ virions. To further test the role of YPO2274 in YpfΦ genome replication, plasmid preparations from the three mutants and from the control strain CO92(YpfΦ::Km) were prepared and introduced by electroporation into *E. coli* Top10. No *E. coli* transformants were obtained when electroporated with the CO92(YpfΦΔ74) plasmid preparation, whereas those from CO92(YpfΦΔ77), CO92(YpfΦΔ79) and CO92(YpfΦ::Km) yielded kanamycin resistant recombinants (data not shown).

Table 2
Infectivity of the phage preparations from various *Y. pestis* mutants.

Strain	Frequency of infection[a]
CO92(YpfΦ::Km)	$1.1\ (\pm 0.2)\times 10^{-6}$
CO92(YpfΦΔ77)	0
CO92(YpfΦΔ79)	0
CO92(YpfΦΔ74)	0

[a] mean frequency of three independent experiments with standard deviations inside parentheses.

Therefore, YPO2274 is necessary for YpfΦ replication, as observed for Ff and ΦLf phages, thus confirming that the YpfΦ module predicted to be involved in phage replication does play this role.

Search for a bacterial YpfΦ receptor

Among the differences that could account for these variations in susceptibility to a YpfΦ infection, one could be that *Y. pestis* has acquired by horizontal transfer a mobile genetic element coding for a phage receptor. This would be reminiscent of the CTXΦ filamentous phage which uses as receptor a type IV pilus (TCP) acquired most likely recently by *V. cholerae* through lateral gene transfer (Faruque and Mekalanos, 2003; Mel and Mekalanos, 1996; Waldor and Mekalanos, 1996). However, none of the few regions acquired by *Y. pestis* after its emergence from *Y. pseudotuberculosis* appears to encode a pilus-like structure (Derbise et al., submitted for publication). This suggests the presence of a phage receptor common to the two species. Some *Y. pseudotuberculosis* strains have been shown to carry a gene cluster that potentially encodes a functional type IV pilus (Collyn et al., 2002). However, since this gene cluster is absent from all *Y. pestis* and most *Y. pseudotuberculosis* strains, their susceptibility to a YpfΦ infection is not due to the production of this pilus. Neither *Y. pestis*, nor *Y. pseudotuberculosis* produce another type IV pilus or an F pilus, usually used as receptors by filamentous phages (Jouravleva et al., 1998; Russel, 1995; Waldor and Mekalanos, 1996). However, the two species synthesize flexible fibrillar organelles known as pH6 antigen (PsA, encoded by YPO1303 in CO92) (Lindler and Tall, 1993). To determine whether this antigen may serve as YpfΦ receptor, a *psa* deletion mutant was constructed by allelic exchange with a kanamycin cassette in *Y. pestis* strain IP550-HC2 (a derivative of IP550 that had lost YpfΦ but that exhibits a high rate of transduction (Table 1)). The IR of IP550-HC2Δ*psA* (2.1×10^{-3}) was similar to that of the parental strain, indicating that this fimbria is not required for an efficient YpfΦ infection. The genomes of *Y. pestis* and *Y. pseudotuberculosis* also carry a locus (YPO3425-27) encoding a putative pilus formed of PilA sub-units. An IP550-HC2Δ*pilA* deletion mutant was therefore constructed and subjected to a YpfΦ infection. This mutant exhibited an IR (1.4×10^{-3}) similar to that of the IP550-HC2 parental strain, showing that PilA is also dispensable for an efficient YpfΦ infection. Our results thus suggest that neither PilA, nor PsaA act as YpfΦ receptors, although the possibility that these two pili are interchangeable cannot be excluded.

Infectivity of YpfΦ for the two other pathogenic Yersinia species

The genus *Yersinia* comprises two other pathogenic species: *Y. pseudotuberculosis,* which is subdivided in five major serotypes (I to V), and *Y. enterocolitica,* which is composed of six biotypes (1A, 1B and 2 to 5). To evaluate the capacity of YpfΦ to infect the various sub-groups of these two species, a panel of isolates covering the most frequently isolated strains of the two species (Table S1) was first screened by PCR for the presence of four phage genes distributed over the YpfΦ genome (YPO2271, YPO2274, YPO2277 and YPO2281, Fig. 1). The seven biotype 4 *Y. enterocolitica* strains tested were found to harbor several open reading frames (ORFs) homologous to YpfΦ genes, suggesting the presence of a filamentous phage of the same family as YpfΦ (Chouikha et al., manuscript in preparation). To avoid any possible bias due to cross-immunity mechanisms, these seven biotype 4 strains were removed from our transduction analysis. The 23 remaining *Y. enterocolitica and Y. pseudotuberculosis* strains did not carry any of the YpfΦ sequences tested and were thus kept for further analyses.

When the ability of the tagged YpfΦ::Tmp phage to infect the species *Y. enterocolitica* was tested, variable levels of infectivity were observed depending on the sub-groups. While the phage failed to infect any of the biotype 1B strains, it was infectious for all biotype 2 and 3 isolates (Table 1). Among them, two strains (IP28109 and IP29127) exhibited remarkably high levels of susceptibility ($IR > 10^{-1}$) to a YpfΦ::Tmp

infection (Table 1). The infectivity of YpfΦ::Tmp for biotype 1A *Y. enterocolitica* was intermediate, with only half of the strains tested infected. Since *Y. enterocolitica* is a genetically heterogeneous species (Lobato et al., 1998), and since only one genome sequence of *Y. enterocolitica* is available (Thomson et al., 2006), it is not possible to identify bacterial genes responsible for these differences in susceptibility to YpfΦ.

Surprisingly, the infectivity of YpfΦ::Tmp for *Y. pseudotuberculosis*, the *Y. pestis* ancestor, was the lowest, with only 2/6 strains infected (Table 1). Furthermore, the phage IR was very low for these two recipient strains (category I). This is consistent with the fact that no natural isolate of *Y. pseudotuberculosis* has been found to harbor YpfΦ. *Y. pestis* and *Y. pseudotuberculosis* are genetically very close, with most of their genes sharing ≥97% nt identity (Chain et al., 2004). The remarkable difference in the infectious capacity of YpfΦ for the two species can thus be attributed to minor genetic modifications that occurred during the transformation of *Y. pseudotuberculosis* into *Y. pestis*.

Altogether, our results thus demonstrate that, although the infectivity potential of YpfΦ is maximal for its natural bacterial host *Y. pestis*, the phage has the capacity to infect all three pathogenic *Yersinia* species, but with variable efficiencies. Its spectrum of infection varies depending on the species and biotype of the recipient strains.

Transduction of E. coli with YpfΦ::Tmp

As the CUS-1 phage of *E. coli* K1 is very similar to YpfΦ, we hypothesized that the *Y. pestis* filamentous phage might infect not only *Yersinia*, but also other enterobacteria species such as *E. coli*. Five *E. coli* strains (Table S1) were selected, checked by PCR for the absence of YpfΦ sequences, and subjected to transduction with this phage. As shown in Table 2, YpfΦ::Tmp was infectious for two strains (TOP10 and ECOR31) out of the five *E. coli* isolates tested. The IR was low for one strain (TOP10), but was of category II for ECOR31. The fact that Top10 is F^- indicates that YpfΦ does not require the F pilus as a bacterial receptor to infect this strain. F^- *E. coli* strains have been shown to retain the capacity to be infected by filamentous phages such as f1 or M13, although at a much lower frequency than their F^+ counterparts (Russel et al., 1988). A low frequency of YpfΦ infection was also observed in several transduced bacteria, including *E. coli* Top10 and various *Yersinia* strains (Table 1). This suggests that YpfΦ uses two strategies to penetrate into bacterial cells: an efficient one through the recognition of a specific receptor, and a much less efficient one via a non-specific receptor or another means of penetration. Our results nonetheless indicate that the infectivity spectrum of YpfΦ is not restricted to members of the genus *Yersinia*.

Location of the acquired YpfΦ genome in the various transductants

In natural isolates of *Y. pestis*, the YpfΦ genome is present mostly as a chromosomally integrated prophage in Orientalis strains and as an extrachromosomal element in the other *Y. pestis* biovars (Derbise et al., 2007). The reason for this difference in location is unknown. We benefited from the availability of transduced strains belonging to various *Yersinia* species and sub-groups to study the fate of the filamentous phage once in a new bacterial host. For this purpose, the undigested DNA of the recipients, before and after transduction, was subjected to Southern hybridization with a Tmp or a YPO2277 probe. The two probes never hybridized with the DNA of the recipient strains prior to transduction (as exemplified in Fig. 3), indicating an absence of background signal, but both probes systematically recognized the same bands in each transductant (data not shown), confirming that the Tmp resistance cassette was acquired with the phage genome.

A signal corresponding to chromosomal DNA (Fig. 3) was detected in all transductant hybridization patterns (Table 3), consistent with the presence of a chromosomally integrated form of the YpfΦ genome in all transduced *Yersinia*. In addition, extra-chromosomal phage DNA molecules, characterized by the presence of several hybridizing fragments of different sizes, were observed in most transductants (Fig. 3). These various forms of the phage genome appeared to be steady since different colonies from the same transductant displayed similar hybridization profiles (data not shown).

Integrated forms of YpfΦ::Tmp were highly abundant in *Y. pestis* (Fig. 3A). This was unexpected because in natural isolates that do not belong to the Orientalis group, YpfΦ is found predominantly (or only) in an extrachromosomal form (Derbise et al., 2007). These results thus demonstrate that in natural *Y. pestis* isolates, the difference between the Orientalis sub-branch (in which the phage is stably integrated into the chromosome), and the other sub-branches is not due to a defect in the bacterial machinery of phage integration. They would rather argue for a more efficient excision of the phage genome in the Antiqua and Medievalis strains, although other hypotheses are possible.

Chromosomal copies of the YpfΦ::Tmp genome were in much lower proportions in the two *Y. pseudotuberculosis* transductants obtained (Fig. 3B). This could be due to less efficient mechanisms of phage chromosomal insertion in this species. Integration of CTXΦ into the *V. cholerae* chromosome has been shown to be mediated by the host tyrosine recombinases XerC and XerD (Huber and Waldor, 2002; McLeod and Waldor, 2004). The difference between *Y. pestis* and *Y. pseudotuberculosis* in efficiency of phage integration is most likely not attributable to a difference in these recombinases as they are highly similar in the two species (99% (XerC) and 100% (XerD) amino acid identity in *Y. pestis* and *Y. pseudotuberculosis*). Alternatively, this

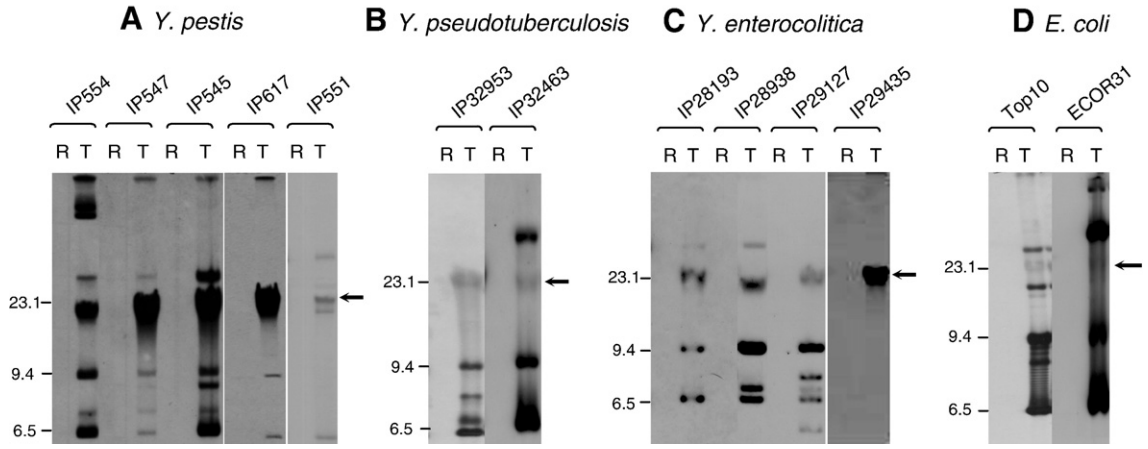

Fig. 3. Sub-cellular location of the acquired phage. Southern blot hybridizations with a Tmp probe of the undigested genomic DNA of various recipient strains, prior to (R) and after (T) transduction with YpfΦ::Tmp. The hybridizing band corresponding to chromosomal DNA is shown by an arrow on the right. Tick marks on the left indicate the molecular sizes of the Lambda-*Hin*dIII marker (in kb).

Table 3
Localization of the acquired YpfΦ::Tmp genome in *Yersinia* and *E. coli* transduced cells.

Transductants	Biovar/ Biotype	Serotype	Southern hybridization	PCR		
				5′ border [a]	3′ border [b]	*dif* site [c]
Y. pestis						
CO92ΔYpfΦ	1.ORI	NA	C + ExtC	+	+	w
IP550-HC2	1.ANT	NA	C + ExtC	+	+	+
IP551	1.ANT	NA	C + ExtC	+	+	+
IP554	1.ANT	NA	C + ExtC	+	+	+
IP547	1.ANT	NA	C + ExtC	+	+	–
IP546	2.ANT	NA	C + ExtC	+	+	+
IP1865-1	2.MED	NA	C + ExtC	+	+	+
IP617	2.MED	NA	C + ExtC	+	+	w
Y. enterocolitica						
IP28938	2	O:9	C + ExtC	+	+	+
IP28193	2	O:9	C + ExtC	+	w	–
IP28109	2	O:9	C	+	+	–
IP29127	3	O:5–27	C + ExtC	+	+	–
IP29435	1A	O:37	C	–	+	–
Y. pseudotuberculosis						
IP32953	NA	I	C + ExtC	+	w	w
IP32463	NA	V	C + ExtC	+	+	w
E. coli						
Top10	NA	NA	C + ExtC	+	w	+
ECOR 31	NA	O79:H43	C + ExtC	+	w	+

–: No amplification, w: weak amplification, +: clear amplification, C: chromosomal form, ExtC: extrachromosomal form.

[a] : Primer pairs 351A/YPO2271F for *Y. pestis* and *Y. pseudotuberculosis*, 719A/YPO2271F for *Y. enterocolitica*, and 718A/YPO2271F for *E. coli*.

[b] : Primer pairs 351B/YPO2281F for *Y. pestis* and *Y. pseudotuberculosis*, 719B/YPO2281F, 752/YPO2281F for *Y. enterocolitica*, and 718B/YPO2281F for *E. coli*.

[c] : Primer pairs 351A/B for *Y. pestis* and *Y. pseudotuberculosis*, 719A/B for *Y. enterocolitica* and 718A/B for *E. coli*.

difference might be the consequence of a higher excision rate of the phage genome in the latter species. Whatever the mechanisms involved, predominantly extrachromosomal forms may lead to a phage instability in *Y. pseudotuberculosis*. Arguing for this hypothesis is the previous observation that when YpfΦ is mainly (or only) in an extrachromosomal form in *Y. pestis*, the virion is lost at high frequencies (Derbise et al., 2007; Li et al., 2008). It is thus possible that the low IR rate observed in *Y. pseudotuberculosis* is not a consequence of a defect in phage penetration but rather of a rapid loss of the virion once inside the host cell.

The YpfΦ::Tmp genome was also found both as an integrated and an extrachromosomal form in several *Y. enterocolitica* transduced strains (Fig. 3C). In these strains, the proportion of integrated versus extrachromosomal forms varied from isolate to isolate, without a marked tendency for a given sub-cellular location. However, *Y. enterocolitica* was the only species in which no extrachromosomal forms of YpfΦ::Tmp was detectable in two strains (IP28109 and IP29435, Fig. 3 and Table 3). The opposite was observed in *E. coli* transductants where the hybridizing signal corresponding to the chromosome was extremely faint, whereas that of non-chromosomal fragments was much stronger (Fig. 3D). This contrasts with the CUS-1 phage, closely related to YpfΦ, which is integrated in the *dif* site of an *E. coli* K1 isolate (Gonzalez et al., 2002).

Altogether these results indicate that the YpfΦ genome has the capacity to integrate into the chromosome of all transduced strains and that extrachromosomal forms of the phage co-exist in most transductants. Since the phage molecule is the same in all transductants, the variable proportions of integrated forms in different strains are due to intrinsic bacterial properties.

Insertion site of YpfΦ::Tmp into the recipient's chromosome

In natural isolates of *Y. pestis*, the YpfΦ prophage is inserted into the *dif* locus (Derbise et al., 2007), a 28 bp site composed of two 11 bp binding sites for XerC and XerD, separated by a 6- to 8-bp spacer (Blakely et al., 1993). This locus is also the integration site for many other filamentous phages such as CTXΦ and VGJΦ of *V. cholerae* (Campos et al., 2003; Davis et al., 1999; Pearson et al., 1993), CUS-1 of *E. coli* K1 (Gonzalez et al., 2002), f237 of *Vibrio parahaemolyticus* (Iida et al., 2002), Cf1c, Cf1t, Cf16v1 and ΦLf of *Xanthomonas campestris* (Lin et al., 2001), and XfΦf1 of *Xylella fastidiosa* (Huber and Waldor, 2002). To determine whether this is also the case for the newly acquired YpfΦ::Tmp genome in the various transduced strains, three pairs of primers (two encompassing the right and left junctions of the phage if integrated at the *dif* site, and one amplifying *dif* only if this site is empty) were used (Fig. 1). Because of the heterogeneity of the sequences flanking *dif* in these strains, different sets of primers were designed (see legend of Table 3).

The positive amplification of the right and left junction fragments in all transduced *Y. pestis* and *Y. pseudotuberculosis* strains tested (Table 3), indicated that YpfΦ::Tmp had inserted itself into their chromosomal *dif* site. Although the results of the PCR analysis was less clear-cut with *Y. enterocolitica* transductants, probably because of an heterogeneity of the sequences flanking *dif* in this species, the fact that at least one of the junction fragments was positive in all strains confirmed the insertion of YpfΦ::Tmp at this site. These results thus demonstrate the propensity of YpfΦ to insert itself into the *dif* site in all three pathogenic *Yersinia* species.

Our observation that YpfΦ may efficiently insert itself into the chromosome of some strains but not of some others might be due to a variability in their *dif* sequence. An analysis of pathogenic *Yersinia* genomes available in the databases showed that all *Y. pestis* strains have an identical *dif* site, whatever their biovar (Supplementary Fig. S1 A). Most *Y. pseudotuberculosis* strains, including the IP32953 isolate studied here, had one nucleotide variation (A/G) in the XerC site (Fig. S1 A), suggesting that this single nucleotide polymorphism might be involved in the lower efficiency of YpfΦ chromosomal integration in IP32953. To further investigate this hypothesis, we sequenced the *dif* site of four strains from this study: two in which a YpfΦ was found exclusively as an integrated prophage (*Y. enterocolitica* IP28109 and IP29435, Table 3), and two in which extrachromosomal forms of YpfΦ were predominant (*Y. enterocolitica* IP29127 and *Y. pseudotuberculosis* IP32463, Fig. 3). The *dif* site of *Y. pseudotuberculosis* IP32463 was identical to that of *Y. pestis* (Fig. S2 B), despite very different efficiencies of YpfΦ integration. Furthermore, the three *Y. enterocolitica* strains studied here had an identical *dif* sequence although the prophage form was in large majority in two strains and in minority in the third one (Fig. S2 B). Therefore, a variation in the *dif* sequence does not appear to be responsible for the different efficiencies of YpfΦ chromosomal integration in different strains of *Yersinia*.

Despite the presence of an integrated prophage, a signal corresponding to an empty *dif* site was also detected in some transductants, mostly of the *Y. pestis* species (Table 3). This indicates a heterogeneous cell population formed of bacteria with and without a chromosomally integrated phage. This heterogeneity suggests either that the phage genome has not integrated itself into the chromosome of all bacterial cells, or that it has excised itself in a portion of the population. In CTXΦ, the integration process has been shown to be irreversible (Val et al., 2005). However, if this is true for YpfΦ, we would then expect to have natural *Y. pestis* clones which would be homogeneously either phage-negative (because they have lost the unstable extrachromosomal form), or phage-positive because the prophage is stably integrated into the bacterial chromosome. Actually, homogeneous populations of Medievalis or Antiqua strains with a stable chromosomally inserted prophage are not observed under natural conditions (Derbise et al., 2007; Li et al., 2008), thus suggesting that, although YpfΦ has the capacity to integrate into the bacterial chromosome, it cannot maintain itself as an integrated prophage in these biovars.

Impact of a resident integrated phage on the location of a newly acquired YpfΦ genome

As shown above, *Y. pestis* strains that naturally harbor YpfΦ can still be infected by the same phage. However, the presence of an

endogenous YpfΦ genome may have an impact on the sub-cellular location of the newly acquired phage. We thus compared the fate of the YpfΦ::Tmp genome upon transduction of wild type CO92 and of its YpfΦ-deleted derivative. Surprisingly, while both chromosomal and extrachromosomal forms of the introduced phage co-existed in the absence of an endogenous YpfΦ, no extra chromosomal form of YpfΦ::Tmp was detected in the transduced CO92 parental strain (naturally YpfΦ-positive) (Fig. 4). This indicates that pre-existing integrated YpfΦ genomes in *Y. pestis* Orientalis do not prevent the subsequent chromosomal insertion of a new incoming copy of the same phage and suggests that the presence of the resident prophage prevents the generation (or maintenance) of extrachromosomal forms.

Filamentous phages are usually inserted in tandem repeats in the *dif* locus on bacterial chromosomes (Davis and Waldor, 2000; Derbise et al., 2007; Mekalanos, 1983). This is also the case for YpfΦ, which forms mostly two tandem copies in the genome of Orientalis strains (Derbise et al., 2007). When the *dif* site is already occupied by a resident prophage, we wondered whether the newly acquired phage would also insert itself at the same chromosomal location and whether this would have an impact on the number of phage repeats. Therefore, the DNA of CO92, before and after transduction, was digested with *Nco*I (an enzyme that does not have any restriction site in the phage, Supplementary Fig. S2A), subjected to pulsed field gel electrophoresis, and hybridized with either a YPO2227 or a Tmp probe. As previously reported, hybridizing fragments with a size corresponding to mostly two, but also three copies of the phage genome were observed in wild type CO92 (Fig. 5A and S1A). In contrast, the number of phage copies varied from three to up to 10 copies in the transduced strain, with a predominant form composed of five tandem repeats (Fig. 5A). Thus, insertion of the incoming phage occurs at the same location as the resident prophage and results in the generation of higher copy numbers of the incoming phage genome. Of note, insertion of the YpfΦ::Tmp led to the replacement of the predominant two-copy form by a tandem composed of a minimum of three copies, as if one and more YpfΦ::Tmp genomes inserted themselves next to the two resident YpfΦ copies. The striking difference in copy numbers between the transduced *Y. pestis* strain and natural isolates may reflect the kinetics of infection. It is possible that at the beginning of a phage infection, integration of numerous phage genomes in the host chromosome occurs. This high prophage number may become a burden for the bacteria, leading to a gradual resolution of these large size tandems, down to an equilibrium of two to three copies, as observed in natural YpfΦ-positive *Y. pestis* isolates.

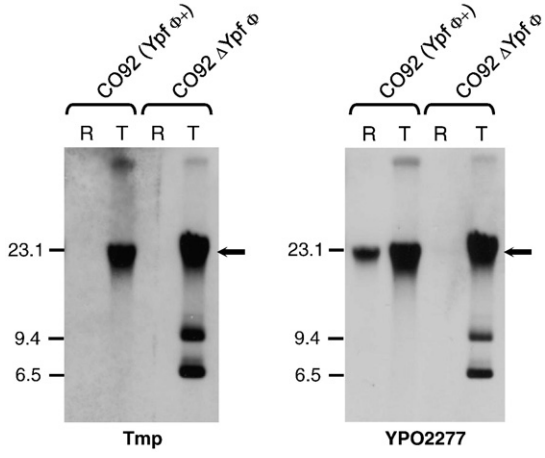

Fig. 4. Fate of the acquired phage in the presence of a resident prophage. Southern blot hybridization with a Tmp and a YPO227 probe of the undigested genomic DNA of *Y. pestis* CO92 before (R) and after (T) YpfΦ::Tmp transduction. The arrows point to chromosomal DNA. Tick marks on the left indicate the molecular size of the Lambda-*Hin*dIII marker (in kb).

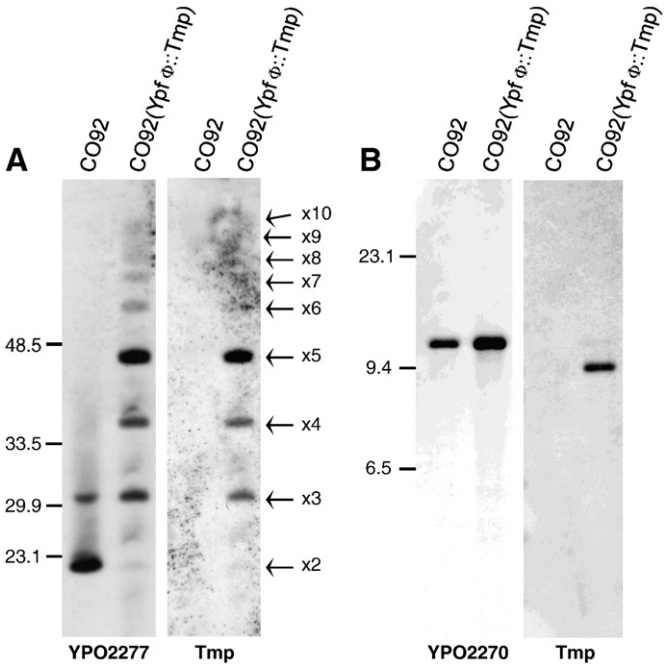

Fig. 5. Insertion of the incoming YpfΦ::Tmp phage in the tandem repeats of the resident YpfΦ phage. Southern blot hybridization of CO92 wild type (naturally YpfΦ-positive) and its transduced derivative CO92(YpfΦ::Tmp). A: Hybridization patterns with a Tmp or YPO2277 probe of the *Nco*I-digested genomic DNA of the two strains subjected to pulse field electrophoresis. The number of phage repeats, as expected from the restriction map shown on Supplementary Fig. S2, is given on the right. Tick marks on the left indicate the molecular size of the 8–48 kb CHEF DNA size standards. B: Hybridization patterns with a Tmp or YPO2270 probe after *Mlu*NI-digestion of the DNA of the two strains. Tick marks on the left indicate the molecular size of the Lambda-*Hin*dIII marker in kb.

The incoming phage has three possible insertion sites in the resident tandem: at its 3′-end, 5′-end, or between two copies of the integrated prophages. In filamentous phages such as CTXΦ and VGJΦ, incoming phage copies have been shown to insert themselves at the 3′ extremity of endogenous phage genomes (Campos et al., 2003; Davis et al., 1999). For YpfΦ, since the resident and the acquired filamentous phages only differ by the presence in the latter of a Tmp cassette at the right-hand extremity of the phage (Fig. 1), we performed a PCR amplification of the fragment encompassing YPO2280a, upstream of the *tmp* gene, and the 3′ bordering chromosomal sequence (primer pair YPO2280aF/351B, Fig. 1) in wild type CO92 and in its transduced derivative. The same amplification product, having the size expected for a wild type phage was obtained in both strains (data not shown), indicating that, in contrast to other filamentous phages, the newly acquired phage is not inserted at the 3′ extremity of the resident prophages.

To determine if the introduced phage genome is integrated at the 5′ end or between two copies of the resident phages, the DNA of the wild type and transductant CO92 strains were digested with *Mlu*NI, an enzyme with a single restriction site in the phage genome (upstream of YPO2281, Fig. S2 B and C). The digested DNA were then subjected to Southern blot hybridizations with the Tmp and YPO2270 probes. If YpfΦ::Tmp is inserted at the 5′ end of the resident phages, the Tmp and YPO2270 probes should both hybridize with the same 11.6 kb fragment (Fig. S2 B). In contrast, if YpfΦ::Tmp is inserted between two copies of the resident phage, the Tmp probe should recognize a smaller fragment of 8.7 kb, corresponding to the size of the phage (Fig. S2 C). As shown in Fig. 5B, YPO2270 hybridized with the expected 11.6 kb band, while Tmp recognized a 8.7 kb fragment. This demonstrates that the site of YpfΦ::Tmp integration is between two copies of the resident prophage. This result is unexpected as the junction between two integrated copies of YpfΦ reconstitutes an imperfect *dif* site (*dif*-like site) which differs from the original locus by a substitution of two nucleotides in the XerD binding site and by a

larger spacer region (11 bp instead of 6 bp) (Supplementary Fig. S3). In other bacterial hosts, the 6–8 bp spacing has been shown to be essential for the interaction between the two recombinases (Blakely et al., 1993; Blakely and Sherratt, 1996). Therefore, this may indicate either that the reconstituted *dif* site located between phage repeats is a better substrate for the *Y. pestis* XerCD recombinases than the natural site, or that the incoming YpfΦ::Tmp phage inserts itself between two copies of the resident phage by a mechanism independent of XerCD, such as a Campbell-like mechanism of recombination with the endogenous prophages.

Conclusion

The *Y. pestis* filamentous phage is a new member of the *Inoviridae* family, but because of its recent description, little is known about its physiology and the events that allowed its acquisition and maintenance in the plague bacillus. Using an experimental transduction approach, we show here that YpfΦ shares several physiological properties common to *Inovirus* phages. The genomes of these phages are organized in three modules involved in replication, encapsidation and secretion of functional virions. YpfΦ has a similar genetic organization and the three corresponding modules are also necessary for phage replication and production of infectious virions. Like other filamentous phages, the YpfΦ genome integrates into the chromosomal *dif* site and forms multiple tandem repeats upon penetration into a *Yersinia* host that does not harbor the phage. Finally, as previously shown for other *Inovirus* phages, the presence of an integrated YpfΦ genome confers some protection against a superinfection with the same phage.

However, YpfΦ has specific physiological characteristics that have not been described or are different from those of other filamentous phages. The infectivity spectrum of this bacteriophage is not limited to the species *Y. pestis* or to the genus *Yersinia*. Although like other filamentous phages, YpfΦ is found both as an integrated prophage and as extrachromosomal replicons upon transduction of the host bacterium, several pieces of evidence suggest that it does not have the capacity to maintain itself in a stable integrated form under natural conditions. Actually, the stabilization of YpfΦ into the genome of a single sub-branch of *Y. pestis*, by an as yet unidentified mechanism, seems to be the exception rather than the rule. Indeed, with the exception of the Orientalis sub-branch, the phage is lost at high frequencies in all other *Y. pestis* strains, and although it has intrinsically the potential to infect the ancestor *Y. pseudotuberculosis*, no isolate of this species has ever been found to harbor the phage. This instability does not seem to be due to a defective integration machinery as all transduced strains contained integrated prophages. The absence of non-Orientalis *Y. pestis* isolates harboring a stably integrated prophage rather argue for an inability of the phage to maintain itself in the bacterial chromosome. If this is the case, it is in striking contrast to the behavior of CTXΦ, which does not have the capacity to excise itself once integrated into the bacterial chromosome. Also in striking contrast to other filamentous phages is the observation that superinfection with the same phage leads to the integration of the incoming genome between two integrated copies, and not at the 3′ end of the tandem. YpfΦ thus seems to use a different and as yet unknown mechanism of insertion.

This study represents the first analysis of physiological properties of the *Y. pestis* filamentous phage. It shows that this phage shares a genetic organization and several features common to the members of the genus *Inovirus*, but it also unraveled some specific characteristics which will deserve further analyses.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study and their characteristics are listed in Supplementary Table S1. The strains were grown in Luria Bertani (LB) or Müller Hinton (MH) broth or on agar plates at 28 °C (*Yersinia*) or 37 °C (*E. coli*). Trimethoprim (Tmp, Sigma Aldrich) or kanamycin (Km, Sigma Aldrich) was added to MH or LB at a concentration of 10 μg/ml and 50 μg/ml, respectively when necessary.

DNA manipulation

Genomic DNA was extracted from cultures grown overnight at 28 °C or 37 °C, using the MasterPure Complete DNA purification kit (Epicentre Biotechnologies) according to the manufacturer's instructions. PCR screening for the presence of YpfΦ sequences in various *Yersinia* strains were done with primer pairs (Table S2) that amplified internal portions of the phage ORFs. PCRs were performed in 50 μl containing 300 nM of the forward and reverse primers (Supplementary Table S2), 200 μM each of deoxynucleoside triphosphate (Roche), 1 unit of Ampli*Taq* polymerase (Invitrogen) or 1 unit of Phusion DNA polymerase (Finnzymes) in the supplier's buffer and 100 ng of DNA as template. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min/kb. The amplification ended with a final extension step at 72 °C for 7 min. Reactions were performed in an iCycler thermocycler (Roche). PCR products were subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide.

Plasmid DNA preparations of YpfΦ::Km replicative forms were extracted from cultures grown overnight at 28 °C, using the Qiagen plasmid mini prep kit according to the manufacturer's instructions. Plasmids were then electroporated into *E. coli* Top10 electrocompetent cells (Invitrogen) and colonies were selected for resistance to kanamycin.

For DNA/DNA hybridizations, undigested or genomic DNAs digested with *Mlu*NI or *Nco*I were subjected to electrophoresis and transferred to Hybond-N+ membranes (Amersham Biosciences). Hybridizations were performed with the direct nucleic acid labelling and detection systems (ECL, Amersham Biosciences), using as probe either a 290-bp portion of YPO2277 (obtained with primers YPO2277F/R), a 216-bp portion of the Dfr cassette (obtained with primers 459A/B from plasmid pGP704N-Dfr (Lesic and Carniel, 2005), or a 529-pb portion of YPO2270 (obtained with primers YPO2270F/R). The PCR products were purified with the QIAquick PCR purification kit (QIAGEN), as specified by the manufacturer.

Pulse field gel electrophoresis of genomic DNA was performed at 14 °C for 22 h in a 0.9% agarose gel in 0.5X Tris/Borate/EDTA buffer using a CHEF-DRIII apparatus (Bio-Rad Laboratories) with an electric field of 6 V/cm, an angle of 120°, and initial and final pulse times of 1 s and 2 s respectively, as previously described (Derbise et al., 2007).

RNA isolation and transcript analysis

RNA extraction was performed with the TRI Reagent® kit (Ambion) on 1.5 ml of *Y. pestis* grown to stationary phase in LB at 28 °C. DNA was removed from the RNA samples by adding 4 U DNase, as described in the DNA-free kit instructions (Ambion). Total RNA was quantified by optical density at OD_{260} and 50 ng were used for reverse transcription and PCR analysis using primer pairs 621/622 (for control of RNA quality and absence of DNA) and YPO2275F/R, YPO2278F/R and YPO2280A/B for the detection of the YPO2275, YPO2278 and YPO2280 specific mRNA transcripts, Table S2).

YpfΦ tagging and mutagenesis of phage and chromosomal genes

Gene deletion and phage labeling were done by allelic exchange with a kanamycin resistance cassette following the SFH procedure (Derbise et al., 2003). The Km resistance cassette was obtained by PCR amplification using primer pair 722A/B and plasmid pGP704N-Km as template. The primers used to generate a portion of each target gene

are listed in Table S2. The resulting PCR products were introduced by electroporation into *Y. pestis* CO92 or IP550-HC2 harboring the pKOBEG-*sacB* plasmid, as described previously (Derbise et al., 2003). Recombinant colonies were selected on Km agar plates. Correct insertion of the antibiotic cassette was verified by PCR with primers pairs encompassing one extremity of the Km cassette (166 or 167) and the DNA region adjacent to the target gene (Table S2).

Phage particle preparation

For phage particle preparation, an overnight bacterial culture was diluted 50-fold in fresh MH-Tmp medium and grown for 24 h at 37 °C with shaking. Cultures were centrifuged for 20 min at 7,500 rpm and the supernatant was sterilized by filtration through 0.22 µm filters (Millipore Corporation, Bedford, MA). The filtrate was mixed with one-third volume of a solution containing 20% polyethylene glycol (PEG-6000) and 10% NaCl, and centrifuged at 12,000*g* and 4 °C to precipitate the phage particles. The precipitate was dissolved in Tris–HCl 20 mM, pH 7.5. The absence of bacterial cells in the phage preparation was checked by streaking on Tmp-LB agar plates. An aliquot of 50 µl was treated with pancreatic DNase I (100 U/ml) and RNase A (50 µg/ml) at 37 °C for 30 min to remove possible nucleic acids from lysed bacterial cells. To increase the production of YpfΦ particles, the antibiotic tagged YpfΦ::Tmp phage (Derbise et al., 2007) was introduced into *E. coli* TOP10 strain that was used to propagate the phage. For the various *Y. pestis* deletion mutants, phage preparations were directly obtained from their bacterial supernatant and used for transduction assays.

Transduction assays

A preliminary evaluation of the effect of DNAse and RNAse on the efficiency of transduction of YpfΦ::Tmp indicated that treatment with these enzymes does not modify the phage infectivity, whatever the level of susceptibility of the recipient strain. Therefore, the phage preparations were not further treated with these enzymes. Transduction experiments were performed by mixing in a final volume of 100 µl in MH broth, 10 µl of the phage preparation with approximately 4×10^8 cfu of various recipient strains grown at 28 °C (*Yersinia*) or 37 °C (*E. coli*) on LB agar plates, corresponding to a multiplicity of infection of 30 phages/bacterial cell. After 2 h incubation at 28 °C or 37 °C, 10-fold serial dilutions of cell suspensions were plated on MH-Tmp plates and Tmp^R colonies were counted. The presence of YpfΦ::Tmp was confirmed in several isolated Tmp^R colonies from each transductant, by PCR amplification of the Tmp resistance cassette and phage adjacent sequences (Fig. 1) with primer pair YPO2280aF/YPO2281R. The infectivity rate (IR) was calculated as the number of Tmp^R colonies on the total number of recipient cells. Transduction experiments were repeated three times for each recipient strain.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.07.048.

Acknowledgments

We thank Liliane Martin for providing the *Y. enterocolitica* and *Y. pseudotuberculosis* strains used in this work.

References

Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A., Carniel, E., 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. U. S. A. 96, 14043–14048.

Achtman, M., Morelli, G., Zhu, P., Wirth, T., Diehl, I., Kusecek, B., Vogler, A.J., Wagner, D.M., Allender, C.J., Easterday, W.R., Chenal-Francisque, V., Worsham, P., Thomson, N.R., Parkhill, J., Lindler, L.E., Carniel, E., Keim, P., 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc. Natl. Acad. Sci. U. S. A. 101, 17837–17842.

Blakely, G., Sherratt, D., 1996. Determinants of selectivity in Xer site-specific recombination. Genes Dev. 10, 762–773.

Blakely, G., May, G., McCulloch, R., Arciszewska, L.K., Burke, M., Lovett, S.T., Sherratt, D.J., 1993. Two related recombinases are required for site-specific recombination at *dif* and *cer* in *E. coli* K12. Cell 75, 351–361.

Brubaker, R.R., 1991. Factors promoting acute and chronic diseases caused by yersiniae. Clin. Microbiol. Rev. 4, 309–324.

Campos, J., Martinez, E., Suzarte, E., Rodriguez, B.L., Marrero, K., Silva, Y., Ledon, T., del Sol, R., Fando, R., 2003. VGJ phi, a novel filamentous phage of *Vibrio cholerae*, integrates into the same chromosomal site as CTX phi. J. Bacteriol. 185, 5685–5696.

Chain, P.S., Carniel, E., Larimer, F.W., Lamerdin, J., Stoutland, P.O., Regala, W.M., Georgescu, A.M., Vergez, L.M., Land, M.L., Motin, V.L., Brubaker, R.R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J.M., Derbise, A., Hauser, L.J., Garcia, E., 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. U. S. A. 101, 13826–13831.

Chang, B., Taniguchi, H., Miyamoto, H., Yoshida, S., 1998. Filamentous bacteriophages of *Vibrio parahaemolyticus* as a possible clue to genetic transmission. J. Bacteriol. 180, 5094–5101.

Collyn, F., Lety, M.A., Nair, S., Escuyer, V., Ben Younes, A., Simonet, M., Marceau, M., 2002. *Yersinia pseudotuberculosis* harbors a type IV pilus gene cluster that contributes to pathogenicity. Infect. Immun. 70, 6196–6205.

Crissman, J.W., Smith, G.P., 1984. Gene-III protein of filamentous phages: evidence for a carboxyl-terminal domain with a role in morphogenesis. Virology 132, 445–455.

Davis, B.M., Waldor, M.K., 2000. CTXphi contains a hybrid genome derived from tandemly integrated elements. Proc. Natl. Acad. Sci. U. S. A. 97, 8572–8577.

Davis, B.M., Waldor, M.K., 2003. Filamentous phages linked to virulence of *Vibrio cholerae*. Curr. Opin. Microbiol. 6, 35–42.

Davis, B.M., Kimsey, H.H., Chang, W., Waldor, M.K., 1999. The *Vibrio cholerae* O139 Calcutta bacteriophage CTXphi is infectious and encodes a novel repressor. J. Bacteriol. 181, 6779–6787.

Davis, B.M., Kimsey, H.H., Kane, A.V., Waldor, M.K., 2002. A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. EMBO J. 21, 4240–4249.

Deng, L.W., Malik, P., Perham, R.N., 1999. Interaction of the globular domains of pIII protein of filamentous bacteriophage fd with the F-pilus of *Escherichia coli*. Virology 253, 271–277.

Derbise, A., Lesic, B., Dacheux, D., Ghigo, J.M., Carniel, E., 2003. A rapid and simple method for inactivating chromosomal genes in *Yersinia*. FEMS Immunol. Med. Microbiol. 38, 113–116.

Derbise, A., Chenal-Francisque, V., Pouillot, F., Fayolle, C., Prevost, M.C., Medigue, C., Hinnebusch, B.J., Carniel, E., 2007. A horizontally acquired filamentous phage contributes to the pathogenicity of the plague bacillus. Mol. Microbiol. 63, 1145–1157.

Dotto, G.P., Enea, V., Zinder, N.D., 1981. Gene II of phage f1: its functions and its products. Proc. Natl. Acad. Sci. U. S. A. 78, 5421–5424.

Faruque, S.M., Mekalanos, J.J., 2003. Pathogenicity islands and phages in *Vibrio cholerae* evolution. Trends Microbiol. 11, 505–510.

Gonzalez, M.D., Lichtensteiger, C.A., Caughlan, R., Vimr, E.R., 2002. Conserved filamentous prophage in *Escherichia coli* O18:K1:H7 and *Yersinia pestis* biovar orientalis. J. Bacteriol. 184, 6050–6055.

Guiyoule, A., Grimont, F., Iteman, I., Grimont, P.A., Lefevre, M., Carniel, E., 1994. Plague pandemics investigated by ribotyping of *Yersinia pestis* strains. J. Clin. Microbiol. 32, 634–641.

Haigh, N.G., Webster, R.E., 1999. The pI and pXI assembly proteins serve separate and essential roles in filamentous phage assembly. J. Mol. Biol. 293, 1017–1027.

Hinchliffe, S.J., Isherwood, K.E., Stabler, R.A., Prentice, M.B., Rakin, A., Nichols, R.A., Oyston, P.C., Hinds, J., Titball, R.W., Wren, B.W., 2003. Application of DNA microarrays to study the evolutionary genomics of *Yersinia pestis* and *Yersinia pseudotuberculosis*. Genome Res. 13, 2018–2029.

Huber, K.E., Waldor, M.K., 2002. Filamentous phage integration requires the host recombinases XerC and XerD. Nature 417, 656–659.

Iida, T., Makino, K., Nasu, H., Yokoyama, K., Tagomori, K., Hattori, A., Okuno, T., Shinagawa, H., Honda, T., 2002. Filamentous bacteriophages of vibrios are integrated into the *dif*-like site of the host chromosome. J. Bacteriol. 184, 4933–4935.

Jacobson, A., 1972. Role of F pili in the penetration of bacteriophage fl. J. Virol. 10, 835–843.

Jouravleva, E.A., McDonald, G.A., Marsh, J.W., Taylor, R.K., Boesman-Finkelstein, M., Finkelstein, R.A., 1998. The *Vibrio cholerae* mannose-sensitive hemagglutinin is the receptor for a filamentous bacteriophage from *V. cholerae* O139. Infect. Immun. 66, 2535–2539.

Kimsey, H.H., Waldor, M.K., 1998. CTXphi immunity: application in the development of cholera vaccines. Proc. Natl. Acad. Sci. U. S. A. 95, 7035–7039.

Lesic, B., Carniel, E., 2005. Horizontal transfer of the high-pathogenicity island of *Yersinia pseudotuberculosis*. J. Bacteriol. 187, 3352–3358.

Lesterlin, C., Barre, F.X., Cornet, F., 2004. Genetic recombination and the cell cycle: what we have learned from chromosome dimers. Mol. Microbiol. 54, 1151–1160.

Li, Y., Dai, E., Cui, Y., Li, M., Zhang, Y., Wu, M., Zhou, D., Guo, Z., Dai, X., Cui, B., Qi, Z., Wang, Z., Wang, H., Dong, X., Song, Z., Zhai, J., Song, Y., Yang, R., 2008. Different region analysis for genotyping *Yersinia pestis* isolates from China. PLoS ONE 3, e2166.

Lin, N.T., Wen, F.S., Tseng, Y.H., 1996. A region of the filamentous phage phi Lf genome that can support autonomous replication and miniphage production. Biochem. Biophys. Res. Commun. 218, 12–16.

Lin, N.T., Chang, R.Y., Lee, S.J., Tseng, Y.H., 2001. Plasmids carrying cloned fragments of RF DNA from the filamentous phage (phi)Lf can be integrated into the host chromosome via site-specific integration and homologous recombination. Mol. Genet. Genomics 266, 425–435.

Lindler, L.E., Tall, B.D., 1993. *Yersinia pestis* pH 6 antigen forms fimbriae and is induced by intracellular association with macrophages. Mol. Microbiol. 8, 311–324.

Lobato, M.J., Landeras, E., Gonzalez-Hevia, M.A., Mendoza, M.C., 1998. Genetic heterogeneity of clinical strains of *Yersinia enterocolitica* traced by ribotyping and relationships between ribotypes, serotypes, and biotypes. J. Clin. Microbiol. 36, 3297–3302.

Marvin, D.A., 1998. Filamentous phage structure, infection and assembly. Curr. Opin. Struct. Biol. 8, 150–158.

McLeod, S.M., Waldor, M.K., 2004. Characterization of XerC- and XerD-dependent CTX phage integration in *Vibrio cholerae*. Mol. Microbiol. 54, 935–947.

Mekalanos, J.J., 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell 35, 253–263.

Mel, S.F., Mekalanos, J.J., 1996. Modulation of horizontal gene transfer in pathogenic bacteria by in vivo signals. Cell 87, 795–798.

Meyer, T.F., Geider, K., 1979a. Bacteriophage fd gene II-protein. I. Purification, involvement in RF replication, and the expression of gene II. J. Biol. Chem. 254, 12636–12641.

Meyer, T.F., Geider, K., 1979b. Bacteriophage fd gene II-protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. J. Biol. Chem. 254, 12642–12646.

Model, P., Russel, M., 1988. Filamentous bacteriophages. In: Calendar, R. (Ed.), The Bacteriophages, vol. 2. Plenum Press, New York, pp. 375–456.

Pearson, G.D., Woods, A., Chiang, S.L., Mekalanos, J.J., 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. U. S. A. 90, 3750–3754.

Rakonjac, J., Model, P., 1998. Roles of pIII in filamentous phage assembly. J. Mol. Biol. 282, 25–41.

Rapoza, M.P., Webster, R.E., 1995. The products of gene I and the overlapping in-frame gene XI are required for filamentous phage assembly. J. Mol. Biol. 248, 627–638.

Russel, M., 1995. Moving through the membrane with filamentous phages. Trends Microbiol. 3, 223–228.

Russel, M., Whirlow, H., Sun, T.P., Webster, R.E., 1988. Low-frequency infection of F-bacteria by transducing particles of filamentous bacteriophages. J. Bacteriol. 170, 5312–5316.

Thomson, N.R., Howard, S., Wren, B.W., Holden, M.T., Crossman, L., Challis, G.L., Churcher, C., Mungall, K., Brooks, K., Chillingworth, T., Feltwell, T., Abdellah, Z., Hauser, H., Jagels, K., Maddison, M., Moule, S., Sanders, M., Whitehead, S., Quail, M.A., Dougan, G., Parkhill, J., Prentice, M.B., 2006. The complete genome sequence and comparative genome analysis of the high pathogenicity *Yersinia enterocolitica* strain 8081. PLoS Genet. 2, e206.

Val, M.E., Bouvier, M., Campos, J., Sherratt, D., Cornet, F., Mazel, D., Barre, F.X., 2005. The single-stranded genome of phage CTX is the form used for integration into the genome of *Vibrio cholerae*. Mol. Cell 19, 559–566.

Waldor, M.K., Mekalanos, J.J., 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272, 1910–1914.

Waldor, M.K., Rubin, E.J., Pearson, G.D., Kimsey, H., Mekalanos, J.J., 1997. Regulation, replication, and integration functions of the *Vibrio cholerae* CTXphi are encoded by region RS2. Mol. Microbiol. 24, 917–926.

Wen, F.S., Tseng, Y.H., 1994. Nucleotide sequence determination, characterization and purification of the single-stranded DNA-binding protein and major coat protein of filamentous phage phi Lf of *Xanthomonas campestris* pv. *campestris*. J. Gen. Virol. 75, 15–22.