The Replication, Partition and *yop* Regulation of the pYV Plasmids Are Highly Conserved in *Yersinia enterocolitica* and *Y. pseudotuberculosis*

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The replication genes (*rep*) of the virulence plasmid pYVe439-80 of *Yersinia enterocolitica* were localized and characterized by restriction endonuclease analysis. Comparison with pIB1, a virulence plasmid of *Y. pseudotuberculosis*, indicates that while the plasmids carry homologous *rep* genes their location with respect to the highly conserved 'calcium region' is different. This replication function is thermosensitive. Mini-derivatives of pYVe439-80 appear to be rather unstable. The region of pYVe439-80 containing homology to the *incD* determinant of F was shown to contain a plasmid-stabilization system (*par*). The region encoding *par* was characterized by restriction endonuclease analysis. pIB1 contained an homologous *par* region but located differently. The pYV plasmids thus underwent rearrangements during their divergent evolution. While the positions of *rep* and *par* in the two plasmids are inverted with respect to the surrounding loci, our determination of the orientation of each locus rules out the hypothesis of a simple inversion of a quadrant of pYV. The gene encoding YOP5, a 26 kDa protein encoded by pIB1, was cloned on a mobilizable vector and introduced in *Y. enterocolitica* W22708 containing pYVe227 (indistinguishable from pYVe439-80), mutated in the homologous gene. The recombinant *Y. enterocolitica* secreted YOP5. Hence, the transcriptional activation and secretion systems of pYVe227 act on a *yop* gene from pIB1 and on its product, indicating that these systems are interchangeable.

INTRODUCTION

Pathogenic species of the genus *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) cause human diseases, with symptoms ranging from enteritis to septicaemia and death, essentially by invading the host tissues. The clinical manifestations of *Y. enterocolitica* infections in man are mostly enteric. The predominant clinical features are abdominal pain, sometimes evoking an appendicitis, and diarrhoea.

The three species carry plasmids of about 70 kb that appear to be necessary for virulence (Gemski *et al.*, 1980*a, b*; Zink *et al.*, 1980; Ferber & Brubaker, 1981; Bakour *et al.*, 1985). These plasmids, generally called pYV plasmids, cause a requirement for calcium for growth at 37 °C: pYV-containing yersiniae form colonies at 28 °C but not at 37 °C on media deprived of calcium, while bacteria cured of pYV plasmids or carrying a mutated plasmid grow at both temperatures on oxalated media (see Cornelis *et al.*, 1987*b*).

In conditions of growth restriction, i.e. at 37 °C in the absence of calcium, pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* direct the secretion of at least nine proteins (Heesemann *et al.*, 1984; Cornelis *et al.*, 1987*a*). Some of these proteins are also inserted in the outer membrane (Portnoy *et al.*, 1984), which explains why these proteins are currently referred to as 'YOPs', for '*Yersinia* outer membrane proteins'. The structural genes of these proteins (*yop* genes) are scattered around the pYV plasmids (Cornelis *et al.*, 1987*a*; Forsberg *et al.*, 1987). At least four transcriptional loci, spanning a contiguous 17 kb region and called *virA*, *virB*, *virC* and *virF*, control the calcium response and the production of the YOPs. Gene *virF* encodes a *trans*-

acting transcription activator. The functions of *virA*, *B* and *C* are not yet known (Cornelis *et al.*, 1986, 1987*a*). The region controlling the calcium response is highly conserved in the pYV plasmids from *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Goguen *et al.*, 1984; Portnoy *et al.*, 1984; Cornelis *et al.*, 1986). However, the *yop* genes are distributed differently in these plasmids (Straley & Bowmer, 1986; Cornelis *et al.*, 1987*a*; Forsberg *et al.*, 1987).

The replication functions are also located differently in the pYV plasmid from *Y. enterocolitica* (Bakour *et al.*, 1983; Portnoy *et al.*, 1984) as compared with the pYV plasmids from *Y. pseudotuberculosis* and *Y. pestis* (Portnoy & Falkow, 1981; Portnoy *et al.*, 1984). Portnoy *et al.* (1984) suggested that the replication functions might be different in these two types and that a recombinational event had occurred between two ancestral plasmids, one encompassing the replication region and another encompassing the calcium-dependence region. The pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* both belong to the incompatibility group FI. The only determinant they have in common with F is *incD*. This determinant could be part of a partition system analogous to that of F (Bakour *et al.*, 1983). The present work is mainly devoted to the ancillary functions of pYV, namely replication and partition.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* C600 and HB101 are described in Maniatis *et al.* (1982). *E. coli* JC6310 and JG112 (*polA*) are described in Balligand *et al.* (1985). *Yersinia* strains and plasmids are listed in Table 1.

Liquid cultures were routinely grown in tryptic soy broth (TSB) and plate cultures were on tryptic soy agar (TSA) or MacConkey agar (MC) containing the relevant selective agents at the following concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; merbromin, 100 µg ml⁻¹; nalidixic acid, 35 µg ml⁻¹; and tetracycline, 10 µg ml⁻¹.

Table 1. *Bacterial strains and plasmids*

Bacterial strain	Relevant characteristics	Reference
<i>Y. enterocolitica</i> W227	Wild-type, serogroup 9	Cornelis & Colson (1975)
<i>Y. enterocolitica</i> W22703	Res ⁻ Mod ⁺ Nal ^R derivative of W227	Cornelis & Colson (1975)
<i>Y. enterocolitica</i> 439-80	Wild-type, serogroup 9	Laroche <i>et al.</i> (1984)
<i>Y. pseudotuberculosis</i> YPIII	Wild-type, serogroup III	Bölin <i>et al.</i> (1982)
Plasmid	Relevant characteristics or derivation	Origin or reference
mini-vir-kan	<i>Bam</i> HI fragment 3 of pYVe439-80 + Km ^R fragment of Tn5	Bakour <i>et al.</i> (1983)
pGC653	Mini-vir-kan deleted from 1.7 kb <i>Bst</i> EII fragment	This study
pGC659	pGC653 deleted from (0.9 kb <i>Hind</i> III fragment + 1.1 kb <i>Bgl</i> II fragment)	This study
pGC660	pGC659 reduced by Tn501 transposition followed by deletion of a <i>Hind</i> III fragment	This study
pGC666	pGC660 deleted from 1.7 kb <i>Sma</i> I fragment; = minimal replicon of pYVe439-80 + Km ^R	This study
pGC680	pGC653 + fragment B1 of pYVe439-80	This study
pGC711	pKT279 inserted in the <i>Eco</i> RI site of pGC666 within pYV DNA	This study site of
pIB1	pYV plasmid from <i>Y. pseudotuberculosis</i> YPIII	Bölin <i>et al.</i> (1982)
pIB588	<i>Bam</i> HI fragment 4 of pIB1 cloned in pBR322	Gift of I. Bölin & H. Wolf-Watz (University of Umeå)
pKT279	<i>bla</i> -negative derivative of pBR322	Talmadge & Gilbert (1980)
pTB1	pTM100 + <i>Eco</i> RI- <i>Bam</i> HI fragment of pIB588 containing <i>yop</i> 5	This study
pTM100	pACYC184 + <i>oriT</i> of RP4; <i>oriT</i> was a gift of D. Helinski	Constructed by T. Michiels in this laboratory
pUC9-B5	pUC9 + <i>Bam</i> HI fragment 5 of pIB1	This study
pYVe227	pYV plasmid from <i>Y. enterocolitica</i> W227	Cornelis <i>et al.</i> (1986)
pYVe439-80	pYV plasmid from <i>Y. enterocolitica</i> 439-80	Laroche <i>et al.</i> (1984)
R388::Tn501		This study

For the analysis of YOP proteins, *yersiniae* were grown in brain heart infusion supplemented with 0.4% glucose, 10 mM-MgCl₂, 10 mM-MgSO₄ and 20 mM-sodium oxalate (BHI-MOX). The inoculum of the culture was adjusted to an optical density (600 nm) of 0.1 and the cultures were incubated with shaking (150 r.p.m.) at room temperature. After 2 h, the cultures were shifted to a 37 °C waterbath (thermal induction) and the incubation was continued for 4 h with shaking. The bacteria were then centrifuged for 30 min at 1875 g.

Analysis of proteins. Proteins present in the supernatant were analysed by SDS-PAGE as described in Cornelis *et al.* (1986).

Genetic methods. Conjugations and transformations were done as described in Balligand *et al.* (1985).

Plasmid stability test. A plasmid-containing colony was picked from a selective plate and grown in TSB, under selective pressure. The culture was then diluted and a volume of 100 µl, containing about 100 cells, was inoculated in a 100 ml flask containing 20 ml antibiotic-free TSB. This culture was incubated at room temperature, at 37 °C or at 40 °C with shaking for 40 h. Dilutions of the culture were plated onto MC. Ninety-six colonies were picked, grown for 6 h in 200 µl TSB and replicated onto MC containing the appropriate antibiotic to detect continued carriage of the original plasmid.

Isolation and in vitro manipulation of plasmid DNA. Plasmid DNA was prepared as described previously (Balligand *et al.*, 1985). *In vitro* restriction fragment deletion, cloning, terminal labelling with DNA polymerase I (Klenow) and hybridizations were done according to Maniatis *et al.* (1982).

RESULTS AND DISCUSSION

Accurate localization of the replication region of the pYV plasmid of Y. enterocolitica

Plasmid mini-vir-kan (11.7 kb) consisted of *Bam*HI fragment 3 (hereafter referred to as B3) from pYVe439-80, linked to the kanamycin (Km) resistance determinant of Tn5 (Bakour *et al.*, 1983). Mini-vir-kan was further reduced in size by *Bst*EII digestion, ligation and transformation of *E. coli* C600. None of the Km^R transformants had lost more than a small 1.7 kb fragment (i.e. ΔI in Fig. 1), suggesting that replication genes or sites span the two other *Bst*EII fragments. One of these deletants was called pGC653. pGC653 was reduced in turn to 8.0 kb (pGC659) by removing a 0.9 kb *Hind*III fragment (i.e. ΔII in Fig. 1) and a 1.1 kb *Bgl*II fragment (i.e. ΔIII in Fig. 1).

The extent of the replication region on the right-hand side of the *Bst*EII site (coordinate 4.8 kb of pGC659) was defined by random insertion of a *Hind*III site followed by the excision of the *Hind*III fragment created by this insertion. Transposon Tn501 was selected to apply this strategy because it contains a single *Hind*III site located 136 bp from one terminus; it also contains two *Eco*RI sites within the inverted repeats, at 13 bp from the termini (Brown *et al.*, 1980; see Fig. 1). Transposition of Tn501 was selected by the mobilization procedure: pGC659 and R388::Tn501 were introduced into *E. coli* C600 and the latter strain was subsequently mated with nalidixic-acid-resistant derivatives of *E. coli* HB101. Selection was applied for resistance to kanamycin, nalidixic acid and merbromin. This strategy is based on the transfer of a cointegrate plasmid (de la Cruz & Grinsted, 1982). The resulting strain thus contains R388::Tn501 and pGC659::Tn501. The latter plasmid is separated from the former by a transformation step. All the pGC659::Tn501 derivatives were pooled. Plasmid DNA was extracted, digested with *Hind*III, ligated and used to transform *E. coli* C600. Among 250 transformants, 90 were sensitive to mercuric ions, suggesting that transposition had occurred in the appropriate orientation. Among these, 37 were analysed by gel electrophoresis. The size of the smallest plasmid, called pGC660, was 5.0 kb (Fig. 1).

Plasmid pGC660 was reduced in size by removing the 1.7 kb *Sma*I fragment on the other side of the replication genes. The resulting plasmid, pGC666, is 3.3 kb long and contains 1.32 kb of DNA from Tn5 encompassing the *neo* gene (Beck *et al.*, 1982) and 0.13 kb from Tn501. The minimal replicon of the virulence plasmid pYVe439-80 is hence no larger than 1.85 kb.

Several attempts to isolate the replication genes on an *Eco*RI fragment of pYVe439-80 were unsuccessful (data not shown), suggesting that the essential region for replication encompasses the *Eco*RI site located 0.2 kb from the junction between pYVe439-80 DNA and Tn5 DNA in plasmid pGC666. In order to verify this assumption, plasmid pKT279 was inserted into pGC666 at this *Eco*RI site after partial digestion. The recombinant plasmid, called pGC711, was used to transform, in parallel, *E. coli* strains C600 and JG112 (*polA*), the latter being a strain in which pKT279 does not replicate. Only the former strain could be transformed by pGC711

Fig. 1. Isolation of the minimal region of pYVe439-80 capable of autonomous replication ('minimal replicon'). Plasmid mini-vir-kan was reduced to pGC659 by three successive *in vitro* deletions (Δ I, Δ II, Δ III). Transposon Tn501 was inserted into pGC659 as described in the text. Plasmid DNA was extracted, digested with HindIII, ligated and used to transform *E. coli* C600. Plasmid pGC660 was the smallest of 37 transformants that were analysed. It was in turn reduced by *in vitro* deletion to give pGC666. Ava, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; Cl, *Cla*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I; *neo*, kanamycin phosphotransferase gene of Tn5; *mer*, mercury resistance gene of Tn501.

while both strains were transformed by pGC666, indicating that insertion of pKT279 at the *Eco*RI site disrupted the replication region of pYVe439-80.

We have previously presented a restriction map of a typical pYV plasmid from serogroup 9 *Y. enterocolitica* strains (Laroche *et al.*, 1984). We propose to recalibrate this map and to use the *Eco*RI site located in the replication region as the origin for the coordinate system (Fig. 2).

Thermosensitivity of the replication function

When grown at 28 °C for 25–30 generations, *E. coli* C600 loses the mini-derivatives of pYVe439-80 (mini-vir-kan, pGC653, pGC660) at frequencies ranging from 10% to 18%. pGC653 was also tested at 37 °C and 40 °C. At 37 °C the rate of segregation was in the same range as that found at 28 °C, but at 40 °C it was greater than 99%. The replication function of pYVe439-80 is thus thermosensitive. This feature is particularly relevant to the biology of the plasmid since the virulence phenotype conferred on *Yersinia* is strongly influenced by temperature.

Analysis of the replication function of a pYV plasmid from *Y. pseudotuberculosis*

The origin of replication of pIB1, the pYV plasmid from *Y. pseudotuberculosis* YPIII, was localized in *Bam*HI fragment 4 by Portnoy *et al.* (1984). This 6.6 kb fragment was cloned into pBR322 to give plasmid pIB588. We constructed the restriction map of this plasmid using enzymes that were found to have sites in pGC666. Comparison of the maps of pGC666 and pIB588 shows that the series of eight sites identified in the pYV moiety of pGC666 are exactly as appear in pIB588 (Fig. 3). In order to check the distances between the sites in the two plasmids,

Fig. 2. *Bam*HI restriction map of pYV plasmids pYVe227 (from *Y. enterocolitica* W227; indistinguishable from pYVe439-80) and pIB1 (from *Y. pseudotuberculosis* YPIII). The relative positions of the replication region (*rep*), partition region (*par*) and the region governing calcium dependence are shown. The map also shows the position of some *yop* genes. The restriction map of the *Y. enterocolitica* plasmid is that of Laroche *et al.* (1984) recalibrated by taking the *Eco*RI site within *rep* as coordinate 0 kb. The nomenclature and positions of *vir* and *yop* genes are from Cornelis *et al.* (1986, 1987a). The numbers identifying *yop* genes correspond to the molecular mass (in kDa) of the protein encoded by the gene. The map and nomenclature of pIB1 is from Forsberg *et al.* (1987). *yop2*, *yop3*, *yop4a* and *yop5* encode proteins of 45, 41, 34 and 26 kDa respectively. *yopA* is the gene encoding the largest YOP. The three hatched fragments are highly conserved in pYV plasmids isolated from members of the genus *Yersinia*. The arrows below *rep* and *par* indicate the orientations, and not transcription directions, of the genes. Arrows below *vir* and *yop* genes indicate directions of transcription. S, *Sal*I; X, *Xba*I; Xh, *Xho*I; for abbreviations of other restriction sites see Fig. 1.

pIB588 DNA and pGC666 or mini-vir-kan DNA were digested with *Pst*I, *Hpa*I, *Bst*EII + *Eco*RI and *Pst*I + *Eco*RI and the digests were electrophoresed in parallel (data not shown). All the fragments derived from between the *Pst*I and *Sma*I sites (i.e. coordinates 6.1–8.05 kb of mini-vir-kan) were indistinguishable in size. Hence, we conclude that the pYV plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis* share very closely related replication functions. However, the homology between fragment B3 from pYVe439-80 and fragment B4 of pIB1 does not extend outside this 1.95 kb region.

The *Bam*HI restriction maps of pYV plasmids from *Y. pseudotuberculosis* and *Y. pestis* are very similar (Portnoy *et al.*, 1984). The replication function of the pYV plasmid from *Y. pestis* O19 is located in *Bam*HI fragment 6 (Portnoy & Falkow, 1981). As compared to the region encoding calcium dependency, this location corresponds to that of the replication function of pIB1. It is thus likely that the pYV plasmids of *Y. pseudotuberculosis* and *Y. pestis* also share closely related replication functions.

Relative orientation of the replication regions in pYVe439-80 and in pIB1

Plasmids pYVe439-80 (from *Y. enterocolitica*) and pIB1 (from *Y. pseudotuberculosis*) have homologous replication functions but these are located differently with respect to other common genes. These plasmids appear to have undergone rearrangements during their evolution from a common ancestor. In order to understand these rearrangements, it was necessary to determine the orientation of the replication regions in both plasmids. The data available for pYVe439-80 (Laroche *et al.*, 1984) allowed us to orientate *rep* unambiguously on this plasmid (Fig. 2). For pIB1, the available *Bam*HI map (Bölin & Wolf-Watz, 1984) was not sufficient to orientate *rep* and the strategy outlined in Fig. 4(a) was devised. pIB1 was cleaved with *Sal*I and *Xba*I. The 17 kb fragment was eluted from the agarose gel. This fragment contains *Bam*HI fragments B4,

Fig. 3. Restriction map of pIB588. For abbreviations of restriction sites, see Fig. 1.

Fig. 4. Determination of the orientation of *rep* on pIB1. (a) Outline of the strategy (see text). The figure shows the 17 kb *SalI*-*XbaI* fragment that was isolated. It also shows the two possible positions of the *BglII* site and the maps of the 11 kb fragment that would be generated by a *BglII* digestion, in both cases. For abbreviations of restriction sites, see Figs 1 and 2. (b) Autoradiograph of an electrophoresis of the *Bam*HI digest of pIB1 (lane 1) and of the 11 kb fragment (lane 2). Fragments were labelled terminally after digestion. B10, B11 and B14 are present in the 11 kb fragment.

B10, B11, B13 and B14. It is known from Fig. 3 that fragment B4 is cleaved by *BglII* into two fragments of 1.2 kb and 5.4 kb, the latter containing *rep*. The *SalI*-*XbaI* fragment was cleaved with *BglII*; this digest gave a fragment of 11 kb and fragments smaller than 5.4 kb. The *rep* gene must be located in the 11 kb fragment, and the order of the sites is *BglII*, *SmaI*, *EcoRI* and *BstEII* (see Fig. 3). The 11 kb fragment was isolated and cleaved with *Bam*HI in order to determine whether it contains B13 or B10, B11 and B14. The restriction fragments were labelled terminally and run in parallel with a *Bam*HI digest of pIB1. As shown in Fig. 4(b), this digest contained B10, B11 and B14, which implies that *rep* is in orientation I in pIB1. Hence the *rep* genes are in the opposite orientation in pIB1 and pYVe439-80 (Fig. 2).

Stabilization of pYVe439-80

Like other low-copy-number plasmids, pYVe439-80 is very stably inherited at low temperature (Bakour *et al.*, 1983). In contrast, the mini-derivatives of pYVe439-80 are lost at 28 °C at a rate of about 10% in 25–30 generations, suggesting that a partitioning system (Nördstrom *et al.*, 1980) was lost during the process of reduction.

Since pYVe439-80 possesses an *incD* incompatibility determinant (Bakour *et al.*, 1983) and since this determinant is involved in the maintenance of F (Phua *et al.*, 1982; Hayakawa *et al.*, 1985), we assumed that the *incD* determinant of pYVe439-80 could exert the same maintenance function. To verify this hypothesis, the largest *Bam*HI fragment (B1) of pYVe439-80 containing *incD* (Bakour *et al.*, 1983), was cloned into the unique *Bam*HI site of pGC653. The ligation mixture was used to transform *E. coli* JC6310(F'*lac*). This allowed an easy screening of the recombinant plasmid because of the incompatibility resulting from the presence of *incD* on both F'*lac* and the recombinant plasmid. The Km^R transformants giving minute colonies were restreaked on MC + kanamycin. Plasmids in lactose-negative clones were analysed by *Bam*HI restriction and the relevant recombinant clone, i.e. pGC653-B1, was called pGC680.

E. coli C600(pGC680) and *E. coli* C600 (pGC653) were subjected in parallel to 14 independent segregation experiments at 28 °C. pGC653 segregated at a rate of $9.5 \pm 3.8\%$ (mean $\pm$ SE), while pGC680 segregated at a rate of only 0.07%. Thus, the insertion of the 13.8 kb B1 fragment reduced the segregation rate by a factor of 135. Hence, we conclude that B1 encodes a partitioning system analogous to that of F. This system most probably involves *incD*.

We localized the determinant *incD* of pYVe439-80 in the *Eco*RI (coordinate 46.7 kb)–*Bam*HI (coordinate 52.4 kb) fragment of B1 by Southern blot hybridization (data not shown) with *Eco*RI fragment 5 of F (called f5), and were surprised to find that the position of these *par* genes in pYVe439-80 corresponded to that of the *rep* genes in pIB1 (see Fig. 2).

Localization of the partitioning system in pIB1

Bakour *et al.* (1983) showed that the pYV plasmid of a *Y. pseudotuberculosis* strain also belongs to the incompatibility group F1, and they speculated that this plasmid also carried an *incD* determinant. We localized this determinant on pIB1 by Southern blot hybridization with f5. This analysis revealed homology with fragment *Bam*HI 5 of pIB1 (called B5) (data not shown). The latter fragment was purified and cloned into pUC9 for characterization. The map of B5 was compared to that of fragment B1 from pYVe439-80. As shown in Fig. 2, there is an identical series of 10 sites in the two fragments, the only differences being two additional *Sal*I and *Hind*III sites near the centre of B5 and one additional *Xba*I site close to the end of B1. Southern blot hybridization experiments (not shown) confirmed the homology between these regions of the two pYV plasmids. Hence, we conclude that both plasmids rely on the same partition function.

It is striking that the position of the *par* genes of pIB1 corresponds to that of the *rep* genes of pYVe439-80 (see Fig. 2). This suggests that an inversion occurred in one of the two plasmids during evolution. This hypothesis is supported by the fact that the *rep* genes are in opposite orientation in the two plasmids. However, the position of *yopA*, determined by Bölin & Wolf-Watz (1984) in pIB1 and by Balligand *et al.* (1985) in pYVe227 can not be explained by this hypothesis. Hence, we decided to determine the relative orientation of the *par* genes in the two plasmids.

The orientation of fragment B1 in the map of pYVe439-80 was determined by comparison with the *Eco*RI, *Bam*HI map of Laroche *et al.* (1984). In order to orientate fragment B5 in the map of pIB1 (Bölin & Wolf-Watz, 1984), we carried out a *Bam*HI digestion on the small *Sal*I fragment isolated from this plasmid (see Fig. 4). This digest was analysed in parallel with a *Sal*I + *Bam*HI digest of pUC9-B5. Both digests generated the small *Sal*I–*Bam*HI subfragment of B5 rather than the large one (data not shown), which allowed us to orientate fragment B5 in pIB1. This orientation agrees with the respective position of the *Sal*I and *Xba*I sites in the map of Bölin & Wolf-Watz (1984) and with the position of *yop5* given by Forsberg *et al.* (1987) (see Fig. 2). Hence, we conclude that the *par* regions of the two plasmids are orientated similarly in the two pYV plasmids. This result confirms that the rearrangement that occurred in these pYV plasmids did not arise simply by the inversion of a large pYV quadrant. This is also confirmed by

Fig. 5. SDS-PAGE of secreted proteins. Lane 1, *Y. enterocolitica* W22703(pGC1256); lane 2, *Y. enterocolitica* W22703(pYVe227); lane 3, *Y. enterocolitica* W22703(pGC1256)(pTB1); lane 4, *Y. pseudotuberculosis* YPIII(pIB1). The arrows point to the 25 and 26 kDa proteins (called YOP25 in *Y. enterocolitica* and YOP5 in *Y. pseudotuberculosis*).

the fact that *yopA* seems to occupy the same locus in both plasmids (see Fig. 2). In the inversion hypothesis, *yopA* would have been included in the inverted segment and hence would be located differently in the two maps. The orientation of *yopA* in either plasmid is not yet known.

The yop regulons of the pYV plasmids from Y. enterocolitica and Y. pseudotuberculosis are functionally related

The pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* govern the synthesis and secretion of YOP proteins, which are encoded by pYV genes called *yop* genes. The production of these proteins requires the activity of at least four other plasmid genes, *virA*, *virB*, *virC* and *virF* (Cornelis *et al.*, 1986, 1987a). We wanted to determine whether a *yop* gene from *Y. pseudotuberculosis* could be expressed in *Y. enterocolitica*.

On plasmid pYVe439-80, gene *yop25*, encoding YOP25 (25 kDa), maps around coordinate 47 kb (Cornelis *et al.*, 1987a), i.e. close to *incD*. In *Y. pseudotuberculosis*, the gene encoding YOP5 (26 kDa) maps in *Bam*HI fragment 5 (Forsberg *et al.*, 1987). According to the restriction maps presented in Fig. 2, the region that appears to be conserved around *par* in both species includes this *yop* gene. The *Bam*HI-*Eco*RI fragment of pIB588 was cloned on pTM100, a mobilizable derivative of pACYC184 (T. Michiels, unpublished construction), and the

recombinant plasmid, pTB1, was introduced into *Y. enterocolitica* W22703(pGC1256). The latter plasmid is a derivative of pYVe227 mutated in *yop25*. We analysed the profile of the proteins secreted by W22703(pGC1256)(pTB1). As shown in Fig. 5, this strain of *Y. enterocolitica* clearly secreted YOP5. Hence the *Y. pseudotuberculosis* gene was expressed and its product was secreted in this *Y. enterocolitica* background.

Thus, the virulence regulon present on pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* is functionally conserved in spite of the fact that some *yop* genes evolved and suffered internal translocations. This evolution is illustrated by the fact that the homologous genes *yop25* (from *Y. enterocolitica*) and *yop5* (from *Y. pseudotuberculosis*) encode proteins of 25 and 26 kDa respectively and map differently on the pYV plasmids.

Concluding remarks

The pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* possess the same replication and partition functions and they carry functionally analogous *yop* regulons. They appear to have originated from a common ancestral plasmid as a result of structural rearrangements.

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