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Identification of Additional Virulence Determinants on the pYV Plasmid of *Yersinia enterocolitica* W227

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This paper describes the mutagenesis of the pYV plasmid from *Yersinia enterocolitica* W22703 (serotype O:9) with Tn*2507*, a new element generating operon fusions. Analysis of the mutants allowed the identification of an additional Yop protein called Yop20 and the mapping of *yop20*, *yop44*, *yop48*, and *lcrV*, the gene encoding the V antigen. The last gene appeared to be part of an operon that also may contain *yop37* and *yop44*. At 37°C, mutants affected in this operon grew poorly, irrespective of the presence of Ca^{2+}, or they even died in the presence of Ca^{2+}. This operon is thus involved in the regulation by Ca^{2+}, and we called it *car*, for Ca^{2+} regulation. It is presumably the *Y. enterocolitica* counterpart of the *lcrGVH* operon of *Yersinia pestis*. Transcription of *yop20* and of the *car* operon was strongly regulated by temperature and only slightly by calcium. Hence, these genes behaved like the other genes of the *yop* regulon. Mutants affected in *yop20* or in *yop48* were markedly less virulent for the desferrioxamine-treated mouse than was the parental strain. Yop20 and Yop48 thus probably are *Yersinia* virulence factors.

The genus *Yersinia* contains three species which are pathogenic for humans. *Yersinia pestis* is the causative agent of plague. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are responsible for gastrointestinal diseases with symptoms ranging from enteritis to mesenteric lymphadenitis sometimes complicated by septicemia.

Tissue invasion is believed to be the first step of the infection, and two genes, *inv* and *ail*, that facilitate invasion of the epithelial cells in vitro have been cloned from the chromosomes of *Y. enterocolitica* and *Y. pseudotuberculosis* (23, 29). Later stages of the infection and, in particular, interaction with the host immune defenses (34) depend on bacterial functions encoded by a 70-kilobase plasmid called pYV (17). *Yersinia* strains harboring this pYV plasmid are calcium dependent (phenotype CD) for growth at 37°C; at this temperature and in the absence of calcium, bacteria stop growing and secrete large amounts of plasmid-encoded proteins called Yops (19; for reviews, see references 6 and 11).

These Yops appear to be highly conserved in the three species. Most of them were shown to have similar molecular masses and to be immunologically related (3, 8, 20, 21, 36). Sequence data show that gene *yop51* from *Y. enterocolitica*, encoding a 50,882-dalton (Da) protein, is 99% homologous with *yopH* (encoding Yop2b), its counterpart from *Y. pseudotuberculosis* (5, 28a). However, this very high homology does not extend outside the *yop* genes, suggesting that the pYV plasmids from the three species contain cassettes of very high homology interspersed by nonhomologous DNA.

Several *yop* genes have been mapped on pCD1 from *Y. pestis* KIM (38), on pIB1 from *Y. pseudotuberculosis* (15), and on pYVe227 (14) or pYV8081 (3) from *Y. enterocolitica*. The localization of the genes appears to be similar in pCD1 and pIB1 but different in pYVe227 and pYV8081. However, pYV plasmids are clearly derived from a common ancestor that suffered rearrangements within a quadrant, as shown by the inverted locations of the replication and partition regions (2).

yop genes are coordinately regulated by a 20-kilobase region of the plasmid called the calcium region or the *lcr* region. This region is highly conserved in the three species. It was shown to contain at least four loci called *virA*, *virB*, *virC*, and *virF* (13, 14) or *lcrA*, *lcrB*, *lcrC*, and *lcrF* (18). The *lcrA* locus was shown later to contain two new loci called *lcrD* and *lcrE* (41). The *yop* genes constitute a temperature-controlled regulon activated by a regulatory gene called *lcrF* (40) or *virF* (14). The product of *virF* was recently shown to be a 30,879-Da transcriptional activator (12) related to AraC, the activator of the arabinose operon (39) and to Rns, the activator of the Cs pili genes (7a). Although temperature seems to play the major role in transcription control, Ca^{2+} was also shown to be involved in the regulation of *yop* genes (5, 16, 40, 41).

Some of the Yops were shown to be essential virulence determinants (5, 16, 34, 37, 38). In particular, Yop2b from *Y. pseudotuberculosis* appeared to be involved in the inhibition of phagocytosis by mouse macrophages (34).

Y. enterocolitica W22703, a typical serotype O:9 strain, was previously shown to release eight Yops, excluding the fibrillar protein P1, also known as Yop1 (1, 24). Five genes have already been mapped (*yop25*, *yop30*, *yop37*, *yop51*, and *yop84*) on pYVe227. In this work, we identify a new Yop called Yop20 and we report the localization of *yop44*, *yop48*, *yop20*, and *lcrV*. We also show that Yop20 and Yop48 are essential virulence determinants in the mouse model. In addition, we show that genes *lcrV* (encoding a protein of 41 kDa known as the V antigen), *yop37* (encoding the 37-kDa Yop and previously called *yopD*), and *yop44* of pYVe227 may be part of a single operon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Y. enterocolitica* W22703(pYVe227) is a nalidixic acid-resistant (Nal^r) restriction mutant (Res^- Mod^+) isolated earlier in this laboratory from strain W227 (10). Plasmid pYVe227, the natural plas-

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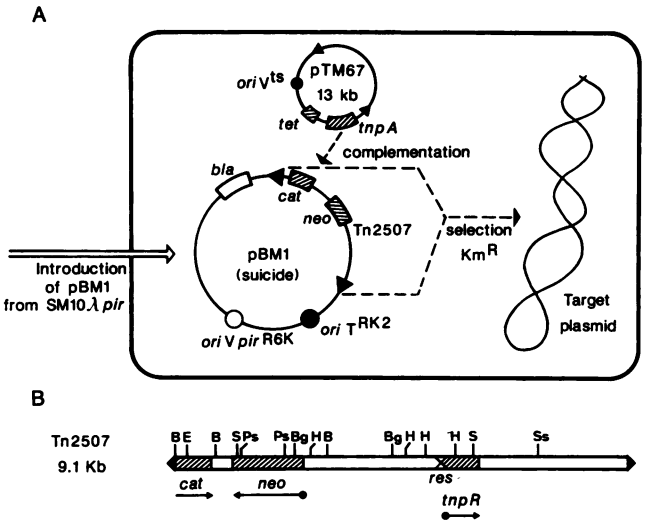

FIG. 1. (A) Strategy of transposition. Tn*2507*, present on the suicide plasmid pBM1, is introduced in the target-containing cell by conjugation, with *E. coli* SM10(λ *pir*$^+$) as a donor. The recipient cells containing the target also contain pTM67 carrying *tnpA* to complement Tn*2507*. After conjugation, transposants are selected on kanamycin. *bla*, β-Lactamase gene; *cat*, CAT gene; *neo*, kanamycin resistance gene (APH 3′); *oriT*, origin of transfer of RK2; *oriV*(*pir*), *pir* mutant, defective, origin of replication R6K; *tet*, tetracycline resistance gene; *tnpA*, transposase gene from Tn*3*. (B) Map of Tn*2507*. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; S, *Sal*I; Ss, *Sst*II; *res* and *tnpR*, resolution site and resolvase gene of Tn*2501*, respectively. The arrows indicate directions of transcription. Kb, Kilobases.

mid of W227, is a typical representative of the pYV plasmids from serotype O:9 *Y. enterocolitica* strains (2, 25). *Y. pseudotuberculosis* YPIII(pIB1) was a gift of H. Wolf-Watz.

Escherichia coli S17.1 (35) was used to mobilize pTM67 into *Y. enterocolitica* W22703. Strain SM10(λ *pir*$^+$) of *E. coli*, constructed by Miller and Mekalanos (30), allows the replication of *pir* mutants of R6K and also mobilizes plasmids containing the origin of transfer of RK2 (35).

Liquid cultures of *Yersinia* spp. were grown on brain heart infusion medium (BHI) supplemented with 0.4% glucose and either 20 mM sodium oxalate–20 mM $MgCl_2$ (BHI-OX) or 5 mM $CaCl_2$ (BHI-Ca^{2+}). Solid cultures were grown on tryptic soy agar (TSA), sometimes supplemented with 20 mM $MgCl_2$ and 20 mM sodium oxalate (MOX). Media were supplemented with relevant selective agents (ampicillin, 100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; nalidixic acid, 35 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}).

Construction of transposon Tn*2507*. We constructed Tn*2507* by cloning the chloramphenicol acetyltransferase (CAT) gene (*cat*) lacking its promoter (9) at one *Bam*HI site of the defective class II transposon Tn*2505* (28). Tn*2507* carries a constitutive kanamycin resistance gene (*neo*) and the *cat* gene oriented to be transcribed from external promoters (Fig. 1B). This element has no *tnpA* gene; its transposition requires the presence of a *trans*-acting *tnpA* gene, but once inserted, it should not promote secondary mutations in the absence of *tnpA*.

Description of the delivery plasmid pBM1 and the complementing plasmid pTM67. Tn*2507* was transposed onto the suicide vector pJM703.1 developed by Miller and Mekalanos (30). This plasmid contains a *pir* mutant replication function of R6K and a β-lactamase gene. It only replicates in strains containing a *trans*-acting *pir* gene, such as *E. coli* SM10(λ *pir*$^+$) (30). Plasmid pJM703.1 contains the origin of transfer of RK2, and it can thus be mobilized from its host strain SM10(λ *pir*$^+$), which contains a copy of RP4 integrated in its chromosome. The recombinant plasmid pJM703.1::Tn*2507* was called pBM1.

The plasmid supplying *tnpA* (22) was pTM67. This replication thermosensitive derivative of pSC101 was constructed from pMBLG2 (28) by deletion of an *Sst*II restriction fragment. Besides *tnpA*, this plasmid contains two termini of Tn*3*—in head-to-tail orientation—which render it immune to Tn*2507* transposition (26). After the transpositional event had occurred, this ancillary plasmid was cured by subculture of the transposants.

Transposition mutagenesis. Transposition of Tn*2507* onto pYVe227 occurred after *E. coli* SM10(λ *pir*$^+$) (pBM1) was mated with the Nal^r *Y. enterocolitica* strain W22703 (pYVe227) (pTM67). Transposants were selected on TSA plates containing kanamycin and nalidixic acid (Fig. 1A). Two types of event could give rise to Km^r recombinants in this system: (i) transposition of Tn*2507* and (ii) homologous recombination between pTM67 and pBM1 because both plasmids contain homologous DNA. The latter event could be identified by the ampicillin-resistant phenotype of the recombinants. About 90% of the recombinants were Ap^s, indicating that transposition occurred rather than homologous recombination. Like its parent transposon Tn*2505* (28), Tn*2507* transposes at a higher frequency (10^4-fold) onto plasmids than onto the chromosome. The transposants were thus assumed to contain plasmid insertions.

Induction of the *yop* regulon, analysis of the Yops, and CAT

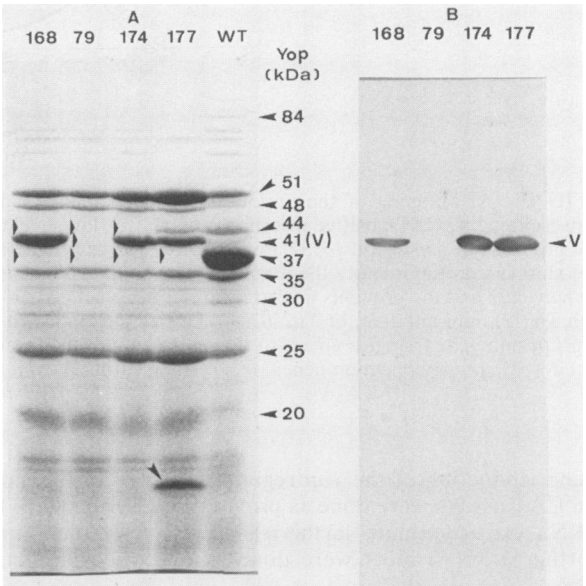

FIG. 2. Analysis of the Yops encoded by the different Gts pBM mutants. (A) SDS-PAGE (Coomassie blue staining) of the supernatant of induced cultures. The numbers on top are the pBM mutant numbers. WT, Wild type (plasmid pYVe227). The short arrows point to missing Yops, while the long arrow in lane pBM177 identifies a truncated protein. The numbers identifying the Yops correspond to their apparent molecular masses. (B) Immunoblot of the same preparations treated with a specific antiserum directed against the V antigen of *Y. pestis*.

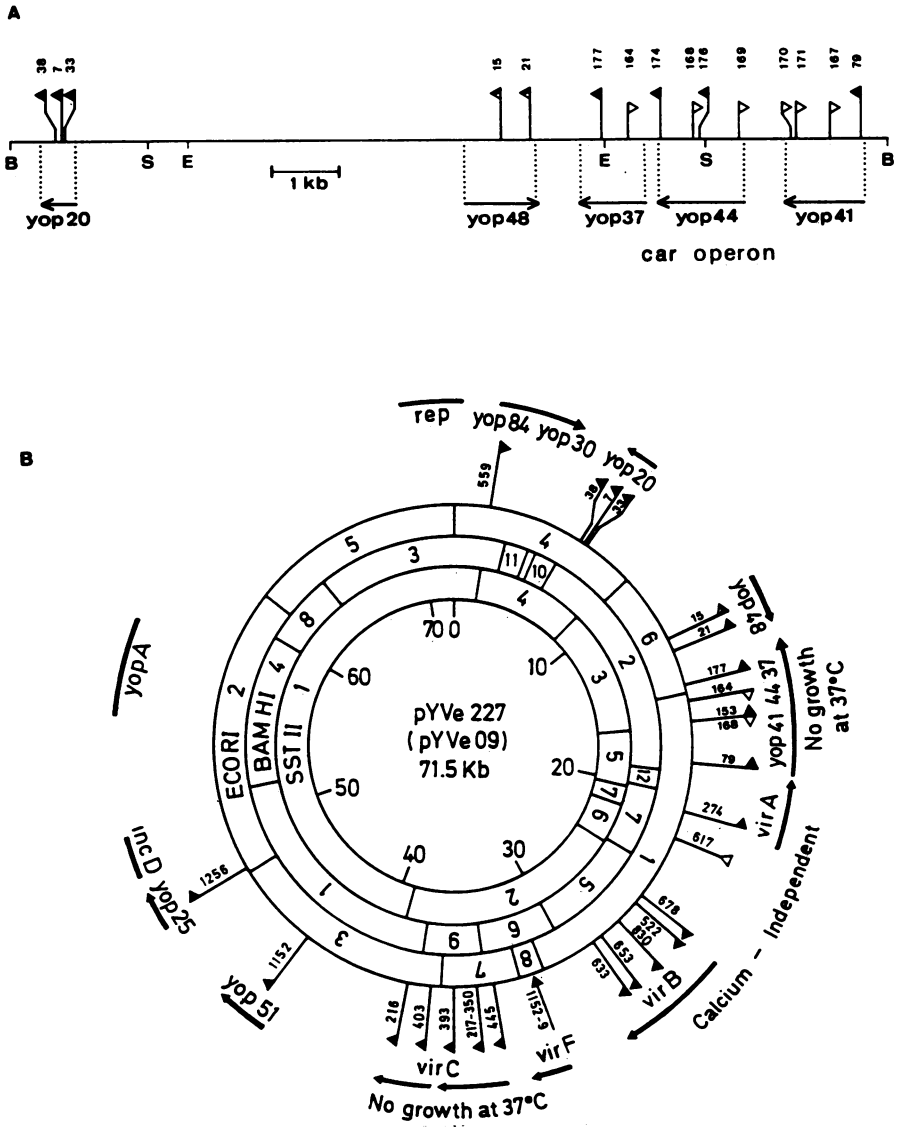

FIG. 3. (A) Mapping of the various insertion points of Tn*2507* within *Bam*HI fragment B2. The triangles point in the direction of transcription. ◀, CAT-positive insertion mutants; ◁, low level of *cat* expression; ▷, lack of *cat* transcription. Arrows symbolize *yop* genes. The positions of *yop48* and *yop37* are supported by the production of truncated peptides; the positions of the other genes are only tentative. The numbers on top identify the mutants (for example, 38 means pBM38). B, *Bam*HI; E, *Eco*RI; S, *Sst*II. (B) Map of pYVe227 integrating the new data and the previous ones (from references 1, 2, 11, 13) to give a complete picture. All the flags identify insertion mutants obtained with Tn*813*, mini-mu d*lac*, or Tn*2507*. ◀, Operon fusion led to expression of the probe gene (*lac* or *cat*); ◁, lack of transcription; ◬, poor transcription. The triangles give also the orientation of the inserted probe genes, while the arrows give the orientation of pYV genes. *yopA* encodes P1. *rep*, Replication genes; *incD*, stabilization region.

assays. Induction of the *yop* regulon, analysis of the Yops, and CAT assays were done as previously described (12, 14).

RNA extraction and Northern blots. RNA extraction and Northern (RNA) blots were done as described previously (28a).

Infection of mice. Specific-pathogen-free ICR female Swiss mice (Charles River Breeding Laboratories, Saint-Aubin-lès-Elbeuf, France), 6 weeks old, were given intraperitoneally 0.5 ml of saline (0.15 M NaCl) containing 20 mg ml^{-1} desferrioxamine (Desferal; CIBA-GEIGY). Twenty-four hours later, mice were inoculated intravenously (i.v.) with 0.5 ml of a *Y. enterocolitica* suspension in saline. For challenges, bacteria were prepared from overnight cultures at room temperature in tryptocasein-soy broth, washed once, and then suspended in saline. Infected animals (groups of 10 mice) were observed for 3 weeks, and the 50% lethal dose (LD_{50}) was determined by the probit method. Growth of bacteria in the spleens and livers of animals was monitored in relation to time after the i.v. injection. Groups of four mice were sacrificed by chloroform anesthesia, and the organs were removed aseptically and homogenized separately in saline; 0.1-ml volumes of serial 10-fold dilutions in saline were spread on TSA, and colonies were counted after incubation for 48 h at room temperature. Minimal detectable limits were 10^2 bacteria $organ^{-1}$. Results were expressed as the $\log_{10}$ of bacterial counts.

Immunoblot analysis. Immunoblot analysis was done as described in reference 37. The antiserum directed against the V antigen of *Y. pestis* was a kind gift of R. Brubaker.

RESULTS

Transposition mutagenesis. A bank of mutants of the pYV plasmid from serotype O:9 *Y. enterocolitica* W22703 was constructed by transposition of Tn*2507*. This new element creates operon fusions with a *cat* gene (Fig. 1B). The delivery plasmid was the mobilizable suicide plasmid pJM703.1, developed by Miller and Mekalanos (30). The strategy of transposition is detailed in Materials and Methods and outlined in Fig. 1A.

A total of 1,920 transposants were screened by replica plating for calcium dependency and chloramphenicol resistance under conditions of variable temperature and calcium concentration. Seventy-eight mutants were calcium independent and were assumed to carry Tn*2507* in the calcium region. The growth of 19 mutants was restricted at 37°C, even in the presence of Ca^{2+}. We call these mutants Gts mutants (growth thermosensitive). This phenotype was encountered earlier (1, 13). Forty-three mutants were calcium dependent and resistant at 37°C to 40 μg of chloramphenicol ml^{-1} but sensitive to this concentration at 28°C. We assumed that, in these mutants, Tn*2507* was inserted into a thermoactivated gene.

The *lcrV yop44 yop37* operon. With respect to Yop production, the 19 Gts mutants fell into three categories (Fig. 2A). (i) The first category contains seven mutants that failed to express only Yop37 (pBM164, pBM172, pBM175, pBM177 [Fig. 2A], pBM178, pBM179, and pBM180). Three of them (pBM164, pBM177, and pBM179) secreted truncated proteins with molecular masses of 12, 15, and 24 kDa, respectively. (ii) The second category contains eight of the Gts mutants that produced neither Yop37 nor Yop44 (pBM162, pBM163, pBM165, pBM168 [Fig. 2A], pBM169, pBM173, pBM174 [Fig. 2A], and pBM176). (iii) The third category contains the remaining four Gts mutants (pBM79 [Fig. 2A], pBM167, pBM170, and pBM171) that failed to produce Yop37, Yop44, and Yop41.

Protein Yop41 was found to react in immunoblot experiments with a specific anti-V serum (Fig. 2B). Moreover, Yop41 appeared to be present in the cytoplasmic fractions of induced cells, as well as in the supernatant of the cultures (data not shown). Hence, we inferred that Yop41 is the V antigen, encoded by *lcrV*.

The existence of these three categories of mutants suggested that *lcrV*, *yop44*, and *yop37* could be part of a single operon. The insertion points of Tn*2507* in 10 Gts mutants were mapped by *Bam*HI, *Eco*RI, and *Sst*II restriction analysis. As expected from the previous mapping of *yop37* (also called *yopD*) (13), all the mutations were clustered on *Bam*HI fragment 2 (called B2). All the mutants expressing *cat* carried Tn*2507* in the same orientation. As shown in Fig. 3A, the order of the insertion points, as well as the direction of transcription of the genes, supported the hypothesis of a single operon transcribed from *lcrV* to *yop37*.

To confirm that all three Yops are produced from a single messenger, RNA from induced cultures of strains carrying pBM79, pBM174, pBM168, and pBM177 was extracted and analyzed by Northern blot hybridization using the *cat* gene of Tn*2507* as a probe. As shown in Fig. 4, the high molecular weights of the major transcripts agreed with the hypothesis of a common promoter localized close to the right end of fragment B2. The probe did not hybridize with RNA from mutant W22703(pBM168) in which Tn*2507* was inserted in inverted orientation with respect to the operon transcription direction. This confirmed that the probe specifically hybridized to *cat* mRNA synthesized from the external promoter.

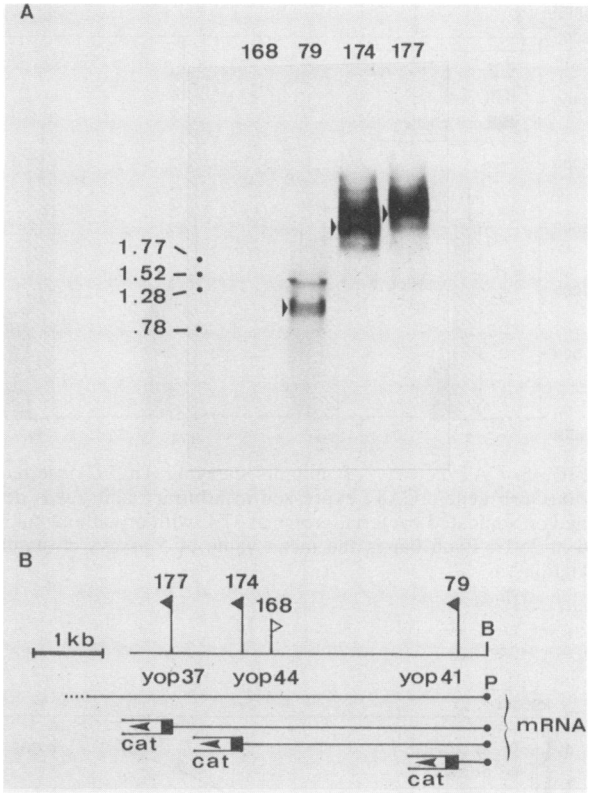

FIG. 4. Analysis of the heat-induced transcripts of the mutants affected in the *lcrV yop44 yop37* operon. (A) Northern blots; the numbers above the figure identify the pBM mutant. The numbers on the left are reference sizes (in kilobases). The probe was ^{32}P-labeled *cat* DNA. RNA was extracted from the cultures that released the Yops presented in Fig. 2. (B) Map of the insertion mutations in fragment B2 and schematic representation of the mRNAs revealed by the Northern blot. B, *Bam*HI; P, promoter; cat, *cat* gene (present at the end of Tn*2507*).

Regulation of transcription throughout the operon was analyzed by monitoring the CAT activity of mutants carrying Tn*2507* in the three loci. Strains with pBM164, pBM168, and pBM170 carrying Tn*2507* inserted in inverted orientation with respect to the operon exhibited a very weak or undetectable activity. Strains carrying either pBM79, pBM174, or pBM177 exhibited a very clear CAT activity at 37°C but not at 25°C. Ca^{2+} seemed to have a two- to fourfold effect on the transcription at 37°C of pBM177 and pBM174 but no effect on the transcription of pBM79 (Fig. 5). This result must be interpreted with care, because Ca^{2+} appeared to be toxic for the former mutants but not for the latter one (see below).

Despite the high level of transcription of these genes at 37°C in the presence of calcium, no Yops were detected in the supernatant of the induced cultures by the Coomassie blue-staining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (data not shown). The Ca^{2+} control of the release of the Yops is thus unaffected in these mutants.

We measured the growth rate of *Y. enterocolitica* W22703 harboring one mutant of each category, namely, pBM79, pBM174, and pBM177. Strain W22703(pBM7) was taken as

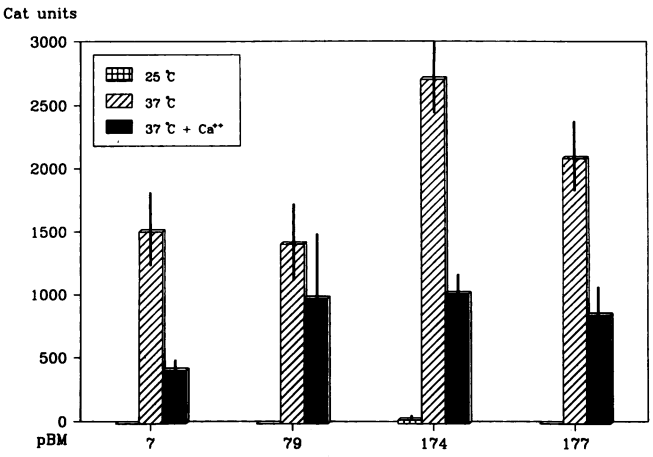

FIG. 5. CAT activity of mutants carrying Tn*2507* inserted in various *yop* genes. CAT, expressed in arbitrary units, was determined on sonicated bacteria, grown at 37°C with or without calcium and at 25°C. Each bar is the mean value of 5 assays ± standard deviation.

a typical Ca^{2+}-dependent control. As shown in Fig. 6, the three mutants failed to grow at 37°C, even in the presence of Ca^{2+} (Gts phenotype). Surprisingly, the viable counts of strains carrying mutants pBM174 and pBM177 decreased after transfer to 37°C in the presence of Ca^{2+}. After a few hours, growth of these strains seemed to resume, but most of the growing bacteria turned out to behave like plasmidless variants (Ca^{2+} independent; CI phenotype). Thus, some Gts mutants appeared to be sensitive to Ca^{2+} for their growth at 37°C. This new phenotype was called CS, for Ca^{2+} sensitive. Since it was not exhibited by the proximal mutant W22703(pBM79), it must have been related to the loss of the distal part of the putative operon.

Mapping of *yop20* and *yop48*. The Yops produced by the 43 CD mutants that acquired a thermoinducible chloramphenicol resistance were analyzed by SDS-PAGE. The pattern of Yops produced by three mutants (pBM7, pBM33, and pBM38) lacked a broad and faint band made of one or more proteins of about 20 kDa that we call Yop20. Two other mutants (pBM15 and pBM21) failed to produce Yop48. Figure 7B shows the Yops released by some of these mutants.

*Bam*HI, *Eco*RI, and *Sst*II restriction analysis of the three *yop20* mutants mapped the Tn*2507* insertions in *Bam*HI

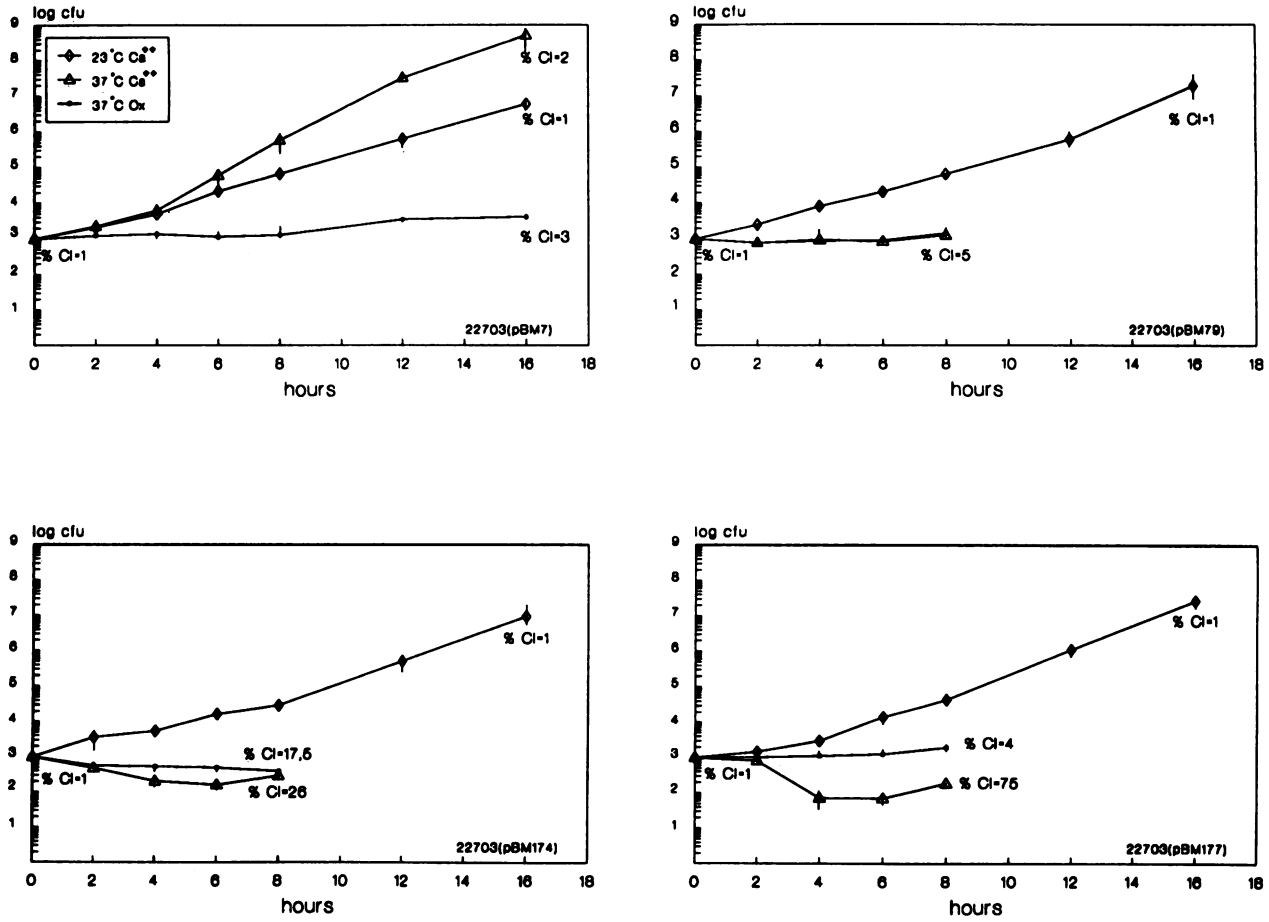

FIG. 6. Growth curves of W22703 carrying pBM7 (Ca^{2+}-dependent phenotype), pBM7, pBM174, and pBM177. Cultures were grown at room temperature in BHI-Ca^{2+}, at 37°C in BHI-Ca^{2+}, and at 37°C in BHI-OX. Bacterial counts were done by plating bacteria on MOX agar at room temperature. For accurate counting, the inoculum of the cultures was about 10^3 CFU ml^{-1} for the cultures grown at room temperature and 10^4 CFU ml^{-1} for the cultures grown at 37°C. The curves have then been normalized to an identical inoculum, for the sake of clarity. The percentage of Ca^{2+}-independent variants (%CI) was measured at the beginning and the end of the curves. Vertical bars give the standard deviations for the counts.

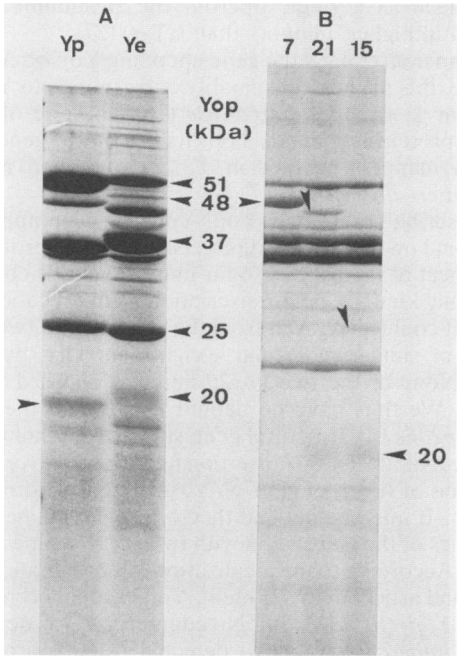

FIG. 7. (A) Comparison of the proteins released by *Y. enterocolitica* W22703 and *Y. pseudotuberculosis* YPIII. The arrows point to the Yops produced by *Y. enterocolitica* and to a *Y. pseudotuberculosis* fuzzy band presumed to correspond to Yop20. (B) SDS-PAGE analysis of the Yops released by mutants affected in *yop48* (pBM15 and pBM21) and *yop20* (pBM7). The numbers on top identify the pBM mutants. The arrows point to Yop48, Yop20, and truncated peptides.

fragment B2 and *Eco*RI fragment E4 (Fig. 3A and 3B). All three mutants expressed *cat* in a thermodependent manner (Fig. 5 gives the values for pBM7). The orientation of Tn*2507* in these mutants indicated that *yop20* is in the same orientation as the *lcrV yop44 yop37* operon. The comparison of the profiles of the Yop proteins released by *Y. enterocolitica* W22703 and *Y. pseudotuberculosis* YPIII showed that *Y. pseudotuberculosis* produced a protein giving a band with the same diffuse appearance as Yop20 but of slightly lower molecular weight (Fig. 7A).

Mutants pBM15 and pBM21 affected in *yop48* released truncated peptides of 28 and 46 kDa, respectively, indicating that insertions occurred in the structural gene (Fig. 7B). The relative positions of Tn*2507* in these two mutants allowed deduction of the orientation and accurate localization of the gene. According to these results, *yop48* maps in fragment B2, between *yop20* and the *lcrV yop44 yop37* operon, but in the opposite orientation (Fig. 3A and 3B). In both mutants, the *cat* gene was weakly expressed in spite of the fact that it was not inserted in the same orientation as *yop48* (Fig. 5).

TABLE 1. LD_{50} of *Y. enterocolitica* W22703 carrying pVYe227 or its mutants affected in *yop20* or *yop48*

pYV plasmid	Genotype	LD_{50} (i.v. route)[a]
pYVe227	Wild type	$10^{3.5}$
pBM7	*yop20*	$10^{5.5}$
pBM21	*yop48*	$10^{6.2}$
None	Cured of pYV	$>10^{8}$

[a] Bacteria $mouse^{-1}$.

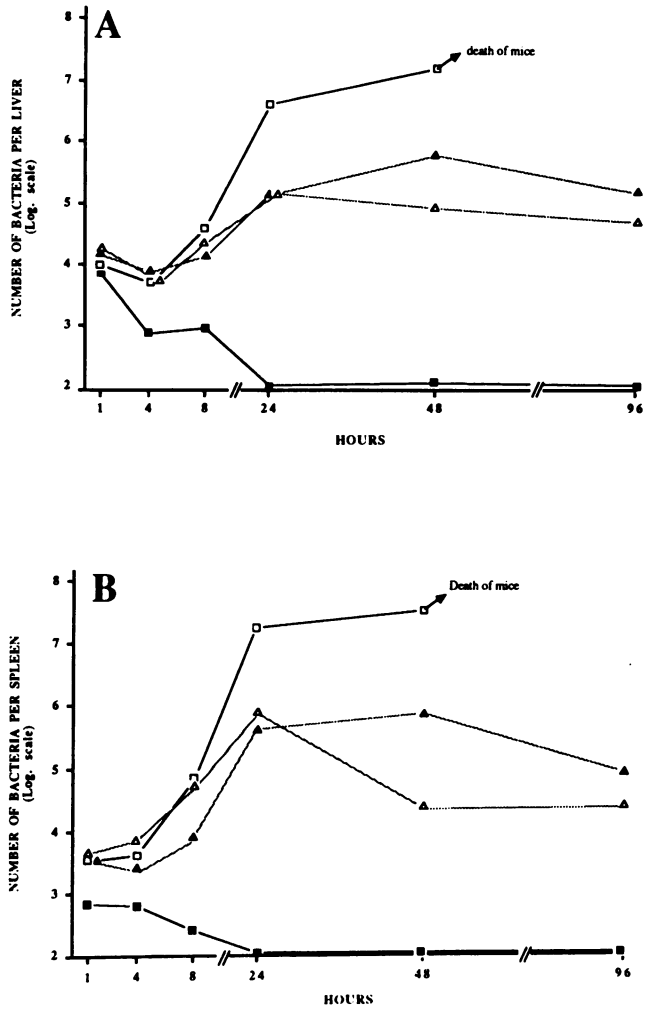

FIG. 8. Growth of *Y. enterocolitica* W22703 carrying pYVe227, pBM7, or pBM21 or cured of pYVe227 in the liver (A) and the spleen (B) of the mouse. Mice were inoculated i.v. with 10^5 bacteria. Bacterial growth was monitored in the liver and spleen during a 4-days period. Each point was the mean value of groups of four mice; the standard deviation was lower than 0.7 $\log_{10}$ bacteria (data not shown). Symbols: □, $pYVe227^+$ bacteria; ■, $pYVe227^-$ bacteria; ▲, $pBM7^+$ bacteria; △, $pBM21^+$ bacteria.

This weak transcription was interpreted to result from residual transcription from the *lcrV* operon.

Role of *yop20* and *yop48* in virulence. The thermosensitive phenotype of the mutants affected in the *lcrV yop44 yop37* operon made it irrelevant to test their virulence in vivo. On the other hand, mutants affected in *yop20* or *yop48* had a normal growth phenotype and Ca^{2+} response. To avoid any interference due to the presence of the plasmid pTM67 supplying *tnpA* for transposition, we first cured this element. Individual colonies were picked and analyzed for the absence of pTM67. Virulence of mutants affected in *yop20* or in *yop48* and lacking pTM67 was then tested in the mouse and compared to that of the parental strain. As shown in Table 1, the LD_{50}s of *yop20* and *yop48* mutants for the desferrioxamine-treated mouse were, respectively, 10^2- or 10^3-fold higher than that of the parental strain. Bacterial counts in the livers and spleens of infected animals (Fig. 8) confirmed that *yop20* and *yop48* mutants had a reduced ability to multiply in the host.

DISCUSSION

This study identifies Yop41 as the V antigen, and it shows that the genes encoding V, Yop44, and Yop37 may be part of a single operon.

Cornelis et al. (13) showed that mutations in locus *yopD* prevented the synthesis of Yop37 and of another Yop with a molecular mass of 40.8 kDa. The work discussed here shows that three proteins are encoded by this region: Yop37, the V antigen (molecular mass, 41 kDa), and Yop44. The last protein is the protein described by Cornelis et al. (13) as Yop40.8. Its molecular mass has been reassessed on the basis of new SDS-PAGE data. Like most of these values, it must be considered provisional, awaiting DNA sequencing data. This Yop44 protein appears to correspond to Yop3 (40 kDa) from *Y. pseudotuberculosis* YPIII (3, 4). Yop37 is also highly conserved, and its counterparts are Yop4a (34 kDa) in *Y. pseudotuberculosis* YPIII (4) and YopD (34.4 kDa) in *Y. pestis* KIM (38).

The V antigen that we identified here as a released protein of 41 kDa was already discovered in *Y. pestis* in the mid-1950s (7). In *Y. pestis* and *Y. pseudotuberculosis*, V is reported to be a 38-kDa protein (15, 32), and its structural gene, *lcrV*, has already been mapped in *Y. pestis* KIM (32), in *Y. pseudotuberculosis* YPIII (15), and in *Y. enterocolitica* 8081 (3). In these three species, *lcrV* is localized similarly with respect to the calcium region. It it thus included in the highly conserved region of pYV. This paper shows that the localization of *lcrV*, *yop37*, and *yop44* is identical in *Y. enterocolitica* W22703 (serotype O:9). However, it provides evidence that in *Y. enterocolitica* W22703, *lcrV*, *yop44*, and *yop37* may be arranged as a single operon transcribed from *yop41* to *yop37*: (i) the three genes are clustered and transcribed in the same orientation; (ii) Tn*2507* insertions in this region provoke polar mutations; (iii) the molecular weight of the messengers in the insertion mutants is in agreement with the hypothesis of a common putative promoter. This operon is the *Y. enterocolitica* counterpart of the *lcrGVH* operon of *Y. pestis* (32), but we did not investigate *lcrG* and *lcrH*.

Transcription of this operon is activated by a temperature shift from 25 to 37°C. At 37°C, Ca^{2+} has a small decreasing effect but transcription still occurs. However, Yops are not released in those conditions. This result confirms previous observations of Yother et al. (40), Straley and Bowmer (38), Perry et al. (31), and Cornelis et al. (13, 14) made with other *yop* genes. As already mentioned by Cornelis et al. (14), these observations suggest that calcium does not act only on transcription of *yop* genes but also controls production of the Yops at a posttranscriptional stage.

The three mutants affected in the operon released Yops at 37°C in the absence of Ca^{2+}. In the presence of Ca^{2+}, they did not release Yops but their growth was nevertheless restricted. This suggests that growth restriction is not simply the consequence of Yop synthesis but rather that a specific growth restriction mechanism is involved. Since Ca^{2+} ions did not relieve the growth restriction at 37°C of these three mutants and since Ca^{2+} had a negative effect on the growth at 37°C of the distal mutants, one may infer that at least one distal gene of this operon is involved in the Ca^{2+} regulation of growth. Hence, we call this operon the *car* operon (for Ca^{2+} regulation).

It is noteworthy that Yop37, which is encoded by the distal gene of the *car* operon, is produced in higher amounts than Yop44. Some regulation of these genes thus also takes place at a posttranscriptional stage. This situation recalls that of the cholera toxin (CT) genes; although *ctxA* and *ctxB* are arranged as a single operon, the B subunit of CT is produced in higher amounts than CT-A (27).

This paper also maps the gene encoding Yop48. According to its size, this protein presumably corresponds to Yop2a (45 kDa) from *Y. pseudotuberculosis* (15). In spite of the fact that this protein is a major Yop, its structural gene had not yet been mapped, neither in *Y. enterocolitica* nor in *Y. pseudotuberculosis*.

We describe here a new Yop, Yop20, which appears as a diffuse band on the SDS-PAGE gels of released proteins. The fuzzy aspect of the band is so far unexplained. It could result from some kind of posttranscriptional modification of the protein. It could also result from the existence of two or more proteins of similar molecular weights encoded by a single operon. None of the two *yop20* mutants released truncated peptides. We thus have no definitive clue that the gene we called *yop20* is the structural gene since we cannot exclude a polar effect of the Tn*2507* insertion mutations on *yop20*. DNA sequencing of this region of pYV, in progress, should settle this point. It must be noticed that *yop20* is regulated like the other genes of the *vir* regulon with respect to temperature and calcium. According to the localization of our mutations and to the size and aspect of this protein, Yop20 could correspond to YopK of *Y. pestis* KIM (38). No counterpart was described in *Y. pseudotuberculosis*, but we detected a similar protein, with a slightly lower molecular mass in *Y. pseudotuberculosis*.

Strains of *Y. enterocolitica* serotype O:9 harboring a pYV plasmid have a low virulence for the mouse, in contrast to those of serotype O:8 (33): that is the case for strain W22703, which displays an LD_{50} (i.v. route) estimated at about $10^{7.5}$ bacteria $mouse^{-1}$ (data not shown). However, virulence of strains O:9 can be enhanced (LD_{50}, by i.v. route, $10^{3.5}$ for strain W22703) when mice are treated 24 h before infection with the iron chelator desferrioxamine, a trihydroxamate siderophore (33). This treatment apparently does not alter the immune defenses in the mouse, since strain W22703 cured of the pYV plasmid was still avirulent (LD_{50}, $>10^8$ bacteria per mouse). Although it does not reproduce the natural infection, the desferrioxamine-treated mouse model allows the study of plasmid-mediated factors involved in the virulence of *Y. enterocolitica* serotype O:9. *Y. enterocolitica* strains affected in *yop48* or in *yop20* clearly had an attenuated virulence for the mouse. Reduction in virulence was also observed for other *yop* mutants in *Y. enterocolitica* (37) as well as in *Y. pseudotuberculosis* (5, 16) and *Y. pestis* (32, 38). However, very little is known, so far, about their individual roles, although Yop2b from *Y. pseudotuberculosis* (corresponding to Yop51 from *Y. enterocolitica*) was shown to be involved in the resistance to phagocytosis by macrophages (34). The analysis of mutants like those described in this paper will certainly help to make clear the individual roles of these important virulence factors.

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