

## Analysis of *virC*, an Operon Involved in the Secretion of Yop Proteins by *Yersinia enterocolitica*

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Received 1 April 1991/Accepted 5 June 1991

Upon incubation at 37°C in the absence of Ca<sup>2+</sup> ions, pathogenic yersiniae release large amounts of pYV plasmid-encoded proteins called Yops that are involved in pathogenesis. *Yersinia enterocolitica* also expresses an outer membrane protein that is considered an adhesin and called YadA (previously called P1 or YopA). The production of Yops is coordinately regulated by a 20-kb region of the plasmid referred to as the Ca<sup>2+</sup> dependence region and containing at least four loci called *virA*, *virB*, *virC*, and *virF*. The *virF* gene encodes a key transcriptional activator of *yop* genes. We have shown here that *virF* is also required for transcription of *yadA* and that *virB* is necessary for full transcription of the *yop* and *yadA* genes. In contrast, mutations in genes *virA* and *virC* had only a weak influence on the transcription of *yop* and *yadA* genes. These mutations did not affect the production of YadA but they completely inhibited the translocation of Yops from the intracellular compartment to the extracellular milieu. We inferred from these data that *virA* and *virC* are involved in the specific transport of Yops. We analyzed the 8.5-kb *virC* region and showed that it is most probably a single operon containing 13 open reading frames called *yscA* to *yscM* (for Yop secretion). Protein YscC has a putative signal sequence and shares significant homology with outer membrane proteins involved in the secretion of pullulanase by *Klebsiella pneumoniae* (PulD) or in the assembly of filamentous bacteriophages (gene IV product). At least the putative products of *yscD*, *yscJ*, and *yscL* were shown to be required for the export of Yops. YscJ turned out to be YlpB, a lipoprotein that we had detected previously. The *yscM* gene shares homology with *yopH*, the adjacent gene on the pYV plasmid. Its product does not appear to be necessary for the production of Yops. Transcription of the *virC* operon was subjected to the same regulation as the *yop* genes.

Human pathogenic yersiniae (*Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*) harbor closely related 70-kb plasmids that are essential for virulence and called pYV (Fig. 1) (for reviews, see references 12, 17, 50, and 54). In response to cultivation at 37°C in the absence of Ca<sup>2+</sup> ions, strains harboring the plasmid cease growing and synthesize a series of plasmid-encoded proteins including 2 outer membrane proteins, YadA and YlpA, and 11 secreted proteins called Yops. YadA (49), an adhesin forming a fibrillar matrix on the cell surface (26), protects *Y. enterocolitica* against the bactericidal action of human serum (1) and promotes the colonization of the mouse intestine (26). YlpA is a lipoprotein related to TraT (10). More spectacular is the extracellular secretion of the Yops (5, 24, 25, 42) in such large amounts that they form visible aggregates in the culture (35). These proteins are essential for pathogenicity (6, 21a, 28, 37, 51). Sequence analysis of *yop* genes (6, 21a, 33, 35) shows that Yops are highly conserved in the genus *Yersinia* but that no homology exists between different Yops in a single species. The functions of individual Yops are now emerging. YopE from *Y. pseudotuberculosis* is cytotoxic to cultured HeLa cells (45), and YopH from *Y. pseudotuberculosis* contributes to the ability of the bacteria to resist phagocytosis by peritoneal macrophages (44). Recently, YopH from *Y. enterocolitica* was found to have tyrosine phosphatase activity (EC 3.1.3.48), which suggests that it interacts with host cell regulation (23). YopM from *Y. pestis*

shares similarities with GPIb $\alpha$  and inhibits platelet aggregation (28, 29).

Coordinate regulation of *yop* gene expression is ensured by a 20-kb region of the pYV plasmid, called the Ca<sup>2+</sup> dependence region. Insertion mutagenesis in this region defined a series of loci called *virA*, *virB*, *virC*, and *virF* in *Y. enterocolitica* (14-16) and *lcrB* and *lcrC* (22), *lcrD* and *lcrE* (57, 64), and *lcrF* (63) in *Y. pestis*. Mutations in *lcrE*, which encodes YopN, allow the cells to secrete the other Yops at 37°C, even in the presence of Ca<sup>2+</sup> ions, suggesting a role of the exported YopN protein in Ca<sup>2+</sup> regulation (21, 45, 57, 64). Mutations in any of the other *vir* or *lcr* genes completely abolish the extracellular Yop production and either make the strains independent on Ca<sup>2+</sup> ions for growth at 37°C (CI phenotype) or unable to grow at that temperature, even in the presence of Ca<sup>2+</sup> ions (Gts phenotype). *virF* mutations have the strongest inhibitory effect on *yop* gene transcription, which led to the discovery that *virF* (corresponding to *lcrF* in *Y. pestis*) encodes a transcriptional activator related to the well-characterized AraC regulator (14). Transcription of *virF* is thermoregulated, which accounts for the thermoregulation of the *yop* regulon (14). The thermoregulation of *virF* is in turn modulated by a chromosome-encoded histone-like protein called YmoA (13).

Secretion of Yops by *Yersinia* species does not involve the cleavage of a classical signal sequence (21a, 35). However, the 48 N-terminal residues of YopH contain the information required for export (34). Coupling the N terminus of YopH to the  $\alpha$ -peptide of  $\beta$ -galactosidase, to the alkaline phosphatase of *Escherichia coli* (34), or to the B subunit of cholera toxin (52) results in efficient extracellular secretion of the hybrid protein. The recognition of YopE and YopQ by

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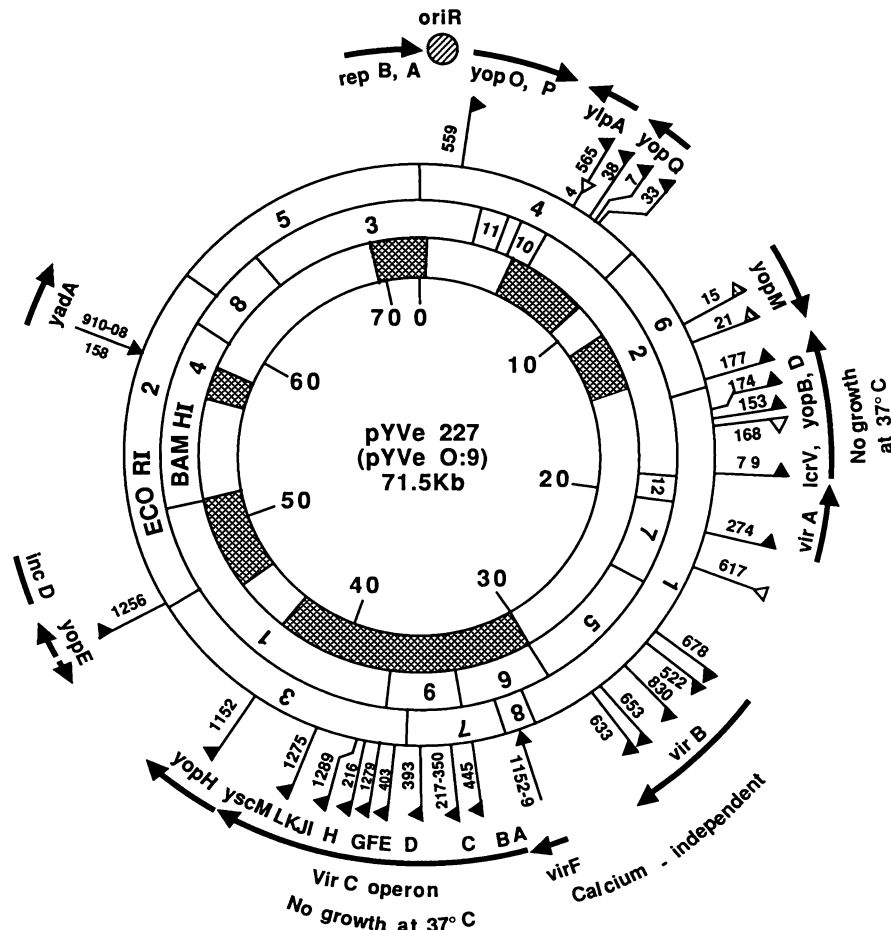


FIG. 1. Map of the pYV plasmid from *Y. enterocolitica* W22703 (serotype O:9) integrating the new data with the data of Balligand et al. (1), Biot and Cornelis (2), Cornelis et al. (14–16), Mulder et al. (37), China et al. (10), and Vanooteghem and Cornelis (56) to give a complete picture. The restriction map is presented for endonucleases *Bam*HI and *Eco*RI. Shaded areas were sequenced. Flags give the localization and orientation of mini-Mud11734 and Tn2507 insertions as follows:  $\blacktriangle$ , strong transcription at 37°C;  $\blacktriangleright$ , weak transcription at 37°C;  $\triangleright$ , no transcription at 37°C. The genes encoding the Yops (*yop* genes) are indicated and oriented by arrows. We adopted the nomenclature uniform for *yop* genes in *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*: *yopH* = *yop51*; *yopE* = *yop25*; *yopO* = *yop84*; *yopP* = *yop30*; *yopQ* = *yop20*; *yopM* = *yop48*; *yopD* = *yop37*; *yopB* = *yop44*; *lcrV* = *yop41* (encoding the V antigen). *yadA* is the gene encoding the outer membrane adhesin previously called P1 or YopA. *ylpA* encodes a lipoprotein related to TraT. The Ca<sup>2+</sup> dependence region contains the *vir* genes and operons involved in the coordinate regulation of Yop production as well as in the low calcium response. The details of the *virC* operon (genes *yscA* to *yscM*) are from this study. *incD* is the partition and stabilization locus; *repB*, *repA*, and *oriR* identify the replicon of the plasmid.

the export apparatus also involves the N-terminal region (34). There is no similarity between the export domains of these proteins with respect to amino acid sequence, hydrophobicity profile, distribution of charged residues, or prediction of secondary structure, suggesting a conformational recognition (34).

In this study, we aimed at identifying the genes involved in the specific export of Yops.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant isolated earlier from W227 (11). The pYV plasmid of that strain (pYVe227) is undistinguishable by restriction analysis from pYVe439-80, the pYV plasmid from *Y. enterocolitica* 439-80, another serotype O:9 strain (2).

*E. coli* JM101 (62) was used for cloning experiments. *E.*

*coli* S17.1 (48) and SM10( $\lambda$  *pir*) (36) were used as mating donors to mobilize pTM200 and its derivatives into *Y. enterocolitica* W22703.

Plasmids are listed in Table 1.

Growth conditions and induction of the *yop* regulon were as described by Michiels et al. (35).

Mini-Mu *d lac* mutagenesis was performed as previously described (15).

**Analysis of the Yops, YadA, and lipoproteins.** The Yops were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots (immunoblots) as previously described (17, 52). Rat monoclonal antibodies (MAb) 6G1 (anti-YopE), 13A4 (anti-YopD), and 6H1 (anti-YopB and -YopN) were used for the detection of proteins (4). For immunofluorescence analysis, the bacteria were heat induced for 2 h in BHI-OX (37). Fifty microliters of culture was applied to each well of a 12-well slide and removed, in order to leave a thin film of culture.

TABLE 1. Bacterial plasmids

Plasmid(s)	Genetic structure	Source or reference
pAG1	pBC19R + <i>EcoRI-XbaI</i> fragment of pYVe439-80 (coordinates, 37.3–43.1 kb)	This study
pAG8	pBC18R + <i>EcoRI-XbaI</i> fragment of pYVe439-80 (coordinates, 37.3–43.1 kb)	This study
pBC5	pBC19R + <i>EcoRI</i> fragment 4 of pYVe439-80 (coordinates, 0–9.6 kb) <i>ylpA</i> <sup>+</sup>	10
pBC18R and pBC19R	pTZ18R and pTZ19R + <i>oriT</i> of RK2	10
pCL4	pBC18R + <i>EcoRI-BamHI</i> fragment of pYVe439-80 (coordinates, 33.7–35.6 kb)	This study
pGC216	pGB63 <i>yscH-216::mini-Mu d1 lac</i>	15; this study
pGC217	pGB63 <i>yscC-217::mini-Mu d1 lac</i>	15; this study
pGC274	pGB63 <i>virA-274::mini-Mu d1 lac</i>	15
pGC393	pGB63 <i>yscD-393::mini-Mu d1 lac</i>	15; this study
pGC403	pGB63 <i>yscD-403::mini-Mu d1 lac</i>	15; this study
pGC445	pGB63 <i>yscC-445::mini-Mu d1 lac</i>	15; this study
pGC633	pGB63 <i>virB-633::mini-Mu d1 lac</i>	15
pGC678	pGB63 <i>virB-678::mini-Mu d1 lac</i>	15
pGC830	pGB63 <i>virB-830::mini-Mu d1 lac</i>	15
pGC1152-9	pGC1152 <i>virF-9::Tn813</i>	16
pGCS334	pBM33 <i>virF-4::pGCS904</i>	27a
pGCS652	pTZ19R + coordinates 32.6–35.6 kb of pYVe439-80	14
pJCV1275	pGB63 <i>yscL-1275::mini-Mu d1 lac</i>	This study
pJCV1279	pGB63 <i>yscG-1279::mini-Mu d1 lac</i>	This study
pJCV1282	pGB63 <i>yscH-1282::mini-Mu d1 lac</i>	This study
pJCV1289	pGB63 <i>yscH-1289::mini-Mu d1 lac</i>	This study
pTM200	pACYC184- <i>oriT</i> + <i>EcoRI</i> fragment 3 of pYVe439-80 (coordinates, 37.3–46.8 kb)	14
pTM262	Filling in of <i>BglII</i> site of pTM200	This study
pTM268	Deletion of <i>BssH1I</i> fragment of pTM200	This study
pTM269	Deletion of <i>MluI</i> fragment of pTM200	This study
pTM270	pTZ18R + <i>BamHI-EcoRI</i> fragment of pYVe439-80 (coordinates, 35.6–37.3 kb)	This study
pTM271	pTZ18R + <i>Sau3A</i> 460 bp from <i>BamHI</i> fragment 9 of pYVe439-80	This study
pTM277	Deletion of <i>BstEII-BglII</i> of pTM200	This study
pTM284	pTZ19R + <i>BamHI-EcoRI</i> fragment of pYVe439-80 (coordinates, 35.6–37.3 kb)	This study
pTZ18R and pTZ19R	<i>ori</i> pBR322 + <i>ori</i> fl <i>lacZ'</i> <i>bla</i> <sup>+</sup>	Pharmacia
pYVe227	pYV plasmid from W22703 (serotype O:9)	2
pYVe439-80	pYV plasmid from 439-80 (serotype O:9)	2

The slide was either dried at room temperature and fixed for a few seconds at 50°C or immersed for 10 min in methanol. It was then immersed for 30 min in phosphate-buffered saline (50 mM phosphate, 150 mM NaCl, pH 7.4) (PBS) containing the anti-YopE 6G1 MAb diluted 1/50 and subsequently washed twice with PBS for 5 min each time. The slides were then incubated in fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulins (DAKO Immunoglobulins, Glostrup, Denmark) diluted 1/50. The slides were then washed in PBS for 5 min and mounted in PBS-glycerol (50% [vol/vol]).

YadA was detected by Western blot analysis of whole-cell extracts, using a rabbit polyclonal antiserum (53).

The lipoproteins were visualized by SDS-PAGE after labelling with [<sup>3</sup>H]palmitic acid as described by China et al. (10).

**DNA sequencing and sequence analysis.** The nucleotide sequence of *virC* was determined on pAG1, pAG8, pGCS652, pCL4, pTM270, and pTM284. Subclones of these plasmids were constructed by cloning or deleting specific restriction fragments. Progressive deletion mutagenesis was performed on some of the clones, either with T4 DNA polymerase (18) by using the Cyclone system of International Biotechnologies, Inc. (New Haven, Conn.), or with exonuclease III (Erase-a-Base; Promega, Madison, Wis.).

Sequences were determined by the dideoxy-chain termination method of Sanger et al. (46) and analyzed as previously described (35). The continuity of the sequence across the junctions of the pYVe439-80 *EcoRI* and *BamHI* fragments was checked by directly sequencing the double-stranded pYV plasmid with synthetic oligonucleotides. The sequence of the mini-Mu *d lac* insertion sites was determined on the pYV mutants with the synthetic oligonucleotide MIPA24 (5' CAGATCCCGAATAATCC 3') derived from the sequence of the right end of bacteriophage Mu (38).

Signal sequences were detected by using the Sigseq program (41), which is based on the rules of von Heijne (58).

Hydrophobicity (27) was calculated by using the FAST program with a window of 10.

**RNA extraction, Northern (RNA) blot analyses, and primer extensions.** These experiments were performed as previously described (35). Unless specified, RNA was extracted after 90 min of induction at 37°C.

## RESULTS

**Influence of the various *vir* loci on transcription and export of the Yops.** The genes required for export of the Yops are most likely contained in the Ca<sup>2+</sup> dependence region of pYV. Because the regulation of *yop* genes is multifactorial

and because the Yops are not expressed when the pYV plasmid is transferred to *E. coli* (16), we decided to identify the secretion genes by mutational analysis in *Y. enterocolitica* rather than by cloning techniques.

We first analyzed *Y. enterocolitica* W22703 strains with various mutations in the *vir* locus (*virA*, *virB*, *virC*, and *virF*) with respect to the expression of the Yops and the adhesin YadA. Since YadA is an outer membrane protein possessing a typical signal sequence, this protein is expected to be targeted to the outer membrane by an export pathway different from that used by the Yops. Thus, mutations in the Yop-specific secretion apparatus were expected to prevent the extracellular appearance of the Yops but not the production of YadA and its insertion in the outer membrane.

We monitored the transcription of *yopE*, *yopH*, and *yadA* in the various *vir* mutants by Northern blotting (Fig. 2). Transcription of the three genes was strong in the wild type and in the *virA* and *virC* mutants, much weaker in the *virB* mutants, and nearly undetectable (if present at all) in the *virF* mutants. We also analyzed the mutants for production of Yops. As expected from our previous work (15, 16), none of the *vir* mutants secreted extracellular Yops as estimated by SDS-PAGE (Table 2). The presence of intracellular Yops was analyzed by immunoblotting, using rat MAb directed against YopE, YopD, and YopB. The detection of YopE in the various *vir* mutants paralleled the transcription pattern: the YopE band was clearly visible in the extracts from *virA* and *virC* mutants, fainter in preparations from *virB* mutants, and not detectable in the *virF* mutants (Fig. 3). The presence of YopB and YopD correlated with the presence of YopE except in the *virA* mutant, in which they were present in much smaller amounts than YopE was. The production of YadA was monitored by SDS-PAGE and immunoblotting, using a polyclonal monospecific rabbit antiserum. The amount of YadA correlated well with the transcriptional level of the gene. *virA* and *virC* mutants produced wild-type levels of YadA, while *virB* mutants produced low levels of YadA. YadA was not detected in *virF* mutants (Table 2). We conclude from these experiments that (i) *virB* and *virF* are involved in a transcriptional regulation pathway common to the *yop* and *yadA* genes and (ii) *virA* and *virC* are involved in a posttranscriptional process specific to the Yops. Both the *virC* mutants and the *virA* mutants produced Yops but did not secrete them, suggesting that these loci are involved in the export of the Yops or in the specific control of the export function. It is worth noting that in none of the mutants tested did the amount of intracellular Yops reach the amounts secreted by the wild-type strain, suggesting that some feedback inhibition of transcription or translation or both occurred when export was prevented, as we previously suggested (16).

In order to know whether *virA* and *virC* mutants accumulate Yops within the cells or at their surface, we examined thermoinduced cultures of the various mutants, by immunofluorescence, using the anti-YopE MAb. When we examined cultures of the wild type, the Yops appeared as strongly fluorescent film particles and the bacteria themselves were poorly fluorescent (Fig. 4A). The cultures of *virA* and *virC* mutants did not contain the fluorescent particles, and the vast majority of the bacteria themselves were not fluorescent. Other preparations of the same cultures were treated with methanol before immunofluorescence staining in order to ensure permeability of the cells to the MAb. In these preparations, the wild-type bacteria were still poorly fluorescent, the *virA* and *virC* mutants were strongly fluorescent (Fig. 4B and C, respectively), and the *virF* mutant remain-

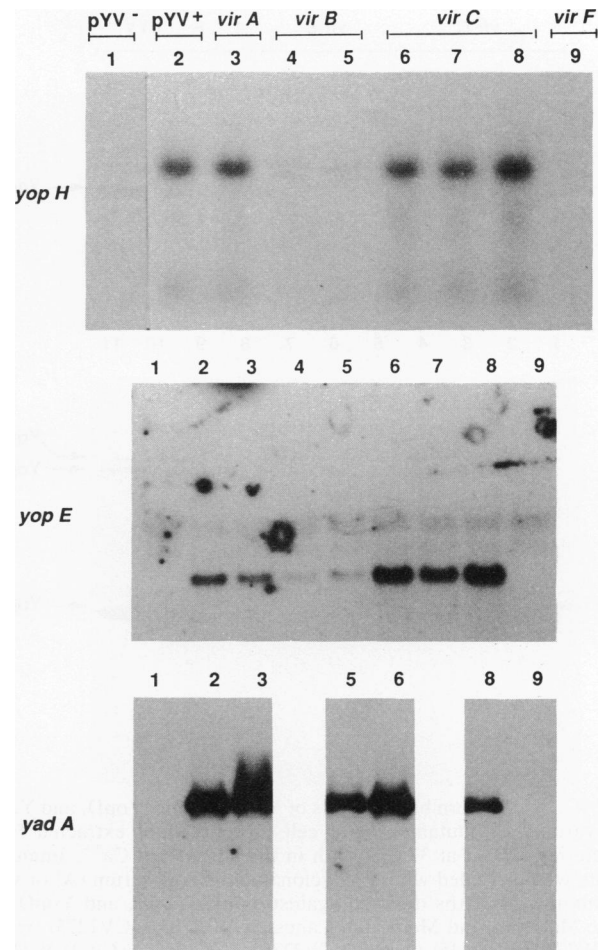


FIG. 2. Northern blot analysis of *yopH*, *yopE*, and *yadA* transcription in various *vir* mutants. RNA was extracted after 1.5- to 2-h induction of the cultures at 37°C in BHI-OX. mRNA from *yopH* was detected with a 264-bp *SspI-XbaI* fragment (coordinates 520 to 784 of the *yopH* sequence of Michiels and Cornelis [33]) <sup>32</sup>P labelled by nick translation. The *yopE* transcript was hybridized with the <sup>32</sup>P-end-labelled oligonucleotide MIPA9 complementary to bases 316 to 330 of the *yopE* sequence of Michiels et al. (35). The *yadA* mRNA was identified with <sup>32</sup>P-labelled oligonucleotide MIPA12 complementary to nucleotides 1178 to 1202 of the *yadA* sequence of *Y. enterocolitica* O:3 determined by M. Skurnik and Wolf-Watz (49). Lanes: 1, *Y. enterocolitica* W22703 (not carrying a pYV plasmid); 2, W22703(pYVe227) (wild type); 3, W22703(pGC274) (*virA*); 4, W22703(pGC633) (*virB*); 5, W22703(pGC830) (*virB*); 6, W22703(pGC216) (*virC*); 7, W22703(pGC445) (*virC*); 8, W22703(pGC1275) (*virC*); 9, W22703(pGCS334) (*virF*).

ed undetectable (Fig. 4D). This result confirmed that YopE indeed accumulated intracellularly in *virA* and *virC* mutants (Table 2).

**Further genetic mapping of the *virC* region.** Five mini-Mu d11734 insertions defined the *virC* locus as a region of ±4 kb with thermodependent unidirectional transcription (15). Insertions in this region abolished the release of Yops and rendered the strains growth thermosensitive (Gts phenotype) (1, 15). In this work, we characterized four additional mini-Mu d *lac* insertion mutants of pYVe227 exhibiting the same properties: pJCV1275, pJCV1279, pJCV1282, and pJCV1289. Plasmid pJCV1275 had a mini-Mud*lac* insertion 2.0 kb downstream of the insertion point of pGC216, the

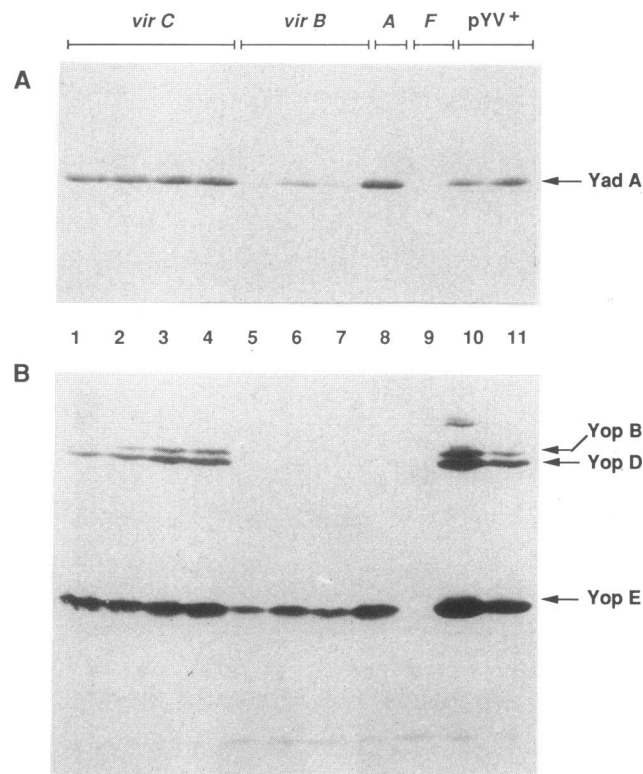


FIG. 3. Western blot analysis of YopE, YopB, YopD, and YadA in various *vir* mutants. Total cell proteins were extracted from bacteria induced at 37°C for 4 h in the absence of Ca<sup>2+</sup>. Immunoblots were revealed with a polyclonal anti-YadA serum (A) or with a mixture of MAbs directed against YopE, YopB, and YopD (B) (see Materials and Methods). Lanes: 1, W22703(pJCV1275) (*virC*); 2, W22703(pGC445) (*virC*); 3, W22703(pGC403) (*virC*); 4, W22703(pGC216) (*virC*); 5, W22703(pGC830) (*virB*); 6, W22703(pGC678) (*virB*); 7, W22703(pGC633) (*virB*); 8, W22703(pGC274) (*virA*); 9, W22703(pGC1152-9) (*virF*); 10, W22703(pGC1152) (*yopH*); 11, W22703(pYVE227) (wild type).

most distal previously described *virC* mutant (15). This indicated that the *virC* region is at least 6 kb long and could extend from *virF* to *yopH* (Table 2).

**Nucleic acid sequence of *virC*.** We sequenced the 8.5-kb region between *virF* and *yopH* (coordinates of 34.0 to 42.5 kb on the map presented in Fig. 1). The sequence presented in Fig. 5 contains 12 open reading frames (ORFs) encoding proteins of at least 60 amino acids and oriented as expected from the previous genetic data (15). An additional 32-codon ORF located in the 5' region was also retained as a potential gene. These putative genes were called *yscA* to *yscM* (*ysc* for Yop secretion). If one takes into account that *yscD* and *yscJ* probably start with a GTG initiation codon, ORFs *yscB* to *yscK* are all contiguous, which strongly suggests that they are part of a single large multicistronic operon.

The insertion point of mini-Mu *lac* in nine different *virC* mutants was sequenced directly on the double-stranded pYV derivative, using an oligonucleotide complementary to the right end of bacteriophage Mu. Mutants pJCV1279 and pJCV1282 turned out to be identical. The seven other mutations were all different from each other and occurred in five different ORFs.

**Relevant features of the Ysc proteins.** All the ORFs of the *virC* region are preceded by potential ribosome binding sequences with good homology to the consensus, which suggests that they all correspond to translated genes. In view of the involvement of the *virC* region in the Yop export process, predicted protein sequences were checked for the presence of a signal sequence and hydrophobic domains that could correspond to membrane anchoring domains. They were also used to screen the GenPept library using the FASTA procedure (39) by electronic mail. The relevant features of the various putative *ysc* gene products are described here below and summarized in Table 3.

(i) **YscA.** The small 32-residue-long hypothetical YscA protein has a hydrophobic C-terminal domain. The presence of a putative ribosome binding sequence and the fact that this region is transcribed (see below) offer support for the hypothesis that this ORF is translated.

(ii) **YscB.** The 137-residue-long YscB protein has neither a putative signal sequence nor any hydrophobic domain.

(iii) **YscC.** Translation of *yscC* yields a 607-residue-long protein with a probable signal sequence of 26 residues.

TABLE 2. Phenotypic characteristics of the *vir* mutants<sup>a</sup>

Plasmid	Mutation	Phenotype	<i>yopH</i> transcription	<i>yopE</i> transcription	<i>yadA</i> transcription	Extracellular Yops	Intracellular YopE	Intracellular YopB and YopD	Production of YadA
None		CI	-	-	-	-	-	-	-
pYVe227		CD	+	+	+	+	+	+	+
pGC274	<i>virA</i>	CI <sup>b</sup>	+	+	+	-	+	±	+
pGC633	<i>virB</i>	CI	±	±	NT	-	±	±	±
pGC678	<i>virB</i>	CI	NT	NT	NT	-	±	±	±
pGC830	<i>virB</i>	CI	±	±	±	-	±	±	±
pGC216	<i>virC</i>	Gts	+	+	+	-	+	+	+
pGC403	<i>virC</i>	Gts	NT	NT	NT	-	+	+	+
pGC445	<i>virC</i>	Gts	+	+	NT	-	+	+	+
pJCV1275	<i>virC</i>	Gts	+	+	+	-	+	+ <sup>c</sup>	+
pGC1152-9	<i>virF</i>	CI	NT	NT	NT	-	-	-	-
pGC334	<i>virF</i>	CI	-	-	-	-	NT	NT	-

<sup>a</sup> Phenotypic characteristics are indicated as follows: +, wild-type level; -, not present; ±, low level; NT, not tested.

<sup>b</sup> As mentioned in Results, this mutant has a reduced growth rate at 37°C in the absence of Ca<sup>2+</sup>.

<sup>c</sup> Only YopD (no YopB, probably because of a point mutation of *yopB*).

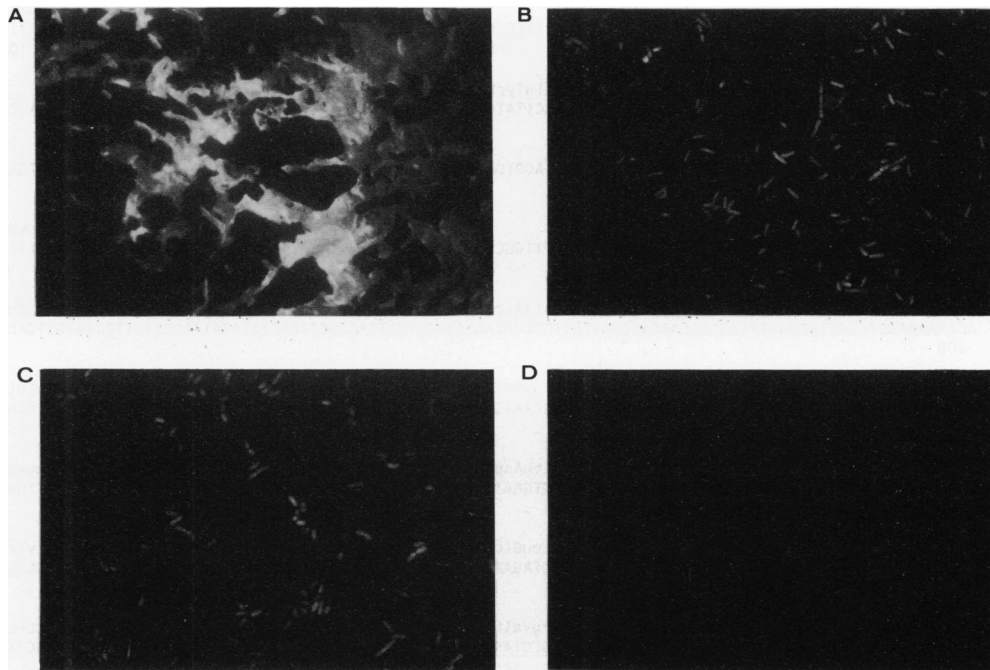


FIG. 4. Immunofluorescence analysis, with anti-YopE MAb 6G1, of various cultures induced at 37°C in absence of  $\text{Ca}^{2+}$  and methanol fixed. (A) W22703(pYVe227) (wild type); (B) W22703(pGC274) (*virA*); (C) W22703(pGC216) (*virC*); (D) W22703(pGCS334) (*virF*).

According to the hydrophobicity analysis, this protein has no obvious transmembrane or membrane anchoring segment. However, YscC shares significant similarity with two described outer membrane proteins: PulD and pIV (Fig. 6). PulD is a factor required for the export of pullulanase by strains of *Klebsiella pneumoniae* (20). Protein pIV, the product of gene IV of the filamentous bacteriophages, is an integral membrane protein required for virus assembly. This protein could be part of the structure through which the assembling phage is extruded (8). Interestingly, the same domain is conserved between the three proteins, suggesting a common function (Fig. 6).

(iv) **YscD.** In view of the adjacency of most *ysc* genes, we assumed that *yscD* initiates at a GTG codon located 49 codons upstream of the first ATG initiation codon, thus encoding a 418-residue-long protein. This protein has no putative signal sequence but contains a highly hydrophobic domain (residues 120 to 130) with a value of 2.9 on the scale of Kyte and Doolittle (27), which suggests an inner membrane localization. Residues 100 to 115 which are contained between two hydrophobic segments are strongly polar and contain five positively charged residues which denote a potential cytoplasmic domain.

(v) **YscE.** The 66-residue-long hypothetical YscE protein has no putative signal sequence. It contains a hydrophobic domain at its C terminus (hydrophobicity value, +1.8).

(vi) **YscF.** *yscF* encodes a 87-amino-acid hypothetical protein that has neither a putative signal sequence nor any strongly hydrophobic or hydrophilic domain.

(vii) **YscG.** The 115-residue-long hypothetical YscG protein has an N-terminal hydrophobic domain preceded by two lysyl residues. However, this region is not predicted to be a cleavable signal sequence, suggesting that this protein could be anchored in the inner membrane by its N terminus.

(viii) **YscH.** The 165-residue-long YscH protein contains two internal domains of average hydrophobicity.

(ix) **YscI.** YscI has no hypothetical signal sequence and no hydrophobic domain.

(x) **YscJ.** As in the case of *yscD*, we assumed that *yscJ* begins at a GTG codon localized 34 residues upstream of the first ATG and thus encodes a 244-residue-long polypeptide. With this hypothesis, the *yscJ* gene product contains a nearly perfect lipoprotein-specific signal sequence. Cleavage by signal peptidase II would occur at the level of the Leu-Thr-Gly  $\blacktriangledown$  Cys sequence instead of the consensus Leu-Ala/Ser/Val-Gly/Ala  $\blacktriangledown$  Cys sequence (61). YscJ contains a hydrophobic C-terminal domain followed by three positively charged residues. This feature is characteristic of a "stop transfer" domain anchoring proteins in the membrane (7). The presence of lysyl residues at the C-terminal end of this lipoprotein also brings to mind the lipoproteins which are covalently bound to peptidoglycan via their C-terminal lysyl residue (55). The region centered at position 130 is extremely hydrophilic and is contained between hydrophobic regions. The similarity search on the GenPept library did not reveal any striking sequence similarity. However, YscJ appeared to have 14.8% identity with Lon protease (9) on a 183-amino-acid stretch starting at the signal cleavage site.

According to its predicted molecular mass, YscJ is a good candidate for being YlpB, the pYV-encoded 27-kDa lipoprotein that we detected previously (10). To verify this hypothesis, we labelled the cultures of two *virC* mini-Mu d *lac* mutants with [ $^3\text{H}$ ]palmitic acid and analyzed the proteins by SDS-PAGE and fluorography. As shown in Fig. 7, YlpB was present in W22703(pJCV1275) (insertion in *yscL*) and missing in W22703(pGC216) (insertion in *yscH*). In view of the polarity of mini-Mu d *lac* insertions, these data show that YlpB is encoded by *yscH*, *-I*, *-J*, or *-K*. The product of *yscJ* is the only one having the expected size and a potential lipoprotein signal sequence. Hence YscJ is indeed YlpB. For the sake of clarity, we propose to use the YscJ designation.

(xi) **YscK.** The 209-residue-long YscK protein does not

1160 of virF seq (J.Bacteriol. 171: 259, 1989) MetSerGlnIleThrThrLysHisIleThrValLeuPhe  
TTTAAAAACACTTTTGGACTATAAAGTAAATACGGGGTAGATTTGAAGATTCAATGGGATGAGCCAAATTACAACGAAACATATAACAGTATTATT  
 1 DraI -35 -10 \* rbs yscA 100

ArgArgTrpMetAlaIleIleCysCysLeuIleIleLysIleAlaTyrLeuAlaTyr\*\*\*  
 TCGCCGCTGGATGGCAATAATATGTTGTTAATAATCAAGATAGCTTATCTGGCTTATTAAGTGGCGTGGCGCAAGAAACGAGGCCATCAATAGAGC  
 100

AAGTCTCATTAGTTCGATTAACGTAATATCATCTATTGCTATATAGTGGTTGATAATTATCACGAACATTTTTTGAATATCTGGAAGTTTGAGCTGA  
 200

ACC GCGAAACCTTATGTCACGATGACATGAAGTAGGTTATTTATTTGGCGCAGGATTACTTAGTTTACATATAACCATCTGAGAAATAATGCAAAATTT  
 300 rbs yscB.

LeuLysAsnLeuAlaThrSerLeuGlyArgLysProPheValAlaAspLysGlnGlyValTyrArgLeuThrIleAspLysHisLeuValMetLeuThr  
 ACTAAAGAACTTGGCTACCAGTTTAGGAAAGAAACCGTTTGTGGCGATAACAAGGTGTTTACCGTTTAACTATAGATAAGCATCTTGTCTGCTGAC  
 400

ProHisGlySerGluLeuValLeuArgThrProIleAspAlaProMetLeuArgGluGlyAsnAsnValAsnValThrLeuLeuArgSerLeuMetGln  
 TCCGATGGTTCAGAACTGGTTTACGCACTCTATTGACGCACCAATGTTACGTGAAGGAAATAACGTTAACGTCACATTGCTTCGCTCCCTAATGCA  
 500

GlnAlaLeuAlaTrpAlaLysArgTyrProGlnThrLeuValLeuAspAspCysGlyGlnLeuValLeuGluAlaArgLeuArgLeuGlnGluLeuAsp  
 ACAAGCGTTGGCATGGCTAAACGTTATCCTCAAACCTTAGTATTGGATGATTGGTCAATTGGTGTGGAGGCGGCTTACGTCACAAAGAGCTTGA  
 600

ThrHisGlyLeuGlnGluValIleAsnLysGlnLeuAlaLeuLeuGluHisLeuIleProGlnLeuThrProPheSerValAlaSerArgValGlyTrp  
 TACTCAGGGATTGCAAGAAGTAATAATAACAACCTGGCTCTGCTAGAACATTTAATCTCAGTTAACGCCATTTTCTGTAGCGTCTCGCTGGGGTG  
 700

Asn\*\*\* MetAlaPheProLeuHisSerPhePheLysArgValLeuThrGlyThrLeuLeuLeuLeuSerSerTyrSerTrpAlaGlnGluLeu  
GAATTAAGTAATATGGCTTTTCCGCTACACTCTTTTTCAAGCGCTACTCACCGGGACGTTACTGTTACTTTCTAGCTATAGCTGGGCGCAAGAACTT  
 rbs 800 yscC signal sequence

AspTrpLeuProIleProTyrValTyrValAlaLysGlyGluSerLeuArgAspLeuLeuThrAspPheGlyAlaAsnTyrAspAlaThrValValVal  
 GATTGGTTGCCTATACCTTATGTTTATGTGGCAAGGGGAAAGTTTACGCGATTTATTAACCTGATTTCGGCGCTAATTATGATGCTACAGTGGTATGA  
 900

SerAspLysIleAsnAspLysValSerGlyGlnPheGluHisAspAsnProGlnAspPheLeuGlnHisIleAlaSerLeuTyrAsnLeuValTrpTyr  
 AGCGATAAGATTATGACAAAGTTTCCGGCCAGTTTGGCATGATAACCTCAGGATTTCTACAGCATATTGCCTCTTTACAATTTGGTTGGTAC  
 1000

TyrAspGlyAsnValLeuTyrIlePheLysAsnSerGluValAlaSerArgLeuIleArgLeuGlnGluSerGluAlaAlaGluLeuLysGlnAlaLeu  
 TATGATGGCAATGTCTCTACATTTTTAAAAATAGTGAGGTAGCGTCTCGTCTATTCTGTTTACAGGAAAGTGAGGCGCAGAGTTAAAGCAGGCATTA  
 1100 DraI

GlnArgSerGlyIleTrpGluProArgPheGlyTrpArgProAspAlaSerAsnArgLeuValTyrValSerGlyProProArgTyrLeuGluLeuVal  
 CAACGTTCTGGTATATGGGAGCCTCGTTTTGGCTGGCGCCTGATGCTAGCAACCGCCTGGTTTACGCTCTGGTCTCCTCGTTATCTTGAATGGTT  
 1200 pGC445

GluGlnThrAlaAlaAlaLeuGluGlnGlnThrGlnIleArgSerGluLysThrGlyAlaLeuAlaIleGluIlePheProLeuLysTyrAlaSerAla  
 GAACAGACCGCAGCCGATTGGAACACAGACGCAAAATTCGCAGTGA AAAAACAGGGGCATTAGCGATTGAGATTTCCCTCTCAAATATGCATCAGCG  
 1300

SerAspArgThrIleHisTyrArgAspAspGluValAlaAlaProGlyValAlaThrIleLeuGlnArgValLeuSerAspAlaThrIleGlnGlnVal  
 AGCGATCGAATATTACCTGATGACGAAGTGGCTGCTCGGGGTTGCAACGATACTTCAACGCGTGTAAAGCGATGCCACTATCAACAAGTG  
 1400

ThrValAspAsnGlnArgIleProGlnAlaAlaThrArgAlaSerAlaGlnAlaArgValGluAlaAspProSerLeuAsnAlaIleIleValArgAsp  
 ACAGTGGATAATCAGAGAATACCGCAGGCCCAACCGGGCTTCAGCTCAAGCCAGAGTTGAAGCGGATCCATCGCTCAATGCGATAATAGTGGCGAT  
 1500 SmaI B6 BamHI B9

SerProGluArgMetProMetTyrGlnArgLeuIleHisAlaLeuAspLysProSerAlaArgIleGluValAlaLeuSerIleValAspIleAsnAla  
 TCTCCTGAGCGTATGCCAATGATCAACGGTTAATTCATGCGCTTGATAAGCCTAGCGCTCGTATTGAAGTGGCGTTATCCATTGTGATATAAATGCC  
 1600

AspGlnLeuThrGluLeuGlyValAspTrpArgValGlyIleArgThrGlyAsnAsnHisGlnValValIleLysThrThrGlyAspGlnSerAsnIle  
 GACCAACTTACTGAATTAGGTGTGGACTGGCGAGTTGGCATTCTGACTGGCAACAATCATCAGTGGTAATAAAAAACCGGGGATCAAAGTAACATC  
 1700

AlaSerAsnGlyAlaLeuGlySerLeuValAspAlaArgGlyLeuAspTyrLeuLeuAlaArgValAsnLeuLeuGluAsnGluGlySerAlaGlnVal  
 GCTTCAAACGGTGCAATGGGTAGTTGGTTGATGCTCGCGGGCTTGACTACCTATTAGCAAGAGTCAATTTACTTGAAAAAGAGGTTCCGGCTCAAGTT  
 1800

ValSerArgProThrLeuLeuThrGlnGluAsnAlaGlnAlaValIleAspHisSerGluThrTyrTyrValLysValThrGlyLysGluValAlaGlu  
 GTTTCACGTCGACCCCTGTTAACACAAGAAATGCCAACGGGTGATTGATCACAGTGAACCTATTACGTCAAAGTGACAGGTAAGGAAAGTGGCTGAA  
 1900

LeuLysGlyIleThrTyrGlyThrMetLeuArgMetThrProArgValLeuThrGlnGlyAspLysSerGluIleSerLeuAsnLeuHisIleGluAsp  
 CTGAAAGGGATCACCTACGGCACTATGCTGCGTATGACGCCAAGGGTCTGACTCAAGGAGATAAGTCAGAAATCAGTCTCAATCTACACATTGAGGAT  
 2000 pGC217

GlyAsnGlnLysProAsnSerSerGlyIleGluGlyIleProThrIleSerArgThrValValAspThrValAlaArgValGlyHisGlyGlnSerLeu  
 GGAACCAAAAACCGAATAGTTTCAAGGATTGAAGGAATCCCCACTATCAGTCTGACGGTCTGATACTGCTCGCTGGGACATGGCCAGAGTTTG  
 2100

FIG. 5



IleIleGlyGlyIleTyrArgAspGluLeuSerValAlaLeuSerLysValProLeuLeuGlyAspIleProTyrIleGlyAlaLeuPheArgArgLys  
 ATTATTGGTGGTATTTATCGTGACGAATTGAGTGTGCTCTTAGTAAGTGCCTTTGCTTGGTGATATTCCTTATATTGGCCACCTTTCCGCCGATAAA  
 2200

SerGluLeuThrArgArgThrValArgLeuPheIleIleGluProArgIleIleAspGluGlyIleAlaHisHisLeuAlaLeuGlyAsnGlyGlnAsp  
 AGTGAGTTAACTCGCGTACGGTACGGCTATTATCATCGAACCCAGGATTATTGACGAAGTATTGCGCATCATTAGCGTTAGGTAATGGTCAGGAT  
 2300

LeuArgThrGlyIleLeuThrValAspGluIleSerAsnGlnSerThrThrLeuAsnLysLeuLeuGlyGlySerGlnCysGlnProLeuAsnLysAla  
 CTACGTAAGTAACTCGCGTACGGTACGGCTATTATCATCGAACCCAGGATTATTGACGAAGTATTAGGTTGGCTCCAGTGTAGCGTTAAACAAGCG  
 2400 DraI

GlnGluValGlnLysTrpLeuSerGlnAsnAsnLysSerSerTyrLeuThrGlnCysLysMetAspLysSerLeuGlyTrpArgValValGluGlyAla  
 CAAGAAGTGCAGAAATGGCTGAGTCAAAAATAAATCATCCTATCTTACTCAGTGAAGTGGACAAAAGTTGGGATGGCGCGTGGTTGAAGTGTCT  
 2500

CysThrProAlaGlnSerTrpCysValSerAlaProLysArgGlyValLeu\*\*\*  
 TGTACTCCCGCGCAATCATGGTGTGTTTCAGCACCTAAGCGTGGCGTATTGTGAGTTGGTCTGTCTTTTATCAAGGGAAGCACCGTGGTGTGAAG  
 2600 rbs yscD MetSerTrpValCysArgPheTyrGlnGlyLysHisArgGlyValGluVal

GluLeuProHisGlyArgCysValPheGlySerAspProLeuGlnSerAspIleValLeuSerAspSerGluIleAlaProValHisLeuValLeuMet  
 TTGAGCTTCTCATGGCGCTGTGTTTTGGCTCAGACCGGTGCAATCAGATATTGTTCTTTCTGACAGCGAAATAGCACCGGTGCATTTAGTGTGA  
 2700

ValAspGluGluGlyIleArgLeuThrAspSerAlaGluProLeuLeuGlnGluGlyLeuProValProLeuGlyThrLeuLeuArgAlaGlyThrCys  
 TGGTCGATGAAGAAGTATTGCGCTAACTGATTCTGCAGAACCTCTACTACAAGAAGGGCTTCCCGTGCCGTTGGGACTCTCTTCGCGCGGCGACTT  
 2800 PstI

LeuGluValGlyPheLeuLeuTrpThrPheValAlaValGlyGlnProLeuProGluThrLeuGlnValProThrGlnArgLysGluProThrAspArg  
 GTCGGAAGTAGGGTTTTACTGTGGACATTTGTCCCGTAGGGCAACCTTTGCCAGAGCGTTACAGGTTCCACGCGAGAGAAAAGCGCAACCGACA  
 2900

LeuProArgSerArgLeuGlyValGlyLeuGlyValLeuSerLeuLeuLeuLeuThrPheLeuGlyMetLeuGlyHisGlyLeuTrpArgGluTyr  
 GGTACTCGTTCACGACTTGGGTTGGGCTGGAGTCTTCTTTATTGTTGCTTTTGACTTTTTGGGGATGCTAGGGCACGGATTGTGGCGCGAGT  
 BstEII 3000 pGC393

AsnGlnAspGlyGlnLeuValGluGlnGluValArgArgLeuLeuAlaThrAlaAlaTyrLysAspValValLeuThrSerProLysGluGlyGluPro  
 ATAACCGATGGCAACTTGTGAGCAAGAAGTACGGCGCTTGTGGCAACTGCTGCCTACAAGGATGTCGTTTTAACATCGCCAAAGAGGGTGAAC  
 3100

TrpLeuLeuThrGlyTyrIleGlnAspAsnHisAlaArgLeuSerLeuGlnAsnPheLeuGluSerHisGlyIleProPheArgLeuGluLeuArgSer  
 CTTGGTTATAAAGTGGTATATCCAGGATAATCATGCCCGCTTGTCACTGCAAAAATTTCTTGAGAGCCATGGCATTCCATTCCGGCTTGAATCGCGCA  
 3200

MetGluGluLeuArgGlnGlyAlaGluPheIleLeuGlnArgLeuGlyTyrHisGlyIleGluValSerLeuAlaProGlnAlaGlyTrpLeuGlnLeu  
 GCATGGAAGAACTCGTCAGGGGCGAGAATTTCATTCTGCAACGGTTGGGATACCATGGAATTGAGGTTCTTTAGCACCGCAAGCGGGATGGCTACAAT  
 E7 EcoRI E3 3310

AsnGlyGluValSerGluGluIleGlnLysGlnLysIleAspSerLeuLeuGlnAlaGluValProGlyLeuLeuGlyValGluAsnLysValArgIle  
 TGAATGGGAAGTGTGAGGAAATCAAAAACAAAATTTGATAGCCTGCTGCAAGCTGAAGTCCAGGGCTGCTTGGTGTAGAAAATAAAGTCCGGA  
 3400

AlaGlyAsnGlnArgLysArgLeuAspAlaLeuLeuGluGlnPheGlyLeuAspSerAspPheThrValAsnValLysGlyGluLeuIleGluLeuArg  
 TTGGCGTAAATCAACGAAGCGGCTTATGATGCAATTAATCAACTACAACAACTTTTCGCCAAGAGTTGGCAATCGACCTAAATAGAACTGGTCAATG  
 3500

GlyGlnValAsnAspGluLysLeuSerSerPheAsnGlnLeuGlnGlnThrPheArgGlnGluPheGlyAsnArgProLysLeuGluLeuValAsnVal  
 GCGGGCAAGTCAATGATGAAAAATGAGTTCATTTAATCAACTACAACAACTTTTCGCCAAGAGTTGGCAATCGACCTAAATAGAACTGGTCAATG  
 3600

GlyGlyGlnProGlnHisAspGluLeuAsnPheGluValGlnAlaIleSerLeuGlyLysValProTyrValValLeuAspAsnHisGlnArgTyrPro  
 TCGGGGGCAACCCAGCATGATGAATGAATTTGAGGTGCAAGCTATCTGCTTAGGAAAGTGCCCTATGTGGTACTCGACAAATCATCAACGCTATC  
 3700 pGC403

GluGlyAlaIleLeuAsnAsnGlyValArgIleLeuAlaIleArgArgAspAlaValIleValSerLysGlyLysArgGluPheValIleGlnLeuAsn  
 CAGAAGCGCCATACTTAACAATGGCGTTCGATTTCTGGCTATTCGACGCGATGCGGTGATTGTGAGTAAAGGAAAACGGGAATTTGTGATCCAGCTCA  
 3800

MetThrGlnLeuGluGluGlnLeuHisAsnValGluThrValArgSerIleThrMetGlnLeuGluMetAlaLeuAlaLysLeu  
 GlyGlyLysProArg\*\*\*  
 ATGGAGGTAACCTCGATGACACAATTAGAGGAGCAACTGCATAACGTGGAGCAGTGCCTCTACTACTGCAACTAGAAATGGCGCTAGCGAAGCT  
 rbs yscE 3900

LysLysAspMetMetArgGlyGlyAspAlaLysGlnTyrGlnValTrpGlnSerGluSerLysAlaIleGluSerAlaIleAlaIleIleHisTyrVal  
 CAAAAAGATATGATGCGCGTGGTATGCCAAGCAGTATCAGGTTGGCAGAGTGAATCTAAAGCTATTGAGTCAGCTATAGCCATTATTCATTATGT  
 4000

AlaGlyGlyLeuLys\*\*\*MetSerAsnPheSerGlyPheThrLysGlyAsnAspIleAlaAspLeuAspAlaValAlaGlnThrLeuLysLysProAla  
 AGCAGGAGTCTAAAAATAATGAGTAATTTCTCGGTTTACAAAAGGGAACGATATCGCTGACTTAGTGGTGGCTCAAACGCTCAAGAAGCCAGC  
 rbs yscF 4100 EcoRV

AspAspAlaAsnLysAlaValAsnAspSerIleAlaAlaLeuLysAspThrProAspAsnProAlaLeuLeuAlaAspLeuGlnHisSerIleAsnLys  
 AGCAGCAGCAAAACAGGCGGTTAATGACTCGATAGCAGCATTGAAAGATACGCTGACACCCGGCGTACTTGTGACTTAAACATTAATATAA  
 4200

FIG. 5—Continued



TrpSerValIleTyrAsnIleSerSerThrIleValArgSerMetLysAspLeuMetGlnGlyIleLeuGlnLysPhePro\*\*\* MetLysTyrLysLeu  
 ATGGTCGGTAATTTACAATAAAGCTCAACCATAGTTCGTAGCATGAAAGACTTAATGCAAGGCATCCTACAGAGTTCCCATATAATGAAATATAAAC  
 . . . . . 4300 . . . . . rbs . . . . . yscG . . . . .  
 AsnValLeuLeuAlaGluIleAlaLeuIleGlyThrGlyAsnHisCysHisGluGluAlaAsnCysIleAlaGluTrpLeuHisLeuLysGlyGluGlu  
 TCAACGTAAGTGTAGCAGAGATTGCTCTGATTGGAACCGGCAACCACTGCCACGAAGAAGCGAATTGCATTGCTGAATGGTTACATTTGAAAGGTGAAG  
 . . . . . pJCV1279 4400 . . . . .  
 GluAlaValGlnLeuIleGlnLeuSerSerLeuMetAsnArgGlyAspTyrAlaSerAlaLeuGlnGlnGlyAsnLysSerThrTyrProAspLeuGlu  
 AAGAGCGGTTCAATTGATTCAGCTTTCTCTTTGATGAACCGTGGGACTACGCAAGCGCTTGCAACAAGGAAATAAATCAACTATCCTGATTTGG  
 . . . . . 4500 . . . . .  
 ProTrpLeuAlaLeuCysGluTyrArgLeuGlyLeuGlyAsnAlaLeuGluSerArgLeuAsnArgLeuAlaThrSerGlnAspProArgIleGlnThr  
 AACCTTGGTTAGCCTTATGTGAATATCGCCTCGGTTGGGGACCCCTTAGAGTCACGTTAAATCGCTCGCAACGAGTCAGGATCCTAGAAATACAGA  
 . . . . . 4600 . . . . . DraI . . . . . B9 BamHI B1 . . . . .  
 MetThrValThrLeuAsnArgGlySerIleThrSerLeuMetSerSerSerGlnAlaValSerThr  
 PheValAsnGlyMetLysGluGlnLeuLysThr\*\*\*  
 CATTGTGGAATGGAATGAAGGAGCAACTAAAAACATGACCGTTACCCTTAATAGAGGTTCCATTACATCGTTGATGTCTTCGCTCAGGCAGTCTCTAC  
 . . . . . rbs . . . . . yscH BstEII 4710 . . . . .  
 LeuGlnProAlaAlaSerGluLeuLysThrGlnLeuGluHisLysLeuLysSerGluSerAlaGluLysThrArgGluValLeuTrpGlnGlnTyrTyr  
 GCTACAACCGCAGCATCTGAGCTGAAACACAACCTGGAGCATAAGCTAAAGTGAATCCGCTGAAAAGACACGGGAAGTTCTGTGGCAGCAATATTA  
 . . . . . 4800 . . . . .  
 AlaSerAsnProProAspHisAlaValLeuGluValLeuAlaThrProValArgGluAlaLeuLeuAlaArgPheGlyGlnHisGlnGlyProValVal  
 TGCCAGTAACCCCTCCTGACCATGCCGTTCTTGAGGTTTTGGCGACGCCCTACGTGAGGCGTTACTGGCGGTTTCGGTCAACATCAAGGGCCTGTTGT  
 . . . . . 4900 . . . . . pGC216 . . . . .  
 ProAlaIleAspLeuProGluLeuArgSerValLeuGlnGlnPheAspSerPheGlyLysArgArgGluAlaIleLeuLeuGlnValLeuGluGlyIle  
 ACCGGCTATAGATTTACCTGAATTCGCTAGTGTATTGCAGCAATTTGACTCGTTTGGTAAGCGCGGGGAAGCAATATTGCTCAAAGTATTAGAGGGTAT  
 . . . . . 5000 . . . . .  
 LysProAsnGluSerGlnValGlyLeuProTyrLeuSerGluLeuIleAsnLysGluLeuMetIleLeuLeuProTyrAsnSerIleValAspSerLeu  
 AAAACCCAAATGAGAGCCAGGTTGGATTACCTTATTTATCAGAGTAAATAAATAAGAAATTAATGATCTATTACCGTATAAATCGATTGTAGATAGCCCT  
 . . . . . 5100 . . . . . pJCV1289 . . . . .  
 LeuHisAsnSerHisGlnIleAspMetGluThr\*\*\*MetProAsnIleGluIleAlaGlnAlaAspGluValIleIleThrThrLeuGluGluLeuGly  
 ACTTCATAACCCATCAAATGATATGGAGACATAAATGCCGAACATAGAAATAGCTCAGGCGGATGAGGTGATCATAACCACCGTGGAGGAATAGG  
 . . . . . rbs . . . . . yscI . . . . . 5200 . . . . .  
 ProValGluProThrThrGluGlnIleMetArgPheAspAlaAlaMetSerGluAspThrGlnGlyLeuGlyHisSerLeuLeuLysGluValSerAsp  
 GCCGGTAGAGCCAAACATGAGCAAAATATGCGCTTTGATGCGGCAATGTCAGAAATACGCAAGGACTGGGCCATTCACTCCTCAAGGAGGTTAGTGA  
 . . . . . 5300 . . . . .  
 IleGlnLysThrPheLysThrAlaLysSerAspLeuHisThrLysLeuAlaValSerValAspAsnProAsnAspLeuMetLeuMetGlnTrpSerLeu  
 TATTGATATCTCAAACGGAAGGCTATCCACACGAGAGTTCACACTAAGCTGGCTGTTTCAGTTGATAATCCCAACGACCTGATGCTAATGCAATGGTCACT  
 . . . . . 5400 . . . . .  
 IleArgIleThrIleGlnGluGluLeuIleAlaLysThrAlaGlyArgMetSerGlnAsnValGluThrLeuSerLysGlyGly\*\*\* MetLys  
 TATCCGTATAACAATCCAAGAAGAACTTATCGCCAAGCAGCCGGCGAATGAGCCAAAATGTTGAAACCTTTCGAAAGGGGGTGAAGAACTAGTGAA  
 . . . . . 5500 . . . . . rbs . . . . . yscJ . . . . .  
 ValLysThrSerThrLeuIleLeuIleLeuPheLeuThrGlyCysLysValAspLeuTyrThrGlyIleSerGlnLysGluGlyAsnGluMet  
 AGTTAAGACTTCACTGCAACATTGATATTAATCTGTGTTTTAACTGGTTGCAAAAGTTGATCTTTATACCGGAATTAGTCAGAAGGAAGGGAACCAAT  
 . . . . . signal . . . . . sequence . . . . . 5600 . . . . .  
 LeuAlaLeuLeuArgGlnGluGlyLeuSerAlaAspLysGluProAspLysAspGlyLysIleLysLeuLeuValGluGluSerAspValAlaGlnAla  
 GCTCGCGCTGTGCGCAAGAGGCGCTTTCGCGAGACAAGAGCCAGACAAGATGGGAAGATTAAAGCTCTGGTTGAGGAGTCAGATGTCGCTCAGGC  
 . . . . . 5700 . . . . .  
 IleAspIleLeuLysArgLysGlyTyrProHisGluSerPheSerThrLeuGlnAspValPheProLysAspGlyLeuIleSerSerProIleGluGlu  
 TATTGATATCTCAAACGGAAGGCTATCCACACGAGAGTTCACACTAAGCTGGCTGTTTCAGTTGATAATCCCAACGACCTGATGCTAATGCAATGGTCACT  
 . . . . . 5800 . . . . . EcoRV . . . . .  
 LeuAlaArgLeuAsnTyrAlaLysAlaGlnGluIleSerArgThrLeuSerGluIleAspGlyValLeuValAlaArgValHisValValLeuProGlu  
 GTTGGCGAGGCTAATATGCCAAGGCGCAAGAGATCTCCCGCACTTTATCTGAAATGACGGGTATTAGTGGCTCGAGTGCATGCTGATTGCTCGA  
 . . . . . BglII . . . . . 5900 . . . . . XhoI . . . . .  
 GluGlnAsnAsnLysGlyLysLysGlyValAlaAlaSerAlaSerValPheIleLysHisAlaAlaAspIleGlnPheAspThrTyrIleProGlnIle  
 AGAGCAAAATAACAAGGTAAGAAGGCGTAGCAGCATCCGCTTCGGTTTTATCAAGCACGCGAGCAGATATTGAGTTGACACCTACATACCTCAGAT  
 . . . . . 6000 . . . . .  
 LysGlnLeuValAsnAsnSerIleGluGlyLeuAlaTyrAspArgIleSerValIleLeuValProSerValAspValArgGlnSerSerHisLeuPro  
 TAAACAATATGTAATAAGTATTGAGGGGCTGGCCTATGATCGCATCAGTGTCATTTTGGTGCCATCGGTAGATGTTGCTCAAAGCTCTCATTTACC  
 . . . . . 6100 . . . . .  
 ArgAsnThrSerIleLeuSerIleGlnValSerGluGluSerLysGlyArgLeuIleGlyLeuLeuSerLeuLeuIleLeuLeuLeuProValThrAsn  
 TCGTAACACGAGCATACTCAATTCAAGTGAGTGAAGAGTCAAAAGGCGCTTATTGGCTTGTGCTGCTTATTTGCTTTTGCCTTTGCCAGTACCA  
 . . . . . 6200 . . . . .  
 MetMetGluAsnTyrIleThrSerPheGlnLeuArgPheCysProAlaAlaTyrLeuHisLeu  
 LeuAlaGlnTyrPheTrpLeuGlnArgLysLys\*\*\*  
 TCTTGTCTCAATTTTTGGTTACAACGCAAGAGTGATGATGGAAAATATATTACCTCTTTTCAATTGCGCTTCTGCCCGCGGCTTATTTGCACCTTG  
 . . . . . rbs . . . . . yscK . . . . . 6300 . . . . .

FIG. 5—Continued

GluGlnLeuProSerLeuTrpArgSerIleLeuProTyrLeuProGlnTrpArgAspSerAlaHisLeuAsnAlaAlaLeuLeuAspGluPheSerLeu  
 GAACAGTTACCATCATTATGGCGTTCAATATTGCCCTACTTACCTCAGTGGCGCATAGTGCTCATCTCAATGCTGCTTTATTGGATGAATTTCTCTT  
 6400

AspThrAspTyrGluGluProHisGlyLeuGlyAlaLeuProLeuGlnProGlnSerGlnLeuGluLeuLeuLeuCysArgLeuGlyLeuValLeuHis  
 GATACCGACTATGAAGAGCCCATGGGTGGGGCGCTGCCTTGGCAGCCCAATCACAGCTCGAACTGTTACTTTGTGCGCTGGATTAGTTCTGCAT  
 6500

GlyGluAlaIleArgArgCysValLeuAlaSerProLeuGlnGlnLeuLeuThrLeuValAsnGlnGluThrLeuArgGlnIleIleValGlnHisGlu  
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 6600

LeuLeuIleGlyProTrpProThrAsnTrpGlnArgProLeuProThrGluIleGluSerArgThrMetIleGlnSerGlyLeuAlaPheTrpLeuAla  
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 6700

AlaMetGluProGlnProGlnAlaTrpCysLysArgLeuSerLeuArgLeuProLeuAlaThrProSerGluProTrpLeuValAlaGluSerGlnArg  
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 6800

ProLeuAlaGlnThrLeuCysHisLysLeuValLysGlnValMetProThrCysSerHisLeuPheLys\*\*\*  
 MetSerGlnThrCysGlnThrGlyTyrAlaTyrMetGlnProPheValGlnIleIleProSerAsnLeuSerLeuAlaCysGly  
 CCACTGGCCAAACTTTATGTCAAACTGTCAACAGGTTATGCTACATGCAGCCATTTGTTCAAATAATACCAAGTAATCTCTCGCTCGTTGCG  
 yscL yscL' 6900 pJCV1275

LeuArgIleLeuArgAlaGluAspTyrGlnSerSerLeuThrThrGluGluLeuIleSerAlaAlaLysGlnAspAlaGluLysIleLeuAlaAspAla  
 GTCTCGTATTTTGCAGCGCAAGATTACCAATCCAGTTAACTACCGAAGAGTTGATTAGTGCCGCAAAACAGGATGCTGAAAAGATCCTGGCTGACG  
 7000

GlnGluValTyrGluGlnGlnLysGlnLeuGlyTrpGlnAlaGlyMetAspGluAlaArgThrLeuGlnAlaThrLeuIleHisGluThrGlnLeuGln  
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 yscL'' 7100

CysGlnGlnPheTyrArgHisValGluGlnGlnMetSerGluValValLeuLeuAlaValArgLysIleLeuAsnAspTyrAspGlnValAspMetThr  
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 7200

LeuGlnValValArgGluAlaLeuAlaLeuValSerAsnGlnLysGlnValValValArgValAsnProAspGlnAlaGlyThrIleArgGluGlnIle  
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 7300

AlaLysValHisLysAspPheProGluIleSerTyrLeuGluValThrAlaAspAlaArgLeuAspGlnGlyGlyCysIleLeuGluThrGluValGly  
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 7400

IleIleAspAlaSerIleAspGlyGlnIleGluAlaLeuSerArgAlaIleSerThrThrLeuGlyGlnMetLysValThrGluGluGlu\*\*\*  
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 7500

CGATTATATTTTTAGTGTTATCTATTATAAGATTGAGTTATCTACATAAATGGATGTTTTCATCCTCGTTTTATGAGAACGATCCCCAGAATAATTTTT  
 Clal 7600

TATTGTGATTTTTCGTTTAAAGCCGATAAAAAATAAATCGTCTACGACAGTGGTTAGCAAAAATAAATAACTTAGAATATCGTAGAGATAATTAT  
 DraI 7700

MetLysIleAsnThrLeuGlnSerLeuIleAsnGlnGlnIleThrGlnValGlyHisGlyGlyGlnAlaGlyArgLeuThrGlu  
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 rbs yscM 7800

ThrAsnProLeuThrGluAsnSerHisGlnIleSerThrAlaGluLysAlaPheAlaSerGluValLeuGluHisValLysAsnThrAlaLeuSerArg  
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 EcoRV 7900

HisAspIleAlaCysLeuLeuProArgValSerAsnLeuGluLeuLysGlnGlyLysAlaGlyGluValIleValThrGlyLeuArgThrGluGlnLeu  
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 8000

SerLeuSerAspAlaLysLeuLeuGluAlaAlaMetArgGlnAspThrAlaAlaAspGly\*\*\*  
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 8100

TAATACTCCCAGGTTGATTTACGTAACATTTTTCAAGAAGTCATGTATCAATTTCCCTTGAGCCAATTTAGAATAATAATACGCCCTCCTCGGTG  
 8200

ATCCCCGAAGTGGGGTATTTATCAGTAGAGTCTGCTCCTCATATAAATTGAAGGAATTAGGATGAAAGATCACATTGTAGCGACTACCGGTTATGG  
 8300

TTATTTTGATGTCATCCCAGGCAATATTCTGTTGCTATCACTGGTATTCTGCAATTACCAGTGGGCTGGCTGACATTTGCCAAAAATAGGGGGGGA  
 8400

TGGATACTTTATTTTTACGCCATGCCGCCAGGGCATTGGAATAAAAATATATTTTCTAAATGATGATGAGTTAAA 8495

1 of yopH seq (Microb. Pathogen 5: 452, 1988) DraI

FIG. 5—Continued

possess a putative signal sequence. It has one domain with a hydrophobicity value of 1.9.

(xii) **YscL.** The longest ORF corresponding to *yscL* is 223 codons long and encodes a 25-kDa protein unlikely to possess a signal sequence. Initiation at the second and third ATG in the same reading frame yields proteins 11 and 76 amino acids smaller and tentatively called YscL' and YscL". Only the initiation codon leading to YscL" is preceded by a potential ribosome binding site.

(xiii) **YscM.** Surprisingly, YscM was found to share significant similarity with YopH, encoded by the neighboring gene on the pYV plasmid. The domain of similarity spans residues 52 to 130 of YopH. This domain is located between the secretion recognition domain (residues 1 to 48) (34) and the tyrosine-phosphatase domain (residues 206 to 468) (23, 33). Since *yscM* and *yopH* are adjacent and in the same orientation on the pYV plasmid, one could speculate that *yscM* arose by tandem duplication of *yopH*.

**Transcription analysis of *virC*.** To find out whether all 13 genes were part of one or more operon, we analyzed the RNA produced by the wild-type strain and by several *virC* mutants by Northern blotting, using different *virC* fragments as probes. As outlined in Fig. 8, all the probes hybridized with large-molecular-size RNA species (>2.5 kb) extracted from the wild-type strain. Detection of the *yscM* transcript was extremely weak, suggesting either that transcription was attenuated before reaching that gene or that degradation of the mRNA proceeds from the 3' end. The probes never hybridized to RNAs extracted from mutants in which mini-Mu *d lac* inserted upstream of the probe, confirming that the mutations were polar. Transcripts of the genes *yscC* to *yscL* were not detected in the RNA extracted from W22703 (pGC445) (insertion in *yscC*), suggesting that genes *yscC* to *yscL* are part of a single operon. Besides this, primer extension experiments indicated that *yscA* and *yscB* are transcribed from the same promoter (Fig. 9). Since, in turn, genes *yscB* and *yscC* are exactly contiguous and hence presumably transcribed together, we conclude that genes *yscA* to *yscL* (and probably also *yscM*) are all contained in the same operon.

**Complementation experiments.** Since mini-Mu insertions caused polar mutations in the *virC* operon and since all the *virC* mutants exhibited the same phenotype, we performed complementation experiments to define the individual role of several of the *virC*-encoded factors. We monitored the secretion of Yops in the mutants containing the complementing plasmid pTM200, a mobilizable derivative of pACYC184 carrying the *EcoRI* fragment 3 of pYVe439-80. Despite the fact that pTM200 does not contain the *virC* promoter, this plasmid was found to complement pGC1279 (*yscG*), pGC216 (*yscH*), and pGC1275 (*yscL*). We thus assumed that transcription occurred from one of the vector's promoters, although this could also occur as the result of the presence of a weak internal *virC* promoter.

TABLE 3. Putative proteins encoded by the *virC* operon

Protein	No. of amino acids	Molecular mass (kDa)	Signal sequence	Relevant features
YscA	32	3,810	—	
YscB	137	15,451	—	
YscC	607	67,133	+ (SPI) <sup>a</sup>	Similarity to PulD and pIV
	581 + 26 <sup>b</sup>	(64,248) <sup>c</sup>		
YscD	418	46,757	—	GTG start codon N-terminal hydrophobic domain
YscE	66	7,440	—	
YscF	87	9,438	—	
YscG	115	12,915	—	N-terminal hydrophobic domain
YscH	165	18,392	—	
YscI	115	12,683	—	
YscJ	244	27,030	+ (SPII) <sup>a</sup>	GTG start codon Previously described as YlpB C-terminal hydrophobic domain
	226 + 18 <sup>b</sup>	(25,072) <sup>c</sup>		
YscK	209	23,970	—	
YscL	223	24,949	—	
YscM	115	12,371	—	Resembles YopH

<sup>a</sup> SPI, signal peptidase I; SPII, signal peptidase II.

<sup>b</sup> Probable signal sequence with the indicated number of amino acids.

<sup>c</sup> Molecular mass of the protein after cleavage of the signal sequence.

Several derivatives of pTM200 containing mutations unlikely to affect downstream genes were constructed in different *ysc* genes either by deletion of restriction fragments or by filling in restriction sites and thus causing frameshift mutations. The plasmids constructed were used in complementation studies with various *virC* mutants. From the data summarized in Fig. 10, it appeared that at least three genes were required for the secretion of Yops: *yscD*, *yscJ*, and *yscL*. According to our complementation analysis, *yscM* was not required for the production of Yops. However, we repeatedly attempted to mutate *yscM* via the integration of a suicide vector containing an internal fragment of the gene. All these attempts failed, suggesting that *yscM* could play an important role.

**Regulation of *virC* expression.** The transcription of the *virC* operon was compared with that of *yopH* with respect to the kinetics of induction at 37°C, the influence of Ca<sup>2+</sup> ions in

FIG. 5. Nucleotide sequence of the *virC* region from pYVe439-80. The sequence presented (coordinates 34.0 to 42.5 kb of the pYVe plasmid) joins the previously sequenced *virF* (14) and *yopH* (33) genes. The translation of hypothetical proteins YscA to YscM is presented. The putative initiation codons of YscD and YscJ are GTG codons. Three possible initiation codons (ATG) for YscL are marked YscL, YscL', and YscL". Predicted signal sequences of YscC and YscJ and the putative ribosome binding sites (rbs) of all the *ysc* genes are underlined. Arrows indicate the insertion site of mini-Mu *d lac* for the various *virC* mutants. The number refers to the corresponding pGC or pJCV plasmid carrying the mutation. The transcriptional start determined by primer extension is indicated by an asterisk. The putative promoter is underlined twice. Some restriction sites are indicated. The region of plasmid pIB1 from *Y. pseudotuberculosis* that corresponds to nucleotides 4637 to 8495 of the sequence presented here is called *lcrK* (45) and has recently been sequenced by M. Rimpiläinen and H. Wolf-Watz (43a). There is a perfect conservation of the ORFs and an overall homology of nearly 90% at the nucleotide level between the corresponding sequences in *Y. enterocolitica* and *Y. pseudotuberculosis*.

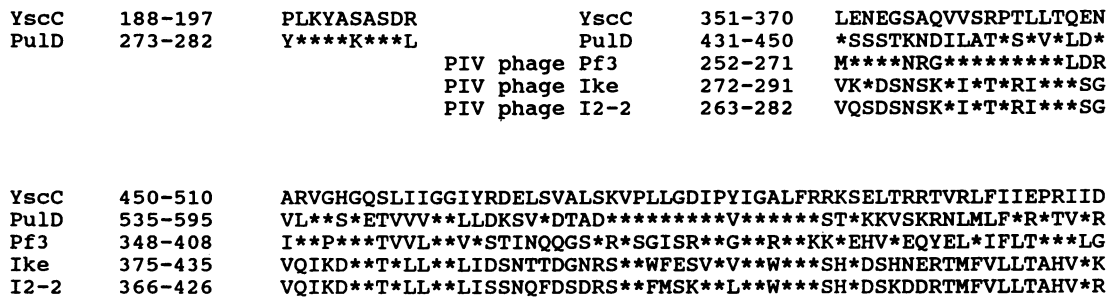


FIG. 6. Alignment of YscC, Pu1D (20), and gene IV (PIV) proteins (30, 40, 47) of bacteriophages Pf3, IKE, and I2-2. Residues identical to YscC are indicated by asterisks.

the culture medium, and the influence of the various *vir* mutations.

Transcription of *virC* and *yopH* was detected after 30-min induction at 37°C and reached a maximum after 1.5 h. However, after 2-h induction at 37°C, the amounts and the sizes of the *virC* messengers decreased while the *yopH* transcripts were still very abundant (Fig. 11). Hence, either transcription of *virC* stops before transcription of *yopH* or the *yopH* messengers are much more stable than those of *virC*. The presence of Ca<sup>2+</sup> ions in the culture medium reduced the transcription of *virC* and *yopH*, although transcription was still readily detectable (data not shown). In conclusion, the transcription of *virC* is subject to the same basic regulation as that of *yop* genes. Very little difference was detected in the kinetics of transcription (or degradation) of the messengers.

Transcription of *virC* was also monitored in *virA* and *virB* mutants. It decreased in a *virB* mutant but not in a *virA* mutant (data not shown).

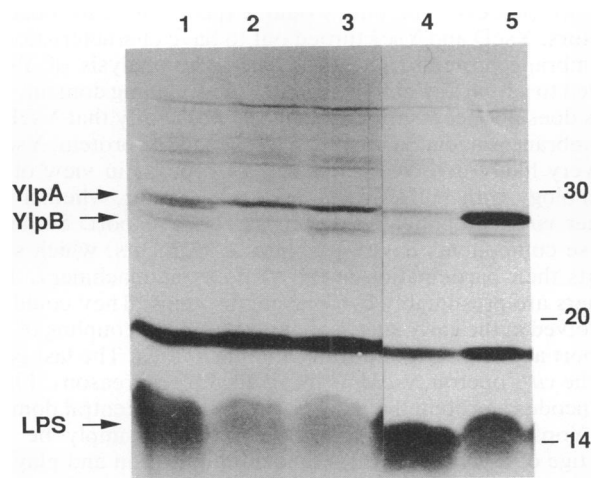


FIG. 7. Identification of YscJ as lipoprotein YlpB. SDS-PAGE and fluorography of [<sup>3</sup>H]palmitic acid-labelled membranes extracted from *Y. enterocolitica* W22703 carrying various pYV plasmid mutants. Lanes: 1, wild-type plasmid pYVe227; 2, *ylpA* and *virC* double mutant plasmid pGC216 (insertion in *yscH*); 3, *ylpA* and *virC* double mutant pJCV1275 (insertion in *yscL*); 4, *ylpA* mutant pYL4; 5, wild-type plasmid pYVe227 and plasmid pBC5 overexpressing *ylpA* (10). Numbers to the right are molecular mass markers (in kilodaltons). Note that YlpA is present in lanes 1 and 5 and that YlpB is present in lanes 1, 3, 4, and 5. LPS, lipopolysaccharide.

DISCUSSION

It has long been known that the appearance of the adhesin YadA occurs only at 37°C and requires neither low Ca<sup>2+</sup> conditions nor an intact Ca<sup>2+</sup> region (1, 15). According to the Northern and immunoblot analyses presented here, there is a good correlation between the transcription of *yadA* and the appearance of the adhesin in the outer membrane. We conclude that translation and export of YadA do not require

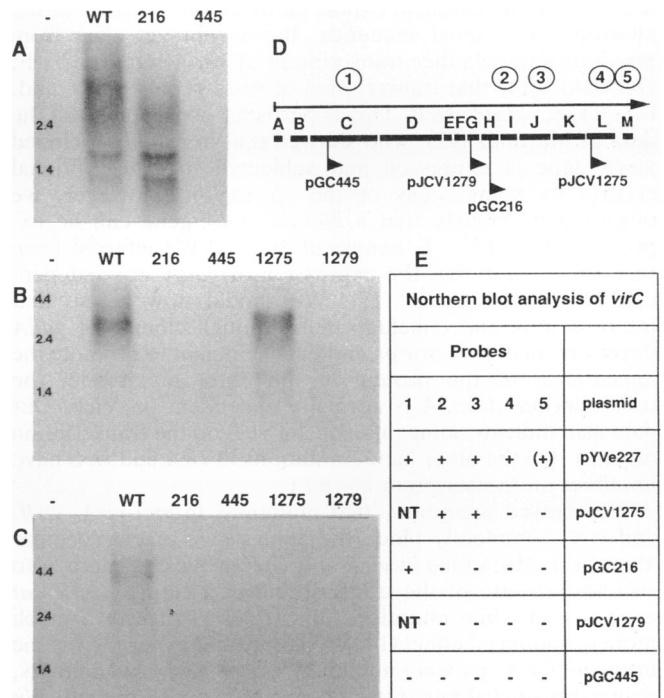


FIG. 8. Northern blot analysis of the *virC* operon. (A, B, and C) Examples of the Northern blots. RNA was extracted after 1.5- to 2-h induction of the cultures at 37°C in BHI-OX from a plasmidless W22703 strain (-) and W22703 strains carrying plasmids pYVe227 (WT), pGC216 (216), pGC445 (445), pJCV1275 (1275), and pJCV 1279 (1279). The probes are identified (circles) in panel D. For panels A, B, and C, the probes were probes 1, 2, and 4, respectively. Note that the large mRNAs are degraded. One should thus focus on the presence or absence of specific mRNAs rather than on their size. Size markers are on the left, in kilobases. (D) Localization of the probes (circles labelled 1 to 5) and of the polar mutations in the *virC* operon. (E) Summary of the results. Note that the results with probes 3 and 5 are not shown. NT, not tested.

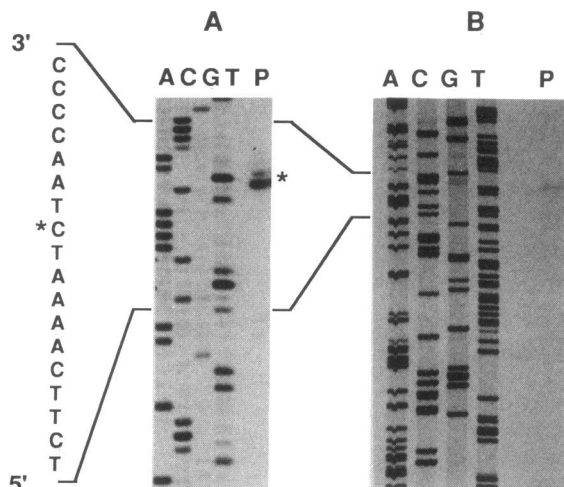


FIG. 9. Determination of the transcriptional start of *virC* by primer extension. (A) Extension from MIPA39 (coordinates 96 to 113 bp of Fig. 5); (B) extension from MIPA40 (coordinates 409 to 426 bp of Fig. 5).

pYV-encoded functions. *YadA* is thus presumably addressed via the classical export pathway on the basis of the cleavage of a signal sequence. It was not yet clear from previous data whether transcription of *yadA* requires *VirF*. Our data show that transcription of *yadA* requires *virF* and, to a lesser extent, *virB*. This is perfectly consistent with the data of Martinez (32), who showed that in *E. coli*, a cloned *yadA* gene is expressed and subjected to transcriptional control by some locus of the *vir* region. However, we observed previously that a cloned *yadA* gene can be expressed in a pYV<sup>-</sup> *Y. enterocolitica* (1). We inferred from this observation that the expression of *yadA* was independent of the *vir* genes (17). We should now revise that interpretation and conclude that although cloning of *yadA* downstream of a vector promoter is sufficient to promote the appearance of the protein in the outer membrane, the transcription of *yadA* is normally dependent on *VirF*. Our data also indicate some influence of *virB* on the transcription of *yadA*. On the other hand, mutations in *virA* and *virC* have no effect on transcription of *yadA*.

We showed previously that mutations in loci *virA*, *virB*, and *virC* completely block the appearance of extracellular Yops (15). Mutations in *virA* and *virC* provoke a mere 5- to 10-fold decrease of the transcription of a cloned *yopH-cat* gene fusion while mutations in *virB* or *virF* have a much more pronounced effect (14). In our present experiments, the intracellular Yops were abundant in *virA* and *virC* mutants, detectable in *virB* mutants, and absent in a *virF* mutant. We conclude from this that *virA* and *virC* are involved in the translocation of Yops across the bacterial membranes or in the specific control of this function. The conclusion that *virA* and *virC* are involved in the same general pathway is somewhat surprising, since we described the *virA* mutant W22703(pGC274) as having the CI phenotype and the *virC* mutants as having the Gts phenotype (15). In view of this, we repeatedly checked the growth rate of W22703(pGC274) at 37°C and we observed that it was reduced, indicating that the phenotype of the *virA* mutant was somehow intermediate between CI and Gts.

For *virB*, we favor a regulatory role. The observation that

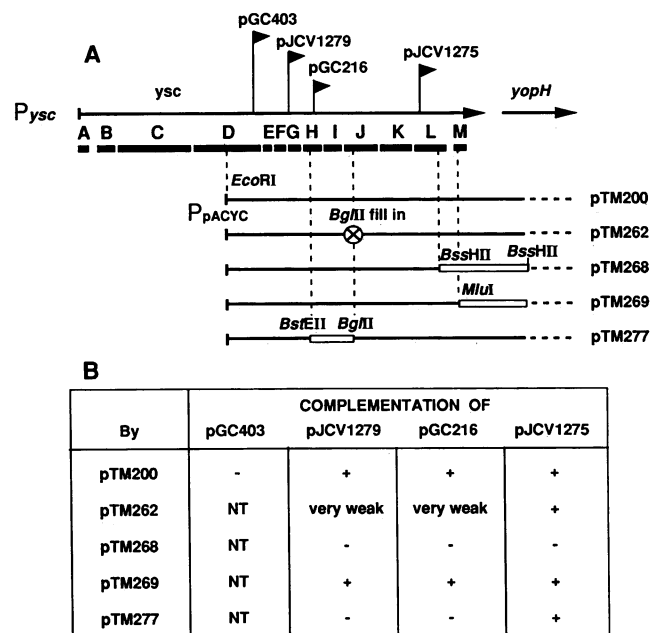


FIG. 10. Complementation of *virC* mutants. (A) Map of *virC* showing the positions of the insertion mutations and the structures of the plasmids used to complement the mutations. Rectangles indicate deletions. (B) Secretion of the Yops by W22703 carrying the four *virC* mutants and pTM200 or its derivatives. NT, not tested.

*virB* mutants also accumulate Yops can be explained by the fact that transcription of *virC* is also reduced in *virB* mutants.

The present study focused on *virC*. This locus was found to be most likely a large single operon containing 13 genes that we called *ysc* for *Yersinia* secretion. Three of the hypothetical proteins encoded by this operon, namely, *YscD*, *YscJ*, and *YscL*, were shown to be required for the export process. As one would expect for translocation factors, *YscD* and *YscJ* turned out to have characteristics of membrane proteins. The hydrophobicity analysis of *YscL* failed to reveal any obvious membrane spanning domain, but this does not necessarily rule out the possibility that *YscL* is membrane associated. A fourth *virC*-encoded protein, *YscC*, is very likely involved in the export process in view of its homology with *PulD*. We do not know, so far, whether the other *virC* components are necessary for export. Some of these components have hydrophobic segments, which supports their participation in the Yop export machinery. The others are presumably cytoplasmic proteins. They could be involved in the early stages of export or in the coupling of the export and transcription-translation activities. The last gene of the *virC* operon, *yscM*, is intriguing for two reasons. First, it encodes a protein that is very similar to the central domain of *YopH*. This suggests that *yscM* could simply be the vestige of a *yopH* ancestral tandem duplication and play no role in the production of Yops. Second, our attempts to disrupt this gene failed, whereas the polar insertion mutants were perfectly viable. One possible interpretation is that *yscM* plays a key role but that it is required only in very small amounts to function and that either mini-Mu mutations do not completely inhibit the transcription of downstream genes or some weak promoter allows the independent transcription of *yscM*.

Data to be presented elsewhere (27a) show that transcription of the *virC* operon is dependent on *VirF*, like transcrip-

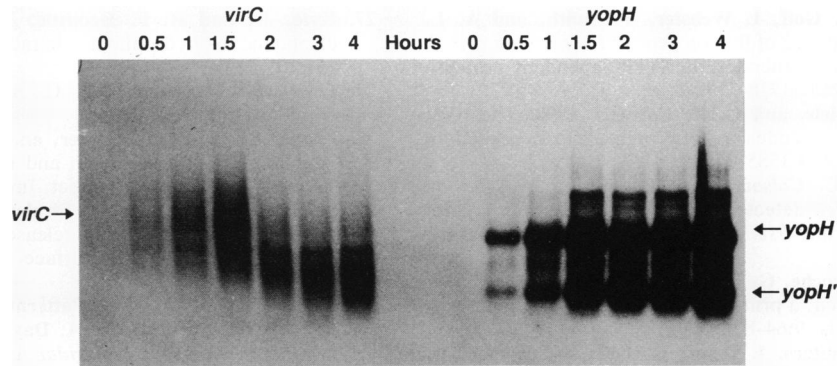


FIG. 11. Transcription regulation of *virC*, in W22703(pYV<sup>+</sup>). Kinetics of transcription of *virC* and *yopH* taken as a control (Northern blots). The time (in hours) after the temperature shift is given on top. The arrows point to the transcripts. The sizes of the two *yopH* transcripts are 1.55 and 0.48 kb (33). The clear space in the middle of the *virC* transcripts presumably corresponds to nonspecific occlusion by rRNA. The probes were the *virC* DNA extracted from pTM271 and *yopH* DNA (the 180-bp *Sau3A-SspI* fragment).

tion of the *yop* genes. This point is in good agreement with the suggested role of *virC* in export of Yops.

The *virC* mutants (1, 15) as well as the corresponding *lcrC* mutant of *Y. pestis* (22) and *lcrK* mutants of *Y. pseudotuberculosis* (45) are growth thermosensitive. The fact that all the Mini-Mu *lac* mutants with mutations in the *virC* operon exhibit the same phenotype indicates that at least one of the distal genes, *yscL* or *yscM*, is involved in this phenotype. As suggested by Forsberg et al. (21), this phenotype can be explained by the lack of secretion of YopN, which is involved in Ca<sup>2+</sup> signal transduction. The same mechanism could account for the reduced growth rate of the *virA* mutant at 37°C.

Secretion of Yop proteins by yersiniae does not fit any of the previously described export mechanisms. As is true for hemolysins (Hly) and related proteins, Yops do not contain a classical signal sequence (21a, 35). However, the Yop export system must be different from the Hly system because it involves the N-terminal domain of the protein rather than the C-terminal one (34, 35). Furthermore, none of the putative secretion factors encoded by *virC* presented similarity with HlyA, HlyB, and TolC or with the factors of related secretion mechanisms (3, 31, 59, 60). The presence of a lipoprotein in the secretion apparatus and the homology of YscC with PulD suggests some similarity between the export pathways of Yops and pullulanase (19, 43). However, apart from YscC, none of the *virC* products was found to share significant homology with any of the described pullulanase export factors. Moreover, the absence of signal sequence in Yops implies that the specific secretion apparatus accounts for the translocation of both inner and outer membranes of the bacteria. All these observations lead to the conclusion that Yops are secreted by a new specific mechanism that is clearly distinct from those previously described, even though YscC resembles PulD.

Interestingly, the domain conserved between YscC and PulD is also conserved in the gene IV-encoded protein (pIV) of filamentous bacteriophages. The fact that these three proteins have signal sequences and similar sizes reinforces the probability that these proteins are indeed related. The suggestion that pIV is part of an exit port involved in the translocation of the phage across the bacterial membrane (8) and the role of PulD in the pullulanase export strongly reinforce our claim that YscC and other Ysc proteins are part of the export machinery of Yops. The possibility that

Yops, pullulanase, and phage proteins export machineries are related raises exciting evolutionary questions.

#### ACKNOWLEDGMENTS

We thank Hans Wolf-Watz and Maria Rimpiläinen for their courtesy in exchanging sequence data prior to publication. We also thank F. Vierendeels for excellent technical assistance, the ICP illustration service for the artwork, and T. Nguyen Bao and J.-P. Cosijns for help in microscopy.

This work was supported by grants from the Belgian Ministry for Sciences (Action concertée 86-91/86), the Belgian Fund for Medical Scientific Research (FRSM, convention 3.4514.83), and the Belgian Fund for Scientific Research (FNRS, Crédit aux Chercheurs 1.5.102.90F). J.-C.V. and B.C. are supported by the Belgian Institute for Scientific Research applied to Industry and Agriculture (IRSIA). T.M. is Senior Research Assistant and C.L. is Research Assistant of the Belgian Fund for Scientific Research (FNRS).

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