Pathogenic bacteria of the genus *Yersinia* (*Yersinia enterocolitica, Y. pseudotuberculosis, and Y. pestis*) cause human diseases, with symptoms ranging from enteritis to septicemia and death, essentially by invading the host tissues. The clinical manifestations of *Y. enterocolitica* infections in humans are mostly enteric. The predominant clinical features are abdominal pain, sometimes resembling appendicitis, and diarrhea (14).

The three species carry plasmids of about 70 kilobases (kb) that are necessary for virulence (2, 20, 23, 24, 55). These plasmids, generally called pYV plasmids, are structurally and functionally related in the three species (3, 15, 16, 25, 30, 36). The pYV plasmids impose a calcium requirement for growth: virulent pYV* virulent* yersiniae form colonies at 28°C but not at 37°C on media deprived of calcium, whereas bacteria cured of pYV or carrying a mutated plasmid emerge on calcium-deprived media at 37°C (calcium response).

Under conditions of growth restriction, i.e., at 37°C in the absence of calcium, pYV plasmids from *Y. enterocolitica* and *Y. pseudotuberculosis* direct the secretion of at least nine proteins (16, 28). Some of these proteins are also inserted in the outer membrane (36), which explains why they are generally referred to as Yops, for *Yersinia* outer membrane proteins. For recent reviews, see references 6 and 14.

The structural genes encoding several of these proteins (*yop* genes) are scattered around pYV (4, 15, 16, 21, 22, 45). At least four transcriptional loci, spanning a contiguous 17-kb region and called *lcrA, lcrB, lcrC, and lcrF* in *Y. pestis* (25, 52) or *virA, virB, virC, and virF* in *Y. enterocolitica* (15, 16), control the calcium dependency and production of the Yops. The *lcrF* locus, called *virF* in *Y. enterocolitica* encodes a *trans-acting* activator of transcription of the *yop* genes (16, 52). *trans-Activation* of the transcription of *yop* genes was also demonstrated for *Y. pseudotuberculosis* (5, 22). A fifth gene, *lcrE*, was described in *Y. pestis* (53). This gene could encode a calcium sensor (53). The functions of *virA, virB,* and *virC* are not yet known (15, 16). The region controlling the calcium response (and hence the transcription of the *yop* genes) appears to be extremely conserved in the pYV plasmids from *Y. enterocolitica, Y. pseudotuberculosis,* and *Y. pestis* (4, 15, 16, 25, 36, 52, 53). However, the *yop* genes are distributed differently in the plasmids from the three species (16, 21, 22, 45). Nevertheless, *Y. pseudotuberculosis* expresses *yop* genes from *Y. pestis* (49), and the transcriptional activation and secretion systems of *Y. enterocolitica* act on a *yop* gene from *Y. pseudotuberculosis* (3). The genetic organization of pYVe227, a typical pYV plasmid from a *Y. enterocolitica* strain of serogroup 9, is given in Fig. 1. In this paper, we focus on the transcriptional activation of the *yop* genes. To understand this regulatory mechanism of virulence functions, we constructed a plasmid carrying an operon fusion between *yop51* and a chloramphenicol acetyltransferase gene. We then cloned *virF* on plasmid vectors compatible with the first plasmid and studied the action of *virF* on *yop51*. We also studied the expression of *virF* itself and determined its nucleotide sequence. The amino acid sequence derived from the sole open reading frame showed that the carboxy-terminal end of *VirF* is about 23% homologous to the C terminus of the arabinose regulatory protein of *Escherichia coli*. This homology with a DNA-binding protein (35) clearly suggests that *VirF* is also a DNA-binding protein and hence presumably not an RNA polymerase σ factor.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res*− Mod*−) isolated previously in this laboratory (13) from wild-type strain W227 (serogroup O:9). This strain was cured of its natural plasmid (pYVe227) to serve as a recipient for the various plasmid constructs. *E. coli* S17.1 is a mobilizing donor strain constructed by Simon et al. (44). It contains an RPltet::Mu neo::Tn7 plasmid integrated into the chromosome. Plasmids are listed in Table 1.
VOL.

encodes protein P1, which forms a fibrillar matrix on the surface of numbers identify the insertion mutations that defined the genes. The incD is the stabilization and incompatibility region; vir are genes described in the text.

version (i.e., supplemented with 20 mM sodium oxalate) or conjugants were selected on MacConkey agar. Selective were supplemented with relevant selective agents (kanamycin, 25 µg/ml; tetracycline, 10 µg/ml; chloramphenicol, 20 µg/ml) to ensure maintenance of the plasmids.

Bacteria were generally grown on brain heart infusion supplemented with 0.4% (wt/vol) glucose, 10 mM MgCl2, and 10 mM MgSO4. This medium was used either in its Mox version (i.e., supplemented with 20 mM sodium oxalate) or in its calcium version (i.e., supplemented with 5 mM calcium). Brain heart infusion-calcium was prepared by adding calcium chloride to autoclaved brain heart infusion. Media were supplemented with relevant selective agents (kanamycin, 25 µg/ml; tetracycline, 10 µg/ml; chloramphenicol, 20 µg/ml) to ensure maintenance of the plasmids.

Conjugations were carried out on tryptic soy agar. Transconjugants were selected on MacConkey agar. Selective agents were nalidixic acid (35 µg/ml), kanamycin (25 µg/ml), and ampicillin (100 µg/ml) to select pUC19, pTZ19R, or pSUP202 derivatives in E. coli; 300 µg/ml to select the same plasmids in Y. enterocolitica [12].

Construction of pTM243. Plasmid pTM243 is a mobilizable derivative of pACYC184 carrying the chloramphenicol acetyltransferase gene placed downstream of the promoter of yopS1. This plasmid was constructed as follows: (i) EcoRI fragment 3 of pYVe227 and a 760-base-pair EcoRI fragment of pTJS82 containing oriT of RK2 were cloned together on pACYC184. The resulting plasmid, pTM200, contains yopS1. (ii) Plasmid pTM200 was mutagenized with transposon Tn2507 by using the delivery system described by Michiels and Cornelis [32]. Transposon Tn2507 is a derivative of Tn2505 [32] containing the chloramphenicol acetyl-

transferase gene (cat) devoid of its promoter. This gene, originating from plasmid pCM4 [11], is located close to the left terminus of Tn2507 and is oriented toward the inside of the transposon. Several insertions of Tn2507 mapped within yopS1. In insertion 43, the cat gene was in the same orientation as yopS1 itself. Plasmid pTM243, containing insertion 43, was selected for the present study. As shown in the Results section, the cat gene of pTM243 is clearly transcribed from the promoter of yopS1, which encodes a truncated peptide of 27,000 daltons which is secreted in pYV-Y. enterocolitica strains (data not shown). The map of pTM243 is given in Fig. 2.

Induction of the yop regulon and analysis of the supernatant proteins. Induction of the yop regulon and analysis of the supernatant proteins were carried out exactly as described by Cornelis et al. [16].

Enzymatic assays. Preparation of the extracts and β-galactosidase assays were as described by Cornelis et al. [15]. Chloramphenicol acetyltransferase was assayed by the spectrophotometric method of Shaw [42]. Results are expressed as the change in optical density at 412 nm per minute at 25°C per optical density of bacteria at 600 nm.

Gene product identification by using the T7 RNA polymerase-promoter system. E. coli C600 containing both pGCS855 or pT7-5 and pGP1-2 was labeled as suggested by S. Tabor (personal communication). Cells were grown in LB with 40 µg of ampicillin per ml and 40 µg of kanamycin per ml at 30°C. At an optical density at 600 nm of 0.5, 1.0 ml of cells was centrifuged, washed in 5.0 ml of M9 medium, and suspended in 1.0 ml of M9 supplemented with 20 µg of thiamine per ml and with 19 amino acids (minus methionine) at 0.01% each. Cells were grown with shaking at 30°C in a Falcon 2059 tube for 60 min. The temperature was then shifted to 42°C for 15 min. Rifampin (Lepetit) (stock solution of 100 µg/ml in dimethylformamide) was added to a final concentration of 200 µg/ml. The cells were left at 42°C for an additional 10 min. The temperature was shifted down to 30°C for 20 min. The samples were then pulsed with 20 µCi of [35S]methionine (NEG-009A) for 5 min at 30°C, and the cells were finally centrifuged, dissolved in sample buffer, and loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide (gradient 14 to 20%) gel.

Extraction of RNA and Northern blot hybridization. RNA was prepared from cells by hot-phenol extraction as described by Derbyshire et al. [18]. Gels for Northern (RNA) blot hybridization were 1.2% agarose—16% formaldehyde and were blotted on Hybond N (Amersham Corp.) membranes. Hybridizations with nick-translated DNA probes were carried out at 55°C in 50% formamide—5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)—50 mM phosphate buffer (pH 6.5)–1× Denhardt solution—0.1% sodium dodecyl sulfate (SDS)—10 µg of denatured salmon sperm DNA per ml. Membranes were washed three times for 5 min each at room temperature in 2× SSC–0.1% SDS and twice for 15 min each in 0.1× SSC–0.1% SDS.

Nucleic acid preparation and analysis. Standard recombinant DNA methods were used for nucleic acid preparation and analysis [31]. DNA restriction enzymes were from Pharmacia, Inc., and Boehringer GmbH.

DNA was sequenced by the dideoxy-chain termination procedure [39] with the DNA polymerase Sequenase (47), the reagents from USB, and [35S]dATP from Du Pont, NEN Research Products. Single-stranded DNA was either from M13 or from phasmid pTZ19R. Overlapping clones were prepared by the method of Dale et al. [17] with the Cyclone FIG. 1. Genetic map of pYVe227, a typical pYV plasmid from a serogroup 9 Y. enterocolitica strain. rep is the replication region; inc is the stabilization and incompatibility region; vir are genes controlling the calcium response and the expression of yop genes; and yop are genes encoding the secreted proteins. The numbers refer to the molecular mass of the proteins (in kilodaltons). yopA encodes protein P1, which forms a fibrillar matrix on the surface of Y. enterocolitica (29, 54). yopD encodes Yop37 (15). The arrows point in the direction of transcription. The flaps and the small numbers identify the insertion mutations that defined the genes. The data are from Laroche et al. [30], Balligand et al. [11], Cornelis et al. (15, 16), and Biot and Cornelis (3). The orientation of virF is described in the text.
system of International Biotechnologies, Inc. Both strands have been sequenced.

Computer analysis. DNA and protein sequences were analyzed on a Micro Vax Computer (Digital Equipment Corp.) with the program package of Claverie (9) and the FastP, FastN, RELATE, and ALIGN programs of the Protein Identification Resource program package.

RESULTS

Influence of the vir loci on transcription of the yop genes. Mutation in any of the vir (i.e., virA, virB, virC, and virF) transcriptional loci results in the disappearance of the calcium requirement for growth and of the secretion of Yops at 37°C. The virF locus was shown to act on transcription of yopS1 (16), but the influence of virA, virB, and virC on transcription of the yop genes has not yet been determined.

To assay the transcription of a given yop gene in various genetic backgrounds, we constructed pTM243, a mobilizable plasmid carrying a cat gene lacking its promoter and fused to yopS1 by in vivo transposition of Tn2507. The map of plasmid pTM243 is given in Fig. 2, and its construction is described in Materials and Methods.

pTM243 was transformed into E. coli S17.1, and S17.1(pTM243) was mated with Y. enterocolitica W22703 carrying pYV plasmids mutated in the various vir loci. Chloramphenicol acetyltransferase was assayed in the various strains after growth at 25°C and after a 4-h shift to 37°C in the presence or absence of calcium (see Materials and Methods).

In the presence of a functional pYV plasmid (e.g., pGCS65), there was strong transcription of cat whereas in absence of a pYV plasmid, there was no detectable transcription (Table 2). In the presence of pYV, transcription of cat was detected only after incubation at 37°C. It was reduced by a factor of about 20 in the presence of calcium, showing that cat is transcribed under the control of the yop regulon (16). This was further confirmed by the fact that Y. enterocolitica W22703 carrying Tn2507 at about the same position in yopS1 but in the opposite orientation did not produce chloramphenicol acetyltransferase at 37°C or at 25°C (data not shown). For the sake of clarity, the transcription unit of pTM243 composed of a truncated yopS1 gene and cat will be referred to as the yopS1-cat unit.

Mutations in virA and virC reduced transcription of the yopS1-cat unit about 10-fold in the absence of calcium. The level of transcription in the presence of calcium was unaf-

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TABLE 1. Bacterial plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genetic structure</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCM4</td>
<td>pBR327 containing cat without promoter as a BamHI fragment</td>
<td>11</td>
</tr>
<tr>
<td>pGC217</td>
<td>pGB63 virC-217::mini-Mu d1 lac</td>
<td>15</td>
</tr>
<tr>
<td>pGC274</td>
<td>pGB63 virA-274::mini-Mu d1 lac</td>
<td>15</td>
</tr>
<tr>
<td>pGC403</td>
<td>pGB63 virC-403::mini-Mu d1 lac</td>
<td>15</td>
</tr>
<tr>
<td>pGC65</td>
<td>pGB63 virB-653::mini-Mu d1 lac</td>
<td>15</td>
</tr>
<tr>
<td>pGC678</td>
<td>pGB63 virC-678::mini-Mu d1 lac</td>
<td>15</td>
</tr>
<tr>
<td>pGC1152</td>
<td>pGB63 yapS1-1152::mini-Mu d1 lac</td>
<td>16</td>
</tr>
<tr>
<td>pGC1152-9</td>
<td>pGC1152 yapE::Tn1201</td>
<td>16</td>
</tr>
<tr>
<td>pGC615</td>
<td>pSUP202-BamHI fragment 6 of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC630</td>
<td>pSUP202-UC19 coordinates 31.0–34.6 kb of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC665</td>
<td>pTZ19R-coordin. 32.6–34.6 kb of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC755</td>
<td>pTZ19R-oriT-coordinates 32.6–34.6 kb of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC756</td>
<td>pTZ19R-oriT-coordinates 33.0–34.6 kb of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC757</td>
<td>cat cloned as a SalI fragment at XhoI site of pGC752 (operon fusion virF-cat)</td>
<td>This paper</td>
</tr>
<tr>
<td>pGC855</td>
<td>pT7.5 + coordinates 33.0–34.6 kb of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pGL1-2</td>
<td>pBR322 encoding c1857 and T7 RNA polymerase from promoter pL</td>
<td>46</td>
</tr>
<tr>
<td>pSUP202</td>
<td>pBR352-mob of RP4</td>
<td>44</td>
</tr>
<tr>
<td>pTJ688</td>
<td>pUC-7 containing oriT of RK2 as a 70-kb-base pair EcoRI fragment</td>
<td>41</td>
</tr>
<tr>
<td>pTM200</td>
<td>pACYC184-oriT + EcoRI fragment 3 of pYVe227</td>
<td>This paper</td>
</tr>
<tr>
<td>pTM243</td>
<td>pTM200 yapS1-43::Tn2507</td>
<td>This paper</td>
</tr>
<tr>
<td>pTJ19R</td>
<td>ori pBR322 + ori f1 lacZ' bla</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pT7-5</td>
<td>pACYC177 containing promoter f10 of phage T7</td>
<td>46</td>
</tr>
<tr>
<td>pUC19</td>
<td>pUC19</td>
<td>51</td>
</tr>
<tr>
<td>pYVe227</td>
<td>Virulence plasmid of Y. enterocolitica W227 (serogroup O:9); typical representative of plasmids from serogroup O:9 strains</td>
<td>30</td>
</tr>
</tbody>
</table>

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FIG. 2. Map of pTM243. (A) Genetic map. Plasmid pTM200 is a pACYC184 derivative containing the origin of transfer of RK2 (oriT) and EcoRI fragment 3 of pYVe227. This fragment contains yopS1. Transposon Tn2507 (9.1 kb) contains a kanamycin resistance gene (neo), the resolvase gene of Tn2505 (inrR) (32), and the cat devoid of its promoter. Plasmid pTM243 is pTM200-43::Tn2507. The insertion occurred in codon 219 of yopS1 (manuscript in preparation). The mutated yop gene encodes a truncated peptide of 27,000 daltons. The cat gene is transcribed from the promoter of yopS1 tet is the tetracycline resistance gene. The arrows point in the direction of transcription. (B) Restriction map of EcoRI fragment 3 (E3) of pYVe227. Restriction sites: B, BamHI; Bg, BgII; E, EcoRI; H, HpaI; Kp, KpnI; Ss, SstII; Xb, XbaI.
fected by mutations in virA or virC. The influence of mutations in virB was about threefold more severe, but there still was a very clear transcription of yop51-cat in the absence of a functional virB locus. In contrast, transcription of yop51-cat became barely detectable when complemented by pGC1152-9, which contains an insertion of Tn813 in virF as well as an operon fusion between lacZ and yop51 (16). Hence, we conclude that mutations in any of the vir loci affect the level of transcription of the yop regulon. However, virF appears to be the major key to transcription of the regulon.

Cloning of virF and complementation of mutant pGC1152-9. BamHI fragment 6 (4.6 kb) of pYVe227 (hereafter referred to as B6) containing the virF locus was cloned into pSUP202. The recombinant plasmid, called pGCS615, was introduced by conjugation in Y. enterocolitica W22703(pGC1152-9). The strain containing pGC1152-9 does not transcribe lacZ or secrete the Yops at 37°C in the absence of calcium (16). In contrast, Y. enterocolitica W22703 carrying both plasmids pGC1152-9 and pGCS615 showed the typical calcium response, produced β-galactosidase in a thermodependent manner, and secreted the Yops after thermal induction (Fig. 3). virF is thus entirely contained within B6, and it acts in trans on the production of all the Yops.

Action of virF on transcription of yop51-cat in the absence of other pYV genes. In the two systems described above, virF acts in trans on yop genes in the presence of all the other pYVe227 genes. We wanted to monitor the effect of an isolated virF gene on the isolated yop51-cat unit. virF was cloned in the EcoRI site of plasmid pSUP202. The recombinant plasmid was called pGCS630 (Fig. 4). Plasmid pGCS630 was introduced into Y. enterocolitica W22703(pTM243), and chloramphenicol acetyltransferase in the recombinant strain was assayed. The yop51-cat unit was expressed in the presence of pGCS630 (Table 3). However, transcription was lower than in a strain containing pYVe227, although the copy number of pGCS630 is higher than that of pYV. This observation is consistent with the fact that virA, virB, and virC also somehow influence transcription (see above).

In the presence of pGCS630 alone, transcription of yop51-cat is no longer regulated by calcium. This is also consistent with the observation that mutations in virA, virB, and virC drastically reduce the regulation by calcium (Table 2). It must be noticed that transcription of yop51-cat is thermodependent even in the presence of virF alone but to a lesser extent than in the presence of a full pYVe227 plasmid.

Localization of the virF gene. The virF gene was subcloned on plasmid pTZ19R as a PstI fragment of pGCS630, giving plasmid pGCS650 (Fig. 4). The latter plasmid was reduced by serial in vitro deletions. The origin of transfer of plasmid RK2 was grafted on the smaller derivatives to make them mobilizable by E. coli S17.1. Plasmid pGCS755 (Fig. 4), containing pYVe227 DNA spanning the coordinates from 32.8 to 34.6 kb, expressed a high activator activity. Plasmid pGCS756 (Fig. 4), containing DNA spanning the coordinates from 33.0 to 34.6 kb, expressed a low virF activity. Hence, the virF structural gene must be contained between the coordinates at 33.0 and 34.6 kb, while the operator-promoter region could be localized around the coordinate at 33 kb and extend somehow toward the coordinate at 32.8 kb. This orientation of virF (5′ Clal-XhoI 3′) is in agreement with the sequence data (see below). To orient virF with respect to the other pYVe227 genes, we hybridized the Southern blot of an EcoRI digest of pYVe227 with labeled DNA from the 200-base-pair EcoRI-XhoI fragment (coordinates 33.7 to 33.9 kb). Hybridization occurred with EcoRI fragment 7 of

**TABLE 2. Influence of vir genes on expression in Y. enterocolitica W22703 of a cat gene placed downstream of the promoter of yop51**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pGC1152 (vir+&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>pGCS65 (vir+&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>pGC274 (virA)</th>
<th>pGC678 (virB)</th>
<th>pGC653 (virB)</th>
<th>pGC217 (virC)</th>
<th>pGC403 (virC)</th>
<th>pGC1152-9 (virF)</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>11 ± 10</td>
<td>4 ± 7</td>
<td>52 ± 35</td>
<td>17 ± 20</td>
<td>3 ± 5</td>
<td>15 ± 13</td>
<td>13 ± 18</td>
<td>7 ± 14</td>
<td>ND</td>
</tr>
<tr>
<td>37°C</td>
<td>8,477 ± 2.009</td>
<td>7,566 ± 3.129</td>
<td>906 ± 218</td>
<td>325 ± 68</td>
<td>265 ± 20</td>
<td>725 ± 134</td>
<td>893 ± 82</td>
<td>51 ± 19</td>
<td>ND</td>
</tr>
<tr>
<td>37°C + Ca&lt;sup&gt;a&lt;/sup&gt;</td>
<td>474 ± 168</td>
<td>552 ± 160</td>
<td>650 ± 321</td>
<td>259 ± 108</td>
<td>147 ± 56</td>
<td>599 ± 125</td>
<td>952 ± 147</td>
<td>35 ± 29</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Results are expressed in arbitrary chloramphenicol acetyltransferase units and are presented as mean ± standard deviation.

<sup>a</sup> ND, Not detectable.
pYVe227, indicating that virF ends within fragment E7 (not E1) and hence that virF is transcribed in the same direction as virB and virC (Fig. 1).

Identification of the virF gene product. Our attempts to detect VirF by using the minicell system turned out to be unsuccessful. This could result from poor transcription of virF in E. coli (see below). However, irrespective of problems of intergenic expression, there are generally few molecules of transcriptional activators per bacterial cell (for a review, see reference 37), and their overproduction may be lethal (43). Hence, we turned to the T7 RNA polymerase-promoter system (46) for the controlled and exclusive expression of virF. virF was cloned as a SalI-Sall fragment of pGCS755 in plasmid pYVe227 with the T7 promoter, and the recombinant plasmid, called pGCS855 (Fig. 4), was used to transform E. coli C600. C600(pGCS855) was subsequently transfected with pGP1-2, a compatible plasmid expressing the T7 RNA polymerase from the p1 promoter. Plasmid pGP1-2 also contains gene c1857 of bacteriophage lambda, which encodes a thermosensitive repressor. The RNA polymerase of T7 is thus produced only at high temperatures (46). C600(pGCS855)(pGP1-2) cells were heat induced at 42°C and incubated in the presence of [35S]methionine, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The cloned DNA governed the production of a major protein of 30,000 daltons, and a fainter band appeared at 34,000 daltons (Fig. 5).

Nucleotide sequence of the virF gene. Both strands of the 1.6-kb BstEII-HpaI fragment were sequenced after subcloning in M13mp vectors or directly from pTZ19R derivatives.

![Diagram of the virF gene and promoter structure](image)

**FIG. 4.** Localization of virF. Restriction map and VirF activity of the various subclones from the BamHI fragment, B6, of pYVe227. The VirF activity was monitored by the ability to activate cat on pTM243. The coordinates are expressed in pYVe227 coordinates. The vertical arrow (top of the figure) localizes the insertion of Tn813 in virF mutant pGCl152-9. Symbols: ■, pYVe227 DNA; ■■, vector DNA or genes. Symbols followed by a prime refer to truncated genes deriving from the constructs. Abbreviations: B, BamHI; Bs, BstEII; Cl, Clal; E, EcoRI; H, HindIII; Hp, Hpall; F, PstI; S, Sall; Sc, SacI; Sm, Smal; X, Xhol. * is the promoter from bacteriophage T7.

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**TABLE 3.** Influence of virF subclones on expression in Y. enterocolitica W22703 and in E. coli of a cat gene placed downstream of the promoter of yop51

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Y. enterocolitica W22703</th>
<th>E. coli S171 (pGCS855)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cat transcription with indicated host and clone</td>
<td></td>
</tr>
<tr>
<td>25°C</td>
<td>pGCS630</td>
<td>pGCS752</td>
</tr>
<tr>
<td></td>
<td>338 ± 69</td>
<td>955 ± 860</td>
</tr>
<tr>
<td>37°C</td>
<td>1,724 ± 249</td>
<td>14,829 ± 6,421</td>
</tr>
<tr>
<td>37°C + Ca²⁺</td>
<td>1,308 ± 271</td>
<td>NT</td>
</tr>
<tr>
<td>37°C</td>
<td>pGCS755</td>
<td>462 ± 273</td>
</tr>
<tr>
<td></td>
<td>57 ± 104</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>pGCS756</td>
<td>13,017 ± 4,938</td>
</tr>
<tr>
<td></td>
<td>1,280 ± 547</td>
<td></td>
</tr>
<tr>
<td>37°C + Ca²⁺</td>
<td>None</td>
<td>NT</td>
</tr>
<tr>
<td>37°C</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>37°C</td>
<td>127 ± 58</td>
<td>NT</td>
</tr>
<tr>
<td>37°C + Ca²⁺</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Results are expressed in arbitrary chloramphenicol acetyltransferase units and are presented as mean ± standard deviation. 

a ND, Not detectable. 

b NT, Not tested.

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J. BACTERIOL.
Deletions were generated either by digestion of double-stranded DNA with appropriate restriction endonucleases and religation or by digestion of single-stranded DNA with T4 DNA polymerase by the method of Dale et al. (17). The sequence is given in Fig. 6.

The region sequenced contains two open reading frames. The first one, extending from the beginning of the sequence to nucleotide 201, presumably consists in the 3' end of the preceding gene, namely, virB. The second large open reading frame starting from an ATG at position 328 and ending at position 1141 corresponds to virF. The translation of this sequence would lead to a 30,879-dalton protein made of 271 amino acids. Since this molecular mass fits that of the observed protein (see above), we assumed that this reading frame encodes VirF. The only possible ribosome-binding site is an AGGA sequence separated by 13 nucleotides from the start codon. This distance is not optimal for protein initiation in E. coli (26), but it is reminiscent of the situation in the yopE gene of Y. pseudotuberculosis (22).

The carboxy-terminal half of VirF appears to be significantly homologous to the C-terminal region of AraC, the regulatory protein of the arabinose operon of E. coli and Salmonella typhimurium. There is 23.9% identity on a stretch of 142 residues with the regulator of the E. coli arabinose operon and 22.9% identity on a stretch of 131 residues with the S. typhimurium regulator. If one takes into consideration the amino acids that are conservatively replaced, the similarity in the same region goes up to 69%. The part of AraC that appears to be homologous to VirF includes the Cro-like DNA-binding domain (amino acids 197 to 217) (19). The same residues appear to be conserved in the three proteins (Fig. 7).

Although the molecular masses of the predicted and observed VirF proteins agree perfectly well, it should be noted that the second ATG in the same reading frame is preceded by a potential ribosome-binding site only 5 nucleotides downstream.
otides away. The protein synthesized from the second ATG would have a molecular mass of 25,293 daltons. Although it seems very unlikely that synthesis of VirF starts at this second ATG, this hypothesis must be kept in mind at this stage.

The first ATG codon is preceded by an A+T-rich stretch of 125 nucleotides in which several imperfect promoterlike structures could be recognized (27). The sequences preceding yopE (22) and yopH (5) of Y. pseudotuberculosis, as well as yopS1, the homologous gene of Y. enterocolitica (T. Michiels and G. Cornelis, Microb. Pathogen., in press), are also very rich in A and T nucleotides. The two short sequences underlined twice in Fig. 6 are homologous to a unique contiguous sequence that appears to be conserved in the regions upstream of yopE (22), yopH (5), and yopS1 (Michiels and Cornelis, in press).

The second ATG in the reading frame is preceded by potential −10 and −35 sequences spaced by the canonical 17 nucleotides (27).

The reading frame is followed by three dyad-symmetry structures that could act as terminators. Two such structures (11 and 8 nucleotides) are localized around nucleotide 1300, and the third (8 nucleotides) occurs after nucleotide 1600.

**Regulation of virF transcription.** In Y. enterocolitica W22703 containing only virF and yopS1-cat, transcription of cat appears to be regulated by temperature but not by calcium (Table 3). To determine whether this regulation results from a regulation of virF itself, we used Northern blot hybridization to measure the effect of temperature and calcium on the transcription of virF. Total RNA from Y. enterocolitica W22703 carrying pGCS1152 or pGCS752 was extracted after culture at 25°C and induction at 37°C. RNA was electrophoresed, blotted onto a nylon membrane, and hybridized with labeled DNA from an EcoRI fragment (spanning 36.2 to 33.7 kb) or an EcoRI-XhoI fragment (spanning 33.7 to 33.9 kb) of pYVE227. Two transcripts (1,100 and 1,400 bases) hybridized with the two probes spanning virF (Fig. 8). The presence of these two transcripts is in good agreement with the presence of two putative terminators in the sequence (Fig. 6). These two transcripts are clearly thermoregulated, suggesting that virF itself is thermoregulated at the transcriptional level. Thermoinduction of transcription occurred not only when virF was part of pYVE227 but also when virF was separate from it. This suggests that transcription of virF is either autoregulated or regulated by a Yersinia chromosomal gene.

**Activity of virF in E. coli.** Plasmid pGCS655 is identical to pGCS755, except that it does not contain oriT. The orientation of the insert is such that it is very unlikely that virF would be expressed from an external E. coli promoter (Fig. 4). We selected this plasmid to monitor the activity of virF in E. coli S17.1. We again used plasmid pTM243 as the source of the target gene. The chloramphenicol acetyltransferase activity of E. coli S17.1(pGCS655(pTM243) was monitored after incubation at 25 and 37°C. The cat activity was much lower in E. coli than in Y. enterocolitica (Table 3). However, this activity remained thermoregulated in E. coli, again suggesting that transcription of virF must be autoregulated. These two results were confirmed (data not shown) by a study of a virF-cat gene fusion present on plasmid pGCS757 (Fig. 4).

**DISCUSSION**

The virF gene encodes a transcriptional activator which was shown to activate transcription of yopS1. Since a clone containing virF was shown to fully complement the pleiotropic mutant pGC1152-9, we conclude that virF is a transcriptional activator of the whole yop regulon. Restriction mapping shows that this gene is presumably homologous to lcrF of Y. pestis (52).

**FIG. 8.** Northern blot analysis of virF. Whole-cell RNA from Y. enterocolitica W22703 carrying pGCS752 (left) or pGCS1152 (right) was extracted from cultures grown at 25°C and from cultures induced for 2 h at 37°C, in the presence or absence of calcium. The DNA probe corresponded to coordinates 33.7 to 33.9 kb (left) or 32.6 to 33.7 kb (right).
A positive control appears to be quite common for functions involved in pathogenicity. In *Vibrio cholerae*, toxR encodes a transcriptional activator controlling cholera toxin, pilus, and outer membrane protein expression (33). In *Bordetella pertussis*, the vir locus is required for expression of the pertussis toxin operon (34). In *Pseudomonas aeruginosa*, a toxR gene exerts a positive control on the transcription of the exotoxin A gene (35). Similarly, the trans-acting positive control element agr regulates the production of a number of staphylococcal extracellular proteins including hemolysins and toxins (38).

The product of virF appears to be a protein of 30,879 daltons. Interestingly, the carboxy-terminal half of this protein is significantly homologous to the regulatory protein of the arabinose operon (8, 48). Miller et al. (33) showed that the cholera toxin transcriptional activator belongs to a family of activators including Ompr, PhoM, PhoB, VirG, and SfrA. AraC and VirF appear as members of a new family of regulators unrelated to the former one. By comparison with AraC, one would anticipate that VirF acts as a DNA-binding protein and not as a σ factor.

Transcription of the yop genes is thermodependent (16, 22, 45). The present work shows that transcription of yopS1 is still thermodependent in the presence of virF only and that transcription of virF itself is thermoderegulated. The fact that thermoderegulation of yopS1 is still present, even in the smallest subclones containing virF, suggests that this regulation does not involve another pYV gene. Since thermoderegulation of virF expression also occurs in *E. coli*, one must conclude that yopS1 is autoregulated. Negative autoregulation is the most frequently encountered type of regulation among positive regulatory genes (37). In particular, this applies to araC (7, 40). Thus, VirF appears to be the major key to the thermal response of yersiniae. One likely hypothesis is that temperature-induced conformational changes modify the DNA-binding properties of this protein.

One can infer from the present data that VirF regulates the expression of the yop genes. We do not know so far whether VirF regulates the vir genes (also called lcr genes) which have also been shown to be thermoderegulated (15, 25). The role of these transcriptional loci is still not known. However, some of them must be involved in exporting the Yops.

When yopS1 is part of pYVa, its transcription is reduced about sixfold in the presence of calcium (16). However, in the presence of virF only, transcription of yopS1-cat is almost unaffected by calcium. Transcription of virF itself seems to be poorly affected by calcium. Reduction of the transcription of yopS1 in the presence of calcium would thus not be the result of a decrease in the production of the activator. Hence, we conclude that the activator of transcription is not itself the sensor that reacts to calcium. This situation is therefore different from that encountered in the cholera *tox* regulon, where the activator of transcription, ToxR, is a transmembrane protein believed to transduce environmental signals (33). This conclusion is in agreement with the results of Yother and Goguen (53), who showed that in *Y. pestis*, another locus, lcrE, is involved in the response to calcium. It also agrees with previous observations that led us to suggest that calcium could act basically at a posttranscriptional stage (16). However, transcription, translation, and export must clearly be coupled (see, e.g., reference 10).

We showed earlier that transcription of the yop genes is weak in *E. coli* (16). The results presented in this paper indicate that the yop results, at least partly, from poor transcription of virF in *E. coli*.

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**ADDITION IN PROOF**

Since this paper was submitted, we have learned of a plasmid-encoded regulatory protein, Rns, which is required for expression of the CS1 and CS2 adhesins of enterotoxigenic *E. coli* and which has partial identity to both AraC and VirF (J. Caron, L. Coffield, and J. R. Scott, Proc. Natl. Acad. Sci. USA, in press).

**LITERATURE CITED**


