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# Homology between VirF, the Transcriptional Activator of the *Yersinia* Virulence Regulon, and AraC, the *Escherichia coli* Arabinose Operon Regulator

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**Virulent yersiniae (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) restrict their growth at 37°C in rich medium deprived of calcium. This property, called calcium dependency, correlates with the secretion of *Yersinia* outer membrane proteins (Yops) and with pathogenicity. It is mediated by a 70-kilobase plasmid called pYV. The structural genes of the Yops (*yop* genes), as well as genes involved in the control of their expression (*vir* genes), have been localized on pYV. In this communication we show that *virF* encodes a transcriptional activator controlling the *yop* regulon. This activator is a 30,879-dalton protein related to AraC, the regulator of the *Escherichia coli* and *Salmonella typhimurium* arabinose operons. We also show in this paper that transcription of *virF* is thermodependent and presumably autoregulated. *virF* is thus responsible for the effect of temperature on the production of the Yops. Finally, we show that *virF* activates transcription of the *yop* genes independently of the presence of calcium ions. The role of calcium therefore remains unaccounted for.**

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 $pYV^+$ 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 currently 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 RP4 *tet*::Mu *neo*::Tn*7* plasmid integrated into the chromosome. Plasmids are listed in Table 1.

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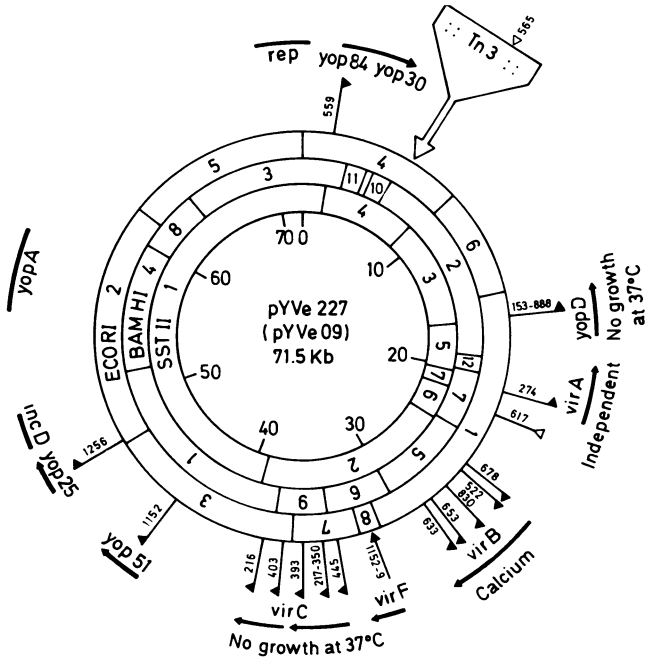



FIG. 1. Genetic map of pYVe227, a typical pYV plasmid from a serogroup 9 *Y. enterocolitica* strain. *rep* is the replication region; *incD* is the stabilization and incompatibility region; *vir* are genes controlling the calcium response and the expression of *yop* genes; and *yop* are genes encoding 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 flags and the small numbers identify the insertion mutations that defined the genes. The data are from Laroche et al. (30), Balligand et al. (1), Cornelis et al. (15, 16), and Biot and Cornelis (3). The orientation of *virF* is described in the text.

Bacteria were generally grown on brain heart infusion supplemented with 0.4% (wt/vol) glucose, 10 mM $MgCl_2$, and 10 mM $MgSO_4$. 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 *yop51*. This plasmid was constructed as follows: (i) *Eco*RI fragment 3 of pYVe227 and a 760-base-pair *Eco*RI fragment of pTJS82 containing *oriT* of RK2 were cloned together on pACYC184. The resulting plasmid, pTM200, contains *yop51*. (ii) Plasmid pTM200 was mutagenized with transposon Tn*2507* by using the delivery system described by Michiels and Cornelis (32). Transposon Tn*2507* is a derivative of Tn*2505* (32) containing the chloramphenicol acetyltransferase gene (*cat*) devoid of its promoter. This gene, originating from plasmid pCM4 (11), is located close to the left terminus of Tn*2507* and is oriented toward the inside of the transposon. Several insertions of Tn*2507* mapped within *yop51*. In insertion 43, the *cat* gene was in the same orientation as *yop51* 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 *yop51*, 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 mg/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 [$^{35}$S]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 [$^{35}$S]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

TABLE 1. Bacterial plasmids

| Plasmid | Genetic structure | Source or reference |
|---|---|---|
| pCM4 | pBR327 containing *cat* without promoter as a *Bam*HI fragment | 11 |
| pGC217 | pGB63 *virC*-217::mini-Mu dl *lac* | 15 |
| pGC274 | pGB63 *virA*-274::mini-Mu dl *lac* | 15 |
| pGC403 | pGB63 *virC*-403::mini-Mu dl *lac* | 15 |
| pGC565 | pGB63-565::mini-Mu dl *lac* | 15 |
| pGC653 | pGB63 *virB*-653::mini-Mu dl *lac* | 15 |
| pGC678 | pGB63 *virB*-678::mini-Mu dl *lac* | 15 |
| pGC1152 | pGB63 *yop51*-1152::mini-Mu dl *lac* | 16 |
| pGC1152-9 | pGC1152 *virF*-9::Tn*813* | 16 |
| pGCS615 | pSUP202-*Bam*HI fragment 6 of pYVe227 | This paper |
| pGCS630 | pSUP202-pUC19-coordinates 31.0–34 kb of pYVe227 | This paper |
| pGCS655 | pTZ19R-coord. 32.8–34.6 kb of pYVe227 | This paper |
| pGCS752 | pTZ19R-*oriT*-coordinates 32.6–35.6 kb of pYVe227 | This paper |
| pGCS755 | pTZ19R-*oriT*-coordinates 32.8–34.6 kb of pYVe227 | This paper |
| pGCS756 | pTZ19R-*oriT*-coordinates 33.0–34.6 kb of pYVe227 | This paper |
| pGCS757 | *cat* cloned as a *Sal*I fragment at *Xho*I site of pGCS752 (operon fusion *virF-cat*) | This paper |
| pGCS855 | pT7.5 + coordinates 33.0–34.6 kb of pYVe227 | This paper |
| pGP1-2 | pBR322 encoding *c*I857 and T7 RNA polymerase from promoter $p_L$ | 46 |
| pSUP202 | pBR325-*mob* of RP4 | 44 |
| pTJS82 | pUC-7 containing *oriT* of RK2 as a 760-base-pair *Eco*RI fragment | 41 |
| pTM200 | pACYC184-*oriT* + *Eco*RI fragment 3 of pYVe227 | This paper |
| pTM243 | pTM200 *yop51*-43::Tn*2507* | This paper |
| pTZ19R | *ori* pBR322 + *ori* f1 *lacZ' bla* | Pharmacia |
| pT7-5 | pACYC177 containing promoter ϕ10 of phage T7 | 46 |
| pUC19 | | 51 |
| pYVe227 | Virulence plasmid of *Y. enterocolitica* W227 (serogroup O:9); typical representative of plasmids from serogroup O:9 strains | 30 |

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 *yop51* (16), but the influence of *virA*, *virB*, and *virC* on transcription of the *yop* genes has not yet been determined.

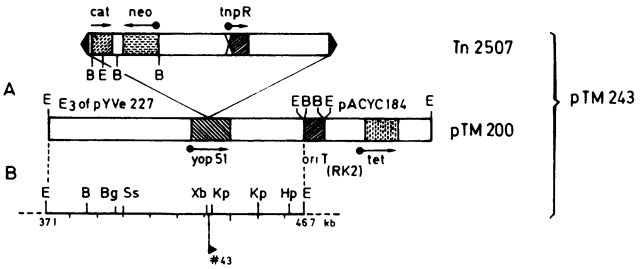



FIG. 2. Map of pTM243. (A) Genetic map. Plasmid pTM200 is a pACYC184 derivative containing the origin of transfer of RK2 (*oriT*) and *Eco*RI fragment 3 of pYVe227. This fragment contains *yop51*. Transposon Tn*2507* (9.1 kb) contains a kanamycin resistance gene (*neo*), the resolvase gene of Tn*2505* (*tnpR*) (32), and the *cat* devoid of its promoter. Plasmid pTM243 is pTM200-43::Tn*2507*. The insertion occurred in codon 219 of *yop51* (manuscript in preparation). The mutated *yop* gene encodes a truncated peptide of 27,000 daltons. The *cat* gene is transcribed from the promoter of *yop51*. *tet* is the tetracycline resistance gene. The arrows point in the direction of transcription. (B) Restriction map of *Eco*RI fragment 3 (E3) of pYVe227. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hp, *Hpa*I; Kp, *Kpn*I; Ss, *Sst*II; Xb, *Xba*I.

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 *yop51* by in vivo transposition of Tn*2507*. 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., pGC565), 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 Tn*2507* at about the same position in *yop51* 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 *yop51* gene and *cat* will be referred to as the *yop51-cat* unit.

Mutations in *virA* and *virC* reduced transcription of the *yop51-cat* unit about 10-fold in the absence of calcium. The level of transcription in the presence of calcium was unaf-

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

| Conditions | *cat* transcription in presence of following pYV plasmid (*vir* mutants)[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pGC1152 (*vir*$^+$) | pGC565 (*vir*$^+$) | pGC274 (*virA*) | pGC678 (*virB*) | pGC653 (*virB*) | pGC217 (*virC*) | pGC403 (*virC*) | pGC1152-9 (*virF*) | None |
| 25°C | 11 ± 10 | 4 ± 7 | 52 ± 35 | 17 ± 20 | 3 ± 5 | 15 ± 13 | 13 ± 18 | 7 ± 14 | ND[b] |
| 37°C | 8,477 ± 2,009 | 7,566 ± 3,129 | 906 ± 218 | 335 ± 68 | 265 ± 20 | 725 ± 134 | 893 ± 82 | 51 ± 19 | 13 ± 18 |
| 37°C + $Ca^{2+}$ | 474 ± 168 | 352 ± 160 | 650 ± 321 | 259 ± 108 | 147 ± 56 | 599 ± 125 | 952 ± 147 | 35 ± 29 | ND |

[a] Results are expressed in arbitrary chloramphenicol acetyltransferase units and are presented as mean ± standard deviation.
[b] ND, Not detectable.

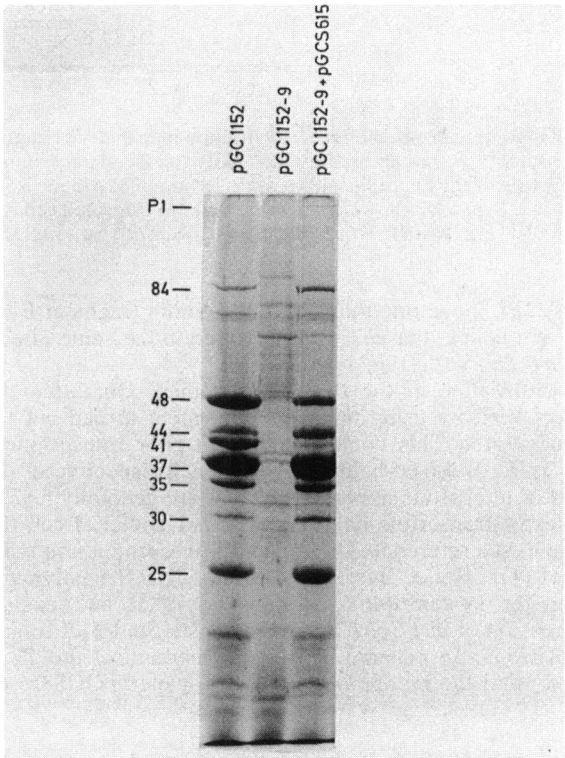
fected by mutations in *virA* or *virC*. The influence of mutations in *virB* was about threefold more severe, but there still was a very clear transcription of *yop51-cat* in the absence of a functional *virB* locus. In contrast, transcription of *yop51-cat* became barely detectable when complemented by pGC1152-9, which contains an insertion of Tn*813* 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.** *Bam*HI 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 *Eco*RI 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 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 *Pst*I 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′ *Cla*I-*Xho*I 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 *Eco*RI digest of pYVe227 with labeled DNA from the 200-base-pair *Eco*RI-*Xho*I fragment (coordinates 33.7 to 33.9 kb). Hybridization occurred with *Eco*RI fragment 7 of




| Plasmid | (pGC1152) | (pGC1152-9) | (pGC1152-9) (pGCS615) |
|---|---|---|---|
| 25°C | 31±15 | 32±20 | 666±445 |
| 37°C | 10549±2297 | 135±39 | 7395±3059 |

FIG. 3. Complementation between pGC1152-9 and pGCS615. (Top) SDS-polyacrylamide gel electrophoresis of the secreted proteins (Yops). (Bottom) β-Galactosidase activity, expressed in arbitrary units. β-Galactosidase activity reflects the transcription of *yop51*. Plasmid pGC1152 carries a mini-Mu (Kan *lac*) insertion in *yop51*. Plasmid 1152-9 is a *virF* mutant of 1152 (16).

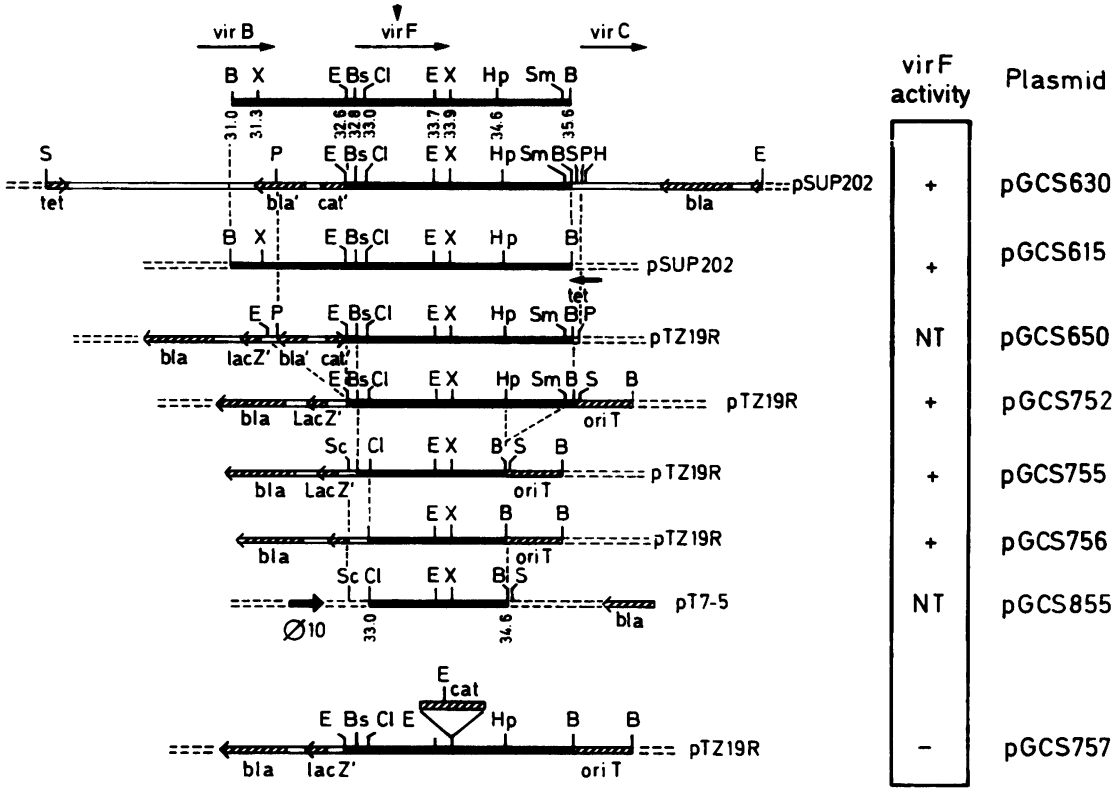



FIG. 4. Localization of *virF*. Restriction map and VirF activity of the various subclones from the *Bam*HI fragment, B6, of pYVe227. The VirF activity was monitored by the ability to activate *cat* on pTM243. The coordinates are expressed in pYVe227 coordinates. The vertical arrow (top of the figure) localizes the insertion of Tn*813* in *virF* mutant pGC1152-9. Symbols: ——, pYVe227 DNA; □, ▨, vector DNA or genes. Symbols followed by a prime refer to truncated genes deriving from the constructs. Abbreviations: B, *Bam*HI; Bs, *Bst*EII; Cl, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I. ϕ is the promoter from bacteriophage T7.

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 intergenus 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 *Sac*I-*Sal*I fragment of pGCS755 in plasmid pT7-5, downstream of the T7 promoter, and the recombinant plasmid, called pGCS855 (Fig. 4), was used to transform *E. coli* C600. C600(pGCS855) was subsequently transformed by pGP1-2, a compatible plasmid expressing the T7 RNA polymerase from the $p_L$ promoter. Plasmid pGP1-2 also contains gene *c*I857 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 [$^{35}$S]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 *Bst*EII-*Hpa*I fragment were sequenced after subcloning in M13mp vectors or directly from pTZ19R derivatives.

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*

| Conditions | *cat* transcription with indicated host and clone[a] | | | | | |
|---|---|---|---|---|---|---|
| | *Y. enterocolitica* W22703 | | | | | *E. coli* S17.1 (pGCS655) |
| | pGCS630 | pGCS752 | pGCS755 | pGCS756 | None | |
| 25°C | 338 ± 69 | 955 ± 860 | 462 ± 273 | 57 ± 104 | ND[b] | ND |
| 37°C | 1,724 ± 249 | 14,829 ± 6,421 | 13,017 ± 4,938 | 1,280 ± 547 | ND | 127 ± 58 |
| 37°C + $Ca^{2+}$ | 1,308 ± 271 | NT[c] | NT | NT | NT | NT |

[a] Results are expressed in arbitrary chloramphenicol acetyltransferase units and are presented as mean ± standard deviation.
[b] ND, Not detectable.
[c] NT, Not tested.

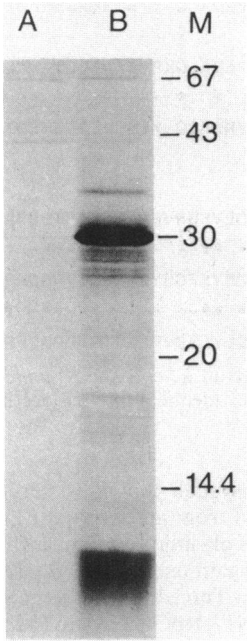



FIG. 5. Expression of VirF with the T7 promoter polymerase system (46). Lanes: A, *E. coli* C600(pT7-5)(pGP1-2); B, *E. coli* C600(pGCS855)(pGP1-2). M, molecular mass markers (in kilodaltons) (see Materials and Methods).

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 nucle-

```
Gly Asn Leu Pro Leu Phe Phe Ser Ile Lys Leu Asn Pro Ala Gln Arg Gly Glu Gly Glu
GGT AAC CTA CCT TTA TTC TTT TCT ATA AAA CTG AAT CCA GCT CAA CGC GGG GAA GGT GAA
 BstEII

Leu Tyr Leu Arg Ser Thr Leu Ser Phe Pro Glu Arg Gly Val Gln Ala Val Ala Gln Gln
CTT TAC CTA CGG TCA ACC CTC TCT TTT CCA GAG CGA GGG GTT CAG GCG GTG GCT CAG CAA
                                                100

Lys Leu Ile Gly Lys Asn Lys Val Val Leu Gln Met Ile Pro Lys Thr Cys Tyr Pro Asn
AAA CTT ATT GGT AAA AAC AAA GTC GTT TTG CAA ATG ATA CCT AAA ACA TGT TAT CCA AAT


Cys Gln Ser Pro Asn Thr Arg ***
TGC CAG TCA CCT AAT ACC AGA TAG GTGATTTATTATATTGGTTTTGGTTGCATTAATCGATGGTTGTACATC
        -------->      200  <-------                              ClaI
                                                                              Met   1
GCACGCATAATAACTCAATACACCTCATTAGATAAATATATACAAGTTTTAGATTTTTAGGACAGTATAACATTT ATG
                                                300          rbs

Ala Ser Leu Glu Ile Ile Lys Leu Glu Trp Ala Thr Pro Ile Phe Lys Val Val Glu His  21
GCA TCA CTA GAG ATT ATT AAA TTA GAA TGG GCC ACA CCT ATA TTT AAG GTT GTT GAG CAT

Ser Gln Asp Gly Leu Tyr Ile Leu Leu Gln Gly Gln Ile Ser Trp Gln Asn Ser Ser Gln  41
TCA CAA GAT GGC CTA TAT ATT CTT TTG CAA GGT CAG ATT TCA TGG CAG AAC AGC AGT CAG
          400

Thr Tyr Asp Leu Asp Glu Gly Asn Met Leu Phe Leu Arg Arg Gly Ser Tyr Ala Val Arg  61
ACA TAT GAT TTA GAT GAG GGG AAT ATG CTG TTT TTG CGT CGT GGC AGC TAT GCT GTT CGA
   NdeI                                                  500

Cys Gly Thr Lys Glu Pro Cys Gln Leu Leu Trp Ile Pro Leu Pro Gly Ser Phe Leu Ser  81
TGT GGT ACA AAA GAA CCC TGC CAA TTA CTT TGG ATT CCA TTA CCA GGC AGT TTT TTG AGT

Thr Phe Leu His Arg Phe Gly Ser Leu Leu Ser Glu Ile Arg Arg Asp Asn Ala Thr Pro 101
ACT TTT TTA CAT CGG TTT GGT TCT TTG CTT AGT GAA ATT AGA CGA GAC AAT GCC ACA CCC
                                600

Lys Pro Leu Leu Ile Phe Asn Ile Ser Pro Ile Leu Ser Gln Ser Ile Gln Asn Leu Cys 121
AAG CCA TTG TTA ATT TTT AAT ATT TCA CCA ATA TTA TCA CAA TCC ATT CAA AAT CTA TGT

Ala Ile Leu Glu Arg Ser Asp Phe Pro Ser Val Leu Thr Gln Leu Arg Ile Glu Glu Leu 141
GCC ATA TTG GAA CGG AGT GAT TTT CCG TCA GTA TTA ACG CAA CTG CGT ATT GAG GAA TTA
          700

Leu Leu Leu Leu Ala Phe Ser Ser Gln Gly Ala Leu Phe Leu Ser Ala Leu Arg His Leu 161
TTG CTT TTG CTT GCC TTT AGC TCG CAA GGG GCT TTA TTC CTC TCG GCT CTG CGC CAT TTA
                                                800

Gly Asn Arg Pro Glu Glu Arg Leu Gln Lys Phe Met Glu Glu Asn Tyr Leu Gln Gly Trp 181
GGC AAC CGC CCA GAA GAA CGG TTG CAG AAA TTT ATG GAG GAA AAT TAT CTA CAA GGG TGG

Lys Leu Ser Lys Phe Ala Arg Glu Phe Gly Met Gly Leu Thr Thr Phe Lys Glu Leu Phe 201
AAA CTA AGC AAA TTT GCG CGA GAA TTC GGC ATG GGA TTA ACC ACA TTC AAA GAA CTG TTT
                        EcoRI  900

Gly Thr Val Tyr Gly Ile Ser Pro Arg Ala Trp Ile Ser Glu Arg Arg Ile Leu Tyr Ala 221
GGT ACA GTT TAT GGC ATT TCA CCA CGC GCC TGG ATA AGC GAG CGA CGT ATT CTC TAT GCT


His Gln Leu Leu Leu Asn Gly Lys Met Ser Ile Val Asp Ile Ala Met Glu Ala Gly Phe 241
CAC CAA TTA CTT CTT AAT GGT AAG ATG AGT ATT GTT GAT ATT GCC ATG GAA GCA GGG TTC
         1000

Ser Ser Gln Ser Tyr Phe Thr Glu Ser Tyr Arg Arg Arg Phe Gly Cys Thr Pro Ser Gln 261
TCG AGT CAG TCT TAT TTC ACT CAA AGT TAT CGA CGT CGC TTC GGA TGC ACT CCC AGC CAA
 XhoI                                                 1100

Ala Arg Leu Thr Lys Ile Ala Thr Thr Gly ***
GCC CGT CTT ACT AAA ATA GCA ACC ACA GGC TAA AATTATCTGTTTTTTTTTTAAAAACACTTTTGGACT


ATAAAGTAAAATACGGGGTTAGATTTTGAAGATTCAATGGGATGAGCCAAATTACAACGAAACATATAACAGTATTATTT
                1200                                                  ---- ------>

CGCCGCTGGATGGCAATAATATGTTGTTTAATAATCAAGATAGCTTATCTGGCTTATTAAGTGCGGTGGCGCAAGAAACG
            <----------  -------  > 1300          < -------

AGCGCCATCAAATAGAGCAAGTCTCATTAGTTCGATTAACGTAATATCATCTATTGCTATATAGGTGGTTGATAATTATC
                                                        1400

ACGAACATTTTTTTGAATATCTGGAAGTTTGAGCTGAACCGCGAAACCTTATGTCACGATGACATGAAGTAGGTTATTTA


TTTGGCGCAGGATTACTTAGTTTACATATAACCATCTGAGAAATAATGCAAAATTTACTAAAAAACTTGGCTACCAGTTT


AGGAAGAAAACCGTTTGTTGCCGATAAACAAGGTGTTTACCGTTTAACTATAGATAAGCATCTTGTCATGCTGACTCCGC
              1600-- ----->  <----- --

ATGGTTCAGAACTGGTTTTACGCACTCCTATTGACGCACCAATGTTACGTGAAGGAAATAACGTTAAC
                                1700                          HpaI
```

FIG. 6. Nucleotide sequence of *virF*. The sequence is numbered starting with the *Bst*EII site at coordinate 32.8 kb of pYVe227. The deduced amino acid sequence of *virF* is numbered on the right side of the figure. Arrows indicate regions of dyad symmetry. A possible ribosome-binding site (rbs) is underlined. The sequences with double underlines in the intergenic region also occur in front of *yopE* and *yopH* of *Y. pseudotuberculosis* (5, 22) as well as in *yop51* of *Y. enterocolitica* (T. Michiels and G. Cornelis, Microb. Pathogen., in press).

```
AraC S.typh  92"    ELFGQIISAGQGEGRYSELLAINLLEQLLLRRMAVINESLHPPMDSRVRDACQ-YISDHLADSHFDIASVAQHVCLSPSR
                                --*   -**  * --- ---  *  -----*  -  * -----  -- ----- *--  -- -
VirF Y.ent.  118'   QNLCAILERSDFPSVLTQLRIEELLLLLAFSSQGALFLSALRHLGNRPEERLQKFMEENYLQG-WKLSKFAREFGMGLTT
                 *-- --  -  --*   -**  * --- -*-  *  ----**  *  * -----  -- ----- *--  -- -
AraC E.coli  92"     DLFGQIINAGQGEGRYSELLAINLLEQLLLRRMEAINESLHPPMDNRVREACQ-YISDHLADSHFDIASVAQHVCLSPSR

AraC S.typh 173"    LSHLFRQQLGISVLSWREDQRISQAKLLLSTTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAGCE
                    ---**    ***  -* ---**  *- ** ---*-*-------**--* **--  ---  * -**- *
VirF Y.ent.  199'   FKELFGTVYGISPRAWISERRILYAHQLLLNGKMSIVDIAMEAGFSSQSYFTQSYRRRFGCTPSQARLTKIATTG
                    ---**    ***  -* ---**  *- ** ---*-*-------**--* **--  ---  * -**- *
AraC E.coli 173"    LSHLFRQQLGISVLSWREDQRISQAKLLLSTTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRAGCEEKVNDVAVK
```

FIG. 7. Amino acid sequence homology of VirF to the activators of the arabinose operons of *E. coli* and *S. typhimurium*. Amino acid residues are aligned to maximize common residues between VirF and AraC (8, 48). Residues identical to VirF are indicated by asterisks between the protein sequences. Conservatively replaced residues are indicated by dashes.

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 *yop51*, 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 *yop51* (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 *yop51-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 pGC1152 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 *Eco*RI fragment (spanning 36.2 to 33.7 kb) or an *Eco*RI-*Xho*I 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 thermodependent, 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 thermodependent 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 *yop51*. 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).

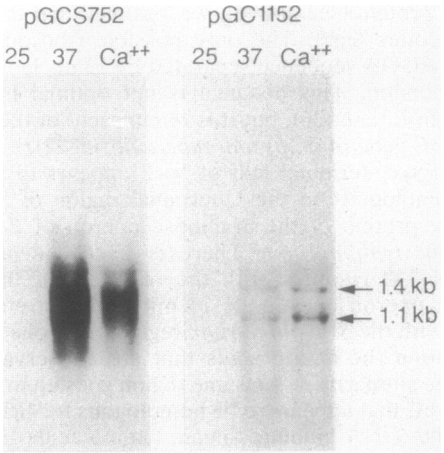



FIG. 8. Northern blot analysis of *virF*. Whole-cell RNA from *Y. enterocolitica* W22703 carrying pGCS752 (left) or pGC1152 (right) was extracted from cultures grown at 25°C and from cultures induced for 2 h at 37°C, in the presence or absence of calcium. The DNA probe corresponded to coordinates 33.7 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 transcription of the pertussis toxin operon (34). In *Pseudomonas aeruginosa*, a *toxR* gene exerts a positive control on the transcription of the exotoxin A gene (50). 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 *yop51* is still thermodependent in the presence of *virF* only and that transcription of *virF* is itself thermoregulated. The fact that thermoregulation of *virF* is still present, even in the smallest subclones containing *virF*, suggests that this regulation does not involve another pYV gene. Since thermoregulation of *virF* expression also occurs in *E. coli*, one must conclude that *virF* 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 thermoregulated (15, 25). The role of these transcriptional loci is still not known. However, some of them must be involved in exporting the Yops.

When *yop51* is part of pYVe, its transcription is reduced about sixfold in the presence of calcium (16). However, in the presence of *virF* only, transcription of *yop51-cat* is almost unaffected by calcium. Transcription of *virF* itself seems to be poorly affected by calcium. Reduction of the transcription of *yop51* 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 this situation results, at least partly, from poor transcription of *virF* in *E. coli*.

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## ADDENDUM 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).

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