JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4230–4237 Vol. 177, No. 15
0021-9193/95/$04.00+0

VirG, a *Yersinia enterocolitica* Lipoprotein Involved in Ca^{2+} Dependency, Is Related to ExsB of *Pseudomonas aeruginosa*

ABDELMOUNAAIM ALLAOUI,† ROBERT SCHEEN, CATHERINE LAMBERT DE ROUVROIT, AND GUY R. CORNELIS*

Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology, and Faculté de Médecine, Université Catholique de Louvain, UCL 74.49, B-1200 Brussels, Belgium

Received 10 March 1995/Accepted 16 May 1995

Pathogenic yersiniae require Ca^{2+} for growth at 37 C. They harbor closely related plasmids of about 70 kb that are essential for virulence. At 37 C and in the absence of Ca^{2+} ions, these plasmids cause a decrease in growth rate and the release of large amounts of proteins called Yops. Here we describe the *virG* gene of *Yersinia enterocolitica*; *virG* is located just upstream of the *virF* gene, which encodes the transcriptional activator of some plasmid virulence factors. Analysis of the VirG amino acid sequence suggested that *virG* encodes a lipoprotein, which was confirmed by [^{3}H]palmitate labeling of VirG-PhoA fusion proteins. A nonpolar *virG* mutant was constructed and found to be Ca^{2+} independent for growth at 37 C but to still secrete Yops. This phenotype was complemented by the introduction of a plasmid harboring an intact *virG* gene. VirG was found to be homologous to ExsB, a protein encoded by a *Pseudomonas aeruginosa* gene located in the locus controlling exoenzyme S synthesis. Interestingly, the *exsA* gene, located just downstream of *exsB*, is also homologous to *virF*.

Pathogenic bacteria of the genus *Yersinia* (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) cause disease in rodents and humans, with symptoms ranging from enteritis to septicemia and death, essentially by invading the host tissues. They harbor closely related plasmids of about 70 kb that are essential for virulence and hence are called pYV (for *Yersinia* virulence). At 37°C and in a medium deprived of Ca^{2+} ions, the pYV plasmid directs secretion of about 10 proteins called Yops. In *Y. enterocolitica* these are YopB, -D, -E, -H, -M, -N, -O, -P, and -Q and LcrV, the protective antigen described in the mid-1950s by Burrows and Bacon (6). For reviews, see references 8, 9, 16, 53, and 54.

Yop secretion is achieved by a type III secretion pathway which is encoded by three loci on the pYV plasmid called *virA*, *virB*, and *virC*. The *virC* locus contains the 12 genes *yscA* to *yscL*, as well as *yscM* (also known as *lcrQ*) (33, 47). The *virB* locus contains the eight genes *yscN* to *yscU* (2, 3, 15, 61), and locus *virA* contains *lcrD* (40). Apart from the secretion apparatus itself, secretion of YopE, YopH, and YopD requires individual cytosolic chaperones called SycE, SycH, and SycD, respectively (58, 59). By binding their cognate Yop, these chaperones either protect the intrabacterial form against folding or association or act as a pilot directing the associated Yop to the secretion machinery.

Yop synthesis and secretion are thermoregulated at the transcriptional level. Some *yop* genes are scattered around the pYV plasmid, while others, like *lcrV*, *yopB*, and *yopD*, form an operon (4, 36, 44). The *yop* genes and operons, as well as some of the *ysc* genes, form a regulon under the positive control of VirF, a transcriptional activator of the AraC family (12, 23, 29, 60) and under the negative control of YmoA, a histone-like global regulator (11, 46). Transcription of the *virF* gene is thermoinduced, which contributes to the thermoregulation of Yop secretion (29). Yop secretion is also regulated by Ca^{2+}: transcription of the *yop* genes is reduced in the presence of millimolar concentrations of Ca^{2+} (13, 14, 18, 44). It is generally believed that this regulation is of the negative type (17), but no classical repressor has been identified so far. Besides these two regulatory circuits, the secretion apparatus itself exerts a feedback regulatory effect on transcription of the *yop* genes. It has been observed for a long time that *yop* transcription is reduced when export is compromised (2, 3, 14, 15, 61). Recent data suggest that LcrQ (YscM in *Y. enterocolitica*) could be an element of this third regulatory circuit (47), but this remains to be demonstrated. Ca^{2+} regulation and feedback inhibition might be related phenomena: if Ca^{2+} prevents the assembly of the secretion machinery in the membranes or prevents secretion itself, then feedback inhibition will lead to the observed reduction of transcription.

pYV^+ yersiniae are unable to grow on oxalated media at 37°C (22). Hence, at 37°C in the absence of Ca^{2+}, they not only secrete Yops but also restrict their growth; they are therefore called Ca^{2+} dependent. Like the exact role of Ca^{2+}, the link between Yop secretion and growth restriction remains elusive. Mutants affected in the *virF* gene as well as those with nonpolar mutations in either the *virA* or *virB* operon are, as expected, unable to secrete Yops, and they grow well at 37°C irrespective of the presence or absence of Ca^{2+}. They are thus Ca^{2+} independent (CI). A nonpolar *lcrV* mutant was also reported to be CI and unable to secrete Yops, supporting the idea that LcrV is a regulatory protein (4, 43).

Mutants with mutations in *lcrE* (*yopN*) secrete all of the Yops except YopN at 37°C, even in the presence of Ca^{2+} ions. Hence, they are "Ca^{2+} blind" for Yop secretion and do not grow at 37°C irrespective of the presence or absence of Ca^{2+} (17, 66). A nonpolar mutation affecting another secreted protein, LcrG, also leads to the same Ca^{2+}-blind phenotype (51). The phenotypes of these two classes of mutants (CI and Ca^{2+} blind) support the hypothesis that growth restriction and Yop production and secretion are linked phenomena.

In the present paper we describe *virG*, a new pYV gene involved in growth restriction. We characterize the gene product and analyze the phenotype of the mutant.

* Corresponding author. Mailing address: Microbial Pathogenesis Unit, Avenue Hippocrate, 74, UCL 74.49, B-1200 Brussels, Belgium. Phone: 32 2 764 74 49. Fax: 32 2 764 74 98. Electronic mail address: Cornelis@mipa.ucl.ac.be.

† Present address: Laboratoire de Recherche sur les Trypanosomatidae, Institut Pasteur, B.P. 245, 59019 Lille, France.

TABLE 1. Plasmids used in this study

Plasmid	Genetic structure	Source or reference
pAA117	pAA67 *yscU* codon 29::Tn*phoA*	2
pBC19R	pTZ19R + *oriT*	7
pKNG101	ori_{R6K} *sacBR* $oriT_{RK2}$ *strAB*	24
pGCS68B	pUC19 + *Bam*HI fragment 6 of pYV227 (coordinates 31.0 to 36.5)	12
pGP1-2	pBR322 + *c*I857 + T7 RNA polymerase from promoter p_L	55
pRS1	pBC19R Δ*Eco*RI	This work
pRS2	pRS1 + 2,460-bp *Pst*I-*Sca*I fragment of pGCS68B	This work
pRS3	pRS2 Δ230-bp *Eco*RI-*Bst*EII + 850-bp *Sma*I fragment containing *aphA-3* gene of pUC18K	This work
pRS5	pKNG101 + 2,143-bp *Pvu*II fragment of pRS3; *virG* mutant	This work
pRS6	pTM100 + 780-bp *Sca*I-*Eco*RI fragment of pGCS68B containing *virG*	This work
pRS6A	pRS6 *virG* codon Cys-121 replaced by Ser	This work
pRS6B	pRS6 *virG* codon Cys-125 replaced by Ser	This work
pRS7	pRS2 Δ1,692-bp *Hin*dIII-*Eco*RI, contains *virG* expressed from both T7 RNA polymerase ϕ10 and *lac* promoters	This work
pRS7A	pRS7 *virG* codon Cys-121 replaced by Ser	This work
pRS7B	pRS7 *virG* codon Cys-125 replaced by Ser	This work
pRS8	pRS7 *virG* codon 64::Tn*phoA*	This work
pRS9	pRS7 *virG* codon 29::Tn*phoA*	This work
pRS11	pRS7 *virG* codon 60::Tn*phoA*	This work
pRS227	pYV227 *virG*::*aphA-3* (nonpolar mutation)	This work
pUC18K	pUC18 + 850-bp DNA fragment containing *aphA-3* gene	31
pTM100	pACYC184 + *oriT* from RK2	32
pTZ19R	pUC19 + T7 RNA polymerase ϕ10 promoter	Pharmacia
pYV227	pYV plasmid from W227 (serotype O:9)	13

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) of the O:9 serotype strain W227 (10). *Y. enterocolitica* KNG22703 is a mutant of strain W22703 in which the *bla* gene encoding β-lactamase A was replaced by the *luxAB* genes (24). *Escherichia coli* LK111, DH5α λ*pir*$^+$, and XL1-blue (Stratagene, La Jolla, Calif.) were used for standard genetic manipulations. *E. coli* Sm10 λ*pir*$^+$ was used to deliver mobilizable plasmids in *Y. enterocolitica* (35). *E. coli* CJ236 (*dut ung*) was used for site-directed mutagenesis (27). The strains were routinely grown in tryptic soy broth (Oxoid, Basingstoke, England) and plated on tryptic soy agar (TSA) (Oxoid), which sometimes was supplemented with 20 mM $MgCl_2$ and 20 mM Na-oxalate (MOX). For induction of the *yop* regulon, *Y. enterocolitica* was grown in brain heart infusion (Difco, Detroit, Mich.) supplemented with 4 mg of glucose ml^{-1}, 20 mM $MgCl_2$, and 20 mM Na-oxalate (BHI-OX). M9 medium ($MgSO_4$, 1 mM; $CaCl_2$, 100 μM; NaCl, 86 mM; Na_2HPO_4, 420 mM; KH_2PO_4, 220 mM; NH_4Cl, 190 mM; and glucose, 4 mg ml^{-1}) was used for the labeling of VirG. Antibiotics were used at the following concentrations: ampicillin, 300 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; nalidixic acid, 35 μg ml^{-1}; and streptomycin, 30 μg ml^{-1}.

The plasmids used are listed in Table 1.

Molecular cloning and sequencing procedures. Plasmid DNA purification, DNA restriction and separation by gel electrophoresis, transfer and hybridization, ligation, and transformation of *E. coli* strains were performed according to standard methods (49). Nucleotide sequences of *virG* and the surrounding region were determined by the dideoxy chain termination procedure with single- or double-stranded DNA (50). Both DNA strands were completely sequenced on plasmids pGCS68B and pRS7.

SDS-PAGE and immunoblotting. Electrophoresis in 10 or 14% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli (28). After electrophoresis, proteins were either stained with Coomassie brilliant blue, visualized by autoradiography, or transferred by electroblotting to a nitrocellulose membrane (56). Immunoblotting procedures were carried out with rat monoclonal antibody 6G1 (anti-YopE), 13A4 (anti-YopD), 7C1 (anti-LcrV) (5), or a rabbit polyclonal serum raised against *E. coli* alkaline phosphatase (kindly provided by Bruno Dupuy, Institut Pasteur, Paris, France).

Induction of the *yop* regulon and analysis of Yops and Ca^{2+} dependency. Yops were prepared and analyzed by SDS-PAGE and Western blotting (immunoblotting) as described by Cornelis et al. (14) and Sory and Cornelis (52). For the analysis of the whole-cell protein extract, the equivalent of 5×10^8 bacteria, induced for Yop synthesis and secretion, were subjected to SDS–14% PAGE. The Ca^{2+} requirement for growth at 37°C was monitored by plating the bacteria in parallel on TSA supplemented with 5 mM $CaCl_2$ and on MOX agar at 37°C.

Identification of the *virG* gene product. Specific labeling of VirG was achieved in *E. coli* LK111 containing pGP1-2 and either pRS7, pRS7A, or pRS7B by using the T7 expression system (55) (see Fig. 3). For more details, see reference 2.

Sequence analysis. Identity scores between proteins were calculated by using the FastA program, based on the method of Pearson and Lipman (39), with a ktuple value of 1. The alignment was made by using the BESTFIT program available in the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison). The isoelectric point was calculated with the same program. The signal sequence was researched by using the SIGSEQ program (41), which is based on the rules of Von Heijne (57).

Engineering of a *virG* mutant. The *virG* gene was inactivated on the pYV plasmid by allelic exchange. First, plasmid pRS2 was constructed by cloning the 2,400-bp *Pst*I-*Sca*I fragment of pGCS68A (Fig. 1; Table 1) into the *Sma*I-*Pst*I sites of pRS1 (pRS1 was obtained by destruction of the *Eco*RI restriction site of the vector pBC19R). We then replaced the 210-bp *Eco*RI-*Bst*EII DNA fragment (from nucleotide 343 to 553) (Fig. 2) of pRS2 (after filling in the end) with an 850-bp *Sma*I DNA fragment containing the *aphA-3* gene from pUC18K (conferring kanamycin resistance) (31). The resultant plasmid was called pRS3 (Fig. 1). In this construction, introduction of the *aphA-3* cassette induces an early stop in *virG* translation and directs in-frame translation of the remaining 3′ end of the mutated gene, avoiding putative polar effects on the transcription of the *virF* gene. Plasmid pRS5 was constructed by inserting the 2,460-bp *Pvu*II fragment of pRS3 into the *Sma*I site of the suicide vector pKNG101 (24) (Fig. 1). Finally, plasmid pRS5 was transferred to *Y. enterocolitica* KNG22703 by conjugal mating, and transconjugants were selected on plates that contained nalidixic acid and kanamycin. Clones in which a double recombinational event had exchanged the wild-type *virG* gene with the mutated copy carried by pRS5 were identified by their sensitivity to streptomycin. The structure of the resultant pYV plasmid carrying the *virG* mutation was confirmed by Southern analysis, and the corresponding pYV plasmid was designated pRS227.

Tn*phoA* mutagenesis. For Tn*phoA* mutagenesis, the target plasmid pRS7 carrying the *virG* gene (Fig. 1) was first introduced in *E. coli* CC102F′ containing Tn*phoA* integrated in the chromosome. Five colonies were separately inoculated in 1 ml of tryptic soy broth. After 1 h of incubation at 37°C, 9 ml of the same medium containing 100 μg of ampicilin ml^{-1} and 400 μg of kanamycin ml^{-1} was added and further incubated overnight. Plasmid DNA was extracted and used to transform *E. coli* KS272 (Δ*phoA*). In-frame fusions of the transposon in pRS7 were identified on plates containing XP (5-bromo-4-chloro-3-indolylphosphate), the chromogenic substrate of alkaline phosphatase, at a final concentration of 40 μg ml^{-1}. Tn*pho*A insertion sites were localized by DNA sequencing with oligonucleotide MIPA 178 (2).

Alkaline phosphatase assay. Alkaline phosphatase activity was assayed by using the substrate *p*-nitrophenylphosphate as described by Manoil and Beckwith (30). The activity was expressed in units of optical density at 420 nm (10^{-3}) per minute per 1.6×10^9 bacteria (34).

In vivo labeling with [^{3}H]palmitate. *Yersinia* strains were inoculated to an optical density at 600 nm of 0.1 in 5 ml of BHI-OX. After 2 h of incubation at room temperature with shaking, 250 μCi of [9,10-^{3}H]palmitic acid (30 Ci $mmol^{-1}$) (Dupont-NEN, Brussels, Belgium) was added to the culture, and bacteria were further incubated for 4 h at 37°C. Proteins were analyzed by SDS-PAGE and autoradiography.

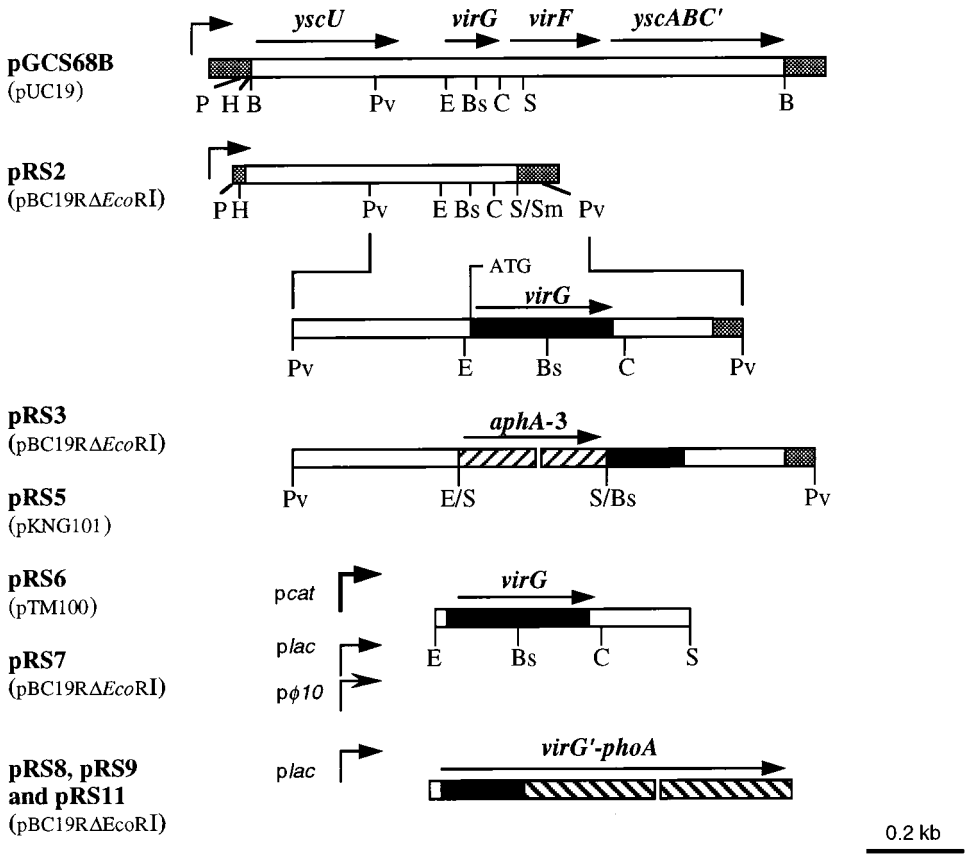

FIG. 1. Structures of plasmids carrying *yscU*, *virG*, *virF*, and the beginning of the *virC* operon (*yscABC'*). The plasmids are shown in a linear form in which *Y. enterocolitica* DNA is indicated by an open or solid bar. Vector DNA in pGCS68B, as well as the *aphA-3* and *phoA* genes (not shown to scale), are indicated by hatched bars; the vector names are indicated in parentheses. Thin arrows represent *lac* and *cat* promoters; thick arrows correspond to the ϕ10 promoter of bacteriophage T7. Horizontal arrows indicate the positions and extents of *yscU*, *virF*, *virG*, and *yscABC'*. The positions of selected restriction sites are shown (abbreviations: B, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sca*I; P, *Pst*I; Sm, *Sma*I; and Pv, *Pvu*II). ATG, start codon of the *virG* gene; /, disturbed restriction site.

Oligonucleotide-directed mutagenesis. Site-directed mutagenesis of *virG* was performed according to the method of Kunkel et al. (27). Oligonucleotide MIPA 197 (5′ 707-GATACCTAAAACTAGCTATCCAAATTGC-734 3′) contained mismatches at positions 719, 720, and 722 (TAGC instead of ATGT), thereby changing cysteine 121 to serine (C121S) (Fig. 2). In oligonucleotide MIPA 198 (5′ 716-AACATGTTATCCGAATTCCCAGTCACCTAA-745 3′), nucleotides A at position 728 and G at position 733 were replaced by G and C, respectively, which introduced an *Eco*RI site (used for checking the mutation) and changed cysteine 125 to serine (C125S) (Fig. 2). To generate these mutations, the *dut ung E. coli* CJ236 strain was transformed with plasmid pRS7 (Fig. 1). A uracil-containing single-stranded pRS7 DNA was isolated and annealed to the phosphorylated oligonucleotides MIPA 197 and MIPA 198; the second DNA strand was synthesized and circularized by using T4 DNA polymerase and T4 DNA ligase. The resulting double-stranded DNA was used to transform *E. coli* XL1-blue. Sequence analysis of plasmids pRS7A and pRS7B confirmed the expected replacements. The mutated *virG* gene was verified by T7 expression with the T7 RNA polymerase system (data not shown). The 235-bp *Bst*EII-*Cla*I DNA fragment of pRS6 was then replaced by the corresponding mutagenized fragments from pRS7A and pRS7B, giving rise to plasmids pRS6A and pRS6B, respectively (Fig. 1). Finally, these plasmids were controlled by restriction and sequence analysis and then used for the trans-complementation experiments with the *virG* mutant (see Results).

Nucleotide sequence accession number. The sequence of *virG* (Fig. 2) has been submitted to the GenBank nucleotide sequence data library under accession number U21297.

RESULTS

Nucleotide sequence of the region upstream of *virF*. We analyzed the 0.8-kb region of the pYV plasmid which is localized between *yscU*, the last gene of the *virB* operon (2), and *virF*, the activator for thermal induction (12). The *yscU* gene is followed by a 400-bp sequence containing stop codons in all three reading frames and a 40-nucleotide perfect palindrome (underlined in Fig. 2) (2). At 176 bp downstream from this putative strong terminator, we detected an open reading frame extending to nucleotide 753, preceded by a fairly good ribosome binding site (AGGGGAGA). Translation of this open reading frame, called *virG*, would result in a polypeptide of 131 amino acid residues with a predicted molecular mass of 14,689 Da and a calculated isoelectric point of 11.06. The *virG* gene is followed by a 7-bp inverted repeat encompassing the stop codon.

Identification of the *virG* gene product. Specific labeling of VirG was achieved in *E. coli* by using the T7 expression system (55). Plasmid pRS7 (Fig. 1) containing *virG* mediated the production of a major protein of about 15 kDa, which is in good agreement with the predicted molecular mass of the *virG* gene product (Fig. 3, lane 2).

Localization of VirG. The N-terminal sequence of the *virG* gene product contains one basic amino acid residue followed by a stretch of noncharged and hydrophobic residues, which are characteristic features of a signal sequence (48), suggesting that VirG could be a protein exported by the general export pathway. To confirm this hypothesis, we carried out Tn*phoA* mutagenesis (30). Plasmid pRS7, expressing *virG* from the *lac* promoter, was mutagenized with Tn*phoA* in *E. coli* (see Materials and Methods). The insertion sites of Tn*phoA* in three mutants (pRS8, -9, and -11) were determined at the nucleotide

```
                                                                    yscU
GGTGATTGTATTTCTCCTGTGCATTTTACAATTAAACATAACTAGCACTAATTAGTATTA      60

ATCTCTTGACTTTTTTTGTTGAATACAAATAATACAGTATTGTCATTACTATTACATGTG      120

TTTGTTTGCTGATTCCGATTCTAACCAAACATCCTTTCTTTCACGAAAGAAAGGATGTTT      180

GGTTTTATACACGCAAGGTGTGATATTGCAGTAGCTAAATTAAATGAGTTCGCGGTGGAC      240

CCGTTGAGCTAATTTGGTATGGGTTGTTATTTTACAATAATAAATTTCACCACATATTGC      300

GTGAACTCGGATTGCTATCATCTGGCTATCTTCTTTGTATGAATTCAAGGGGAGACTGGC      360
                                          EcoR1       SD
virG
ATGAGTCGTATTATAGCACTCATCATTTCTTTTCTATTAGTGGGGTGCGCCACCCCCCCA      420
M  S  R  I  I  A  L  I  I  S  F  L  L  V  G  C  A  T  P  P       20

ATGCCAGCTCAGCGTATTGTGGGGGAGGTGCGTATGTCACGACCATTATCTCGCACAGCA      480
M  P  A  Q  R  I  V  G  E  V  R  M  S  R  P  L  S  R  T  A       40

CACATTGATGTTAGTATATTTGGGTTGTATGAGGGGAAAGTTCGAGAGGTTCAGCGCACT      540
H  I  D  V  S  I  F  G  L  Y  E  G  K  V  R  E  V  Q  R  T       60

          BstEII
CGTTTCGAAACAGGTAACCTACCTTTATTCTTTTCTATAAAACTGAATCCAGCTCAACGC      600
R  F  E  T  G  N  L  P  L  F  F  S  I  K  L  N  P  A  Q  R       80

GGGGAAGGTGAACTTTACCTACGGTCAACCCTCTCTTTTCCAGAGCGAGGGGTTCAGGCG      660
G  E  G  E  L  Y  L  R  S  T  L  S  F  P  E  R  G  V  Q  A      100

GTGGCTCAGCAAAAACTTATTGGTAAAAACAAAGTCGTTTTGCAAATGATACCTAAAACA      720
V  A  Q  Q  K  L  I  G  K  N  K  V  V  L  Q  M  I  P  K  T      120

TGTTATCCAAATTGCCAGTCACCTAATACCAGATAGGTGATTTATTATATTGGTTTTGGT      780
C  Y  P  N  C  Q  S  P  N  T  R  <        131

TGCATTAATCGAT    793  virF
      ClaI
```

FIG. 2. Nucleotide sequence of the region between the *virB* operon (ending with the *yscU* gene [accession number U08019]) (in 5′) and *virF* (accession number M22781) (in 3′). The sequence of *yscU* presented in reference 2 ends at the *Eco*RI site, while the sequence of *virF* presented in reference 12 begins at the *Bst*EII site. SD, putative Shine-Dalgarno sequence. Putative terminators are underlined. Important restriction endonuclease sites are shown. The amino acid sequence of VirG, including the signal sequence, is given under the nucleotide sequence. The signal peptidase II cleavage site (63) is underlined.

level, and the hybrid VirG-PhoA proteins were analyzed by Western blotting with an antiserum directed against *E. coli* alkaline phosphatase (Fig. 4B). The three hybrid proteins had sizes of about 53 kDa, values which were in agreement with the determined insertion sites. We then assayed the alkaline phosphatase activity in extracts of *E. coli* KS278 (Δ*phoA*) and *Y. enterocolitica* KNG22703 containing plasmid pRS8, -9, or -11 (Table 2). All six extracts had high enzymatic activities, indicating that the PhoA moieties of the hybrid proteins were at least exported to the periplasm. VirG is thus an exported protein.

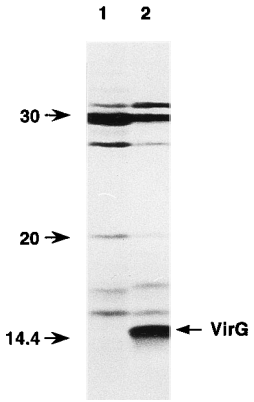

FIG. 3. Expression of the *virG* gene by T7 RNA polymerase (55). Lane 1, *E. coli* LK111(pBC19R)(pGP1-2); lane 2, *E. coli* LK111(pRS7)(pGP1-2). The positions of molecular mass markers (in kilodaltons) are indicated on the left.

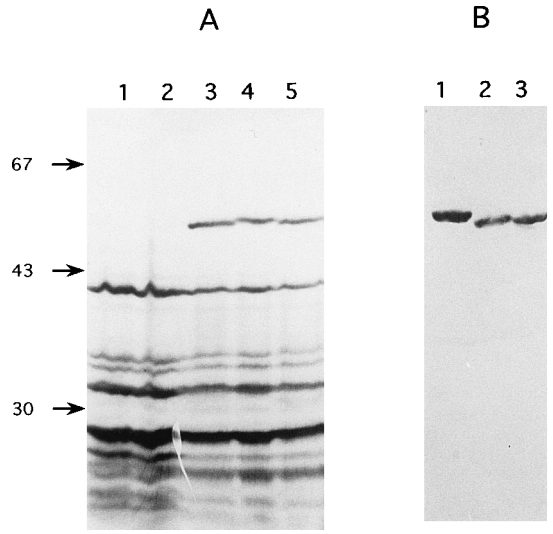

FIG. 4. (A) Identification of lipoproteins encoded by *Y. enterocolitica* KNG22703 and its derivatives. [^{3}H]palmitic acid-labeled whole-cell extracts were analyzed by SDS-PAGE and autoradiography. Lane 1, KNG22703(pYV$^-$); lane 2, KNG22703(pYV$^+$); lanes 3, 4, and 5, KNG22703(pYV$^-$) harboring pRS8, pRS9, or pRS11, respectively. (B) Immunoblot analysis of VirG-PhoA hybrid proteins. Whole-cell extracts from *Y. enterocolitica* KNG22703 harboring either pRS8 (lane 1), pRS9 (lane 2), or pRS11 (lane 3) were separated by SDS–10% PAGE. Proteins were transferred to nitrocellulose and incubated with rabbit anti-PhoA polyclonal antibodies. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

VirG is a lipoprotein. The signal sequence of VirG ends with Leu-Xaa-Gly-Cys, a motif which is characteristic of the processing site of lipoproteins (63) (Fig. 2). Prolipoproteins are subjected to a cascade of modifications that give rise to mature lipoproteins having an N-terminal Cys residue modified by two fatty acyl groups through ester linkages and by a third fatty acyl group via an amide linkage (63). These acylations can be detected after growth in the presence of radioactive palmitate. To verify whether VirG is indeed a lipoprotein, we labeled strain KNG22703 (pYV$^+$) and its pYV$^-$ cured derivative in vivo with [^{3}H]palmitate and analyzed whole-cell extracts by SDS–10% PAGE and autoradiography. Several proteins in the range of

TABLE 2. Alkaline phosphatase activities expressed by *E. coli* and *Y. enterocolitica* strains containing *virG-phoA* gene fusions[a]

Plasmid (hybrid protein)	PhoA (U)	
	E. coli	*Y. enterocolitica*
pRS7 (VirG)	ND	ND
pSF22 (MxiJ-PhoA)	390 ± 25	NT
pAA117 (YscU-PhoA)	31 ± 5	NT
pRS8 (VirG64-PhoA)	227 ± 12	141 ± 6
pRS9 (VirG29-PhoA)	320 ± 47	246 ± 26
pRS11 (VirG60-PhoA)	286 ± 18	201 ± 37

[a] Alkaline phosphatase activities in transformants of *E. coli* KS272 (Δ*phoA*) and *Y. enterocolitica* grown to stationary phase were assayed as described previously (30). Values are means ± standard deviations for three independent experiments. For each plasmid, the last amino acid of the VirG moiety of the hybrid PhoA is indicated. Plasmids pSF22 and pAA117 encode the *Shigella flexneri* hybrid lipoprotein MxiJ-PhoA and the recently reported *Y. enterocolitica* hybrid protein. YscU-PhoA, which expressed high and low alkaline phosphatase activities, respectively (1,2). ND, not detected; NT, not tested.

```
ExsB   1 MRCWMLLPLALLLGGCVSQPAPMSPKVTVGGSVGGVSLQARQAQLRLRLY 50
VirG   1 MSRIIALIISFLLVGCATPPMPAQR...IVGEVRMSRPLSRTAHIDVSIF 47

ExsB  51 AVVQGRMQTIAERRYWVSGLPLRYAFDLEVDRLEGEALYLRTELSWVGVA 100
VirG  48 GLYEGKVREVQRTRFETGNLPLFFSIKLNPAQRGEGELYLRSTLSFPE.R 96

ExsB 101 AVQASAWQQVAAGVDERVRLVRRDCFPNCTAARPEERSGND 141
VirG  97 GVQAVAQQKLIGKNKVVLQMIPKTCYPNCQSPNTR 131
```

FIG. 5. Comparison between VirG and *P. aeruginosa* ExsB, translated from the GTG codon corresponding to the start of VirG (with addition to ExsB of the four amino acid residues MRCW [boldface]) (19). The alignment was made by using the BESTFIT program available in the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis package software.

10 to 30 kDa were labeled, but there was no clear difference between the patterns of the two strains (data not shown). We then took advantage of the *virG-phoA* gene fusions described above because their larger size would allow easy detection. We assumed that the fused C-terminal PhoA moiety would not interfere with processing and addition of the acylation. Labeled proteins of approximately 53 kDa were detected in KNG22703 carrying plasmid pRS8, -9, or -11 (Fig. 4A, lanes 3 to 5) but not in the plasmidless host strain (Fig. 4A, lanes 1 and 2). The size of those proteins is consistent with the size expected for the PhoA fusion proteins. From these results we concluded that VirG is a lipoprotein.

VirG is homologous to ExsB. The sequence of VirG was compared with the protein sequences in the GenBank library. This comparison revealed extensive similarity (26.2% identity in a 126-amino-acid overlap) between VirG and ExsB, a 137-amino acid *Pseudomonas aeruginosa* polypeptide encoded by a trans-regulatory locus controlling exoenzyme S (ExoS) synthesis (19). The alignment (Fig. 5) shows the conservation of the Leu-Xaa-Gly-Cys motif in the amino-terminal part of ExsB (underlined), which suggests that ExsB is also a lipoprotein. The signal sequence of ExsB is very short, but the proposed start codon of ExsB is preceded by a possible GTG start codon (19). The use of this upstream start codon made the alignment more complete and improved the signal sequence of ExsB by adding four amino acid residues, MRCW (indicated in boldface) (Fig. 5). The size of ExsB could thus be revised. Interestingly, an even stronger similarity exists between the proteins encoded by the genes located downstream from *virG* and *exsB*, namely *virF* and *exsA*, respectively, as has been reported by Frank and Iglewski (19). ExsA and VirF have 56% identity over their entire length, with their carboxy-terminal regions being almost identical. Both proteins belong to the AraC family of regulators.

Effect of the *virG* mutation on Yop synthesis and release. In order to investigate the role of VirG, we engineered a *virG* mutation in pYV227. The 210-bp *Eco*RI-*Bst*EII fragment of pRS2 (from nucleotide 343 to 553) was replaced by the nonpolar *aphA-3* cassette (31), giving pRS227 (see Materials and Methods and Fig. 1). *Y. enterocolitica* W22703(pRS227) was first tested for its capacity to produce and secrete the Yops. Upon thermal induction, the *virG* mutant secreted Yops. While no difference in YopH secretion was detected between wild-type and *virG* mutant strains, the amounts of the others Yops were reduced, especially those of YopB and YopD (Fig. 6, lanes 1 and 2, respectively) and LcrV (not visible in Fig. 6). The level of reduction of these Yops was quite variable from one experiment to another. Moreover, secretion of LcrV was more difficult to quantify in supernatants because of its higher solubility and possible losses during preparation. To ensure

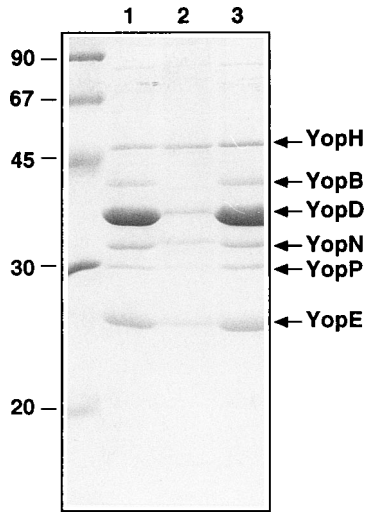

FIG. 6. Analysis of Yop secretion and synthesis. Proteins from a culture supernatant of *Y. enterocolitica* W22703 mutants KNG22703(pYV$^+$) (lane 1), KNG22703(pRS227) (*virG* mutant) (lane 2), or KNG22703(pRS227)(pRS6) (lane 3) were separated by SDS-PAGE and stained with Coomassie brilliant blue. The positions of YopH, YopB, YopD, YopN, YopP, and YopE are indicated by arrows. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

that the defect in Yop secretion was due to the *virG* mutation itself and not to a polar effect on the *virF* gene, we introduced pRS6 (Fig. 1), a derivative of pTM100 containing only *virG*, into the mutant. Strain W22703(pRS227)(pRS6) was able to secrete Yops as well as the parental strain (Fig. 6, lane 3). Thus, the defect resulted from the lack of the *virG* product. To decide whether the synthesis or secretion of YopB and YopD was compromised, we monitored the presence of YopD, YopE, and LcrV among the intracellular proteins by immunoblotting with monoclonal antibodies (Fig. 7). YopD, YopE, and LcrV were detected in the *virG* mutant, and the signal was of approximately the same intensity as that observed in crude extracts of the wild-type strain (Fig. 7). This result showed that export of these Yops is certainly affected. However, the fact that these Yops did not accumulate intracellularly indicates that their synthesis could also be reduced.

Effect of the *virG* mutation on growth restriction at 37 C in the absence of Ca^{2+}. Surprisingly, the *virG* mutant gave the same efficiency of plating at 37°C on MOX agar and on TSA. Hence, it was CI for growth at 37°C. The wild-type Ca^{2+}-dependent phenotype was restored after the introduction of

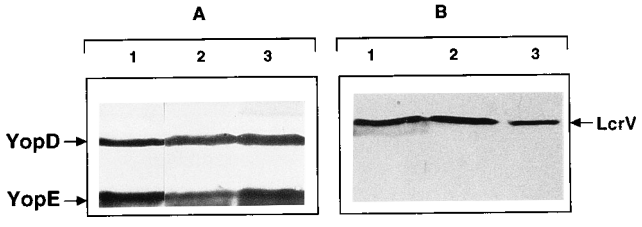

FIG. 7. Western blot analysis of YopD, YopE, and LcrV. Whole-cell protein extracts of *Y. enterocolitica* W22703 mutants KNG22703(pYV$^+$) (lane 1), KNG22703(pRS227) (*virG* mutant) (lane 2), and KNG22703(pRS227)(pRS6) (lane 3) were transferred onto nitrocellulose (after SDS-PAGE) and incubated with a mixture of monoclonal antibodies directed against YopD and YopE (A) or with a monoclonal antibody against LcrV (B). The positions of YopD, YopE, and LcrV are indicated by arrows.

plasmid pRS6 but not after introduction of pTM100, the vector used for the construction of pRS6 and derivatives (data not shown). The cloned *virG* gene complemented the mutation, confirming that the CI phenotype was exclusively due to the inactivation of the *virG* gene. To ensure that the ability to grow on MOX plates at 37°C was not the result of an increased frequency of loss of the pYV plasmid or mutations, we analyzed the plasmid profiles of six colonies recovered from MOX agar and six colonies isolated from TSA and grown at 37°C. We were unable to detect any difference in the plasmid contents and restriction profiles (not shown). Moreover, colonies isolated from MOX agar were used for trans-complementation experiments by introduction of pRS6. The transformants were unable to grow at 37°C on MOX medium and showed the same profile of secreted proteins as the wild-type strain. The *virG* mutant was thus phenotypically CI although it secreted Yops. Although Ca^{2+} did not prevent growth of the *virG* mutant, it still prevented Yop secretion (data not shown). The *virG* mutant is thus not Ca^{2+} blind.

Site-directed mutagenesis of Cys-121 and Cys-125 of VirG. Cysteine residues are known to be involved in dimerization of some proteins by formation of disulfide bonds. Cysteines 121 and 125 are conserved among VirG and ExsB (Fig. 5), suggesting that they may play a functional role in the VirG protein. To test this hypothesis, we replaced the codon for Cys-121 or Cys-125 with a serine codon on plasmid pRS7 and introduced both single mutations on the low-copy-number plasmid pRS6, giving rise to pRS6A (C121S) and pRS6B (C125S) (see Materials and Methods). pRS6A and pRS6B were then used to transform the *virG* mutant, and the phenotype of the *virG* mutant carrying either pRS6, pRS6A, or pRS6B was tested for Ca^{2+} dependency and for Yop secretion. No difference in the phenotypes of these three strains was detected (data not shown), indicating that individual mutation of these cysteines does not influence the function of VirG, at least in our in vitro assays.

DISCUSSION

In the present study, we characterized *virG*, a new pYV-related gene located immediately upstream of the *virF* gene. Tn*phoA* mutagenesis generated three VirG-PhoA fusion proteins endowed with high levels of alkaline phosphatase activity, showing that VirG is at least exported to the periplasm. The presence of the motif Leu-Xaa-Gly-Cys at the end of the putative signal sequence of VirG suggested that VirG might be a lipoprotein (63). Detection of VirG-PhoA fusion proteins after labeling with [^{3}H]palmitate confirmed that VirG is indeed a lipoprotein. Localization in the outer membrane of the *Klebsiella pneumoniae* lipoprotein pullulanase and of *E. coli* lipoproteins is dependent on the absence of an aspartate amino acid residue in position +2, following the fatty acylated amino-terminal cysteine. Replacement of the serine residue (position +2) by an aspartic acid directs hybrid lipo-β-lactamase and pullulanase to fractionate with the inner membrane (21, 42, 45). According to these observations, VirG, which contains an alanine residue in position +2 of the mature protein and no charged amino acid residue between +2 and +5, is most likely located in the outer membrane.

To investigate the function of VirG, we generated a *virG* nonpolar mutation on the pYV plasmid and studied the phenotype of the resulting strain. The *virG* mutant secreted a smaller amount of Yops and was CI for growth at 37°C. The amounts of YopB, YopD, and LcrV were strongly reduced among the secreted Yops, but they could still be detected intracellularly (not tested for YopB). This phenotype could be complemented by the introduction of a plasmid harboring an intact copy of the *virG* gene into the mutant, confirming that this mutation resulted only in the lack of functional VirG.

If one judges from the phenotype, VirG does not seem primarily to regulate Yop production. The reduced release of YopD, YopB, and LcrV tends to suggest that VirG could be a nonessential component of the secretion machinery. The loss of the Ca^{2+} requirement for growth, however, is impossible to interpret at this stage. First, it should be remembered that our *virG* mutants are not Ca^{2+} blind like the *yopN* (17) and *lcrG* (51) mutants: although they have lost the Ca^{2+} requirement for growth, they do not secrete Yops in the presence of Ca^{2+}. This result is in agreement with the fact that YopN (LcrE), which has been reported to be a Ca^{2+} sensor (17), is secreted by the *virG* mutants. The present report is the first characterization of a CI mutant which still secretes Yops. This particular phenotype was previously described by Wolf-Watz et al. (62) for a *Y. pseudotuberculosis* mutant carrying an insertion in the same region of the pYV plasmid, but no gene was described in that early paper. One could hypothesize that VirG is an element of a regulatory pathway that determines growth arrest under low-Ca^{2+} conditions. Alternatively, one could envision that the lack of a Ca^{2+} requirement results from the poor secretion of YopB, YopD, and LcrV. However, since *lcrV* and *yopBD* polar mutants are not CI but are unable to grow at 37°C irrespective of the Ca^{2+} concentration (4, 36, 44), it is unlikely that the CI phenotype results simply from reduced secretion of YopB and YopD. Similarly, since nonpolar *lcrV* mutants are CI and unable to secrete Yops (43), one can also conclude that the phenotype does not result simply from reduced secretion of LcrV. Finally, one could hypothesize that reduced secretion of LcrV, YopB, and YopD could negatively influence the expression of another gene that is responsible for Ca^{2+} dependency. We are thus still far from understanding the role of Ca^{2+} in yersiniae. Here we simply add a new element of the puzzle.

VirG is similar to ExsB from *P. aeruginosa*. This similarity extends over the entire length of the proteins, which strongly suggests that they have similar localizations and functions. The motif Leu-Xaa-Gly-Cys is also present at the amino-terminal part of ExsB, which indicates that it could also be a lipoprotein. *exsB* is the second gene of an operon consisting of three genes, *exsC*, *exsB*, and *exsA* (64). The three genes of the *exs* operon are referred to as a trans-regulatory locus required for ExoS synthesis (19, 20). ExoS is an ADP-ribosyltransferase that plays a major role in the dissemination of *P. aeruginosa* from the initial colonization site in the skin to the bloodstream of infected animals (25, 37, 38). The amino-terminal half of ExoS was recently shown to have 27% identity to YopE and to be controlled at the transcriptional level by ExsA (65). The product of the gene (*orf1*) located just upstream of the *exoS* gene has 44% identity to SycE, the specific protein chaperone required for YopE secretion (65). Like the Yops, ExoS is secreted without cleavage of an amino-terminal sequence (26). Moreover, expression and secretion of ExoS are also induced by growing *P. aeruginosa* in a medium containing a Ca^{2+}-chelating agent such as nitrilotriacetic acid or EDTA (65). To summarize, VirG and ExsB are probably involved in the synthesis or secretion of virulence proteins. Their function remains to be determined. Type III secretion mechanism are now known to exist in various pathogenic bacteria, and the similarity in genetic organization and regulation of *virG-virF* and *exsB-exsA* as well as *sycE-yopE* and *orf1-exoS* indicates that such a secretion system is also present in *P. aeruginosa.*

ACKNOWLEDGMENTS

We are pleased to acknowledge Bruno Dupuy (Institut Pasteur, Paris, France) for the generous gift of rabbit polyclonal serum raised against *E. coli* alkaline phosphatase; Ralph Shulte, Margot Koster, and Marie-Paule Sory for a critical reading of the manuscript; and the International Institute of Cellular and Molecular Pathology (ICP) illustration service for the artwork.

A.A. was the recipient of a fellowship from the ICP. This work was supported by grants from the Belgian FRSM (Fonds National de la Recherche Scientifique Médicale; convention 3.4627.93), the Belgian FNRS (Fonds de la Recherche Scientifique; crédit aux chercheurs 1.5.025.94F) and the Belgian program on interuniversity poles of attraction initiated by the Belgian State, Prime Minister's Office, Sciences Policy Programming. This work was also supported by the Human Capital and Mobility Program from the European Community (contract ERBCHRXCT930171).

REFERENCES

1. **Allaoui, A., P. J. Sansonetti, and C. Parsot.** 1992. MxiJ, a lipoprotein involved in secretion of *Shigella* Ipa invasins, is homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. J. Bacteriol. **174:**7661–7669.
2. **Allaoui, A., S. Woestyn, C. Sluiters, and G. R. Cornelis.** 1994. YscU, a *Yersinia enterocolitica* inner membrane protein involved in Yop secretion. J. Bacteriol. **176:**4534–4542.
3. **Bergman, T., K. Erikson, E. Galyov, C. Persson, and H. Wolf-Watz.** 1994. The *lcrB* (*yscN*/*U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. J. Bacteriol. **176:**2619–2626.
4. **Bergman, T., S. Hakansson, A. Forsberg, L. Norlander, A. Macellaro, A. Backman, I. Bolin, and H. Wolf-Watz.** 1991. Analysis of the V antigen *lcrGVH-yopBD* operon of *Yersinia pseudotuberculosis*: evidence for the regulatory role of LcrH and LcrV. J. Bacteriol. **173:**1607–1616.
5. **Bodeus, M., M. P. Sory, J. C. Fang, M. Janssens, N. Delferriere, G. Cornelis, G. Wauters, and G. Burtonboy.** 1990. Production of rat hybridomas directed against *Yersinia enterocolitica*, p. 335–338. *In* H. Bazin (ed.), Rat hybridoma and rat monoclonal antibodies. CRC Press, Inc., Boca Raton, Fla.
6. **Burrows, T., and G. A. Bacon.** 1956. The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. Br. J. Exp. Pathol. **37:**481–493.
7. **China, B., T. Michiels, and G. R. Cornelis.** 1990. The pYV plasmid of *Yersinia* encodes a lipoprotein, YlpA, related to TraT. Mol. Microbiol. **4:**1585–1593.
8. **Cornelis, G. R.** 1992. Yersiniae, finely tuned pathogens, p. 231–265. *In* C. E. Hormaeche, C. W. Penn, and C. J. Symth (ed.), Molecular biology of bacterial infection. SGM symposium no. 49. Cambridge University Press, Cambridge.
9. **Cornelis, G. R.** 1994. *Yersinia* pathogenicity factors. Curr. Top. Microbiol. Immunol. **192:**243–263.
10. **Cornelis, G. R., and C. Colson.** 1975. Restriction of DNA in *Yersinia enterocolitica* detected by recipient ability for a derepressed R factor from *Escherichia coli*. J. Gen. Microbiol. **87:**285–291.
11. **Cornelis, G. R., C. Sluiters, I. Delor, D. Geib, K. Kaniga, C. Lambert de Rouvroit, M. P. Sory, J. C. Vanooteghem, and T. Michiels.** 1991. *ymoA*, a *Yersinia enterocolitica* chromosomal gene modulating the expression of virulence functions. Mol. Microbiol. **5:**1023–1034.
12. **Cornelis, G. R., C. Sluiters, C. Lambert de Rouvroit, and T. Michiels.** 1989. Homology between VirF, the transcriptional activator of the *Yersinia* virulence regulon, and the *Escherichia coli* arabinose operon regulator. J. Bacteriol. **171:**254–262.
13. **Cornelis, G. R., M. P. Sory, Y. Laroche, and I. Derclaye.** 1986. Genetic analysis of the plasmid region controlling virulence in *Yersinia enterocolitica* O:9 by mini-Mu insertions and *lac* gene fusions. Microb. Pathog. **1:**349–359.
14. **Cornelis, G. R., J. C. Vanooteghem, and C. Sluiters.** 1987. Transcription of the *yop* regulon from *Y. enterocolitica* requires trans-acting pYV and chromosomal genes. Microb. Pathog. **2:**367–379.
15. **Fields, K. A., G. V. Plano, and S. C. Straley.** 1994. A low-Ca^{2+} response (LCR) secretion (*ysc*) locus lies within the *lcrB* region of the LCR plasmid in *Yersinia pestis*. J. Bacteriol. **176:**569–579.
16. **Forsberg, A., R. Rosqvist, and H. Wolf-Watz.** 1994. Regulation and polarized transfer of the *Yersinia* outer proteins (Yops) involved in antiphagocytosis. Trends Microbiol. **2:**14–19.
17. **Forsberg, A., A. M. Viitanen, M. Skurnik, and H. Wolf-Watz.** 1991. The surface-located YopN protein involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol. Microbiol. **5:**977–986.
18. **Forsberg, A., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-pIB1-encoded trans-acting elements controlled by temperature and calcium. Mol. Microbiol. **2:**121–133.
19. **Frank, D. W., and B. H. Iglewski.** 1991. Cloning and sequence analysis of a trans-regulatory locus required for exoenzyme S synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**6460–6468.
20. **Frank, D. W., G. Nair, and P. S. Herbert.** 1994. Construction and characterization of chromosomal insertion mutations of the *Pseudomonas aeruginosa* exoenzyme S trans-regulatory locus. Infect. Immun. **62:**554–563.
21. **Gennity, J. M., and M. Inoue.** 1991. The protein sequence responsible for lipoprotein membrane localization in *Escherichia coli* exhibits remarkable specificity. J. Biol. Chem. **266:**16458–16464.
22. **Higuchi, K., L. L. Kupferberg, and J. L. Smith.** 1959. Studies on the nutrition and physiology of *Pasteurella pestis*. III. Effects of calcium ions in the growth of virulent and avirulent strains of *Pasteurella pestis*. J. Bacteriol. **77:**317–321.
23. **Hoe, N. P., and J. D. Goguen.** 1993. Temperature sensing in *Yersinia pestis*: translation of the LcrF activator protein is thermally regulated. J. Bacteriol. **175:**7901–7909.
24. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137–141.
25. **Kulich, S. M., D. W. Frank, and J. T. Barbieri.** 1995. Expression of recombinant exoenzyme S of *Pseudomonas aeruginosa*. Infect. Immun. **63:**1–8.
26. **Kulich, S. M., T. L. Yahr, L. M. Mende-Mueller, J. T. Barbieri, and D. W. Frank.** 1994. Cloning the structural gene for the 49-kDa form of exoenzyme S (exoS) from *Pseudomonas aeruginosa* strain 388. J. Biol. Chemistry. **269:**10431–10437.
27. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
29. **Lambert de Rouvroit, C., C. Sluiters, and G. R. Cornelis.** 1992. Role of the transcriptional activator, VirF in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol. Microbiol. **6:**395–409.
30. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
31. **Menard, R., P. J. Sansonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899–5906.
32. **Michiels, T., and G. R. Cornelis.** 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173:**1677–1685.
33. **Michiels, T., J. C. Vanooteghem, C. Lambert de Rouvroit, B. China, A. Gustin, P. Boudry, and G. R. Cornelis.** 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. **173:**4994–5009.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y.
35. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
36. **Mulder, B., T. Michiels, M. Simonet, M. P. Sory, and G. R. Cornelis.** 1989. Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. Infect. Immun. **57:**2534–2541.
37. **Nicas, T. I., J. Bradely, J. E. Lochner, and B. H. Iglewski.** 1985. The role of exoenzyme S in infections with *Pseudomonas aeruginosa*. J. Infect. Dis. **152:**716–721.
38. **Nicas, T. I., and B. H. Iglewski.** 1985. Contribution of exoenzyme S to the virulence of *Pseudomonas aeruginosa*. Antibiot. Chemother. (Basel) **36:**40–48.
39. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
40. **Plano, G. V., S. S. Barve, and S. C. Straley.** 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173:**7293–7303.
41. **Popowicz, A. M., and P. F. Dash.** 1989. SIGSEQ version 1.0. Prediction of signal sequence cleavage sites based on the method of G. Von Heijne. Copyrights. The Rockefeller University, New York, N.Y.
42. **Poquet, I., M. G. Kornacker, and A. P. Pugsley.** 1993. The role of the lipoprotein sorting signal (aspartate +2) in pullulanase secretion. Mol. Microbiol. **9:**1061–1069.
43. **Price, S. T., C. Cowan, R. D. Perry, and S. C. Straley.** 1991. The *Yersinia pestis* V antigen is a regulatory protein necessary for Ca^{2+}-dependent growth and maximal expression of low-Ca^{2+} response virulence genes. J. Bacteriol. **173:**2649–2657.
44. **Price, S. T., K. Y. Leung, S. S. Barve, and S. C. Straley.** 1989. Molecular analysis of LcrGVH, the V antigen operon of *Yersinia pestis*. J. Bacteriol. **171:**5646–5653.
45. **Pugsley, A. P., and M. G. Kornacker.** 1991. Secretion of the cell surface lipoprotein pullulanase in *Escherichia coli*. J. Biol. Chem. **266:**13640–13645.
46. **Rhode, J. R., J. M. Fox, and S. A. Minnich.** 1994. Thermoregulation in *Yersinia enterocolitica* is coincident with changes in DNA supercoiling. Mol. Microbiol. **12:**187–199.
47. **Rimpiläinen, M., A. Forsberg, and H. Wolf-Watz.** 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. **174:**3355–3363.

48. **Saier, M. H., P. K. Werner, and M. Müller.** 1989. Insertion of proteins into bacterial membrane: mechanism, characteristics, and comparisons with the eucaryotic process. Microbiol. Rev. **53:**333–336.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
51. **Skrzypek, E., and S. C. Straley.** 1993. LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. J. Bacteriol. **175:**3520–3528.
52. **Sory, M. P., and G. R. Cornelis.** 1988. *Yersinia enterocolitica* O:9 as a potential live oral carrier for protective antigens. Microb. Pathog. **4:**431–442.
53. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol. Microbiol. **8:**1005–1010.
54. **Straley, S. C., E. Skrzypek, G. V. Plano, and J. B. Bliska.** 1993. Yops of *Yersinia* spp. pathogenic for humans. Infect. Immun. **61:**3105–3110.
55. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
56. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
57. **Von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
58. **Wattiau, P., B. Bernier, P. Deslee, T. Michiels, and G. R. Cornelis.** 1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA **91:**1074–1078.
59. **Wattiau, P., and G. R. Cornelis.** 1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. Mol. Microbiol. **8:**123–131.
60. **Wattiau, P., and G. R. Cornelis.** 1994. Identification of DNA sequences recognized by VirF, the transcriptional activator of the *Yersinia* Yop regulon. J. Bacteriol. **176:**3878–3884.
61. **Woestyn, S., A. Allaoui, P. Wattiau, and G. R. Cornelis.** 1994. YscN, the putative energizer of the *Yersinia* Yop secretion machinery. J. Bacteriol. **176:**1561–1569.
62. **Wolf-Watz, H., D. A. Portnoy, I. Bölin, and S. Falkow.** 1985. Transfer of the virulence plasmid of *Yersinia pestis* to *Yersinia pseudotuberculosis*. Infect. Immun. **48:**241–243.
63. **Wu, H. C., and M. Tokunaga.** 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Micobiol. Immunol. **125:**127–157.
64. **Yahr, T. L., and D. W. Frank.** 1994. Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3832–3838.
65. **Yahr, T. L., A. K. Hovey, S. M. Kulich, and D. W. Frank.** 1995. Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. J. Bacteriol. **177:**1169–1178.
66. **Yother, J., and J. D. Goguen.** 1985. Isolation and characterization of Ca^{2+}-blind mutants of *Yersinia pestis*. J. Bacteriol. **164:**704–711.