JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1561–1569
0021-9193/94/$04.00+0

Vol. 176, No. 6

YscN, the Putative Energizer of the *Yersinia* Yop Secretion Machinery

SOPHIE WOESTYN, ABDELMOUNAAÏM ALLAOUI, PIERRE WATTIAU, AND GUY R. CORNELIS*

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

Received 13 October 1993/Accepted 10 January 1994

Pathogenic yersiniae secrete a set of 11 antihost proteins called Yops. Yop secretion appears as the archetype of the type III secretion pathway. Several components of this machinery are encoded by the *virA* (*lcrA*) and *virC* (*lcrC*) loci of the 70-kb pYV plasmid. In this paper, we describe *yscN*, another gene involved in this pathway. It is the first gene of the *virB* locus. It encodes a 47.8-kDa protein similar to the catalytic subunits of F_0F_1 and related ATPases, as well as to products of other genes presumed to be involved in a type III secretion pathway. YscN contains the two consensus nucleotide-binding motifs (boxes A and B) described by Walker et al. (J. E. Walker, M. Saraste, M. J. Runswick, and N. J. Gay, EMBO J. 1:945–951, 1982). We engineered a pYV mutant encoding a modified YscN protein lacking box A. This mutant, impaired in Yop secretion, can be complemented in *trans* by a cloned *yscN* gene. We conclude that YscN is a component of the Yop secretion machinery using ATP. We hypothesize that it is either the energizer of this machinery or a part of it.

Three species of the genus *Yersinia*, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, have adapted themselves to multiply at the expense of a host that is still alive. Although they cause syndromes as different as gastroenteritis (*Y. enterocolitica*) and plague (*Y. pestis*), they share a common capacity to escape nonspecific host defenses.

This capacity depends on the highly conserved pYV plasmid which codes for the secretion of about 11 antihost proteins called Yops (for some recent reviews, see references 7, 12, and 56). The secretion of Yops by yersiniae does not involve the cleavage of a classical N-terminal signal sequence (21, 39, 48). The addressing signal is nevertheless localized in the N-terminal domain of the Yops. The secretion domains of these proteins do not show any similarity with respect to amino acid sequence, hydrophobicity profile, distribution of charged residues, or prediction of secondary structure (37). The Yop secretion pathway is thus different from the two pathways exemplified by the *Escherichia coli* hemolysin (29) and the *Klebsiella oxytoca* pullulanase (47) systems. Several genes involved in the Yop transport system have been identified on the pYV plasmid, in the *virC* operon and in the *virA* (*lcrA*) locus. The 8.5-kb *virC* operon is composed of 13 genes, called *yscA* to *yscM* (for Yop secretion) (38). So far, only *yscD*, *yscJ*, and *yscL* have been shown to be required for secretion (38). The inner membrane protein LcrD, encoded by the *virA* locus, is probably another component of the secretion machinery (43, 44). One of the peculiarities of the Yop secretion system is that it makes use of cytoplasmic chaperones that are specific for individual Yops (66). These chaperones, called Syc (for specific Yop chaperone), could recognize the secretion domain of their nascent Yop partner proteins and lead them to the translocon (66). They would thus be the counterparts of SecB in the Sec-dependent pathway (17).

Homologs of *yscC*, *-F*, and *-J* and *lcrD* genes have been discovered not only in other animal pathogens such as *Shigella flexneri* (2, 3, 4) and *Salmonella typhimurium* (24) but also in plant pathogens such as *Xanthomonas campestris* (19), *Pseudomonas solanacearum* (25), and *Pseudomonas syringae* (67). The Yop secretion system thus turns out to be the archetype of a new pathway that we will here call type III (50). For a short review of this new family, see reference 59.

None of the yersinia *vir* genes that have been characterized so far encodes an ATP-binding protein which could act as the energizer of the secretion system. The present paper describes *yscN*, which codes for such a protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res^- Mod^+) of the serotype O:9 strain W227 (13). *Y. enterocolitica* KNG22703 is a *blaA* mutant of strain W22703 in which the gene encoding β-lactamase A was replaced by the *luxAB* genes (32). *E. coli* LK111 received from M. Zabeau (Ghent, Belgium) and *E. coli* XL1-blue (Stratagene, La Jolla, Calif.) were used for standard genetic manipulations. *E. coli* CJ236 (*dut ung*) (34) was used for site-directed mutagenesis. *E. coli* SM10 λ *pir*$^+$ constructed by Miller and Mekalanos (40) was used to deliver the mobilizable plasmids in *Y. enterocolitica*. This strain allows replication of *pir* mutants of R6K, and it mobilizes plasmids containing the origin of transfer of RK2. The plasmids used in this study are listed in Table 1.

The strains were routinely grown in tryptic soy broth (TSB) (Oxoid, Basingstoke, England) and plated on tryptic soy agar (TSA) (Oxoid), sometimes supplemented with 20 mM $MgCl_2$ and 20 mM sodium oxalate (MOX). For the 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. M9 medium (1 mM $MgSO_4$, 100 μM $CaCl_2$, 86 mM NaCl, 420 mM Na_2HPO_4, 220 mM KH_2PO_4, 190 mM NH_4Cl, and 4 mg of glucose ml^{-1}) was used for the labeling of YscN. All media were supplemented with the relevant selective agents. Unless otherwise specified, the concentrations were as follows: ampicillin, 400 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; and nalidixic acid, 35 μg ml^{-1}.

PCR amplification of *yscN*. Gene *yscN* was amplified by PCR

* 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.

TABLE 1. Plasmids used in this study

Plasmid	Genotype or description	Origin or reference
pAA16	pBC18R + 1.9-kb *Hin*dIII fragment of pYV227 (oriI): *yscN* + first 118 codons of *lcrE*	This study
pAA17	pBC18R + 1.9-kb *Hin*dIII fragment of pYV227 (oriII): *yscN* + first 118 codons of *lcrE*, downstream from p*lac*	This study
pAA31	pAA17 with a 224-bp *Pst*I internal deletion ($YscN^+$)	This study
pBC18R	pTZ18R + $oriT_{RK2}$	11
pGC445	pGB63 *yscC445*::mini-Mu dI *lac*	14
pGC678	pGB63 *virB678*::mini-Mu dI *lac*	14
pGC1152-9	pGC1152 *virF9*::Tn*813*	15
pGP1-2	pBR322 + cI857 + T7 RNA polymerase gene downstream from promoter pL	57
pKNG101	ori_{R6K} *sacBR* $oriT_{RK2}$ *strAB*	32
pSW2	pBC18R + PCR-synthesized 2.1-kb fragment containing *yscN* gene	This study
pSW4	pAA31, *yscNΔ169-177*	This study
pSW6	pKNG101 + *yscNΔ169-177* allele of pSW4 cloned as a *Sal*I-*Pvu*II fragment	This study
pSW2276	pYV227 with *yscNΔ169-177* allele of pSW6	This study
pYV227	pYV plasmid from W227 (serotype O:9)	14

(49) carried out on pGC678 DNA either with primers MIPA 117 and MIPA 116 or with primers MIPA 117 and MIPA 118 (Fig. 1A). MIPA 117 (5′-GGGAATTCTTCGTGCGCCGC TTC-3′) is identical, from nucleotides 9 to 23, to the right terminus of bacteriophage Mu (26). Nucleotides 9 to 26 of MIPA 116 (5′-TTGTCGACTACCACACCACTTTCTTT-3′) correspond to nucleotides 516 to 533 of *yscN* (Fig. 2), while MIPA 118 (5′-TTGTCGACCGTCATAACTACTCCCT-3′) is complementary to nucleotides 637 to 653 of the coding strand of *lcrE*, the gene located immediately upstream from *yscN* (61). A *Sal*I restriction site was included in MIPA 116 and MIPA 118, while an *Eco*RI restriction site was added to MIPA 117. The PCR protocol was that described by Lambert de Rouvroit et al. (35). The *Taq* DNA polymerase was from Amersham (Little Chalfont, England). The first cycle was 5 min at 93°C, 2 min at 40°C, and 3 min at 70°C. Cycles 2 through 29 were 1 min at 93°C, 1 min at 57°C, and 3 min at 70°C. The last cycle was 1 min at 93°C, 1 min at 57°C, and 10 min at 70°C.

Construction of plasmid pSW2276. The 27 bp encoding the Walker box A of YscN was deleted from plasmid pAA31 (Fig. 1B) by site-directed mutagenesis as described by Kunkel et al. (34). A uracil-containing single-stranded pAA31 was produced from *dut ung E. coli* CJ236. Oligonucleotide MIPA 146 (5′-AGAATGGGCATCTTCCTGCTTGCTTCGCTTAT-3′), identical to the 15 bases upstream and to the 17 bases downstream from the sequence encoding box A (Fig. 2), was purified and phosphorylated in the 5′ end. This synthetic 32-mer was then hybridized with the uracil-containing single-stranded pAA31 to prime complementary-strand synthesis in vitro according to the Muta-Gene Phagemid Kit protocol (Bio-Rad, Nazareth Eke, Belgium). The resulting double-stranded DNA was introduced into *E. coli* XL1-blue. The expected mutated plasmid was called pSW4 (Fig. 1B). The mutated allele of *yscN*, called *yscNΔ169-177*, was then subcloned as a *Sal*I-*Pvu*II fragment into the *Sal*I-*Sma*I sites of suicide vector pKNG101, yielding pSW6. The recombinant plasmid was crossed in *Y. enterocolitica* W22703(pYV227), and allelic exchange was selected as described by Kaniga et al. (32). The mutated pYV plasmid was called pSW2276.

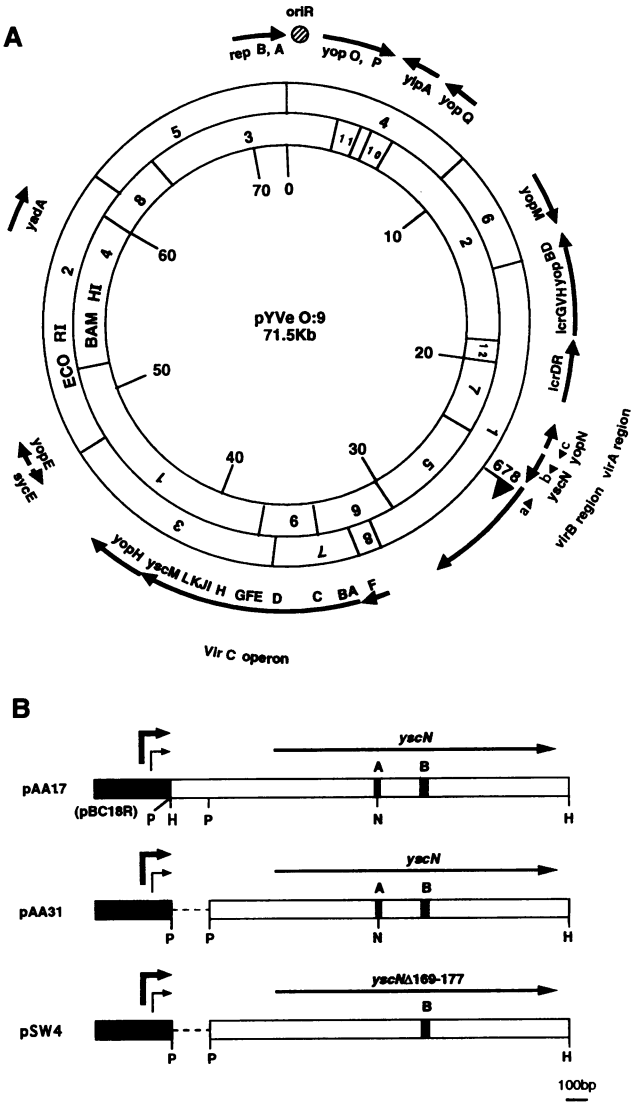

FIG. 1. (A) Genetic map of the pYV227 plasmid integrating previous data (for a review, see reference 12). Note that *lcrE* corresponds to *yopN* (20, 61). *yscN* encodes the putative energizer of the Yop secretion system described in this study. The flag indicates the position and orientation of the mini-Mu dI 1734 insertion in pGC678 (14). The arrowheads indicate the locations and orientations of primers MIPA 117 (a), MIPA 116 (b), and MIPA 118 (c). (B) Structure of plasmids carrying *yscN* and *yscNΔ169-177*. *Y. enterocolitica* DNA is indicated by an open bar, and the vector (pBC18R) DNA (not shown to scale) is shown by a shaded bar. H, *Hin*dIII; P, *Pst*I; N, *Not*I. Boxes A and B represent the nucleotide sequences encoding the corresponding Walker nucleotide-binding motifs. The dashed lines indicate deletions. The thick arrows represent the *lac* promoter, and the thin arrows correspond to the Φ10 promoter of bacteriophage T7.

Induction of the *yop* regulon, analysis of the Yops, Ca^{2+} dependency, and genetic conjugation. Yop production was analyzed as described by Wattiau and Cornelis (66). The Yops were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) as described by Cornelis et al. (15) and by Sory and Cornelis (53). For the analysis of the intracellular proteins, $7 \cdot 10^{10}$ bacteria were disrupted by son-

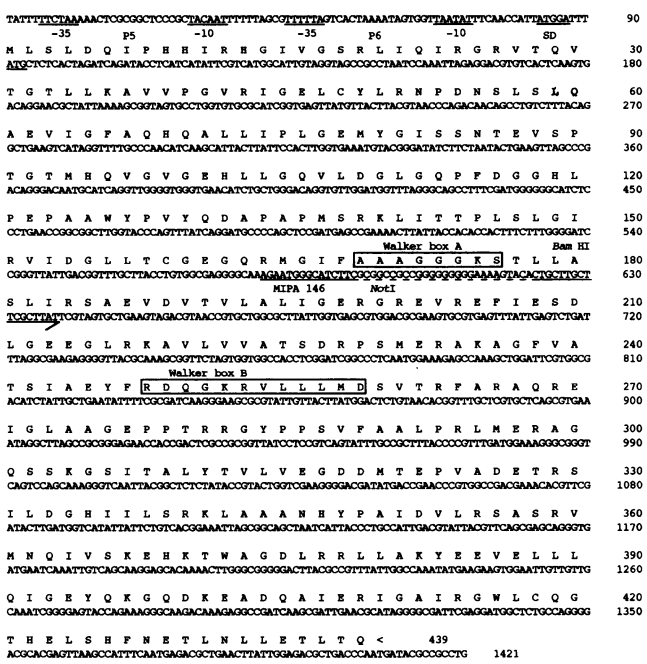

FIG. 2. Nucleotide sequence of *yscN* and deduced amino acid sequence of YscN. P5 and P6 are two hypothetical promoters identified by Viitanen et al. (61). SD indicates a putative Shine-Dalgarno sequence. The nucleotide-binding motifs A and B described by Walker et al. (65) are shown boxed. Important restriction endonuclease cleavage sites are shown. The half-arrow underlines the nucleotide sequence of primer MIPA 146, which was used to mutate the *yscN* gene by deletion of the sequence encoding the Walker box A (dashed line).

ication in 800 μl of phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl [pH 7.4]). Undisrupted cells were removed by three rapid centrifugations at 10,000 × *g*. Membranes were then removed by a 30-min centrifugation at 10,000 × *g*. An aliquot of the soluble fraction, containing the cytoplasmic and periplasmic proteins of $5 \cdot 10^9$ bacteria, was applied on an SDS-PAGE (14% polyacrylamide) gel.

Rat monoclonal antibodies 6G1 (anti-YopE) and 13A4 (anti-YopD) are described by Bodeus et al. (5).

The Ca^{2+} requirement for growth at 37°C was monitored by plating the bacteria on TSA supplemented with 5 mM $CaCl_2$ and on MOX agar at 37°C.

To introduce a plasmid in *Y. enterocolitica* by conjugation, the plasmid was first introduced in *E. coli* SM10 λ *pir*$^+$ by electroporation. This donor strain and the recipient *Y. enterocolitica* were then mated overnight on TSA at 32°C. The selective agents were ampicillin at 300 μg ml^{-1} and nalidixic acid.

Identification of the *yscN* gene product. Exclusive labeling of YscN was done in *E. coli* by using the T7 expression system (57). *E. coli* LK111 containing both pGP1-2 and pBC18R, pAA31, or pSW4 was grown in 10 ml of TSB with ampicillin and kanamycin. At an optical density at 600 nm of 0.5, 1 ml of culture was centrifuged and bacteria were washed in 1 ml of M9 medium. Cells were then resuspended in 5 ml of M9 supplemented with thiamine at 20 μg ml^{-1}; 19 amino acids (minus methionine), each at a final concentration of 100 μg ml^{-1}; ampicillin at 200 μg ml^{-1}; and kanamycin and incubated with shaking at 28°C in a 100-ml conical flask for 180 min. The temperature was then shifted to 42°C for 15 min. Rifampin (Lepetit, Milan, Italy) was subsequently added to a final concentration of 400 μg ml^{-1}. The incubation temperature was maintained at 42°C for an additional 10 min and afterwards shifted down to 37°C for 20 min. The samples were then labeled with 20 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Brussels, Belgium) for 25 min at 37°C, and the cells were finally centrifuged, dissolved in sample buffer, and loaded on an SDS-PAGE (14% polyacrylamide) gel.

Sequence analysis. Identity scores between proteins were calculated by using the FastA program according to the method of Pearson and Lipman (41), with a ktuple value of 1. The multiple alignments were made by using the MULTALIN program with the Dayhoff matrix (16). The isoelectric point was calculated by using the GCG sequence analysis software package (Genetics Computer Group, University of Wisconsin, Madison) computer program. The signal sequence was researched by using the SIGSEQ program (45) according to the rules of von Heijne (63).

Nucleotide sequence accession number. The sequence shown in Fig. 2 will appear in GenBank under accession no. U02499.

RESULTS

Identification of a gene encoding a putative energizer. Since all the genes involved in Yop secretion that have been described so far are located on the pYV plasmid (*virC* and *virA* loci), we assumed that the energizer of the system should also be encoded by the pYV plasmid. We examined the available sequences of the *virA* and *virB* loci for the presence of an open reading frame (ORF) encoding a protein resembling a known ATPase or ATP-binding protein. ORF6, localized at the beginning of *virB* (*lcrB*) and described by Viitanen et al. (61), encodes a truncated peptide similar to a domain of FliI of *S. typhimurium*, a recently described ATP-binding protein (18). We thus considered that ORF6 could encode the amino-terminal domain of the putative Ysc ATPase. We called the gene encoding this putative ATPase *yscN*.

Cloning of *yscN*. Since only the proximal sequence of *virB* was available, we decided to amplify *yscN* from pGC678, a pYV plasmid carrying a mini-Mu dI *lac* insertion downstream from *yscN* (14) (Fig. 1A). This would allow amplification of the stretch of DNA encompassed between the known terminus of Mu (26) and the beginning of ORF6 sequenced by Viitanen et al. (61). Surprisingly, we noticed that the right terminus of mini-Mu dI 1734 (10) contains less than 90 bp of the Mu *attR* site. Hence we selected an amplimer identical to the beginning of *attR*, but this primer (MIPA 117) presented some unavoidable homology with *attL*. An *Eco*RI restriction site was added to the 5′ extremity of the downstream amplimer. To ensure that the amplified fragment was the expected one, we designed two different upstream amplimers. MIPA 116 was complementary to *yscN*, and MIPA 118 was complementary to a sequence of *lcrE*, the gene located immediately upstream from *yscN* (61). The pYV sequences complementary to MIPA 116 and MIPA 118 are 610 bp distant from each other. Both amplimers contained a *Sal*I restriction site at their 5′ end. The amplification reactions carried out with MIPA 117 plus MIPA 116 and with MIPA 117 plus MIPA 118 gave several products. Some products of the two reactions were of the same size and hence did not correspond to *yscN*. Two products differed by about 600 bp and could thus correspond to *yscN*. The larger band was eluted, labeled, and used to probe the total pYV plasmid digested with *Bam*HI. Only *Bam*HI fragments 5 and 7 hybridized with the probe, which confirmed that this amplified fragment was the expected one.

The PCR fragment was cloned in the *Eco*RI-*Sal*I sites of pBC18R, giving pSW2. One strand of the amplified fragment was then sequenced, and a *Hin*dIII site was identified downstream from *yscN*. According to the restriction map of Straley and Bowmer (55), this site is conserved in the pYV plasmid of *Y. pestis* KIM5(pCD1).

In order to avoid any sequence mistake due to PCR amplification, we decided to isolate and clone the native *yscN* gene. A 1.9-kb fragment, encompassed by this newly identified *Hin*dIII site and the *Hin*dIII site previously identified by Viitanen et al. (61) in *lcrE*, was extracted from the pYV plasmid and cloned in both directions in pBC18R, giving pAA16 and pAA17. In pAA17, *yscN* is situated downstream from both the Φ10 promoter of bacteriophage T7 and the *lac* promoter (Fig. 1B).

Sequence analysis of *yscN*. One strand of the amplified *yscN* and both strands of the native *yscN* carried by plasmids pAA16 and pAA17 were sequenced. The sequence of the amplified fragment differed from the native sequence at three positions. The sequence of the native DNA, given in Fig. 2, contains a single ORF starting with ORF6 described by Viitanen et al. (61) and extending to codon 439. The calculated molecular mass of the protein is 47.8 kDa. Its isoelectric point is predicted to be 6.7. According to the von Heijne algorithm (63), no classical N-terminal signal sequence is present in this putative YscN protein. As already pointed out by Viitanen et al. (61), a nearly canonical Shine-Dalgarno sequence precedes the start codon but the spacing (3 bp) is rather short. The two potential promoters (P5 and P6) identified by Viitanen et al. (61) are perfectly conserved.

Visualization of YscN. In order to visualize the predicted 47.8-kDa YscN protein, we used pAA31, a plasmid in which *yscN* is cloned downstream from the promoter Φ10 from bacteriophage T7 (Fig. 1B). As expected, the induction of the T7 RNA polymerase led to the exclusive labeling of β-lactamase and a protein of about 48 kDa encoded by the cloned gene (see Fig. 4). Another band was visible just below the former one. According to its molecular weight, this band could result from a second initiation at codon 30 (GUG), but it could also represent a degradation product of YscN.

Similarity analysis of YscN. YscN is 60% identical to HrpB6 from *X. campestris* (19) and 57% identical to HrpE from *P. solanacearum* (58), two proteins involved in plant pathogenesis and in the induction of the so-called hypersensitive reaction. YscN is 43% identical to Spa47 from *S. flexneri*, a component of the machinery involved in the transport of the Ipa proteins or in their presentation at the cell surface (60). YscN is also 52% identical to the known part of SpaL, the *S. typhimurium* counterpart of Spa47 (27). As expected from our early observations, YscN is related to FliI, a component of the flagellar assembly apparatus from *Bacillus subtilis* (ORF4 of the *flaA* locus) (49% identity) (1) and from *S. typhimurium* (43% identity) (62).

Finally, YscN was found to have 29% identity to the β-subunit and 25% identity to the α-subunit of the bacterial F_0F_1 proton-translocating ATPase (64). This membrane-bound F_0F_1 ATPase of *E. coli* is composed of F_1, a water-soluble component (α_3, β_3, γ, δ, and ε), and F_0, a membrane-embedded component (a, b_2, and c_{10}). It couples the synthesis of ATP by F_1 with proton flux through F_0 (reviewed in reference 51). The catalytic sites of F_1 are present on the β-subunits. YscN also has some similarity to the catalytic subunits of the related eukaryotic vacuolar ATPases (29% for *Neurospora crassa*) (6) and archaebacterial ATPases (35% for *Methanosarcina barkeri*) (31).

The amino acid sequences of several nucleotide-binding proteins, including the F_1 β-subunit, are conserved in two particular regions of the proteins, approximately 100 amino acids distant from each other (65). According to the X-ray crystallographic analysis of the adenylate kinase, these two regions form structural elements within the nucleotide-binding domains (23). The first conserved region, called Walker box A, consists of the sequence GXXXXGKT/S (65) (for a recent review, see reference 42), where X is commonly a small amino acid residue. According to the X-ray analysis of crystals of four nucleotide-binding proteins, box A forms a phosphate-binding loop (54). Genetic studies suggest that this glycine-rich sequence forms part of the catalytic site of the F_1 β-subunit (reviewed in reference 51) (Fig. 3), in which the lysine residue could bind the γ-phosphate of ATP (52). The second conserved region, called Walker box B, consists of RX_nh_4D (42, 65), a sequence of hydrophobic residues followed by an aspartate which is required for the catalytic activity and thought to be involved in Mg^{2+} binding (54).

The alignments between YscN and the F_1 β-subunit, FliI, HrpE, HrpB6, and Spa47 are presented in Fig. 3. As expected, YscN shares the nucleotide-binding boxes A and B with its homologs. As shown in Fig. 2, a *Not*I restriction site is partially included in the sequence encoding box A.

Loop-directed mutagenesis of *yscN*. In order to demonstrate the involvement of this putative ATPase in the secretion of the Yops, we engineered a mutation encoding a protein deprived of Walker box A (amino acids 169 to 177). We first carried out loop-directed mutagenesis of plasmid pAA31, a high-copy-number plasmid in which *yscN* is placed under the control of the *lac* promoter (Fig. 1B). The mutation was monitored by the loss of the *Not*I restriction site and by sequence analysis. The integrity of the modified *yscN* reading frame, called *yscNΔ169-177*, was checked by SDS-PAGE after overexpression by the T7 RNA polymerase (Fig. 4). Finally, the DNA fragments flanking *yscN* were also checked by sequencing. The plasmid encoding the modified YscN protein ($YscN^{\Delta 169\text{-}177}$) was called pSW4 (Fig. 1B). The *Sal*I-*Pvu*II fragment of pSW4 was extracted and cloned into the suicide vector pKNG101, containing *sacBR* genes for selection purposes. The resultant plasmid, called pSW6, was crossed into the ampicillin-sensitive *Y. enterocolitica* KNG22703. The integration of pSW6 in the pYV plasmid by a single crossover led to the appearance of the merodiploid *yscN-yscNΔ169-177*. Excision of the suicide vector with the intact copy of *yscN* was selected on sucrose and monitored by *Not*I restriction and Southern blot analysis (Fig. 5). The engineered pYV plasmid encoding $YscN^{\Delta 169\text{-}177}$ was called pSW2276.

We compared the Ca^{2+} requirements and the Yop secretion capacities of KNG22703(pYV227) and KNG22703(pSW2276). The *yscN* mutant appeared to be independent of Ca^{2+} for growth at 37°C and did not secrete any Yop (Fig. 6A), a phenotype resembling that of the *virF* mutants. We then monitored the presence of YopD and YopE among the intracellular proteins by an immunoblot using monoclonal antibodies. We analyzed in parallel the wild-type KNG22703 (pYV227), the *virF* mutant W22703(pGC1152-9), the polar *yscC* mutant W22703(pGC445), and the *yscN* mutant KNG 22703(pSW2276). As expected from our previous work (35, 38), YopD and YopE could be detected in the extracts from the wild type and from the *virC* mutant but they could not be detected in the *virF* mutant (Fig. 6B). The extract of the engineered *yscN* mutant did contain YopD and YopE, but signals were weaker than for the extracts of the wild type and the *virC* mutant (Fig. 6B). Surprisingly, YopD seemed to be present in smaller amounts than YopE. We inferred from

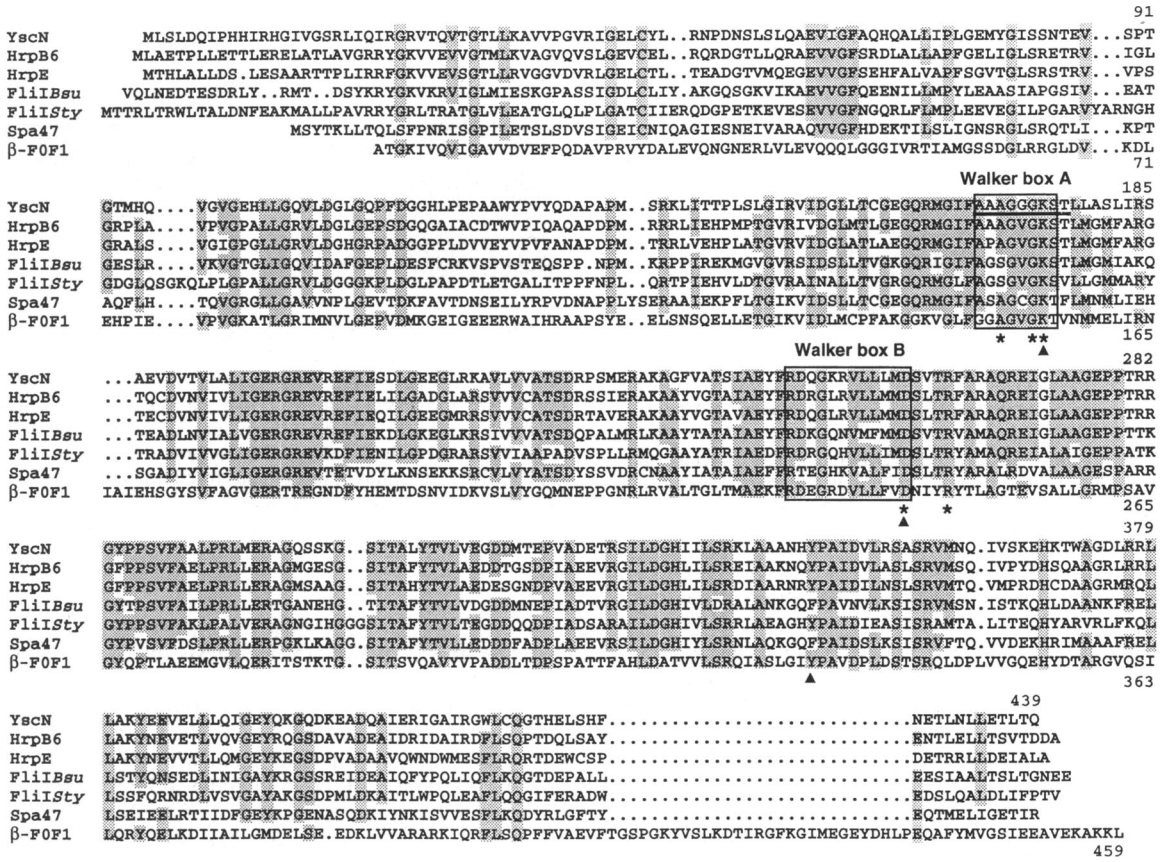

FIG. 3. Amino acid sequence alignment of YscN, HrpB6 (*X. campestris*) (19), HrpE (*P. solanacearum*) (58), FliI (*S. typhimurium*) (FliI*Sty*) (62), FliI (*B. subtilis*) (FliI*Bsu*) (1), Spa47 (*S. flexneri*) (60), and the *E. coli* F_1 β-subunit (β-F0F1) (64). The alignment was done by using the MULTALIN program (16) with a gap penalty of 8. Residues conserved in at least five proteins are shaded. Nucleotide-binding motifs A and B described by Walker et al. (65) are shown boxed. Asterisks indicate mutation sites in the β-subunit of F_0F_1 that impair catalysis (reviewed in reference 51). Triangles indicate mutation sites in FliI of *S. typhimurium* that result in loss of flagellation (18). The sequence underlined in YscN (amino acids 169 to 177) and containing Walker box A has been deleted in $YscN^{\Delta 169\text{-}177}$

these observations that the *yscN* mutant was impaired in secretion.

Complementation of pSW2276. In order to confirm that the phenotype of KNG22703(pSW2276) was due only to the *yscNΔ169-177* mutation and not to a polar effect on another gene, we tried to complement the mutation with plasmid pAA31. As shown in Fig. 6, the introduction of pAA31 in KNG22703(pSW2276) restored the secretion of the Yops. The cloned *yscN* gene thus complemented the lack of secretion of the Yops, which allowed us to conclude that YscN is indeed required for Yop secretion. This observation also confirmed that Walker box A is a key element in the catalytic activity of YscN. The recombinant strain grew slowly at 37°C; thus, we could not study the dependence on Ca^{2+} for growth at this temperature. This phenotype was not too surprising. Indeed, for some unknown reason, strain KNG22703(pYV227) harboring the multicopy plasmid pBC18R, the vector used to construct pAA31, also grew poorly at 37°C.

We wondered whether the overproduction of the modified YscN protein would alter the secretion machinery in a wild-type strain. We thus introduced the multicopy plasmid pSW4 carrying the *yscNΔ169-177* mutation into KNG22703 and monitored the Yop production. The introduction of pSW4 did not impair Yop secretion (data not shown), indicating the absence of negative complementation.

DISCUSSION

The Yop secretion process has been shown to involve the *virC* operon (*yscA-yscM*) and *lcrD*, a gene of the *virA* locus. Neither LcrD nor any of the 13 *ysc* gene products possesses the sequence signature of a putative ATPase. This is surprising, since all the known export systems seem to use at least ATP as an energy source. SecA is the ATPase feeding the classical Sec-dependent pathway which translocates proteins across the cytoplasmic membrane (36). In the system secreting pullulanase, the energy required to cross the outer membrane could be supplied by PulE, which contains the two Walker boxes (46). In the hemolysin secretion system, the HlyB transporter protein, which belongs to the ATP-binding cassette superfamily of transporters (30), has been shown to hydrolyze ATP (33). We thus assumed that the inventory of the genes involved in Yop secretion was not yet complete. This working hypothesis was supported by the fact that several genes of the *mxi-spa* cluster of *S. flexneri* and of the *hrp* region of *X. campestris* or *P. solanacearum* had no counterpart yet among pYV genes suspected to be involved in secretion. Among these, *spa47* and *hrpB6* or *hrpE* encode a protein with a putative ATP-binding domain. We thus searched for such a gene in the *vir* region of *Y. enterocolitica*, and we found *yscN*. The similarity between

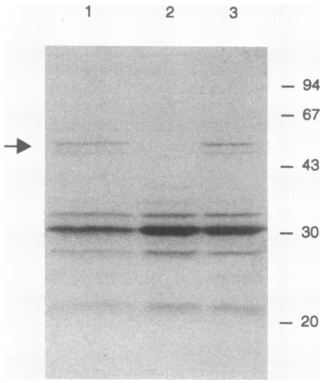

FIG. 4. Expression of the wild-type *yscN* and mutant *yscNΔ169-177* genes with T7 RNA polymerase. Lanes: 1, *E. coli* LK111(pAA31)(pGP1-2); 2, *E. coli* LK111(pBC18R)(pGP1-2); 3, *E. coli* LK111(pSW4)(pGP1-2). The positions of molecular size markers (in kilodaltons) are indicated. The arrow points to YscN and $YscN^{\Delta 169-177}$.

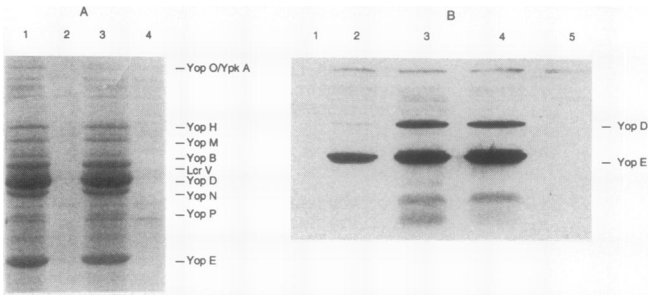

FIG. 6. Analysis of Yop expression. (A) SDS-PAGE of the Yops secreted by *Y. enterocolitica* KNG22703 harboring wild-type pYV227 (lane 1), pSW2276 (lane 2), pSW2276 and pAA31 (lane 3), and pSW2276 and pSW4 (lane 4). (B) Western blot analysis of YopD and YopE. Lanes: 1, culture supernatant of *yscN* mutant KNG22703 (pSW2276); 2, soluble subcellular fraction of *yscN* mutant KNG22703 (pSW2276); 3, soluble subcellular fraction of wild-type KNG22703 (pYV227); 4, soluble subcellular fraction of *virC* mutant W22703 (pGC445); 5, soluble subcellular fraction of *virF* mutant W22703 (pGC1152-9).

YscN, HrpB6, HrpE, and Spa47 suggested that it belongs to the secretion pathway.

In order to prove the involvement of YscN in the process of secretion and to investigate its possible role as an energizer, we engineered a mutant producing a protein altered in the putative ATP-binding domain. This mutant was not polar, and, as expected, it did not secrete the Yops but synthesized at least YopE and YopD. This phenotype resembles that observed with *virC* secretion mutants, but it is slightly different: the *yscN* mutant contains less cytoplasmic YopE and even less YopD than does the *virC* mutant. The reason for this reduction of YopD is unknown. We nevertheless conclude that the main deficiency is secretion, but we hypothesize that YscN may also be involved in a feedback inhibition mechanism preventing active synthesis of the Yops when secretion is compromised. We already postulated the existence of such a mechanism some years ago (15). It could also apply to the synthesis of the Yop secretion apparatus.

The analysis of our site-specific mutant provides the first demonstration of the role of an ATP-binding motif in a type III secretion system. The similarity between YscN and the catalytic β-subunit of F_0F_1 (51) suggests that YscN could work as an ATPase.

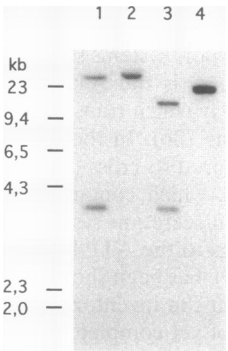

FIG. 5. Southern blot analysis of wild-type pYV and mutant pSW2276 plasmids. Lanes: 1, pYV digested with *Not*I; 2, pSW2276 digested with *Not*I; 3, pYV digested with *Not*I and *Sma*I; 4, pSW2276 digested with *Not*I and *Sma*I. Molecular size markers (in kilobases) are phage λ DNA digested with *Hin*dIII. The probe consisted of the *Pvu*II-*Sal*I insert of pSW4. Note that the *yscNΔ169-177* mutation removes the *Not*I restriction site.

We wanted to complete our study by demonstrating the binding of radioactive ATP to YscN. Unfortunately, we could not carry out such an experiment with crude extracts because of the existence of housekeeping ATPases with molecular weights in the same range as that of YscN. Even the use of an *E. coli unc* mutant lacking the F_0F_1 proton-translocating ATPase did not circumvent the difficulty. The experiment will thus have to await the purification of YscN.

The similarity analysis indicated that YscN is very close to HrpB6, HrpE, Spa47, and SpaL, suggesting that these components of class III secretion systems constitute a new family of ATPases. Alignments also suggest that this family includes ATPases involved in the secretion or assembly of flagella in *B. subtilis* (1) and *S. typhimurium* (18, 62). The similarity between those two systems is not restricted to ATPases, since LcrD (43) is related to FlhA of *B. subtilis* (9). As a hallmark, all the members of this new family of ATPases have an alanine instead of the first glycine in Walker motif A and most of them conserve important catalytic residues of the F_1 β-subunit (reviewed in reference 51) (Fig. 3). Some of these residues, namely, Lys-155 and Asp-242 of the *E. coli* F_1 β-subunit, have been shown to be essential in the function of FliI from *S. typhimurium* (18) (Fig. 3).

In *S. flexneri* and *S. typhimurium*, *spa47* and *spaL*, respectively, are localized at the 5′ end of homologous loci consisting of eight genes involved in the transport or presentation of invasion antigens. Since these loci have the same size as the *virB* locus, one can hypothesize that *virB* is made of related genes involved in Yop secretion.

The site-directed mutation on the pYV plasmid could be complemented in *trans*, and the introduction of multicopy plasmid pSW4 carrying the mutated *yscN* gene did not impair secretion. This lack of negative complementation was somehow surprising because ATPases involved in secretion, such as HlyB (33) and SecA (8), as well as the *E. coli* F_1 β-subunit (22), are known or presumed to form dimers or multimers. It is also surprising because YscN would be expected to interact with other components of the secretion machinery. This lack of negative complementation has, however, a precedent: site-directed mutants of FliI can also be complemented (18). This

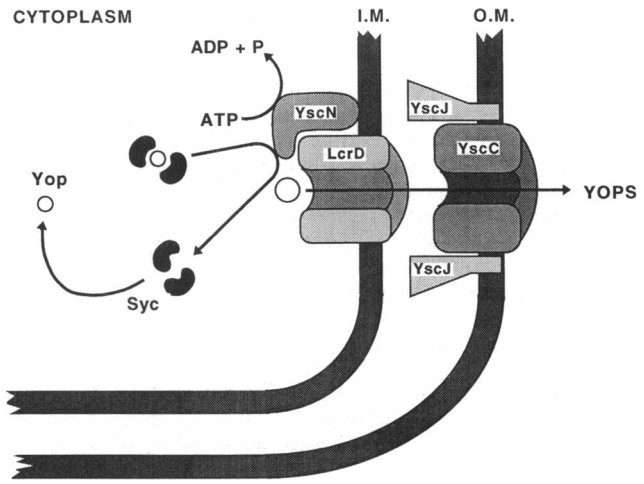

FIG. 7. Tentative model for Yop secretion. Protein YscC and lipoprotein YscJ are presumed to be located in the outer membrane (O.M.) (38). LcrD is an inner membrane (I.M.) protein (43). We hypothesize that YscN is cytoplasmic but connected to the inner membrane, possibly through LcrD. The Syc chaperones would lead the Yops to the translocon by contacting YscN.

observation suggests either that YscN does not form dimers or that heterodimers made of only one functional ATPase can fulfill the function. The lack of negative complementation could also be explained by the fact that hydrolysis of ATP could be required for the interaction with the other components of the system.

The information is still insufficient to draw a general model. Either YscN is associated with a proton channel like the β-subunit of F_0F_1 or, like SecA, it is independent of such a channel. YscN could supply the energy, either for the secretion of the Yops or for the assembly of the secretion apparatus itself. We favor the first hypothesis, and we would consider YscN as the functional counterpart of SecA. To demonstrate this, it will be necessary to localize YscN in the bacterial cell and to identify the other components of the system which contact YscN. It is tempting to speculate that YscN interacts with the inner membrane protein LcrD. It is also tempting to speculate that YscN recognizes the Yops with their Syc chaperones in the way that SecA binds the SecB-preprotein complex (28). This hypothesis is presently being tested. Figure 7 gives a simple tentative model of the role of YscN in Yop secretion.

ACKNOWLEDGMENTS

We thank Jean-Claude Vanooteghem for assistance in computing and Frederique Van Ghijsegem and Christian Boucher (INRA, Toulouse, France) for constant exchange of information during the progress of this work. We thank Alvydas Mikulskis, Ralf Schulte, and M.-P. Sory for critical reading of the manuscript and the ICP illustration service for the artwork.

A.A. is the recipient of a fellowship from the ICP, and P.W. was the recipient of a scholarship from the Belgian IRSIA (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture). 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, Science Policy Programming.

REFERENCES

1. **Albertini, A. M., T. Caramori, W. D. Crabb, F. Scoffone, and A. Galizzi.** 1991. The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. **173**:3573–3579.
2. **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.
3. **Allaoui, A., P. J. Sansonetti, and C. Parsot.** 1993. MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins. Mol. Microbiol. **7**:59–68.
4. **Andrews, G. P., and A. T. Maurelli.** 1992. *mxiA* of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium-response protein, LcrD, of *Yersinia pestis*. Infect. Immun. **60**:3287–3295.
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. **Bowman, E. J., K. Tenney, and B. J. Bowman.** 1988. Isolation of genes encoding the *Neurospora* vacuolar ATPase. J. Biol. Chem. **263**:13994–14001.
7. **Brubaker, R. R.** 1991. Factors promoting acute and chronic diseases caused by yersiniae. Clin. Microbiol. Rev. **4**:309–324.
8. **Cabelli, R. J., K. M. Dolan, L. Qian, and D. B. Oliver.** 1991. Characterization of membrane-associated and soluble states of SecA protein from wild-type and *SecA51(TS)* mutant strains of *Escherichia coli*. J. Biol. Chem. **266**:24420–24427.
9. **Carpenter, P. B., and G. W. Ordal.** 1993. *Bacillus subtilis* FlhA: a flagellar protein related to a new family of signal transducing receptors. Mol. Microbiol. **7**:735–743.
10. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
11. **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.
12. **Cornelis, G. R.** 1992. Yersiniae, finely tuned pathogens, p. 231–265. *In* C. E. Hormacche, C. W. Penn, and C. J. Smyth (ed.), Molecular biology of bacterial infection. Cambridge University Press, Cambridge.
13. **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.
14. **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 insertion and *lac* gene fusions. Microb. Pathog. **1**:349–359.
15. **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.
16. **Corpet, F.** 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**:10881–10890.
17. **de Cock, H., and J. Tommassen.** 1992. SecB-binding does not maintain the translocation-competent state of prePhoE. Mol. Microbiol. **6**:599–604.
18. **Dreyfus, G., A. W. Williams, I. Kawagishi, and R. M. Macnab.** 1993. Genetic and biochemical analysis of *Salmonella typhimurium* FliI, a flagellar protein related to the catalytic subunit of the F_0F_1 ATPase and to virulence proteins of mammalian and plant pathogens. J. Bacteriol. **175**:3131–3138.
19. **Fenselau, S., I. Balbo, and U. Bonas.** 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. **5**:390–396.
20. **Forsberg, Å., A.-M. Viitanen, M. Skurnik, and H. Wolf-Watz.** 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol. Microbiol. **5**:977–986.

21. **Forsberg, Å., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at the transcriptional level by plasmid-pIB1-encoded *trans*-acting elements controlled by temperature and calcium. Mol. Microbiol. **2:**121–133.
22. **Foster, D. L., and R. H. Fillingame.** 1982. Stoichiometry of subunits in the H^+-ATPase complex of *Escherichia coli*. J. Biol. Chem. **257:**2009–2015.
23. **Fry, D. C., S. A. Kuby, and A. S. Mildvan.** 1986. ATP-binding site of adenylate kinase: mechanistic implications of its homology with *ras*-encoded p21, F_1-ATPase, and other nucleotide-binding proteins. Proc. Natl. Acad. Sci. USA **83:**907–911.
24. **Galan, J. E., C. Ginocchio, and P. Costeas.** 1992. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174:**4338–4349.
25. **Gough, C. L., S. Genin, V. Lopes, and C. A. Boucher.** 1993. Homology between the HrpO protein of *Pseudomonas solanacearum* and bacterial proteins implicated in a signal peptide-independent secretion mechanism. Mol. Gen. Genet. **239:**378–392.
26. **Groenen, M. A. M., E. Timmers, and P. van de Putte.** 1985. DNA sequences at the ends of the genome of bacteriophage Mu essential for transposition. Proc. Natl. Acad. Sci. USA **82:**2087–2091.
27. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. **12:**3779–3787.
28. **Hartl, F.-U., S. Lecker, E. Schiebel, J. P. Hendrick, and W. Wickner.** 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane. Cell **63:**269–279.
29. **Holland, I. B., B. Kenny, and M. Blight.** 1990. Haemolysin secretion from *E. coli*. Biochimie **72:**131–141.
30. **Hyde, S. C., P. Emsley, M. J. Hartshorn, M. M. Mimmack, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard, and C. F. Higgins.** 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature (London) **346:**362–365.
31. **Inatomi, K.-I., S. Eya, M. Maeda, and M. Futai.** 1989. Amino acid sequence of the α and β subunits of *Methanosarcina barkeri* ATPase deduced from cloned genes. J. Biol. Chem. **264:**10954–10959.
32. **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.
33. **Koronakis, V., C. Hughes, and E. Koronakis.** 1993. ATPase activity and ATP/ADP-induced conformational change in the soluble domain of the bacterial protein translocator HlyB. Mol. Microbiol. **8:**1163–1175.
34. **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.
35. **Lambert de Rouvroit, C., C. Sluiters, and G. R. Cornelis.** 1992. Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol. Microbiol. **6:**395–409.
36. **Lill, R., K. Cunningham, L. A. Brundage, K. Ito, D. Oliver, and W. Wickner.** 1989. SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. EMBO J. **8:**961–966.
37. **Michiels, T., and G. R. Cornelis.** 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173:**1677–1685.
38. **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.
39. **Michiels, T., P. Wattiau, R. Brasseur, J.-M. Ruysschaert, and G. Cornelis.** 1990. Secretion of Yop proteins by yersiniae. Infect. Immun. **58:**2840–2849.
40. **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.
41. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
42. **Pedersen, P. L., and L. M. Amzel.** 1993. ATP synthases. Structure, reaction center, mechanism, and regulation of one of nature's most unique machines. J. Biol. Chem. **268:**9937–9940.
43. **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.
44. **Plano, G. V., and S. C. Straley.** 1993. Multiple effects of *lcrD* mutations in *Yersinia pestis*. J. Bacteriol. **175:**3536–3545.
45. **Popowiecz, M., and P. F. Dash.** 1989. Prediction of signal sequence cleavage sites based on the method of G. von Heijne. The Rockefeller University, New York. (Program available by electronic mail [NETSER@EMBL.BITNET].)
46. **Possot, O., C. d'Enfert, I. Reyss, and A. P. Pugsley.** 1992. Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol. Microbiol. **6:**95–105.
47. **Pugsley, A. P., C. d'Enfert, I. Reyss, and M. G. Kornacker.** 1990. Genetics of extracellular protein secretion by Gram-negative bacteria. Annu. Rev. Genet. **24:**67–90.
48. **Reisner, B. S., and S. C. Straley.** 1992. *Yersinia pestis* YopM: thrombin binding and overexpression. Infect. Immun. **60:**5242–5252.
49. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
50. **Salmond, G. P. C., and P. J. Reeves.** 1993. Membrane traffic wardens and protein secretion in Gram-negative bacteria. Trends Biochem. Sci. **18:**7–12.
51. **Senior, A. E.** 1988. ATP synthesis by oxidative phosphorylation. Physiol. Rev. **68:**177–231.
52. **Senior, A. E., S. Wilke-Mounts, and M. K. Al-Shawi.** 1993. Lysine 155 in β-subunit is a catalytic residue of *Escherichia coli* F_1 ATPase. J. Biol. Chem. **268:**6989–6994.
53. **Sory, M.-P., and G. R. Cornelis.** 1988. *Yersinia enterocolotica* O:9 as a potential live oral carrier for protective antigens. Microb. Pathog. **4:**431–442.
54. **Story, R. M., and T. A. Steitz.** 1992. Structure of the recA protein-ADP complex. Nature (London) **355:**374–376.
55. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51:**445–454.
56. **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.
57. **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.
58. **Van Gijsegem, F., M. Arlat, S. Genin, C. L. Gough, C. Zischek, P. A. Barberis, and C. Boucher.** 1993. Genes governing the secretion of factors involved in host-bacteria interactions are conserved among animal and plant pathogenic bacteria, p. 627–640. *In* C. I. Kado and J. H. Crosa (ed.), Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Amsterdam.
59. **Van Gijsegem, F., S. Genin, and C. Boucher.** 1993. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol. **1:**175–180.
60. **Venkatesan, M. M., J. M. Buysse, and E. V. Oaks.** 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. J. Bacteriol. **174:**1990–2001.
61. **Viitanen, A.-M., P. Toivanen, and M. Skurnik.** 1990. The *lcrE* gene is part of an operon in the *lcr* region of *Yersinia enterocolitica* O:3. J. Bacteriol. **172:**3152–3162.
62. **Vogler, A. P., M. Homma, V. M. Irikura, and R. M. Macnab.** 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and

archaebacterial ATPase subunits. J. Bacteriol. **173:**3564–3572.
63. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
64. **Walker, J. E., M. Saraste, and N. J. Gay.** 1984. The *unc* operon nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta **768:**164–200.
65. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
66. **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.
67. **Xiao, Y., Y. Lu, S. Heu, and S. W. Hutcheson.** 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. syringae 61 *hrp* cluster. J. Bacteriol. **174:**1734–1741.