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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*

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Virulent bacteria of the genus *Yersinia* secrete a number of virulence determinants called Yops. These proteins lack typical signal sequences and are not posttranslationally processed. Two gene loci have been identified as being involved in the specific Yop secretion system (G. Cornelis, p. 231–265, *In* C. E. Hormache, C. W. Penn, and C. J. Smythe, ed., *Molecular Biology of Bacterial Infection*, 1992; S. C. Straley, G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields, Mol. Microbiol. 8:1005–1010, 1993). Here, we have shown that the *lcrB/virB* locus (*yscN* to *yscU*) encodes gene products essential for Yop secretion. As in previously described secretion apparatus mutants, expression of the Yop proteins was decreased in the *yscN/U* mutants. An *lcrH yscR* double mutant expressed the Yops at an increased level but did not secrete Yops into the culture supernatant. The block in Yop expression of the *ysc* mutants was also circumvented by overexpression of the activator LcrF in *trans*. Although the Yops were expressed in elevated amounts, the Yops were still not exported. This analysis showed that the *ysc* mutants were unable to secrete Yops and that they were also affected in the negative Ca^{2+}-regulated loop. The *yscN/U* genes showed remarkably high homology to the *spa* genes of *Shigella flexneri* and *Salmonella typhimurium* with respect to both individual genes and gene organization. These findings indicate that the genes originated from a common ancestor.

Pathogenic *Yersinia* spp. are able to express a class of secreted proteins, Yops, known to be important in the virulence process (for reviews, see references 10 and 54). The Yop proteins are encoded by a 70-kb plasmid common to virulent yersiniae (for reviews, see references 10, 19, 53, and 54). In addition, the plasmid harbors regulatory genes as well as genes involved in the secretion of the Yops. Two regulatory loops regulate Yop expression: one positive, temperature-controlled pathway and one negative pathway controlled by calcium (21). In response to an increase in temperature, an activator, VirF (LcrF), that activates the transcription of *yop* genes at 37°C is expressed (11, 28, 31). The VirF (LcrF) activity is counteracted by a negative element, which is either active (high Ca^{2+} [>1 mM]) or inactive (low Ca^{2+} [<0.1 mM]), depending on the level of Ca^{2+} in the growth medium (4, 20, 43). Maximal expression and subsequent secretion of Yops in vitro occur at 37°C in a medium devoid of Ca^{2+} (10, 53).

Yop secretion is also regulated by the concentration of Ca^{2+} in the culture medium. In media containing low levels of Ca^{2+}, the Yops are secreted, while at high Ca^{2+} concentrations, Yop secretion is blocked (10, 53). The Yops lack typical signal sequences and are not processed during transfer (21, 35); nevertheless, the Yops carry an undefined secretion signal at the N-terminal end of the protein (33). The *lcrDR* (5, 41, 42) and *yscA/M* (26, 34, 46) gene loci have been identified as being involved in Yop secretion. The LcrD protein is a 75-kDa cytoplasmic membrane-bound protein (41) that shows high homology to the flagellum-biosynthetic proteins of several different genera (8, 36, 44, 49; for a review, see reference 55), including MxiA of *Shigella flexneri* (3), *InvA* of *Salmonella typhimurium* (22), HrpC2 of *Xanthomonas campestris* (16), and HrpO of *Pseudomonas solanacearum* (24). All of these related proteins have been suggested to play an essential role in the surface presentation of proteins (55). The genes of the *yscA/M* operon of *Yersinia* spp. are also related to proteins involved in protein secretion in other bacterial genera (26, 34, 45, 55). The YscC protein shows homology to PulD of *Klebsiella pneumoniae* (13), MxiD of *S. flexneri* (2), and the Hrp proteins of different plant pathogens (55). *lcrD* mutants (5, 41, 42) as well as *yscA/M* mutants are defective in the secretion of Yops (26, 34, 46). In addition, they are also affected in the regulation of Yop expression. This double phenotype has hampered analysis of Yop secretion in yersiniae, making it difficult to show that the export process is indeed blocked in these mutants.

We show here that the *lcrB/virB* locus (*yscN/U*) is involved in Yop secretion. Mutants carrying mutations in these genes are also affected in the expression of Yops. By genetic analysis, we have been able to demonstrate that the mutants are unable to secrete the Yops and that they are also influenced in the negative Ca^{2+}-controlled pathway. The *ysc* genes show remarkable homology to genes of *S. flexneri* and *S. typhimurium* involved in surface presentation of molecules necessary for triggering of the invasive phenotype of these pathogens.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Yersinia pseudotuberculosis* YPIII (7) carrying the different plasmids indicated in Table 1 was used. The *Escherichia coli* strains used were DH5α (27) and SM10(λ*pir*) (37). The liquid growth medium for *Yersinia* strains consisted of brain heart infusion (BHI) broth (Oxoid; Unipath Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with either 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 20 mM $MgCl_2$ (BHI minus Ca^{2+}) or 2.5 mM $CaCl_2$ (BHI plus

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TABLE 1. Plasmids used

Plasmid	Description	Reference
pIB1	Wild-type virulence plasmid	7
pIB102	*yadA*::Tn*5*	21
pIB102F::LUX	Transcriptional fusion between *lcrF* and *luxAB* in pIB102	This study
pIB13	GeneBlock (kanamycin) insertion mutant of *lcrH*	4
pIB61	*yscT*::Tn*5* (Fig. 1)	21
pIB61F::LUX	Transcriptional fusion between *lcrF* and *luxAB* in pIB61	This study
pIB66	*yscR* polar mutant; pNQR1 integrated into the *yscR* gene of pIB102 (Fig. 1)	This study
pIB67	*yscR* in-frame deletion mutant (Fig. 1)	This study
pIB68	*yscS* in-frame deletion mutant (Fig. 1)	This study
pIB1366	*lcrH yscR* double mutant; pNQR1 integrated into the *yscR* gene of pIB13 at the same position as in pIB66	This study
pKE76	*Bam*HI fragment 7 and parts of fragment 6 of pIB1 cloned into pACYC184 (Fig. 1)	This study
pKE7	*Bam*HI fragment 7 of pIB1 cloned into pACYC184 (Fig. 1)	This study
pTB300	*Hin*dIII-*Xba*I fragment containing the *yscR* gene of pIB1 cloned into pACYC184 (Fig. 1)	This study
pACYC184	Cloning vector	9
pGP704	Suicide vector; Ap^r; based on the oriR6K replicon	37
pNQ705	Suicide vector; Cm^r; derivative of pGP704	39
pNQR1	*yscR* mutation vector; internal *yscR* fragment cloned into pNQ705	This study
pCH257	Suicide vector; derivative of pNQ705 containing the *luxAB* gene used to generate *luxAB* fusions	18

Ca^{2+}). The solid medium was blood agar base containing 20 mM sodium oxalate, 20 mM $MgCl_2$, and 0.2% glucose (MOX plates; Ca^{2+} free) or blood agar base supplemented with only 2.5 mM Ca^{2+}. *E. coli* strains were grown in Luria broth or on Luria agar.

DNA methods. Preparation of plasmid DNA, restriction enzyme digests, ligation, and transformation of *E. coli* were performed essentially as described by Sambrook et al. (48). Transformation of *Y. pseudotuberculosis* was performed as described previously (21).

DNA sequencing. DNA fragments encoding the *lcrB* locus were subcloned into the pBluescript SK vector (Stratagene, La Jolla, Calif.). These subclones were subjected to DNA sequencing by the dideoxy chain termination method (50). T7 DNA polymerase (Pharmacia AB, Uppsala, Sweden) was used for the sequencing reactions, with [α-^{35}S]dATP (Amersham Corp., Little Chalfont, United Kingdom) used as a label.

DNA and protein sequences were analyzed by using the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis software package (14) and PC Gene (Intelligenetics, Inc., Mountain View, Calif.) computer programs.

Analysis of Yop expression. *Yersinia* strains were grown in the media indicated above at 26°C to a cell density of approximately 2×10^8 cells per ml. The cultures were then shifted to 37°C and grown for an additional 3 h before being harvested. The secreted proteins were precipitated with 10% trichloroacetic acid and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels as described earlier (17). After SDS-PAGE, proteins in gels were electroblotted onto nitrocellulose membranes (Schleicher and Schuell), and immunoblotting analysis with a specific anti-Yop rabbit antiserum was carried out as described previously (17).

Determination and definition of phenotypes. The strains tested were diluted in a physiological concentration of NaCl. The serial dilutions were spread on agar plates containing 2.5 mM Ca^{2+} ions or deprived of Ca^{2+} ions (composition of plates is given above). The plates were then incubated at 26 or 37°C for about 40 h. The number of bacteria able to form colonies under the appropriate growth conditions was calculated, and the result was presented as a ratio of the titer of the cells growing at 37°C to that at 26°C.

Strains unable to grow at 37°C on MOX plates without the addition of Ca^{2+} are defined as calcium dependent (CD), which is the wild-type phenotype of *Yersinia* spp. Calcium-independent (CI) mutants are able to grow at 37°C irrespective of the Ca^{2+} concentration in the growth medium. Strains unable to grow at 37°C are defined as temperature sensitive (TS) for growth.

Construction of mutants. The YPIII(pIB66) polar *yscR* mutant was constructed by cloning a PCR-derived internal fragment of the *yscR* gene into the *Sac*I and *Sal*I sites of the suicide vector pNQ705 (39), generating the mutation vector pNQR1. The PCR primers used were 5′-TTCTTGAGCTCG AATATGCTGCTTAAGGAACAT-3′, containing an engineered *Sac*I site, and 5′-CTTCCATCGATTTCGGTAATG GCTAAATCGTTTG-3′, containing an engineered *Cla*I site. pNQR1 was subsequently introduced into YPIII(pIB102) and integrated into the *yscR* gene by homologous recombination as described earlier (45). This resulted in an integration of vector DNA in the *yscR* gene 340 bp downstream of the start codon, which leads to a disruption of the gene. The double *lcrH yscR* mutant YPIII(pIB1366) was constructed as above except that the *lcrH* mutant YPIII(pIB13) was used as the recipient strain.

The *yscR* in-frame deletion mutant YPIII(pIB67) was constructed as follows. A DNA fragment of the *yscR* gene carrying a 240-bp in-frame deletion of bp 341 to 580 was generated by PCR with a set of four specifically designed oligonucleotide primers. The first step involved the separate PCR amplification of two fragments of the *yscR* gene. Fragment one contained the 5′ end of the gene, bp 58 to 340, generated by PCR with primers 5′-CTTCCATCGATTTCGGTAATGGCTAAATCG TTTG-3′ and 5′-CCAACAAGATATATTTCTCAACAGAT TCTATGTTCGTG-3′. Fragment two contained the 3′ end of the gene, bp 581 to 105 downstream of the *yscR* gene, and was generated by PCR with primers 5′-CTGTTGAGAAACATA TCTTGTTGGCAATGGGGATG-3′ and 5′-ACCAGAGCTC CTACCACCGCAGCCACTAA-3′. These two fragments carried a 12-bp overlap which was complementary to the other fragment. In the second round of PCR, the two fragments were mixed together with the 5′-end primer and the 3′-end primer. This resulted in a *yscR* gene fragment deleted of 240 bp, which carried an engineered *Sac*I site at the 5′ end and a *Cla*I site at the 3′ end of the fragment. The fragment was subsequently cloned into the *Cla*I and *Sac*I sites of the suicide vector pNQ705. The resulting hybrid plasmid was then introduced into strain YPIII(pIB102) and integrated into the *yscR* gene by homologous recombination as described earlier (45). Bacteria that were found to carry the integrated plasmid were subjected to three rounds of ampicillin enrichment in the presence of chloramphenicol. This treatment selected bacteria that had lost the integrated plasmid and the chloramphenicol resistance conferred by pNQ705 by homologous recombination. PCR

analysis showed that several clones carried the deleted allele of the *yscR* gene. Sequencing of the *yscR* gene in one of these clones confirmed that it carried the in-frame deletion, and this mutant was denoted YPIII(pIB67).

Exactly the same strategy was used for the construction of the *yscS* mutant strain YPIII(pIB68), which carries a 120-bp in-frame deletion of bp 55 to 174 of the *yscS* gene. The PCR primers used were 5′-CGAGGAGCTCTTTACTTAACACT GG-3′, 5′-ACAGTGTGACCACTAGCACCAGCCATAAT GCCTG-3′, 5′-TCTCATCGATTTACCATTTATCGTCATT-3′, and 5′-GGCTGGTGCTAGTGGTCACACTGTTTGCTA CCGCC-3′.

Construction of *lcrF-luxAB* transcriptional fusion strains. A transcriptional fusion between *lcrF* and the promoterless *luxAB* genes, originating from *Vibrio harveyi* (40), was constructed as follows. A 139-bp DNA fragment containing nucleotides 1021 to 1160 of the *lcrF* sequence (11) was amplified with Amplitaq *Taq* polymerase (Cetus). The template was pKE7. The primers were 5′-GAAGCAGGGTTCTCGAGT CAGTC-3′ and 5′-AAAAAAAAGAGCTCATTTTAGCCT GTGGT-3′, corresponding to nucleotides 1021 to 1061 and 1131 to 1160, respectively, and they contained *Xba*I and *Sac*I sites to facilitate subsequent cloning. The amplified DNA fragment was cloned into the *Xba*I and *Sac*I sites of the suicide plasmid pCH257 (18) to generate an *lcrF-luxAB* (pNQF::LUX) fusion.

To integrate *luxAB* into the virulence plasmid, the *E. coli* strain SM10(λ*pir*) containing plasmid pNQF::LUX was conjugated with either YPIII(pIB102) or YPIII(pIB61). The offspring were selected on LA plates containing kanamycin and chloramphenicol. Since plasmid pNQF::LUX is dependent on the λ Pir protein for replication, this plasmid cannot replicate in *Y. pseudotuberculosis*; therefore, for pNQF::LUX to be maintained in this strain, it must integrate into the genome of the host cell. This integration occurs most frequently by a single homologous recombination event, generating an operon fusion. The *luxAB* gene was inserted 60 bp downstream of the *lcrF* stop codon. Successful integration of pNQF::LUX was confirmed by PCR analysis. The first amplimer was derived from the *lcrF* upstream region between positions 313 and 336 (5′-GACAGTATAACATTTATGGCATC-3′), far outside of the presumed duplicated region (11). The second amplimer was within the *luxAB* gene, corresponding to the sequence from codon 2 to the first nucleotide of codon 19 (5′-CGGT CTGAGATAGCTCAGGTGGCTG-3′) (40). The constructed strains were named YPIII(pIB102F::LUX) and YPIII (pIB61F::LUX), respectively.

Measurement of luciferase activity. YPIII(pIB102F::LUX) and YPIII(pIB61F::LUX) were grown at 26°C for 60 min in BHI medium containing 2.5 mM Ca^{2+}. The cultures were then shifted to 37°C and grown for an additional 90 min. Samples were taken out at different time points. Samples (10 to 100 μl) of the cultures were placed directly in a luminometer (LKB 1250). *n*-Decanal substrate (Sigma D-7384), as a sonicated 1:1,000 dilution in water, was then injected into the luminometer. The total light produced during the first 10 s after injection of the substrate was recorded. The recorded in vivo activity is given as light units per milliliter of cells at an optical density at 495 nm (OD_{495}) of 1.0 (40).

Nucleotide sequence accession number. The nucleotide sequence of the *yscN/U* genes of *Y. pseudotuberculosis* strain YPIII(pIB1) has been submitted to the EMBL data library. The *yscN* gene was assigned accession number L23522, and the *yscO*, *P*, *Q*, *R*, *S*, *T*, and *U* genes were assigned accession number L25667.

RESULTS

Conservation of genes of *Yersinia* and *Salmonella/Shigella spa* loci. Earlier studies had shown that mutants carrying mutations in the *lcrB/virB* loci of the virulence plasmid of *Yersinia* spp. were unable to express the Yops, and these mutants showed a calcium-independent (CI) phenotype (21, 23, 34). When this region of *Y. pseudotuberculosis* was sequenced and analyzed for the presence of open reading frames, we found that the locus contained eight potential structural genes (Fig. 1).

The *lcrB* gene cluster showed striking homology to the *spa* loci of *S. flexneri* and *S. typhimurium* (Fig. 1). The *spa* gene products of *S. flexneri* (51, 56) have been shown to be involved in the secretion of the Ipa proteins, and *spa* mutants of *S. typhimurium* are unable to invade cultured epithelial cells, probably because of an inability to present effector proteins on the surface of the pathogen (25). In addition to the remarkable conservation of these gene clusters, the gene order was also maintained between the species (Fig. 1). On the basis of sequence homology, the conserved gene products are likely to exhibit similar functions. We therefore named the genes *yscN* to *yscU* (for Yop secretion), based on the nomenclature suggested by Michiels et al. for genes involved in Yop secretion (34). The *ysc* genes were also found to be similar to genes involved in the assembly or secretion of flagella of different bacterial genera, including *Bacillus subtilis*, *S. typhimurium*, *E. coli*, and *Erwinia carotovora* (Fig. 1). In addition, *yscR* was homologous (52% identity) to orf2 of *X. campestris*, known to be involved in pathogenicity (Fig. 1).

***ysc* mutants are calcium independent and are affected in the negative Ca^{2+}-controlled loop.** Previously, we isolated a number of *yscN/U* gene cluster mutants that all showed a calcium-independent phenotype, indicating that they were expressing the Yops at a low level at 37°C (21). These mutants carried insertion mutations causing polar effects on downstream genes. To avoid polarity, in-frame deletion mutations of the *yscR* and *yscS* genes were constructed. These mutants also showed a CI phenotype. Like mutants with polar insertion mutations of this gene cluster, the *yscR* mutant YPIII(pIB67) expressed the Yops at a low level, corresponding to the level of the wild-type strain YPIII(pIB102) grown in the presence of Ca^{2+} (Fig. 2). Moreover, the nonpolar mutant did not secrete Yops into the culture supernatant (Fig. 2).

The *yscR* gene was cloned into the vector pACYC184, generating pTB300 (Fig. 1). This plasmid construct was put into the nonpolar *yscR* mutant. This strain, YPIII(pIB67, pTB300), was found to secrete Yops into the culture supernatant when incubated in a Ca^{2+}-free medium, while no Yop proteins were present in the culture supernatant of cells incubated in Ca^{2+}-containing medium (Fig. 2). This result showed that the *yscR* mutation was indeed nonpolar and that the mutation could be complemented in *trans*. Identical results were obtained for the *yscS* mutant YPIII(pIB68) (data not shown).

Thus, both polar and nonpolar mutants were found to be CI and affected in Yop expression and secretion. Since the *yop* transcriptional activator, LcrF, is expressed from a gene located downstream of the *yscN/U* gene cluster (11, 28) (Fig. 1), it was possible that transcription of *lcrF* was affected by the mutations in the upstream genes. To measure *lcrF* transcription, a promoterless *luxAB* gene (40) was fused downstream of *lcrF*, resulting in a transcriptional fusion which expressed wild-type levels of LcrF and allowing the monitoring of *lcrF* expression via the LuxAB reporter protein. These fusions were put into the wild-type strain YPIII(pIB102) as well as into the polar *yscT* insertion mutant YPIII(pIB61). Luciferase activity

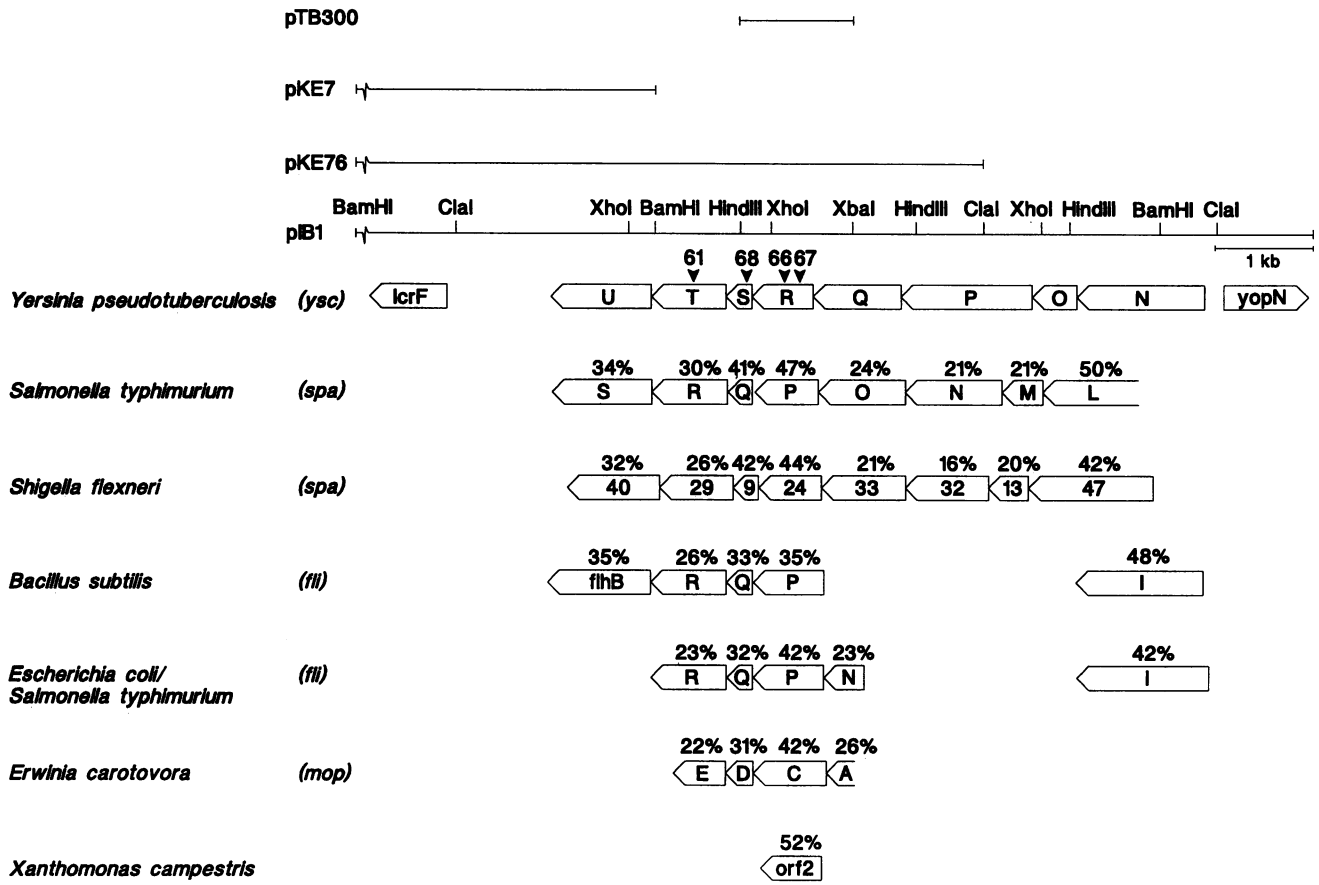

FIG. 1. Organization of the *yscN/U* gene cluster and homologous genes involved in protein secretion. Shown at the top is a map of the *yscN/U* genes of *Y. pseudotuberculosis* YPIII(pIB1) with restriction enzyme sites indicated. The positions of the YPIII(pIB61), YPIII(pIB68), YPIII(pIB66), and YPIII(pIB67) mutations are indicated by arrowheads. The DNA fragments cloned into pKE7, pKE76, and pTB300 are shown at the top. Homologous genes have been aligned, and the deduced amino acid sequences were compared with those of the corresponding *ysc* gene products by using the Bestfit program of the Genetics Computer Group software package. The results from the Bestfit analysis are shown above each gene as the percentage of identical amino acid residues. Data for the *S. typhimurium spa* genes (25); *S. flexneri spa47*, *13*, *32*, *33*, and *24* (56) and *spa9*, *29*, and *40* genes (51); *B. subtilis fliI* (1), *fliP,Q* (6), *fliR* (EMBL accession number X74122), and *flhB* (EMBL accession number X74121) genes; *S. typhimurium fliI* gene (57); *E. coli fliN* (32) and *fliP,Q,R* (EMBL accession number L22182) genes; *E. carotovora mopA*, *C*, *D*, and *E* genes (38); and *X. campestris orf2* (29) were obtained from the indicated references.

was determined in a luminometer with *n*-decanal as the coinducer. Both strains showed temperature-dependent regulation, and no difference in their ability to express LcrF was noticed (Fig. 3). These results showed that the polar insertion mutant YPIII(pIB61) expressed LcrF at the wild-type level, and consequently, the CI phenotype could not be explained by polar effects on LcrF expression, indicating that the positive control loop was not affected in the mutant.

It has been suggested that a negative element regulates Yop expression in response to the Ca^{2+} concentration in the growth medium. Thus, at high Ca^{2+} concentrations, a putative repressor which represses Yop transcription is active, and at low Ca^{2+} concentrations, this repressor is inactive (19, 21). The expected phenotype of repressor-negative mutants is high expression of Yops in Ca^{2+}-containing media and temperature-sensitive growth at 37°C. Since *lcrH* mutants have been shown to exhibit this phenotype (4, 43), we constructed a *yscR lcrH* double mutant, YPIII(pIB1366), to examine whether the *yscR* mutant was affected in the negative Ca^{2+}-controlled regulatory loop. This mutant showed the same temperature-sensitive phenotype (inability to grow at 37°C) as the *lcrH* mutant. When the *yscR lcrH* double mutant was analyzed for the ability to express the Yop proteins, it was found that the strain expressed the Yops at a higher level than the *yscR* mutant (Fig. 4), suggesting that the CI phenotype of *yscN/U* mutants involves the negative regulatory loop.

***yscN/U* gene cluster is involved in Yop secretion.** The fact that *yscN/U* gene cluster mutants expressed the Yops at a low level made it difficult to analyze their Yop secretion phenotype. We took advantage, however, of the finding that the *yscR lcrH* double mutant showed increased Yop expression at 37°C. When this mutant, YPIII(pIB1366), was tested after incubation at either 26 or 37°C in the presence or absence of Ca^{2+}, it was found that the mutant expressed the Yops in high amounts at 37°C but showed no Yop expression at 26°C. However, the mutant was unable to secrete the Yops into the culture medium at 37°C irrespective of the Ca^{2+} concentration. Although the expression of Yops was elevated, the Yops were found to be associated solely with the whole-cell fraction (Fig. 4), indicating that at least some of the *ysc* genes are involved in secretion.

When the LcrF activator is overexpressed from an inducible

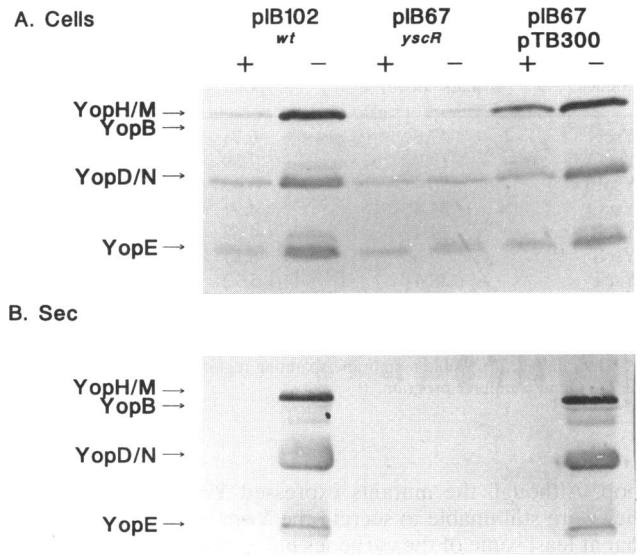

FIG. 2. Western blotting (immunoblotting) analysis of Yop expression and secretion in the wild-type (wt) YPIII(pIB102) and *yscR* mutants YPIII(pIB67) and YPIII(pIB67) *trans*-complemented with plasmid pTB300. The bacteria were grown at 37°C for 3 h in Ca^{2+}-containing (+) or Ca^{2+}-depleted (−) medium. Samples obtained from the same number of bacteria were subjected to immunoblotting analysis with a specific anti-Yop rabbit antiserum. (A) Whole-cell fractions; (B) trichloroacetic acid-precipitated proteins from the culture supernatant. The positions of the Yops are indicated to the left.

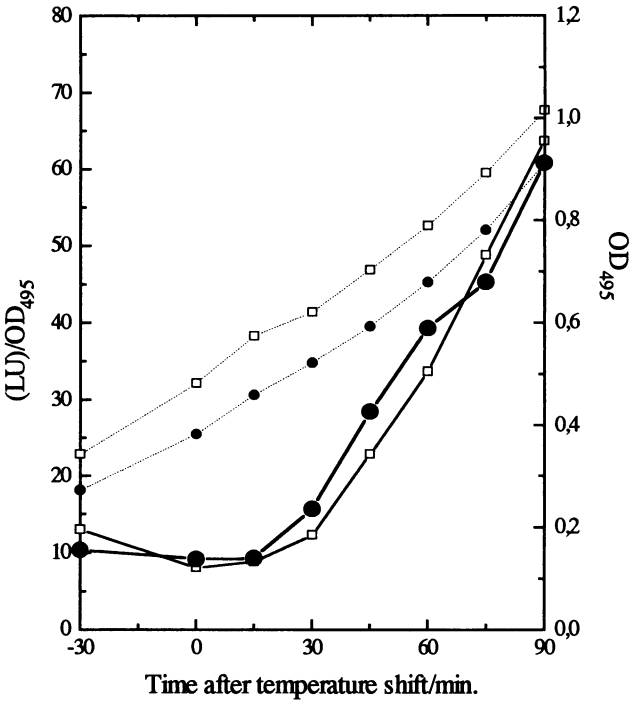

FIG. 3. Transcription of the *lcrF* gene in the wild-type YPIII (pIB102F::LUX) and in the polar *yscT* mutant YPIII(pIB61F::LUX). The *luxAB* gene was inserted downstream of the *lcrF* gene, generating *lcrF-luxAB* transcriptional fusions, creating strains YPIII(pIB102F::LUX) and YPIII(pIB61F::LUX). The transcription of the *lcrF-luxAB* transcriptional fusion was measured as the activity of the *luxAB* gene product. The strains were first grown in BHI containing 2.5 mM Ca^{2+} at 26°C and then shifted to 37°C. Samples were taken every 15 min. *n*-Decanal was added to the samples, and luciferase activity was measured in a luminometer. ●, YPIII(pIB61F::LUX); □, YPIII (pIB102F::LUX); ....., growth curves; ——, luciferase activities. LU, light units.

promoter such as the *tac* promoter, Yop proteins are expressed in elevated amounts in cells grown in a Ca^{2+}-containing medium (15). This increased Yop expression can also be obtained by simply having the *lcrF* gene with its intrinsic promoter present on a plasmid vector, e.g., pACYC184 (9), in *trans*. The hybrid plasmid pKE7, which contained the *lcrF* gene carried by pACYC184, was introduced into the *yscT* mutant YPIII(pIB61), and Yop expression was analyzed. It was found that strain YPIII(pIB61, pKE7) showed increased levels of Yops in the whole-cell fraction after incubation at 37°C, while no secretion of Yops could be observed (Fig. 5). The insert in the hybrid plasmid was increased to include the *yscT* gene (pKE76) (see Fig. 1). pKE76 was put into the *yscT* mutant YPIII(pIB61), and an experiment similar to that described above was carried out. Not only was Yop expression increased, but the Yops were also secreted into the culture supernatant in a Ca^{2+}-controlled manner (Fig. 5). Thus, the mutation in the *yscT* gene and its possible polar effect on downstream genes could be complemented in *trans* with respect to Yop secretion. Taken together, these results strongly suggest that the *ysc* gene products are involved in secretion of the Yops.

Characteristics of the *yscN/U* gene products. The characteristics of the different gene products of the *yscN/U* gene cluster are summarized in Table 2. No protein exhibited a sequence motif of classical signal peptides, but four of the proteins, YscR to YscU, had two or more hydrophobic domains, suggesting that some of the proteins are integral membrane proteins. The YscN protein showed a protein signature characteristic of ATP/GTP-binding proteins. Moreover, YscN is homologous to protein-translocating ATPases and shows high sequence homology to Spa47 (56) and SpaL (25), which both carry similar sequence motifs. YscN is also homologous to the FliI proteins of *B. subtilis* (1) and *S. typhimurium* (57), which likely function as ATPases involved in the assembly of flagella. In addition, YscN is homologous to the β subunit of the F_0F_1 proton-translocating ATPase of *E. coli* (30).

DISCUSSION

We have shown here that the *lcrB/virB* locus of the virulence plasmid of *Y. pseudotuberculosis* comprises a set of genes involved in the secretion of Yops. We have named these genes *yscN* to *yscU*, using the nomenclature suggested by Michiels and coworkers for genes involved in Yop secretion (34). The gene organization suggests that the *yscN* to *yscU* genes are contained within one operon, since there are no intervening noncoding stretches of DNA. Polar insertion mutants grown in a medium lacking Ca^{2+} show decreased Yop expression and express the Yops at the same low level as the wild-type strain grown in a medium containing Ca^{2+}, i.e., the mutants were calcium independent. Since the activator LcrF (11, 28) is encoded by a gene located downstream of the *yscN/U* locus, one possibility for the lower Yop expression in the *ysc* mutants was that the insertions had polar effects on the expression of *lcrF*. This hypothesis could be rejected, since nonpolar *ysc* mutants expressed the Yops at a low level and since the transcription of *lcrF* was unaffected in polar *ysc* mutants.

LcrH acts at the end of the Ca^{2+}-regulated negative loop, and it has been suggested that LcrH is the yop repressor or works close to the repressor (4, 43). Since one class of CI

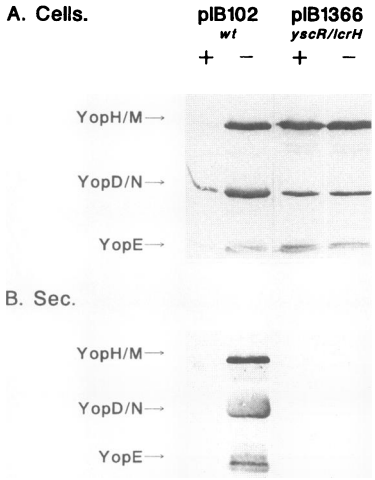

FIG. 4. Western blotting analysis of Yop expression and secretion in the wild-type (wt) YPIII(pIB102) and the *lcrH yscR* double mutant YPIII(pIB1366). The bacteria were grown at 37°C for 3 h in Ca^{2+}-containing (+) or Ca^{2+}-depleted (−) medium. Samples obtained from the same number of bacteria were subjected to immunoblotting analysis with a specific anti-Yop rabbit antiserum. (A) Whole-cell fractions; (B) trichloroacetic acid-precipitated proteins from the culture supernatant. The positions of the Yops are indicated to the left.

mutants exhibit a constitutive high level of repressor not affected by the Ca^{2+} concentration and since we have previously shown that some *ysc* mutants likely belong to this class (19, 21), we constructed an *lcrH yscR* double mutant. This mutant showed higher Yop expression than a *yscR* mutant and was temperature sensitive for growth at 37°C, showing that *ysc* mutants are affected in the regulation of the negative control

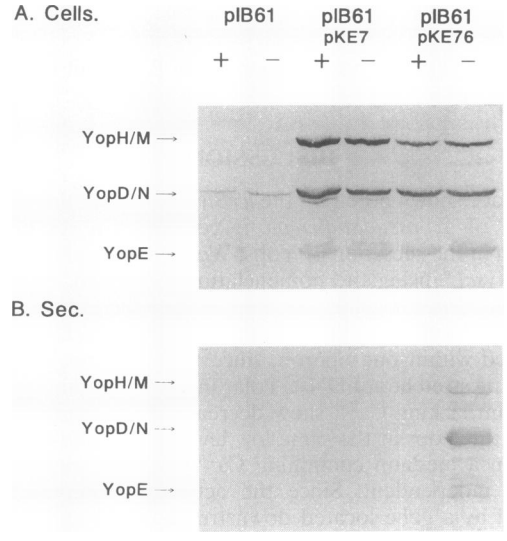

FIG. 5. Western blotting analysis of Yop expression and secretion in the YPIII(pIB61) *yscT* mutant and YPIII(pIB61) containing plasmid pKE7 or pKE76. The bacteria were grown at 37°C for 3 h in Ca^{2+}-containing (+) or Ca^{2+}-depleted (−) medium. Samples obtained from the same number of bacteria were subjected to immunoblotting analysis with a specific anti-Yop rabbit antiserum. (A) Whole-cell fractions; (B) trichloroacetic acid-precipitated proteins from the culture supernatant. The positions of the Yops are indicated to the left.

TABLE 2. Characteristics of the YscN/U proteins

Gene product	Molecular mass[a] (kDa)	Isoelectric point[a]	Membrane-spanning regions[b]
YscN	47.8	6.71	0
YscO	19.0	7.89	0
YscP	50.4	5.43	0
YscQ	34.4	4.91	0
YscR	24.4	4.63	3
YscS	9.6	6.51	2
YscT	28.4	5.72	3
YscU	40.4	9.38	4

[a] Calculated by using the Genetics Computer Group computer program (14).
[b] The number of possible membrane-spanning regions was predicted by using the PC Gene computer program.

loop. Although the mutants expressed Yops at a high level, they were still unable to secrete the Yops, supporting the idea that at least some of the *ysc* genes have a role in Yop secretion.

When LcrF is expressed in elevated amounts, the negative effect of Ca^{2+} on Yop expression can be partially circumvented (15). We thus overexpressed LcrF in the *yscT* mutant, which resulted in increased expression of the Yops; however, no Yop secretion could be observed. On the other hand, when a hybrid plasmid that contained several genes of the *ysc* operon, including *yscT*, as well as the downstream-located *lcrF* (see map in Fig. 1) was introduced into the mutant, Yop secretion was obtained. Moreover, nonpolar *yscR* and *yscS* mutants expressed the Yops at a low level and did not secrete the Yops into the culture supernatant. Both mutations could be complemented in *trans* by introduction of the corresponding wild-type gene. Thus, *ysc* mutants could be complemented in *trans* with respect to secretion. Taken together, these experiments strongly indicate that at least some of the *ysc* genes play an essential role in the secretion of Yops.

How can *ysc* mutants be affected in the regulation of Yop expression? Cornelis and coworkers were the first to suggest that Yop secretion was regulated by a feedback control mechanism that senses the level of secretion (12). This hypothesis was later supported by the observation that the LcrQ protein was not only secreted but was also a constituent of the negative control loop (*lcrQ* mutants are derepressed for Yop expression and temperature sensitive for growth at 37°C) (45). On the basis of these results, we suggested that LcrQ could be the actual feedback regulator by itself being a negative regulator of Yop expression as well as a substrate for secretion by the specific Yop secretion apparatus (45). Thus, secretion-competent bacteria would also secrete LcrQ and thereby the intracellular concentration of LcrQ would be lowered, allowing elevated Yop expression. Interestingly, it was recently shown by Skrzypek and Straley that the negative regulator LcrG is also exported to the growth medium under conditions that favor Yop secretion (52). Thus, there exist two candidate proteins, LcrQ and LcrG, which may be involved in a mechanism that allows the pathogen to sense its secretion competence and regulate the expression of Yops by secretion of a negative regulator.

Two additional loci on the virulence plasmid have been implicated in the export of Yops. The LcrD protein (encoded by the *lcrDR* operon) shows homology to proteins involved in surface presentation of proteins of several genera (5, 41, 55). The *yscA/M* (*lcrC/virC*) operon (26, 34, 45) maps immediately downstream of *lcrF*. This gene cluster contains 13 genes, of which some show homology to genes known to be involved in

protein secretion in different bacterial genera, including *S. flexneri* (2, 55).

Thus, 22 gene products have been identified, of which the majority are likely to be involved in Yop secretion, and it seems plausible that they interact to form some sort of secretion organelle. The secretion apparatus works in conjunction with regulatory proteins in a finely tuned, tightly regulated system to deliver the effector Yop proteins to the proper targets. Yop expression in vivo is stimulated by the intimate interaction between the pathogen and the surface of the target cell (19, 47). This interaction results not only in increased expression of Yops but also in the polarized transfer of, at least, YopE through the cell membrane of the target cell (47). This implies that the secretion apparatus is accessible for Yop secretion only at the site of interaction between the target cell and the pathogen, indicating that the Yop secretion system is integrated as an active part of the specific virulence behavior of *Yersinia* spp. In this context, the homology between the *ysc* genes and genes involved in the biosynthesis and assembly of flagella is of considerable interest, since both the transfer of the Yops and the assembly of flagella are polarized processes. It is therefore plausible that the *ysc* ancestral genes have been recruited from genes essential for the secretion of flagella.

In conclusion, we have identified a new gene cluster that is involved in Yop secretion. Future work will focus on understanding how the proteins involved in Yop secretion interact and how they are regulated to form the secretion apparatus.

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The first two authors contributed equally to this work.

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ADDENDUM IN PROOF

In a recent paper by Fields et al., the DNA sequences of the *yscQ*, *yscR*, and *yscS* genes from *Yersinia pestis* were presented (K. A. Fields, G. V. Plano, and S. C. Straley, J. Bacteriol, 176:569–579). The DNA sequences of these genes are very similar to those of the corresponding *yscQ*, *yscR*, and *yscS* genes from *Y. pseudotuberculosis*.

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