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Identification of SycN, YscX, and YscY, Three New Elements of the *Yersinia* Yop Virulon

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The Yop virulon allows *Yersinia* spp. to resist the immune response of the host by injecting harmful proteins into host cells. We identified three new elements of the Yop virulon: SycN, required for normal secretion of YopN, and YscX and YscY, two new components of the secretion machinery.

Pathogenic *Yersinia* spp. (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) share a tropism for lymphoid tissues and the capacity to resist the primary immune response of the host. The apparatus conferring this resistance is called the Yop virulon and is encoded by a 70-kb plasmid called pYV in *Y. enterocolitica*. The Yop virulon allows extracellular bacteria to inject harmful bacterial proteins straight into the cytosols of the cells of the immune system (for review, see references 11 and 19). The Yop virulon products comprise the Yop proteins, their secretion machinery (called Ysc), and the cytosolic Syc chaperones required for secretion of specific Yops. The Yop proteins can be classified into two groups. The first group includes the effectors (YopE, YopH, YopM, YopO/YpkA, YopP/YopJ, and YopT), which are injected into the cytosol of the target cells and disarm these cells (6, 7, 18, 20, 28, 29, 31, 33, 36, 37), while the second one includes the proteins that form the apparatus allowing the delivery of the effectors into the target cell (YopB, YopD, and LcrV) (for review, see references 11 and 14). The Ysc secretion machinery is encoded by four loci: *virC*, including *yscABCDEFGHIJKLM* (2, 25, 27, 41), *virG* (encoding YscW) (1), *virB*, including *yscNOPQRSTU* (3, 5, 21, 44), and *virA*, including *yopN*, *tyeA*, *yscV* (formerly called *lcrD*), and *lcrR* (4, 22, 32, 34, 35). The Syc chaperones are small cytosolic proteins with an acidic isoelectric point and a putative α-helix in the C terminus. They specifically serve one or two Yops, and they are encoded by the gene neighboring the *yop* gene. Four such chaperones have been identified: SycE for YopE, SycH for YopH, SycD/LcrH for YopD and YopB, and SycT for YopT (20, 30; for review, see reference 43). In addition, it has been shown recently that YscB behaves as a chaperone for YopN: it is specifically required for normal secretion of YopN and it binds to YopN, but unlike the Syc chaperones, it has a basic pI (23). In vitro, *Yersinia* secretes Yops when they are incubated at 37°C in a medium deprived of Ca^{2+}. Under these conditions, they cease growing. Mutants with mutations in *yopN*, *tyeA*, and *lcrG* are "Ca^{2+} blind." This means that, unlike the wild-type strain, they secrete Yops in the presence as well as in the absence of Ca^{2+}, and they cannot grow at 37°C, irrespective of the presence of Ca^{2+} (8, 16, 22, 38, 39). YopN is a secreted protein (16, 22). TyeA is a 10.8-kDa surface-exposed protein that binds to the second coil-coiled domain of YopN, and it is required for delivery of YopE and YopH (22). LcrG, which binds to heparin sulfate proteoglycans (9), is a nonsecreted bacterial protein required for efficient translocation of all the Yop effectors (38). YopN, LcrG, and TyeA are thus required for the control of Yop release, and they are thought to form a stop valve closing the secretion channel (8, 16, 22, 38, 39). The *virA* locus contains, between *tyeA* and *yscV* (*lcrD*), three putative open reading frames (ORFs) that have not been characterized yet (16, 21, 22, 42). In this work we analyzed the role of these three genes in the *Yersinia* Yop virulon. We show that the ORF situated immediately downstream of *tyeA* encodes SycN, a protein required for normal secretion of YopN, and that the other ORFs encode YscX and YscY, two new proteins of the Ysc secretion machinery.

SycN, a putative chaperone for YopN. The ORF located immediately downstream from *yopN* and *tyeA* (Fig. 1) (previously called ORF2; now called *sycN*) encodes a putative protein of 123 amino acids (16, 42; also this work). The size of this protein (13.6 kDa), its acidic pI (5.2), and the hydrophobic moment plot (15) evoke those of the chaperones SycE, SycH, and SycT (20; for review see reference 43). As is the case for SycE, SycH, and SycT, the C terminus is predicted to form an amphiphilic α-helix (Fig. 2).

To investigate the role of *sycN* we constructed a nonpolar mutant by directed mutagenesis (26) with oligonucleotide MIPA 320 (5′-CGCTAACCACAGTGTCTGGCCAAAATG GGA-3′) and plasmid pIM180 carrying *sycN* as a template. (The plasmids used in this study are listed in Table 1.) This primer hybridizes to nucleotides 25 to 39 (with a mismatch at nucleotides 33 and 34 to introduce a *Bal*I site) and to nucleotides 139 to 153 of *sycN*. The mutated allele $sycN_{\Delta 14–46}$ was verified by sequencing, cloned into a suicide vector (24), and introduced into strain E40(pYV40) to obtain strain E40 (pIM404). To facilitate the complementation experiments, we also inactivated the chromosomal gene encoding β-lactamase A (10) by inserting the *luxAB* genes using the mutator plasmid pKNG105 as described by Kaniga et al. (24) to obtain MIE40 (pIM404). The $sycN_{\Delta 14–46}$ mutant was restricted for growth at 37°C and was Ca^{2+} blind for Yop secretion. Like the *yopN* and *tyeA* mutants it secreted all the Yops in the presence as well as in the absence of Ca^{2+} (Fig. 3). The amount of YopN secreted was, however, significantly reduced compared to that of the wild type. The mutant could be fully complemented by a plasmid containing the gene cloned downstream from P_{lac} (pIML246) (Fig. 3), indicating that the phenotype observed was due to the lack of *sycN* and not to an effect on a downstream gene. As shown in Fig. 4, there was no significant dif-

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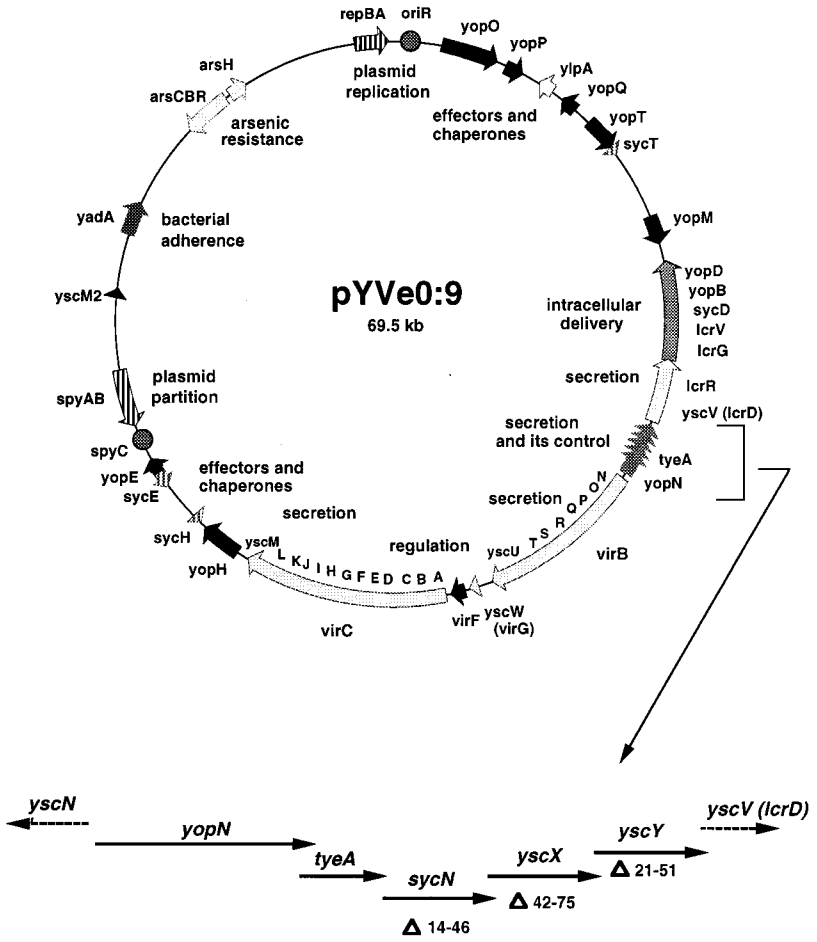

FIG. 1. Detailed genetic map of the pYV plasmid of *Y. enterocolitica* W22703. The *virA* locus is shown in more detail below the map. The codons deleted in each gene are indicated.

ference between the amounts of YopN found in the total cells of the wild-type strain and the *sycN* mutant, but degradation products appeared in the mutant. Under permissive conditions (minus Ca^{2+}), transcription of *yopN* in the *sycN* mutant was similar to that in the wild type. Under nonpermissive conditions (plus Ca^{2+}), transcription of *yopN* was up-regulated, as expected from the fact that secretion of all the Yops was deregulated (Fig. 4C). *sycN* was thus necessary for normal secretion of YopN. The deregulated phenotype of the mutant affected in *sycN* can be explained by the fact that *sycN* is

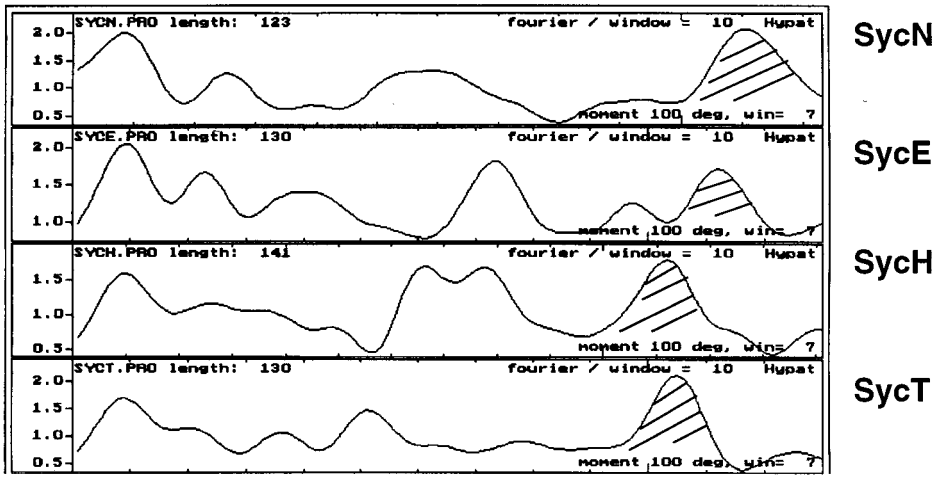

FIG. 2. Similarity between SycN and the other chaperones of the SycE family. Shown are hydrophobic moment plots of SycN, SycE, SycH, and SycT.

TABLE 1. Plasmids used in this study

Plasmid	Genotype or description	Reference
pYV derivatives		
pYV40	Wild-type pYV plasmid from strain E40	40
pIM41	pYV40 *yopN*$_{45}$ (encodes a truncated YopN of 45 amino acids)	8
pIM404	pYV40 *sycN*$_{\Delta 14–46}$ (pYV40 mutated with pIM212)	This work
pIM405	pYV40 *yscX*$_{\Delta 42–75}$ (pYV40 mutated with pIM211)	This work
pIM406	pYV40 *yscY*$_{\Delta 21–51}$ (pYV40 mutated with pIM209)	This work
pIM413	pYV40 *yopN*$_{45}$ *yscX*$_{\Delta 42–75}$	This work
pIM414	pYV40 *yopN*$_{45}$ *yscY*$_{\Delta 21–51}$	This work
pMSL41	pYV40 *yscN*$_{\Delta 169–177}$	40, 44
Clones		
pIM180	pIM176$_{\Delta 1167bp}$ *Bam*HI-*Bgl*II (contains 52 codons of *yopN*, *tyeA*, *sycN*, *yscX*, and *yscY*)	22
pIM192	*Hin*dIII deletion of pIM188; pSelect *ori*T$_{RK2}$ P$_{lac}$ *yscX*, *yscY*	This work
pIML236	pSelect *ori*T$_{RK2}$ P$_{lac}$ *yscY*	This work
pIML246	pBC18R P$_{lac}$ *tyeA sycN*	This work
pSI57	pTM100 P$_{lac}$ *sycH yopH*	41
Suicides and mutators		
pIM150	pKNG101 + *Sal*I-*Xba*I fragment of pIM149 (encodes YopN$_{45}$)	8
pIM209	pKNG101 + *Sal*I-*Xba*I fragment of pIM204 (encodes YscY$_{\Delta 21–51}$)	This work
pIM211	pKNG101 + *Sal*I-*Xba*I fragment of pIM207 (encodes YscX$_{\Delta 42–75}$)	This work
pIM212	pKNG101 + *Sal*I-*Xba*I fragment of pIM202 (encodes SycN$_{\Delta 14–46}$)	This work
pKNG101	*ori*$_{R6K}$ *sacBR ori*T$_{RK2}$ *strAB*	24
pKNG105	*ori*$_{R6K}$ *sacBR ori*T$_{RK2}$ *strAB blaA′ luxAB blaA″*	24

required for the export of the YopN plug. Together with the physical characteristics of the protein, this suggested that *sycN* encodes a chaperone specific for YopN. Unfortunately, we could not obtain evidence for direct binding of SycN to YopN, but others mention the existence of this binding in a recent paper (23).

A homologue of SycN, called Pcr2, has been identified in the type III secretion system of *Pseudomonas aeruginosa*. SycN and Pcr2 are 67% similar (45). The presence of a homologue in another type III secretion system reinforces our view that SycN has an important role to play in the Yop virulon.

YscX and YscY, two new proteins required for Yop secretion. ORF3 (which we now rename *yscX*) (42, also this work), situated immediately downstream from *sycN*, encodes a protein of 122 amino acids (13.6 kDa) with a calculated isoelectric point of 6.5. The GTG start codon of *yscX* overlaps with the TGA stop codon of *sycN*. Immediately downstream from *yscX* lies *yscY* (42; also this work), which encodes a protein of 114 amino acids (13.1 kDa) with a calculated isoelectric point of 7.0. The stop codon of *yscX* overlaps with the start codon of *yscY*. The function of these genes has not yet been investigated in any *Yersinia* spp.

We constructed mutants with nonpolar mutations in both genes. The mutation in *yscX* consisted of an in-frame deletion spanning codons 42 to 75 and was constructed by directed mutagenesis (26) with oligonucleotide MIPA 321 (5′-GTT CGCGCCGATATTCGACTGGATGCCC-3′) and plasmid pIM180 carrying *yscX*. This primer hybridized to nucleotides 109 to 123 and to nucleotides 226 to 238 of *yscX*. The mutated allele (*yscX*$_{\Delta 42–75}$) was verified by sequencing, cloned into the suicide vector, and introduced into strain E40(pYV40) to create strain E40(pIM405). The mutation in *yscY* consisted in an in-frame deletion of codons 21 to 51, and it was constructed with oligonucleotide MIPA 322 (5′-TTAAGTAGCGCGACT TGTAACCAACCGTT-3′) and plasmid pIM180 carrying *yscY*. This primer hybridized to nucleotides 46 to 60 and to nucleotides 154 to 167 of *yscY*. The mutant strain carrying *yscY*$_{\Delta 21–51}$ was called E40(pIM406).

Both mutants were unable to secrete Yops under permissive conditions (Fig. 5A, lanes 2 and 3). Secretion by the *yscY* mutant was restored after introduction of plasmid pIML236 bearing *yscY* under the control of the P$_{lac}$ promoter (Fig. 5A, lane 9). To complement the secretion defect of the *yscX* mutant, we used plasmid pIM192 bearing *yscX* and *yscY* and plasmid pIML236 bearing *yscY* only. As shown in Fig. 5A,

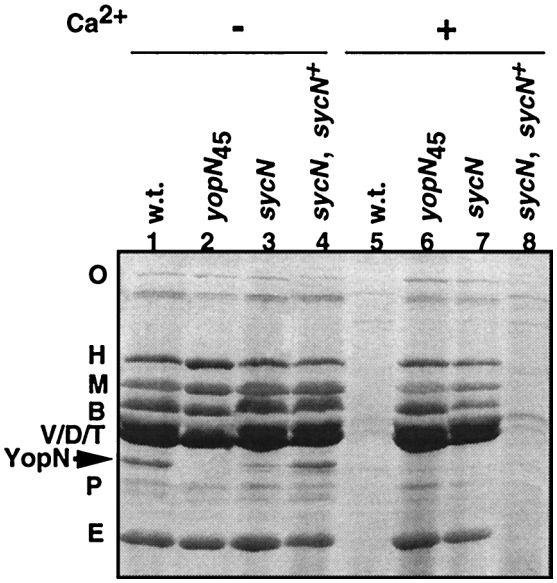

FIG. 3. Yop secretion patterns of the wild-type strain (w.t.), the *yopN*$_{45}$ mutant, and the ORF2 (*sycN*) mutant. Lanes 1 and 5, β-lactamase mutant of the wild-type strain [E40(pYV40)] (38); lanes 2 and 6, *yopN*$_{45}$ mutant E40(pIM41); lanes 3 and 7, ORF2 (*sycN*) mutant E40(pIM404); lanes 4 and 8, E40(pIM404) (pIML246 [= P$_{lac}$ *sycN*]). The strains were grown in brain heart infusion-OX (without Ca^{2+}) or brain heart infusion-Ca^{2+} (with Ca^{2+}). Culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Letters on the left identify the different Yops.

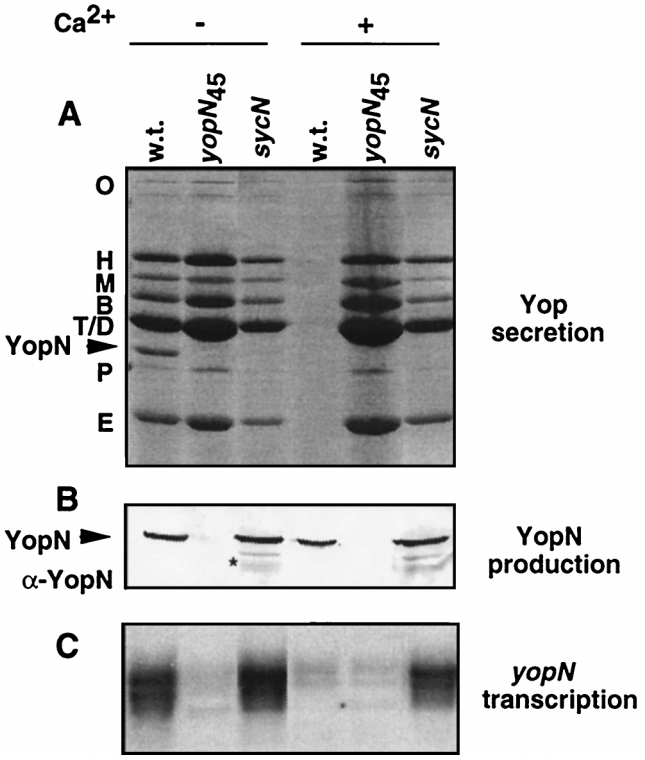

FIG. 4. Phenotype of the *sycN* mutant. (A) Culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Letters on the left identify the different Yops. (B) Western blot analysis of total cell (90 μl of a suspension with an optical density at 600 nm of 1) using a polyclonal antibody against YopN (α-YopN). The asterisk indicates degradation products of YopN. (C) Northern blot analysis carried out on the same culture with a *yopN* probe (internal *Pst*I fragment of *yopN*). The strains were grown in brain heart infusion-OX (without Ca^{2+}) or brain heart infusion-Ca^{2+} (with Ca^{2+}). Lane 1, wild type [E40(pYV40)] (w.t.); lane 2, E40(pIM41); lane 3, E40(pIM404).

plasmid pIM192 restored secretion (lane 6) but plasmid pIML236 did not (lane 7). This indicates that the absence of Yop secretion is due to the lack of either *yscX* or *yscY* and not to an effect on the downstream gene *yscV* (*lcrD*), which is known to be required for Yop secretion (34, 35).

Yop expression is subject to a feedback inhibition of transcription when secretion is compromised (13; for review, see reference 12). To confirm that the lack of Yop proteins in the culture supernatant of the *yscX* and *yscY* mutants was due to a defect in Yop secretion and not to a defect in *yop* transcription, we introduced plasmid pSI57, containing *sycH* and *yopH* under the control of the P_{lac} promoter (41), into the *yscN* (40, 44), *yscX*, and *yscY* mutants, and we analyzed the pattern of protein secretion. As shown in Fig. 5B and C, YopH was secreted only by the wild-type strain, although it was present in the total cells of the wild type and the *yscN*, *yscX*, and *yscY* mutants. We concluded from this experiment that YscX and YscY are involved in Yop secretion.

To gain further insight into the function of *yscX* and *yscY*, we analyzed the production of YopN by mutants affected in these genes. As seen in Fig. 6A, YopN was present among the intracellular proteins of the mutant bacteria, in agreement with previous results showing that YopN is not subject to the feedback inhibition of transcription that occurs when secretion is compromised (16; for review, see reference 12). Taking into account that *yscX* and *yscY* form an operon with *yopN* and that they are not involved in the production of YopN, we wondered

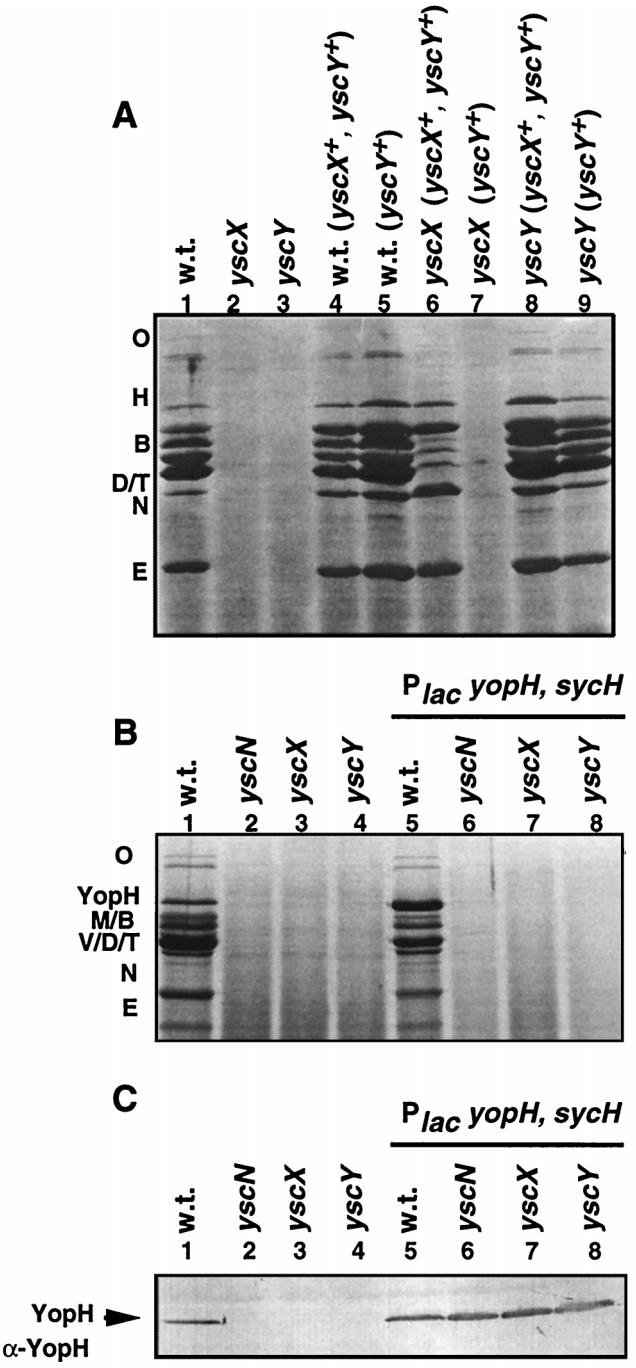

FIG. 5. Analysis of the *yscX* and *yscY* mutants. (A) Effect on Yop secretion. Shown are results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the secreted Yops (Coomassie blue staining). Lane 1, wild type [E40(pYV40)] (w.t.); lane 2, E40(pIM405); lane 3, E40(pIM406); lane 4, E40(pYV40)(pIM192); lane 5, E40(pYV40)(pIML236); lane 6, E40(pIM405)(pIM192); lane 7, E40(pIM405)(pIML236); lane 8, E40(pIM406)(pIM192); lane 9, E40(pIM406)(pIML236). (B) Effect on secretion of overproduced YopH. Shown are results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the secreted Yops (Coomassie blue staining). Lane 1, wild type [E40(pYV40)] (w.t.); lane 2, E40(pMSL41); lane 3, E40(pIM405); lane 4, E40(pIM406); lane 5, E40(pYV40)(pSI57); lane 6, E40(pMSL41)(pSI57); lane 7, E40(pIM405)(pSI57); lane 8, E40(pIM406)(pSI57). (C) Analysis of intrabacterial overproduced YopH. Total cells (90 μl of a suspension with an optical density at 600 nm of 1) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the presence of YopH was detected with a polyclonal antibody against YopH (α-YopH). The lanes are the same as in panel B.

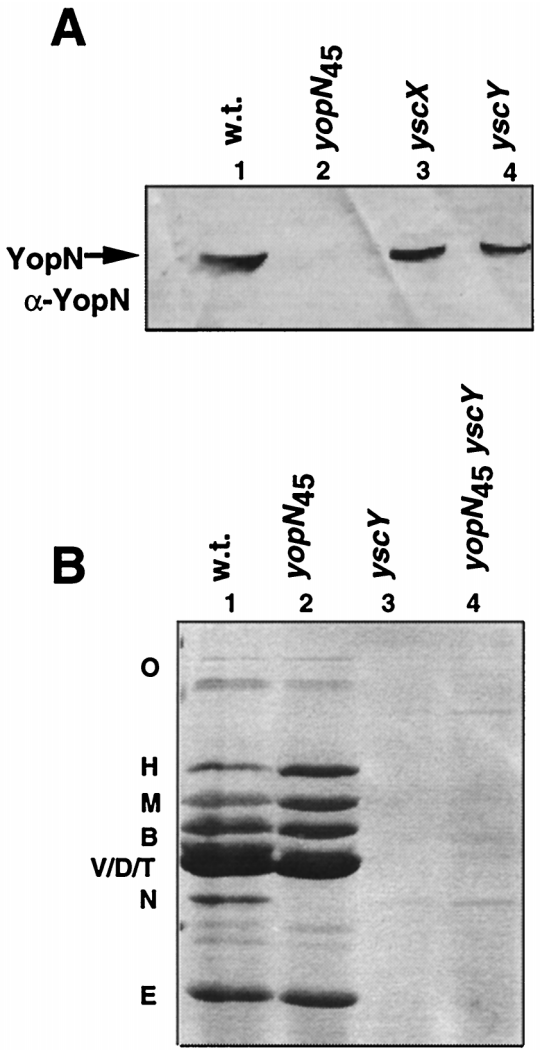

FIG. 6. Role of YscX and YscY in YopN synthesis and operation. (A) Analysis of the intrabacterial YopN. Total cells (90 μl of a suspension with an optical density at 600 nm of 1) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the presence of YopN was detected with a polyclonal antibody against YopN (α-YopN). Lane 1, wild type [E40(pYV40)] (w.t.); lane 2, E40(pIM41); lane 3, E40(pIM405); lane 4, E40 (pIM406). (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the Yops secreted. Lane 1, wild type [E40(pYV40)] (w.t.); lane 2, E40 (pIM41); lane 3, E40(pIM405); lane 4, E40(pIM414).

whether their products could be required to remove the stop valve YopN. We constructed a nonpolar *yopN*$_{45}$-*yscX* double mutant [MIE40(pIM413)] and a nonpolar *yopN*$_{45}$-*yscY* double mutant [MIE40(pIM414)]. If the role of YscX and YscY were solely to remove the stop valve YopN, then a double mutant should be able to secrete Yops. However, the double *yopN*$_{45}$-*yscY* and *yopN*$_{45}$-*yscX* mutants did not secrete Yops (Fig. 6B and data not shown), ruling out this hypothesis. In conclusion, these results indicate that the proteins YscX and YscY are part of the secretion machinery, independently of the function of YopN.

Homologues of YscX and YscY have been found in the type III secretion system of *P. aeruginosa* (45): YscX is 64% similar to Pcr3, and YscY is 61% similar to Pcr4. Surprisingly, YscY is, in addition, 47% similar to the central part of Lpm1, a 1,119-amino-acid surface-located membrane protein of *Borrelia burgdorferi* (17), an organism not known to form a type III secretion apparatus.

In conclusion, this work allowed us to assign functions to the last genes of the *virA* locus: one encodes SycN, a putative chaperone for YopN, and the other two encode YscX and YscY, two new proteins of the secretion machinery. If we consider YopN and TyeA, which form the plug (16, 22), to belong to the Ysc apparatus, then the whole *virA* locus appears to be devoted to the Ysc apparatus, like *virB, virC*, and *virG*. In total, this apparatus thus requires 28 genes, with the possible exception of *yscH*, encoding YopR.

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