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

MAITE IRIARTE AND GUY R. CORNELIS*

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

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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 cytosol 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 Yse), and the cytosolic Ysc 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 Yse secretion machinery is encoded by four loci: virC, including yscABCD/EGHJKL/M (2, 25, 27, 41), virG (encoding YscW) (1), virB, including yscNOPQRSTU (3, 5, 21, 44), and virA, including yopN, tyeA, yseV (formerly called lcrD), and lcrR (4, 22, 32, 34, 35). The Ysc 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 Ysc chaperones, it has a basic pI (23). In vitro, Yersinia secretes Yops when they are incubated at 37°C in a medium deprived of Ca²⁺. Under these conditions, they cease growing. Mutants with mutations in yopN, tyeA, and lcrG are “Ca²⁺ blind.” This means that, unlike the wild-type strain, they secrete Yops in the presence as well as in the absence of Ca²⁺, and they cannot grow at 37°C, irrespective of the presence of Ca²⁺ (8, 16, 22, 38, 39). YopN is a secreted protein (13.6 kDa), its acidic pI (5.2), and the hydrophobic face-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 yseV (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 components of the Yse secretion machinery.

SycN, a putative chaperone for YopN. The ORF located immediately downstream from yopN and tyeA (Fig. 1) (previously called ORF2; now called yscN) 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 yscN we constructed a nonpolar mutant by directed mutagenesis (26) with oligonucleotide MIPA 320 (5′-CGCTAACCACGTGCTGGCCTTATG GGA-3′) and plasmid pIM180 carrying yscN 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 BalI site) and to nucleotides 139 to 153 of yscN. The mutated allele yscN14–46 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 yscN14–46 mutant was restricted for growth at 37°C and was Ca²⁺ 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 Ca²⁺ (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 sacrific (pIML246) (Fig. 3), indicating that the phenotype observed was due to the lack of yscN and not to an effect on a downstream gene. As shown in Fig. 4, there was no significant dif-
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. 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.

FIG. 2. Similarity between SycN and the other chaperones of the SycE family. Shown are hydrophobic moment plots of SycN, SycE, SycH, and SycT.
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 *type III secretion system of Pseudomonas aeruginosa* paper (23).

**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 *yopN* (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 *yopN*. 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′-TATCGCCGCGTACTTGACGATGCCC-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*<sub>42-75</sub>) was verified by sequencing, cloned into the suicide vector, and introduced into strain E40(pYV40) to create strain E40(pIM405). The mutation in *yopN* consisted of an in-frame deletion of codons 21 to 51, and it was constructed with oligonucleotide MIPA 322 (5′-TTAATGACCGGACTTGTAACACCGTT-3′) and plasmid pIM180 carrying *yopN*. This primer hybridized to nucleotides 46 to 60 and to nucleotides 154 to 167 of *yopN*. The mutant strain carrying *yopN<sub>21-51</sub>* was called E40(pIM406).

Both mutants were unable to secrete Yops under permissive conditions (Fig. 5A, lanes 2 and 3). Secretion by the *yopN*<sub>45</sub> mutant was restored after introduction of plasmid pIML236 bearing *yscX* under the control of the P<sub>lac</sub> promoter (Fig. 5A, lane 9). To complement the secretion defect of the *yscX* mutant, we used plasmid pIM192 bearing *yscX* and *yopN* and plasmid pIML236 bearing *yscX* only. As shown in Fig. 5A,
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 yscH and yopH under the control of the P<sub>lac</sub> 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...
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 YscN, 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 excH, encoding YopR.

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REFERENCES


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-polycrylamide 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-polycrylamide 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).


