Identification of Substrates and Chaperone from the *Yersinia enterocolitica* 1B Ysa Type III Secretion System

Boris Foultier,1 Paul Troisfontaines,2 Didier Vertommen,3 Marie-Noëlle Marenne,1 Mark Rider,3 Claude Parsot,4 and Guy R. Cornelis1,2,*

Microbial Pathogenesis Unit1 and Hormone and Metabolic Research Unit,3 Christian de Duve Institute of Cellular Pathology and Faculté de Médecine, Université Catholique de Louvain, B1200 Brussels, Belgium; Biozentrum der Universität Basel, CH 4056 Basel, Switzerland; and Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, 75724 Paris, France

Received 11 June 2002/Returned for modification 31 July 2002/Accepted 23 October 2002

All pathogenic *Yersinia enterocolitica* strains carry the pYV plasmid encoding the Ysc-Yop type III secretion (TTS) system, which operates at 37°C. In addition, biovar 1B *Y. enterocolitica* strains possess a second, chromosomally encoded, TTS system called Ysa, which operates, at least in vitro, under low-temperature and high-salt (LTHTS) conditions. Six open reading frames, *sycB*, *yspB*, *yspC*, *yspD*, *yspA*, and *acpY*, neighbor the *ysa* genes encoding the Ysa TTS apparatus. Here we show that *YspA*, *YspB*, *YspC*, and *YspD* are secreted by the Ysa TTS system under LTHTS conditions. *SycB* is a chaperone for *YspB* and *YspC* and stabilizes *YspB*. *YspB*, *YspC*, and *SycB* share some similarity with TTS substrates and the chaperone encoded by the Mxi-Spa locus of *Shigella flexneri* and SPI-1 of *Salmonella enterica*. In addition, *Ysa* also secretes the pYV-encoded YopE under LTHTS conditions, indicating that YopE is a potential effector of both *Y. enterocolitica* TTS systems. *YspC* could also be secreted by *S. flexneri*, but no functional complementation of *ipaC* was observed, which indicates that despite their similarity the Ysa and the Mxi-Spa systems are not interchangeable. When expressed from the *yopE* promoter, *YspB* and *YspC* could also be secreted via the Ysc injectosome. However, they could not form detectable pores in eukaryotic target cells and could not substitute for YopB and YopD for translocation of Yop effectors.

The genus *Yersinia* contains three species that are pathogenic for rodents and humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. Although they infect their host via different routes and cause diseases of different severities, bacteria from these three species are organotropic for lymphatic tissue, where they proliferate as extracellular pathogens in spite of the presence of immune cells (7, 15). The capacity of pathogenic *Yersinia* spp. to overcome the primary immune response of the host is primarily dependent on the presence of the *Ysc-Yop* type III secretion (TTS) system (TTSS), encoded on a 70-kb plasmid (8).

*Y. enterocolitica* bacteria enter the gastrointestinal tract after ingestion of contaminated food or water. They cross the intestinal epithelium through M cells and reach the underlying lymphoid tissue, where they multiply (13). Pathogenic *Y. enterocolitica* strains are classified into a series of biotypes (1B to 5) on the basis of metabolic properties and epidemiological observations (60). Most of the *Y. enterocolitica* strains obtained from human clinical material worldwide belong to biotype 4, serotype O:3 (22, 25). In contrast, strains from biotype 1B are usually isolated from patients in the United States (9, 56), although they have also been found recently in Europe and Asia (18, 27). They are lethal for mice orally infected while bacteria from the other biotypes are not. This high virulence can be explained by the presence, in addition to the pYV plasmid, of a pathogenicity island encoding an iron uptake system (4, 44).

Recently, genes encoding a second TTSS, called Ysa, have been characterized on the chromosome of biotype 1B *Y. enterocolitica* strains 8081 and A127/90 and detected on the chromosome of the nine biotype 1B strains that have been tested previously (11, 14). The *ysa* locus is not present in the low-virulence strains of *Y. enterocolitica* (11). According to gene sequence and organization, the Ysa system is closely related to the Mxi-Spa TTSS of *Shigella flexneri* and to the SPI-1-encoded TTSS of *Salmonella enterica* (11).

*S. flexneri* is an enteropathogenic bacterium with a lifestyle different from that of *Y. enterocolitica*. When *S. flexneri* bacteria reach the colon, they are transported through the epithelial barrier by way of M cells (46, 59). They infect the resident macrophages and induce cell death (65). *S. flexneri* bacteria released from killed macrophages enter enterocytes from the basolateral surface by inducing membrane ruffling and macropinocytosis (39). Following entry, the membrane of the vacuole that contains bacteria is rapidly disrupted and bacteria escape into the cytoplasm (48), where they multiply (1, 26). These features are associated with the presence of the Mxi-Spa TTS apparatus; the effector proteins *IpaA*, -B, -C, and -D; *IpgB* and *IpgD*; and the chaperones *IpgC*, *IpgE*, and *Spa15* (3, 41, 50, 51). The main effectors of entry into epithelial cells are *IpaB* and *IpaC* (29), which are also needed for escape from the phagosome (17, 64) and for lysis of the membrane surrounding bacteria during the process of cell-to-cell dissemination (40). In addition, *IpaB* is responsible for the induction of the apoptotic process in macrophages (5, 65). Before secretion, *IpaB*
and IpA cpe associate independently with the chaperone IpG C (31).

Products of the ysc locus called yscB, yspB, yspC, and yspD share limited sequence similarity to the products of siaA, siaB, sipC, and sipD from the Salmonella SPI-1 TTSS and to the products of ipyC, ipaB, ipaC, and ipaD from Shigella Mxi-Spa TTSS.

In this study we characterize the products of the yscB, yspB, yspC, yspD, and yspA genes, and we identify YspA, YspB, YspC, and YspD as secreted proteins and SecB as a chaperone for YspB and YspC. We also show that under low-temperature and high-salt (LTHS) conditions the pYV-encoded YopE protein is secreted by the Ysa apparatus together with the Ysp proteins. Although YspC and probably YspB proteins could be secreted by the Shigella Mxi-Spa apparatus, we did not observe any functional complementation of ipyB or ipyC mutants. Additionally, they could not form detectable pores in eukaryotic target cells and could not substitute for YopB and YopD for translocation of Yop effectors.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli Top10 and XL-1 Blue were used for standard genetic manipulation. E. coli was grown in tryptic soy broth (TSB) (Oxoid) and plated on tryptic soy agar. For induction of secretion by the Ysa injection, Y. enterocolitica was grown overnight at room temperature in brain heart infusion (BHI) (Remel, Lenexa, Kans.) and inoculated to an optical density at 600 nm of 0.1 in 10 ml of fresh BHI supplemented with 4 mM glucose/ml, 20 mM MgCl2, and 20 mM sodium oxalate (BHI-Ox). Cultures were incubated for 2 h at room temperature and then shifted for 3 h to 37°C. For induction of secretion by the Ysa system, Y. enterocolitica was grown overnight at room temperature in Luria broth (LB) ( Gibco-BRL Life Technologies), inoculated to an optical density of 0.1 in 10 ml of fresh LB supplemented with 0.49 mM NaCl, and incubated at 26°C for 18 h (14). To monitor YspB-(His)6 secretion by Y. enterocolitica 8081, Y. enterocolitica 8081(pBF23) (pMM0100), (100 mM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside) was added prior to the 18-h incubation at 26°C. To monitor secretion by S. flexneri, an overnight culture at 37°C in TSB was diluted 1/10 in 30 ml of fresh TSB and grown for 4 h at 37°C. The following selective agents were used at the indicated concentrations: nalidixic acid, 35 µg/ml; streptomycin, 50 µg/ml; ampicillin, 200 µg/ml; chloramphenicol, 20 µg/ml; kanamycin, 50 µg/ml; and sodium arsenite, 0.4 mM. The bacterial strains used in this study are listed in Table 1.

Construction of recombinant plasmids. (i) Construction of pCNR26-derived recombinant plasmids. The yscB, yspB-yopH, or yscB-yspB-yspC genes were PCR amplified with CoS23 DNA as a template with amplifiers Mipa 1143 (5’-GAATTCTCGGGTCGCTGAA-3’) and Mipa 1144 (5’-CCCAAGCTTCCGGGCTGC-3’) and cloned in the Ndel-HindIII sites of pCNR26, giving pBF15. pBF15 DNA was used as a template for reverse PCR from ysaN-yopA and yspA-yopH, leading to the pPT14 with the oligonucleotides Mipa 1197 (5’-CTTGCTCGAGTTAACCCTTAACAATCC-3’) and Mipa 1250 (5’-CCGGCATCGAAAGAGGAAACATG-3’) and cloned into BamHI-XhoI sites of pGEX-6P-1, giving pBF21, and pBF22, respectively. A 1,062-bp segment of yspB and surrounding bases was amplified by PCR with the oligonucleotides Mipa 1257 (5’-CCGGGATCCACCAACAGCATTGACATCTCTGGG-3’) and Mipa 1258 (5’-CCGGAATTCCGACCAATGCC-3’) and cloned in the BamHIEcoRI sites of pGEX-4T-3 (Amersham Biosciences), giving pFT14. The plasmids used in this study are listed in Table 1.

YsaN, yspA, yspB, and yspD mutagenesis. A 399-bp segment of yspB was amplified by PCR with oligonucleotides Mipa 922 (5’-ATGTTGGGCTGACGCACCA-3’) and Mipa 923 (5’-TTAGGGCTAACAAGGGC-3’) and blunt end cloned in the EcoRV site of suicide vector pKNG90A (23), giving pBF19. A 402-bp segment of ysaN was amplified by PCR with oligonucleotides Mipa 841 (5’-GAGCCTGAGTATACCGCC-3’) and Mipa 842 (5’-CCGGCGTTTGAGGG-3’) and blunt end cloned in the EcoRV site of pKNG90A suicide vector, giving pBF22. A 510-bp segment of yspB was amplified by PCR with pFT14 with the oligonucleotides Mipa 1259 (5’-GCTTCTGACAGTAGGTGACGAGCA-3’) and Mipa 1260 (5’-GCTCTAGATCTCTCTGATGATTGGAG-3’) and blunt end cloned in the XbaI site of the suicide vector pFT17, leading to the pFT15 yspB mutant. A 501-bp segment of yspA was amplified by PCR with the oligonucleotides Mipa 1261 (5’-GCTCTATTGCTAGATCTCGACGAGCA-3’) and Mipa 1262 (5’-GCTGCTAGATCTCACTTCTGAGG-3’) and cloned in the XbaI site of the suicide vector pFT17, leading to the pFT16 yspA mutant. The suicide plasmids were introduced into Y. enterocolitica strain 8081 by electroporation. Transformants were selected for their ability to grow on a medium containing streptomycin.

The insertion of the suicide plasmid was checked by PCR with the Expand long template system (Boehringer Mannheim GmbH).

Analysis of proteins from culture supernatants. Y. enterocolitica or S. flexneri was harvested from centrifugation at 2,500 × g for 15 min. Proteins from the culture supernatant were precipitated with trichloroacetic acid (TCA) at a 10% (wt/vol) final concentration and resuspended in Laemmli buffer at concentrations indicated in the figure legends. For Congo red-induced secretion of S. flexneri, the bacterial pellet of a 4-h culture was resuspended in phosphate-buffered saline (PBS). Bacteria were then incubated in the presence of 0.1 µg/ml of Congo red/ml for 30 min at 37°C. Samples were centrifuged for 10 min at 14,000 × g, and proteins present in the supernatant were TCA precipitated and resuspended in Laemmli buffer. Proteins from the crude extracts of E. coli, Y. enterocolitica, or S. flexneri were resuspended in Laemmli buffer, boiled for 5 min at 95°C, and loaded on sodium dodecyl sulfate (SDS)-12% (wt/vol) polyacrylamide gels (SDS-polyacrylamide gel electrophoresis). After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane. Immunoblotting was carried out with anti-GST polyclonal antibody (Amersham Pharmacia Biotech) and anti-His (C-terminal) monoclonal antibody (Invitrogen) as recommended by the manufacturer. Anti-YspC polyclonal antibody was used at a dilution of 1:5,000, and anti-YspD polyclonal antibody was used at a dilution of 1:2,000. YopE and SycE were detected with polyclonal antibodies used at dilutions of 1:15,000 and 1:500, respectively. Supersignal chemiluminescent substrates (Pierce) were used for chemiluminescence detection. The production and purification of YspC and YspD were performed according to the protocol supplied by Amersham Pharmacia Biotech. YspC and YspD were eluted by Plession protease cleavage. One milligram of the purified YspC or YspD was used to immunize a rabbit.

SycB, YspB, and YspC binding and stabilization experiments. GST-SycB plus YspB-(His)6, and GST-SycB-yspB-(His)6, production was induced with 0.5 mM IPTG when the optical density at 600 nm reached 0.5, and the mixture was incubated further for 80 min. Bacteria (5 × 109) were harvested at 22,000 × g for 5 min. The bacterial pellet was resuspended in 1 ml of PBS (pH 7.4) containing 0.5 µg of 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) per ml and sonicated. The soluble fraction was recovered by a 15-min centrifugation step (14,000 × g) at 4°C. For coimmunoprecipitation, polyclonal anti-GST antibody (Amersham Biosciences) was added at a 1:200 dilution with the mixture just before centrifugation. The pellet was harvested by addition of 50 µl of protein A-Sepharose CL-4B (50% slurry in PBS). The complex was then gently rocked overnight at 4°C, collected by cen-
### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> Top 10</td>
<td>F- mcrA Δ(mvr mutant hisG mutant mcrBC) 680lacZΔM15 ΔlacY74 deoR recA1 araD139 Δ araL leu7697 galU galK rpsL endA1 supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>(F' proAB lacF' ΔZΔM15 Tn10)ΔY2444 hisD217 recA1 endA1 gyrA46 thi relA1 lac</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> A127/90</td>
<td>Clinical isolate from acute gastroenteritis (Japan); serotype O:8, biotype 1B</td>
<td>18</td>
</tr>
<tr>
<td>8081</td>
<td>Clinical isolate from fatal septicemia (Ohio); serotype O:8, biotype 1B</td>
<td>43</td>
</tr>
<tr>
<td>ΔHOPEMT</td>
<td>Invasin mutant derivative from strain MRS40(pIML421)</td>
<td>This work</td>
</tr>
<tr>
<td>8081BF19</td>
<td>Integration of the suicide plasmid pBF19 into yspB</td>
<td>This work</td>
</tr>
<tr>
<td>8081FM2</td>
<td>Integration of the suicide plasmid pFM2 into yspN</td>
<td>This work</td>
</tr>
<tr>
<td>8081PT16</td>
<td>Integration of the suicide plasmid pPT16 into yspA</td>
<td>This work</td>
</tr>
<tr>
<td>8081PT15</td>
<td>Integration of the suicide plasmid pPT15 into yspD</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. flexneri</em> M90T</td>
<td>Clinical isolate</td>
<td>47</td>
</tr>
<tr>
<td>RM97</td>
<td>ipaC</td>
<td>31</td>
</tr>
<tr>
<td>RM221</td>
<td>ipaB</td>
<td>32</td>
</tr>
<tr>
<td>SF1068</td>
<td>ipaB ipaC</td>
<td>28</td>
</tr>
<tr>
<td>RM81</td>
<td>ipaC</td>
<td>32</td>
</tr>
<tr>
<td><strong>Plasmids</strong> pYV plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pYV40</td>
<td>Wild-type pYV plasmid of <em>Y. enterocolitica</em> E40</td>
<td>Sory and Cornelis, unpublished data</td>
</tr>
<tr>
<td>pABL403</td>
<td>pYV40 yopEΔ170, yopHΔ351-352, yopOΔ655-658, yopPΔ351, called ΔHOPEM</td>
<td>52</td>
</tr>
<tr>
<td>pML421</td>
<td>pYV40 yopEΔ170, yopHΔ351-352, yopPΔ217, yopMΔ23, called ΔHOPEMT</td>
<td>2</td>
</tr>
<tr>
<td>pMSK50</td>
<td>pYV40 yopEΔ170, yopHΔ351-352, yopOΔ655-658, yopPΔ217, yopDΔ121, called ΔHOPEMYscN</td>
<td>19</td>
</tr>
<tr>
<td>pAB409</td>
<td>pYV40 yopEΔ170, yopHΔ351-352, yopOΔ655-658, yopPΔ217, yopMΔ23, called ΔHOPEMB</td>
<td>Neyt and Cornelis, unpublished data</td>
</tr>
<tr>
<td>pCNK4004</td>
<td>pYV40 yopEΔ170, yopHΔ351-352, yopOΔ655-658, yopPΔ217, yopMΔ23, called ΔHOPEMB, obtained by allelic exchange between ΔHOPEMB and pMSL19</td>
<td>2</td>
</tr>
<tr>
<td>pCNK4005</td>
<td>Introduction of pMSL19 into yopBΔ9-217 mutant bacteria, giving yopBΔ9-217 yopDΔ121-165</td>
<td>Neyt and Cornelis, unpublished data</td>
</tr>
<tr>
<td><strong>Expression plasmids and vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBF15</td>
<td>pCNR26 yopE yscB+ yspB+ yspC+ (from A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF17</td>
<td>pBF15 yscB+ yspB(His)+ yspC+ (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF35</td>
<td>pGEX-6P-1 yscB+ yscB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF27</td>
<td>pGEX-6P-1 yscB+ yspB+ (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF28</td>
<td>pGEX-6P-1 yscB– yspB+ (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF29</td>
<td>pGEX-6P-1 yscB+ yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF30</td>
<td>pGEX-6P-1 yscB+ yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF31</td>
<td>pGEX-6P-1 yscB+ yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF32</td>
<td>pGEX-6P-1 yscB– yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF33</td>
<td>pGEX-6P-1 yscB+ yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF34</td>
<td>pGEX-6P-1 yscB– yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF35</td>
<td>pGEX-6P-1 yscB+ yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF36</td>
<td>pGEX-6P-1 yscB– yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF37</td>
<td>pBluescriptSk+ yscB+ yspB+ (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF38</td>
<td>pBluescriptSk+ yscB– yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pBF39</td>
<td>pBluescriptSk+ yscB– yspB– (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pMM100</td>
<td>pACYC184 lacI857+</td>
<td>41</td>
</tr>
<tr>
<td>pPT14</td>
<td>pGEX-4T-3 yspD+ (A127/90)</td>
<td>This work</td>
</tr>
<tr>
<td>pCNR26</td>
<td>Cloning vector with yopE and optimized ribosome binding site</td>
<td>49</td>
</tr>
<tr>
<td>pGEX-4T-3</td>
<td>Cloning vector, for generation of fusion with GST</td>
<td>Pharmacia Biotech</td>
</tr>
<tr>
<td>pGEX-6P-1</td>
<td>Cloning vector, for generation of fusion with GST</td>
<td>Pharmacia Biotech</td>
</tr>
<tr>
<td>pBluescriptSk+(−)</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Suicide vectors and mutator plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBF19</td>
<td>399 bp of yspB (A127/90) cloned into pKNG90A</td>
<td>This work</td>
</tr>
<tr>
<td>pFM2</td>
<td>402 bp of ysaN (A127/90) cloned into pKNG90A</td>
<td>This work</td>
</tr>
<tr>
<td>pPT15</td>
<td>510 bp of yspD (A127/90) cloned into pPT7</td>
<td>This work</td>
</tr>
<tr>
<td>pPT16</td>
<td>501 bp of yspI (A127/90) cloned into pPT7</td>
<td>This work</td>
</tr>
<tr>
<td>pKNG90A</td>
<td>Suicide vector</td>
<td>23</td>
</tr>
<tr>
<td>pMS154</td>
<td>inv mutator</td>
<td>53</td>
</tr>
<tr>
<td>pMSL19</td>
<td>pKNG101 yopDΔ121-165</td>
<td>35</td>
</tr>
<tr>
<td>pPT7</td>
<td>Derivative of pKNG101 without PstI sites and sacBR</td>
<td>This work</td>
</tr>
</tbody>
</table>
trifugation at 600 × g, washed five times in PBS, and eluted from the protein A-Sepharose CL-4B by being boiled in sample buffer. The eluted proteins were analyzed by SDS-PAGE.

For GST affinity-binding experiments, 50 μl of glutathione-Sepharose CL-4B (50% cross-linked in PBS) was added to the soluble fraction and incubated overnight with gentle rocking at 4°C. The washing and elution steps were the same as for coprecipitation. For GST-Syb-yscC binding, GST-SybC production was induced with 0.5 mM IPTG for 80 min. Bacteria (5 × 10^9) were centrifuged at 15,000 rpm for 5 min. The bacterial pellet was resuspended in 1 ml of PBS (pH 7.4)-0.5 mg of CHAPS per ml and sonicated. The soluble fraction was recovered by a 15,000 rpm centrifugation step (14,000 × g) at 4°C. Twenty micrograms of purified YscC was added to the soluble fraction and incubated for 1 h at 4°C. Fifty microliters of glutathione-Sepharose CL-4B (50% slurry in PBS) was added to the soluble fraction and incubated overnight with gentle rocking at 4°C. The washing and elution steps were the same as described above.

For YspB stabilization experiments, GST-SybC + YspB-(His)₆, and GST-SybC-(His)₆ production was induced as described above. After 80 min, protein synthesis was stopped by adding 20 μg of chloramphenicol per ml to the culture and an initial 500-μl aliquot was taken. Similar aliquots were then taken every 40 min. The content of 5 × 10^10 bacteria was analyzed by SDS-PAGE.

BCECF release assay. J774 mouse monocyte macrophage cell lines were grown routinely in RPMI 1640 medium (Gibco) supplemented with 2 mM l-glutamine (Seromed), 10% (vol/vol) fetal bovine serum (Gibco), 100 U of penicillin (Gibco) per ml, and 100 μg of streptomycin per ml at 37°C under 5% CO₂. At 20 h before infection, cells (4 × 10^6 cells per ml) were seeded in 24-well tissue culture plates (1 ml per well). Just before infection, cells were washed twice with 1 ml of PBS and labeled by incubation for 20 min at 37°C with 10 μM BCECF-AM (2′,7′-bis-(carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxyethyl ester; Molecular Probes). Cells were then washed twice with RPMI with 2 mM l-glutamine (Seromed) and incubated further with 1 ml of the same medium containing bacteria. Y. enterocolitica strains were grown for 90 min at room temperature in BHI-Ox and then transferred to 37°C for 2 h to induce Yop synthesis. Bacteria were then washed and resuspended in prewarmed saline. Cells were infected for 1 h at a multiplicity of infection of 125. To facilitate contact between bacteria and cells, the plates were centrifuged for 5 min at 400 × g. After 1 h of infection, the macrophages were pelleted by centrifugation at 250 × g for 5 min. Aliquots of the cell culture supernatant (150 μl) were transferred into a 96-multiwell plate, and BCECF fluorescence was measured with a fluorescence microplate reader (Perkin-Elmer HTS 7000+) with an excitation wavelength of 440 nm and an emission wavelength of 520 nm. The amount of BCECF released by cells treated with Triton X-100 (0.1%) for 1 h was taken as 100% of release. The percentage of lysis was calculated by the following formula: percent lysis = (sample uninfected)/(Triton uninfected).

YspE injection. The J774 cells were grown routinely as indicated above. Twenty hours before infection, six-well plates were seeded with 5 × 10^5 cells per well. Prior to infections, freshly grown Y. enterocolitica strains were preincubated at room temperature for 2 h and at 37°C for 2 h, washed in RPMI, and added to the cells at a multiplicity of infection of 50. After 2 and 4 h of infection, cells and bacteria were scraped from the plate, collected in 2-ml tubes, washed twice with PBS, and lysed in 100 μl of 0.1% Triton X-100 in PBS supplemented with 100 μM phenylmethylsulfonyl fluoride, 1 μg of apolipoprotein per ml, and 1 M leupeptin. Lysates were then centrifuged for 10 min at 20,800 × g. The supernatants, corresponding to the cellular cytoplasmic fractions, were recovered and added to 25 μl of 5% Laemmli buffer. The pellet (bacteria and J774 membranes) was resuspended in 120 μl of water and 30 μl of Laemmli buffer. Both fractions were separated on SDSPBS-12% polyacrylamide gels and analyzed by Western blotting.

Immunofluorescence experiments and invasion assay. To distinguish between intracellular and extracellular Y. enterocolitica bacteria, the double-immunofluorescence test was performed as described by Rosqvist et al. (45). S. flexneri invasion of HeLa cells was tested by the gentamicin protection assay (20). For immunofluorescence microscopy examinations, infected cells were treated as described in references 39 and 57.

Mass spectrometry (MS). (i) In-gel tryptic digestion. Stained protein bands were cut from the SDS-polyacrylamide gel and washed with high-pressure liquid chromatography-grade water. The gel was cut into 1-mm cubes, which were washed twice for 15 min with 50% acetonitrile-0.1 M ammonium bicarbonate in an Eppendorf tube. The gel pieces were then dried under vacuum. Sequencing-grade trypsin was added to the dried gel pieces (10 μl of 0.1 mg/ml in 0.1 M ammonium bicarbonate). The tube was left on ice for 20 min, then an additional 50 μl of 0.1 M ammonium bicarbonate was added, and gel pieces were incubated overnight at 30°C. The supernatant was collected in a clean Eppendorf tube, and the gel was extracted once more by adding 50 μl of 60% acetonitrile-0.1% trifluoroacetic acid. The supernatants were combined, and the volume was reduced to 1 to 2 μl under vacuum and stored at −20°C for MS analysis. (ii) MS. Solutions from in-gel digestions were acidified with 10 μl of 0.1% trifluoroacetic acid, adsorbed on a pipette tip (ZapTip C₁₂, Millipore), desalted by being washed eluted with 1 to 4 μl of 50% acetonitrile-0.5% acetic acid. Peptides were analyzed by nanoelectrospray ionization-tandem MS (MS/MS). Briefly, 2 μl of the eluate was analyzed in an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, Calif.) fitted with a nanoelectrospray probe (61). Spectra were taken in full MS and zoom scan mode to determine parent masses and their charge state. The source voltage was set at 0.8 kV with a cone voltage of 36 V. Selected peptides were fragmented by collision-induced dissociation to generate tandem mass spectra. The collision energy was adjusted to the minimum needed for fragmentation. For the identification of proteins, fragment masses from collision-induced mass spectra were compared by use of the computer program MS-Tag (6) to predicted spectra from protein sequence databases. If no positive identification was found, peptide sequences were derived de novo from MS/MS spectra by following the fragmentation path of y-ion series and b-ion series.

Nucleotide sequence accession number. The nucleotide sequence of the yscB-yspBCDA-acpY locus has been deposited in the GenBank nucleotide sequence database under accession number AY100449.

RESULTS

Analysis of the sequence of proteins encoded by the ysc-ysp locus. The organization of the ysc-ysp locus, which consists of the yscB, yspB, yspC, yspD, yspA, and acpY genes, suggests that it constitutes an independent operon, since yscB is separated by 98 nucleotides from ysaU and only short intergenic regions separate the six coding regions. The organization of the locus is presented in Fig. 1A. yscB encodes a 170-residue polypeptide with a predicted molecular mass of 19 kDa, an acidic pI of 4.4, 46% identity with IpgC from S. flexneri, and 53% identity with SicA from S. enterica. yspB encodes a polypeptide of 580 residues with a predicted molecular mass of 67 kDa and a low similarity (20% identity) to IpaB from S. flexneri. However, identity rises to 40% in a hydrophobic central stretch of 119 residues (amino acids [aa] 370 to 489) that corresponds to the central domain of IpaB (aa 287 to 414). Transmembrane domain prediction analysis revealed two possible transmembrane α-helices spanning residues 348 to 376 and 429 to 447, yspC encodes a polypeptide of 382 residues with a predicted molecular mass of 48 kDa and 16% identity with IpaC. This percentage of identity increases between aa 150 and 227 to reach 25%. The transmembrane domain prediction algorithm revealed one possible transmembrane α-helix between aa 241 and 259, yspD encodes a polypeptide of 348 aa with a predicted molecular mass of 38.7 kDa, 23% identity with IpaD of S. flexneri, and 18% identity with SipD of S. enterica. Although the carboxy-terminal parts of IpaD and SipD exhibit 73% identity in comparison with the 40% global identity (24), the C-terminal part of YspD does not appear to be more conserved than the N-terminal part. yspA encodes a polypeptide of 644 aa with a predicted molecular mass of 68.7 kDa and no significant identity with IpaA or SipA. acpY encodes a polypeptide of 83 aa that presents a low similarity with acyl carrier proteins. Putative acyl carrier proteins are also encoded downstream from ipaA in S. flexneri and sipA in S. enterica. However, AcpY from Y. enterocolitica does not share any identity with Acp from S. flexneri or with IacP from S. enterica, which are themselves 31% identical.

Analysis of the phenotype of Y. enterocolitica strain 8081 ysaU::yfpFM2. A set of approximately 10 proteins called YsapA to -K has been detected in the culture medium of Y. enterocolitica.
strain 8081 after growth at 28°C for 18 h in LB containing 490 mM NaCl (14) or at 26°C for 18 h in LB containing 290 mM NaCl (63). To determine which proteins were released by the Ysa apparatus, we created a polar insertion mutant of *Y. enterocolitica* strain 8081 by integrating the suicide plasmid pFM2 into ysaN, the putative energizer of the Ysa TTS apparatus. This mutation led to the disappearance of eight bands in the pattern of proteins secreted under LTHS conditions (Fig. 1B). The most likely explanation is that secretion of these eight proteins depends on the presence of a functional Ysa TTS apparatus.

Identification of the 76-, 48-, and 23-kDa proteins as YspA, YspC, and YopE, respectively. To identify proteins whose secretion was dependent on the Ysa TTS apparatus, we analyzed the five major proteins (76, 48, 46, 44, and 23 kDa) secreted by Ysa and recovered from the supernatant of *Y. enterocolitica* strain 8081 (Fig. 1B). The 76-kDa protein was in-gel trypsin digested, and the resulting fragments were analyzed by MS. The sequence of three peptides could be determined: a/TQVILD, b/I/LSAD, and c/QGTSFA, where I/L represents an indeterminate result on the mass between leucine and isoleucine. All three peptides are present in the amino acid sequence translated from yspA, corresponding to residues 103 to 107, 216 to 219, and 514 to 519, respectively. However the size of the protein (76 kDa) did not correspond to that predicted for the yspA product (69 kDa). To elucidate that point, we created a yspA mutant by integrating plasmid pPT16 into yspA. When Ysa-mediated secretion was induced in *Y. enterocolitica* strain 8081 yspA::pPT16, the 76-kDa band disappeared (Fig. 1B), confirming that the protein migrating at the apparent molecular mass of 76 kDa was indeed YspA.

Following trypsin digestion of the 48-kDa protein, two peptides were identified by MS: I/LNSI/L-(R/K) and I/LDAI/LI/LADDTEQR, where — represents an undetermined residue before the trypsin cleavage site represented by (R/K). The first peptide matched exactly the sequence of residues 427 to 430 of YspC from *Y. enterocolitica* strain A127/90, and the second one corresponded to residues 128 to 139 of YspC, except for the Thr instead of an Ala in position 136 that was predicted from the nucleotide sequence determined from strain A127/90. This change from Thr to Ala fits with a difference of one base (GCC to ACC) between the yspC sequences from strains 8081 and A127/90. YspC from *Y. enterocolitica* strain A127/90 was cloned in an expression vector, giving pBF23 (yspB(His)6) grown at 26°C for 18 h in LB containing 490 mM NaCl. When necessary, IPTG at a 1 mM final concentration was added prior to the 18-h incubation. Numbers at right are molecular weights (MW) in thousands.

FIG. 1. Identification of proteins secreted by the Ysa TTSS. (A) Detail of the organization of the sycB-yspB-yspC-yspD-yspA operon located 98 bp downstream of ysaU (white arrow). (B) Culture supernatants of 1.5 × 10⁸ *Y. enterocolitica* 8081 bacteria grown at 26°C for 18 h in LB containing 490 mM NaCl were precipitated with TCA (10%), loaded on an SDS–12% polyacrylamide gel, and stained with Coomassie blue. WT, wild-type strain 8081; ysaN, 8081FM2; yspD, 8081PT15; yspB, 8081BF19; yspA, 8081PT16. Proteins secreted by the Ysa TTS apparatus are labeled by arrows, and their apparent molecular weights (MW, in thousands) are indicated. NI, nonidentified proteins. (C) Western blot of proteins from culture supernatants and bacterial extracts with rabbit polyclonal antibodies directed against YspC (48 kDa), YspD (38 kDa), YopE (23 kDa), and SycE (15 kDa); the same strains as in panel B are shown, with (+) or without (−) their pYV plasmid. (D) Western blot of proteins from the culture supernatant of *Y. enterocolitica* 8081 carrying (+) or not (−) pMM100 (lacI) and pBF23 (yspB(His)6) grown at 26°C for 18 h in LB containing 490 mM NaCl. When necessary, IPTG at a 1 mM final concentration was added prior to the 18-h incubation. Numbers at right are molecular weights (MW) in thousands.
The sequences of two peptides were obtained from the analysis of the 23-kDa protein: NHDOQFATGSGPLR and SGVDI/LTQAAEEL/K. The sequence of these peptides corresponds to residues 132 to 144 and 194 to 206, respectively, of the pYV-encoded YopE protein. Indeed, a polyclonal antibody raised against YopE reacted against the 23-kDa protein secreted by Y. enterocolitica strain 8081. In addition, the 23-kDa protein was absent from the culture supernatant of a derivative of Y. enterocolitica strain 8081. Indeed, a polyclonal antibody raised against YopE reacted against the 23-kDa protein secreted by Y. enterocolitica strain 8081. In addition, the 23-kDa protein was absent from the culture supernatant of a derivative of Y. enterocolitica strain 8081.

In previous reports, the letter code for the Ysp proteins had been assigned according to their molecular weight (14, 63). In the Foutlier et al. paper, a letter code consistent with Ipa and Sip nomenclature was introduced (11). Since the Ysp proteins are so clearly related to the Ipa and Sip proteins (see above), we continue to adopt the uniform ipa-sip nomenclature to avoid future confusion.

Attempts to deregulate Ysa secretion. A classical although not understood way to deregulate secretion by the Mxi-Spa TTSS of S. flexneri is to add Congo red to the culture medium (42). To check whether Congo red could have the same effect on Ysa, we added Congo red (3 mg/ml) to the culture of Y. enterocolitica strain 8081 and monitored the level of Ysps in the supernatant during both exponential and stationary growth phases. Congo red did not increase the level of Ysps in the culture supernatant (data not shown).

Another way to deregulate the Mxi-Spa TTSS of S. flexneri consists in inactivating ipaB or ipaD. This leads to enhanced secretion of the remaining Ips as well as secretion of a set of about 15 other proteins (30, 42). We thus analyzed the culture supernatant of our yspB and yspD mutants. In both cases, the level of secretion of the remaining Ysp was similar to that observed with the wild-type strain, and no additional bands could be observed in an SDS-polyacrylamide gel (Fig. 1B). This suggests that regulation of secretion by the Ysa TTSS is different from that of the Mxi-Spa TTSS.

SycB binds YspB and YspC in E. coli. To analyze the ability of SycB to bind YspB and YspC as suggested by its homology with IpgC (31), we constructed plasmid pBF23 carrying a bicistronic gst-sycB yspB(His)₆ ptac-dependent operon. To serve as a control, we used plasmid pBF24 encoding truncated GST-SycB₁₋₂₁ and YspB-(His)₆ (Fig. 2A). After 80 min of induction by IPTG, cleared extracts of E. coli were mixed either with glutathione-Sepharose beads or with an anti-GST antibody absorbed on protein A-Sepharose beads. After washing, proteins absorbed on the beads were analyzed by Western blotting. YspB-(His)₆ coeluted with GST-SycB but not with GST-SycB₁₋₂₁. Similarly YspB was coimmunoprecipitated with GST-SycB (Fig. 2B). This indicated that GST-SycB is able to bind specifically YspB-(His)₆.

To investigate whether SycB was also able to bind YspC, purified YspC was incubated with glutathione-Sepharose beads in the presence of cleared lysates of bacteria overexpressing GST-SycB or GST. After washing, proteins were eluted from the matrix and analyzed on an SDS-polyacrylamide gel. A 48-kDa protein copurified with GST-SycB but not with GST (Fig. 3A); The polyclonal anti-YspC antibody recognized this 48-kDa band (Fig. 3B), indicating that SycB was also able to bind YspC.

SycB stabilizes YspB in E. coli. To test whether binding of SycB to YspB could help to stabilize YspB, expression of GST-SycB was induced by IPTG and the bacterial content of E. coli was analyzed by SDS-PAGE and Western blotting at various time points after inhibition of protein synthesis by the addition of chloramphenicol to the culture medium. The YspB-(His)₆ content was stable up to 320 min in the presence of GST-SycB (Fig. 4A), whereas it immediately decreased after addition of chloramphenicol to completely disappear after 160 min of incubation in the presence of GST-SycB₁₋₂₁ (Fig. 4B).
It thus appeared that GST-SycB stabilizes YspB-(His)_6 in E. coli.

**YspB and YspC can be secreted via the Ysc injectisome.** As shown above the pYV-encoded YopE protein is secreted via the Ysa apparatus following growth of bacteria under LTHS conditions, indicating that some Yops can be secreted by more than one TTS apparatus. We wondered whether YspB and YspC could be recognized as substrates by the Ysc apparatus. Firstly, the sycB, yspB, and yspC genes were cloned into vector pCNR26 under the control of the yopE promoter, giving pBF15. This promoter ensured a coordinate expression of sycB, yspB, and yspC with the activity of the Ysc TTS apparatus. Then, we fused a (His)_6 tag at the C terminus of YspB on pBF15, giving pBF17. Plasmid pBF17 was introduced in the polymutant *Y. enterocolitica* ΔHOPEM, a derivative of the E40 biotype 2 strain that does not carry any ysa locus (11). When Yop secretion was induced, *Y. enterocolitica* ΔHOPEM bearing pBF17 produced both YspB-(His)_6 and YspC as seen by a Western blot analysis of the crude extracts (Fig. 5A). Moreover, both YspC and YspB-(His)_6 were detected in the supernatant of the culture (Fig. 5A). To know if this presence was due to Ysc-mediated secretion and not to secretion mediated by the flagellar TTS apparatus at 37°C or simply to bacterial lysis, we introduced pBF17 in the ΔHOPEM/YscN strain. This strain lacks the YscN ATPase and is defective for Yop secretion. When secretion by the Ysc injectisome was induced,
YspB and YspC do not complement YopB and YopD for pore formation and effector translocation in J774 macrophages. The facts that YspB and YspC can be secreted by the Ysc injectisome and that they are homologous to the pore-forming proteins YopB and YopD led us to investigate whether they could form pores in J774 macrophage cells. Experimentally, we monitored pore formation by the BCECF release method used by Neyt and Cornelis (37). Prior to infection, macrophages were first loaded with BCECF-AM, a membrane-permeant dye that becomes fluorescent and membrane impermeant after cleavage by intracellular esterases. As expected, BCECF was released from macrophages infected with the ΔHOPEM strain but not with the ΔHOPEMBD strain (Fig. 5B). However, when pBF15 coding for sycB, yspB, and yspC was introduced in trans into ΔHOPEMBD, no BCECF was released from the infected macrophages. Although it was not possible in the experiment to determine whether YspB and YspC were released upon contact with macrophages, we again assumed that it was the case on the basis of our in vitro secretion experiment (Fig. 5C). This observation suggests that, despite their homology with YopB and YopD, YspB and YspC alone are not able to form a pore when secreted by the Ysc machinery.

In addition we tested if pBF15 could complement a yopB yopD mutant of Y. enterocolitica E40 for translocation of Yop proteins. J774 cells were infected with a yopB yopD mutant bacterial strain carrying pBF15, and the injection of YopE was tested. After 2 and 4 h of infection the infected macrophages were fractionated and the cytosolic fraction was tested with an anti-YopE antibody. Although YspC was detected in the Triton-insoluble fraction, YopE was not recovered in the cytosolic fraction, indicating that pBF15 could not complement the yopB yopD mutation (data not shown). In addition, macrophages did not undergo apoptosis, indicating that YopP was not injected (data not shown).

**Complementation of S. flexneri mutants.** To investigate if the function of SycB, YspB, and YspC was conserved during evolution, we tried to complement a series of *S. flexneri* mutants defective in the corresponding homologs. IgC is required for the stability of IpaB in the cytoplasm of *S. flexneri* (31), and inactivation of *igC* results in a decreased amount of IpaB (Fig. 6A). To test whether SycB could replace IgC in its role of chaperone for IpaB, plasmid pBF25, which constitutively expresses sycB from a lac promoter, was introduced into the *S. flexneri* strain RM97 (igC). We analyzed IpaB in the supernatant of *S. flexneri* bacteria after induction of Mxi-Spa secretion. There was no difference in the IpaB content between the supernatant of *igC* mutant bacteria and the supernatant of *igC plac-sycB*+ bacteria (Fig. 6A). Production of SycB from pBF25 in the *igC* mutant bacteria was detectable by SDS-PAGE and Coomassie blue staining (data not shown), but the presence of SycB did not result in an increased production of IpaB, indicating that SycB could not act as a chaperone for IpaB (Fig. 6A). In addition to its role as a chaperone for IpaB and IpaC, IgC acts as a coactivator of MxiE, the transcriptional activator involved in expression of genes that are regulated by the activity of the Mxi-Spa TTS apparatus (28). These genes include members of the *ipaH* family, which are expressed in an *ipaB* mutant as a consequence of the deregulated activity of the Mxi-Spa apparatus (10). In contrast to an *ipaB* mutant,
an ipaB ipgC mutant produces little IpaH (Fig. 6B). Expression
of SycB in the ipaB ipgC mutant did not restore production of
IpaH proteins, indicating that SycB could not act as a coacti-
vator for MxiE. As indicated above, inactivation of ipaB leads
to deregulated secretion, and increased amounts of Ipa proteins
are secreted by an ipaB mutant compared to those secreted by the
wild-type strain. To test whether YspB could complement the
ipaB mutant for this phenotype of deregulated secretion, we
introduced plasmid pBF26, which expresses both sycB and
yspB, into an ipaB mutant and analyzed by SDS-PAGE and
Coomassie blue staining the proteins secreted by the recombinant
strain (Fig. 6C). Similar amounts of proteins were secreted by the
ipaB mutant and its derivative expressing SycB and YspB, indi-
cating that YspB was not functionally equivalent to IpaB for regu-
ulating the Mxi-Spa apparatus.

Inactivation of the ipaC gene is associated with a defect in
entry into nonphagocytic cells (32). We introduced plasmid
pBF29, which constitutively expresses both sycB and yspC, into
S. flexneri RM81 (ipaC). The defect in entry of the ipaC mutant
was not complemented by YspC as monitored by a gentamicin
protection assay and immuno
fluorescence microscopy (data
not shown). Since this result could be the consequence of a
lack of secretion of YspC, we analyzed by Western blotting the
supernatant of S. flexneri RM81(pBF29) grown for 4 h of cul-
ture in tryptic soy broth at 37°C. YspC was recovered when
secretion was induced by addition of Congo red (Fig. 6D). The
polyclonal anti-YspC antibody did not cross-react with pro-
teins secreted by S. flexneri, because no signal was observed in
the supernatant of S. flexneri RM81 (Fig. 6D). We conclude
from this experiment that, even if YspC is recognized as a
substrate for the Mxi-Spa apparatus, it does not promote in-
vansion of eukaryotic cells. This can be explained in two ways:
either YspC does not interact with the other proteins of the
entry complex or it is not secreted in sufficient amounts. How-
ever, one cannot rule out the possibility that YspC is not linked
to cell invasion.

DISCUSSION

We had previously identified four open reading frames, ysp-
BCDA, located within the ysa locus and close to genes speci-
fying the Ysa TTS apparatus (11). Based on gene organization
and primary structure comparisons, we hypothesized that their
products could be substrates for secretion by the Ysa machin-
ery. An MS analysis after trypsin digestion of the five major
proteins that were secreted under LTHS conditions by wild-type *Y. enterocolitica* 8081 bacteria and not by the *ysaN* mutant bacteria allowed us to identify two of these proteins as YspA (76 kDa) and YspC (48 kDa). Phenotypical analysis of the *yspB* and *yspD* mutants and generation of anti-YspC and anti-YspD sera indicated that YspB and YspD are also secreted by the Ysa apparatus. Thus, the *yspA* to *-D* genes encode four proteins that are secreted by the Ysa apparatus. These four proteins are the first Ysa-specific substrates identified so far. None of the three other proteins that were secreted by *Y. enterocolitica* 8081 but not by the *ysaN* mutant bacteria are encoded by the *ysa* locus. Two did not correspond to any known proteins. These proteins are probably encoded by the chromosome but outside the *ysa* locus, as they were still secreted by a derivative of *Y. enterocolitica* strain 8081 that had been cured of the virulence plasmid. This situation is reminiscent of the SopE protein of *S. enterica* SspH1 protein, which is secreted by both SPI-1- and SPI-2-encoded TTSSs (33). To understand this recruitment, it will be interesting to determine whether secretion of YopE under LTHS conditions requires VirF or another, as yet unknown, transcription activator. Since Ysa is present only in biotype 1B strains, it would be interesting to study the expression of YopE under LTHS conditions, comparing strains from biotype 1B to strains from the low-virulence biotypes. The recruitment of YopE by Ysa also raises the question of the physiological significance of this dual role. Indeed the YspB and YspD proteins resemble the invasion-promoting IpaB and IpaD or SipB and SipD, while YopE has an anti-internalization activity. This does not seem to be consistent unless the YspB and YspD proteins are not invasion-promoting proteins, as suggested by our cell invasion experi-

FIG. 6. Lack of complementation of *S. flexneri* mutations. (A) Protein contents of cultures of *S. flexneri* wild-type (M90T), *ipgC* (RM97), *ipgC* carrying *plac* 
*yscB* (pBF15), and *ipaB* (RM221) strains grown for 4 h at 37°C were analyzed by Western blotting with monoclonal anti-IpaB antibody. (B) Protein contents of cultures of the *S. flexneri* *ipaB* strain, wild-type strain, *ipaB* *ipgC* (SF1068) strain, and *ipaB* *ipgC* 
*yscB* strain carrying pBF25 grown for 4 h at 37°C were analyzed by Western blotting with polyclonal anti-IpaH antibody. (C) Protein contents of culture supernatants of the *S. flexneri* wild-type strain, *ipaB* strain, and *ipaB* *yscB* *yspB* strain carrying pBF26 grown for 4 h at 37°C were determined by staining with Coomassie blue. (D) Protein contents of supernatants of the *S. flexneri* *ipaC* (RMS81) strain and *ipaC* *yscB* *yspC* strain carrying pBF29 after Congo red induction were analyzed by Western blotting with polyclonal anti-IpaB antibody. WT, wild type; MW, molecular weights in thousands.
ments. Alternatively, YopE could play in this context a role different from its anti-internalization one. Answers to these questions will probably await some progress in the identification of the target organism or cell for the Ysa system. Secretion of Yop proteins by the Ysa system represents a new example of the promiscuity of TTS effectors in Yersinia. In addition, we demonstrate that YspB and YspC can also be secreted via the Ysc TTSS. To complete the picture, it should be remembered that YplA, a protein secreted by the flagellar TTS apparatus of Yersinia, could also be secreted by Ysc and Ysa TTSSs (63).

Because of their putative transmembrane structure, YspB and YspC could interact with the eukaryotic cell membrane and behave as translocators for other secreted proteins such as YspD, YspA, and YopE (8). However, the type of contact that induces secretion by the Ysa system is still not known. In order to investigate the role of YspB and YspC in contact with eukaryotic cells, we took advantage of the fact that they can be secreted via the Ysc apparatus, and we tested whether they could complement YopB and YopD for pore-forming and effector translocation activity. YspB and YspC did not complement YopB and YopD, suggesting either that they are not pore formers or that they are not in the appropriate amount or conformation when secreted by the Ysc apparatus. By analogy with the Shigella and Salmonella systems, we also tested whether YspB and YspC secreted by the Ysc apparatus could promote entry into eukaryotic cells. This hypothesis was tested with an invasion-negative Y. enterocolitica strain, but again no conclusive result was obtained. However, this does not rule out the possibility that YspB and YspC are part of an entry mechanism, because other proteins encoded by this operon could be involved in the process. This could be solved by expressing the whole yecB-yspBCDA-acpY operon downstream from the yopE promoter. Addressing this question would presumably shed some light on the role of the second TTSS in Y. enterocolitica.

The last open reading frame identified was called acpY. AcpY resembles acyl carrier proteins involved in the biosynthesis of fatty acid chains. Interestingly, the activation step of toxins from the RTX family is accomplished by fatty acid acylation and requires the participation of an acyl carrier protein. Acyl groups are key elements in the attachment of an RTX toxin to the cell membrane (21, 54). When the biological function of Ysa is understood, it will be worthwhile testing the effect of a mutation in acpY on this function.

In consideration of the small size of SycB, its acidic pI, and its similarity to IgPC and SicA, there was a strong presumption that it might constitute a chaperone for YspB and YspC. By using a GST pull-down assay and coimmunoprecipitation experiments, we demonstrated that indeed SycB belongs in the family of bimodular TTS chaperones, which include IgPC, the chaperone of IpaB and IpaC from S. flexneri (29, 58); SicA (SipB and SipC) from S. enterica (16, 24); SycD (YopB and YopD) from Y. enterocolitica (38); and PcrH (PopB and PopD) from Pseudomonas aeruginosa (12). The role of these chaperones could be to maintain their substrates in a stable or TTS-competent state, to prevent cytoplasmic interaction between the two secreted partners, and to protect the bacterium from membrane toxicity (31, 38, 55). Our results demonstrate the validity of the third hypothesis, without ruling out the others.

ACKNOWLEDGMENTS

We thank Nathalie Sauvonnet for discussions and critical reading of the manuscript. We thank N. Grosdent for providing us Y. enterocolitica ΔHOPETMiv, C. Neyt for the gift of Y. enterocolitica ΔHOPEMBD, and M. P. Sory for construction of Y. enterocolitica ΔHOPEMTYscN.

P.T. and M.-N.M. are funded by the Belgian “Fond pour la formation à la Recherche dans l’Industrie et dans l’Agriculture (FRIA).” This work was supported by the Belgian “Fonds National de la Recherche Scientifique Médicale” (Convention 3.4595.97), by the “Direction générale de la Recherche Scientifique Communauté Française de Belgique” (Action de Recherche concertée 99/03-236), and by the EU TMR network (FMRX-CT98-0164), and by the Swiss National Science Foundation (grant 32-65393.1).

REFERENCES


