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

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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 (LTHS) 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 LTHS 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 LTHS 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 injectisome. 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

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and IpaC associate independently with the chaperone IpgC (31).

Products of the *ysa* locus called *sycB*, *yspB*, *yspC*, and *yspD* share limited sequence similarity to the products of *sicA*, *sipB*, *sipC*, and *sipD* from the *Salmonella* SPI-1 TTSS and to the products of *ipgC*, *ipaB*, *ipaC*, and *ipaD* from *Shigella* Mxi-Spa TTSS.

In this study we characterize the products of the *sycB*, *yspB*, *yspC*, *yspD*, and *yspA* genes, and we identify YspA, YspB, YspC, and YspD as secreted proteins and SycB 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 *ipaB* or *ipaC* 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 Ysc injectisome, *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 mg of glucose/ml, 20 mM $MgCl_2$, 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 at 600 nm of 0.1 in 10 ml of fresh LB supplemented with 0.49 M NaCl, and incubated at 26°C for 18 h (14). To monitor YspB-$(His)_6$ secretion by *Y. enterocolitica* 8081(pBF23)(pMM100), 1 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 *sycB-yspB-yspC* genes were amplified by PCR with Cos23 DNA as a template (11) with amplimers Mipa 1143 (5′-GGAATTCCATATGAACCAGAAACATGATGCG-3′) and Mipa 1144 (5′-CCCAAGCTTTTAACCCTTAACAATGGCC-3′) and cloned in the *Nde*I-*Hin*dIII sites of pCNR26, giving pBF15. pBF15 DNA was used as a template for reverse PCR amplification with oligonucleotides Mipa 1169 (5′-ACGTCTAGATTTCAGGAGAAACTATTTATGACC-3′) and Mipa 1170 (5′-ACGTCTAGATTAATGATGATGATGATGAGCAAAACTGCTTTTTATTAT-3′). The amplification product was then digested with *Xba*I and self-ligated, giving pBF17 encoding SycB, YspB-$(His)_6$, and YspC.

(ii) Construction of pBluescript SK(−)-derived recombinant plasmids. The *sycB*, *sycB-yspB*, or *sycB-yspB-yspC* genes were PCR amplified with Cos23 DNA as a template with amplimers Mipa 1251 (5′-CGCTCTAGAATAAAGGAGAGTATATGAACCAG-3′) and Mipa 1198 (5′-CCGCTCGAGTTATCCTTCTGCTGG-3′) or Mipa 1184 (5′-CCGCTCGAGTTAAGCAAAACTGCTTTT-3′) or Mipa 1186 (5′-CCGCTCGAGTTAACCCTTAACAATGGC-3′) and cloned into the *Xba*I-*Xho*I sites of pBluescript SK(−), yielding plasmids pBF25, pBF26, and pBF27, respectively. For construction of pBF29, pBF27 was digested with *Pst*I and *Nru*I, resulting in a 969-bp in-frame deletion in *yspB*. The linear DNA fragment was treated with T4 DNA polymerase and blunt end ligated to give pBF29.

(iii) Construction of pGEX-6P-1-derived recombinant plasmids. The *sycB-yspB*$(His)_6$ genes were PCR amplified with pBF17 DNA as template with oligonucleotides Mipa 1197 (5′-GCGGATCCAACCAGAAACATG-3′) and Mipa 1200 (5′-CCGCTCGAGTTAATGATGATGATGATG-3′) and cloned into *Bam*HI-*Xho*I sites of pGEX-6P-1 vector, giving the pBF23 plasmid encoding glutathione *S*-transferase (GST)–SycB YspB-$(His)_6$. When we sequenced our recombinant clones, we observed that one clone had suffered a point mutation (GAA→TAA) in codon 21 of *sycB*, giving the truncated *gst-sycB*$_{1-21}$ gene. This clone encoding GST-$SycB_{1-21}$ YspB-$(His)_6$ has been named pBF24. The *sycB*, *yspB*, and *yspC* genes were PCR amplified with Cos23 DNA as a template with amplimers Mipa 1197 (5′-GCGGATCCAACCAGAAACATG-3′) and Mipa 1198 (5′-CCGCTCGAGTTATCCTTCTGCTGG-3′), Mipa 1183 (5′-GCGGATCCGAACAGGAGAAACATCACC-3′) and Mipa 1184 (5′-CCGCTCGAGTTAAGCAAAACTGCTTTT-3′), and Mipa 1185 (5′-GCGGATCCACCACTATTCAACAAGCC-3′) and Mipa 1186 (5′-CCGCTCGAGTTAACCCTTAACAATGGC-3′) and cloned into *Bam*HI-*Xho*I sites of pGEX-6P-1 vector, giving pBF20, pBF21, and pBF22, respectively. A 1,062-bp segment of *yspD* and surrounding bases was amplified by PCR with the oligonucleotides Mipa 1257 (5′-CGCGGATCCACAACAGCGATTATTTCAC-3′) and Mipa 1258 (5′-CCGGAATTCTGACCATAGCCCATA-3′) and cloned in the *Bam*HI-*Eco*RI sites of pGEX-4T-3 (Amersham Biosciences), giving pPT14. The plasmids used in this study are listed in Table 1.

***ysaN*, *yspA*, *yspB*, and *ysaD* mutagenesis.** A 399-bp segment of *yspB* was amplified by PCR with oligonucleotides Mipa 922 (5′-ATGTTGGCCGATCAGGAC-3′) and Mipa 923 (5′-TTAGGGCATACCAACCG-3′) and blunt end cloned in the *Eco*RV site of suicide vector pKNG90A (23), giving pBF19. A 402-bp segment of *ysaN* was amplified by PCR with oligonucleotides Mipa 841 (5′-GCGAAGAGGAATCCGACCC-3′) and Mipa 842 (5′-CGCCGTTGTGTGAGGCTC-3′) and blunt end cloned in the *Eco*RV site of pKNG90A suicide vector, giving pFM2. A 510-bp segment of *yspD* was amplified by PCR from pPT14 with the oligonucleotides Mipa 1259 (5′-GCTCTAGACGAAGTGACGACAGCTA-3′) and Mipa 1260 (5′-GCTCTAGATCTCTGTGATGATGGGAG-3′) and cloned in the *Xba*I site of the suicide vector pPT7, leading to the pPT15 *yspD* mutator. A 501-bp segment of *yspA* was amplified by PCR with the oligonucleotides Mipa 1261 (5′-GCTCTAATGCCTGATATCACGGCA-3′) and Mipa 1262 (5′-GCTCTAGATCACTCAATCTCTGTGGGT-3′) and cloned in the *Xba*I site of pPT7, leading to the pPT16 *yspA* mutator. 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 plasmids 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 by 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 7 μg 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 sample 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 Prescission 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-$SycB_{1-21}$ plus 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 (5×10^8) were centrifuged at 22,000 × *g* for 5 min. The bacterial pellet was resuspended in 1 ml of PBS (pH 7.4) containing 0.5 mg 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. After 30 min of gentle rocking at 4°C, the immunocomplexes were 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

Strain or plasmid	Characteristic(s)	Reference or source
Bacterial strains		
E. coli		
Top 10	F$^-$ *mcrA* Δ(*mrr* mutant *hsoRMS* mutant *mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*araA leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
XL-1 Blue	(F′ *proAB lacI^q Z*Δ*M15* Tn*10*)*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*	Stratagene
Y. enterocolitica		
A127/90	Clinical isolate from acute gastroenteritis (Japan); serotype O:8, biotype 1B	18
8081	Clinical isolate from fatal septicemia (Ohio); serotype O:8, biotype 1B	43
ΔHOPEMT*inv*	Invasin mutant derivative from strain MRS40(pIML421)	This work
8081BF19	Integration of the suicide plasmid pBF19 into *yspB*	This work
8081FM2	Integration of the suicide plasmid pFM2 into *ysaN*	This work
8081PT16	Integration of the suicide plasmid pPT16 into *yspA*	This work
8081PT15	Integration of the suicide plasmid pPT15 into *yspD*	This work
S. flexneri		
M90T	Clinical isolate	47
RM97	*ipgC*	31
RM221	*ipaB*	32
SF1068	*ipaB ipgC*	28
RM81	*ipaC*	32
Plasmids		
pYV plasmids		
pYV40	Wild-type pYV plasmid of *Y. enterocolitica* E40	52
pABL403	pYV40 $yopE_{21}$ $yopH_{\Delta 1-352}$ $yopO_{\Delta 65-558}$ $yopP_{23}$ $yopM_{23}$, called ΔHOPEM	2
pIML421	pYV40 $yopE_{21}$ $yopH_{\Delta 1-352}$ $yopP_{23}$ $yopM_{23}$ $yopT_{135}$ $yopO_{\Delta 65-558}$, called ΔHOPEMT	19
pMSK50	pYV40 $yopE_{21}$ $yopH_{\Delta 1-352}$ $yopO_{\Delta 65-558}$ $yopP_{23}$ $yopM_{23}$ $yscN_{\Delta 169-177}$, called ΔHOPEMYscN	Sory and Cornelis, unpublished data
pAB409	pYV40 $yopE_{21}$ $yopH_{\Delta 1-352}$ $yopO_{\Delta 65-558}$ $yopP_{23}$ $yopM_{23}$ $yopB_{\Delta 89-217}$, called ΔHOPEMB	2
pCNK4004	pYV40 $yopE_{21}$ $yopH_{\Delta 1-352}$ $yopO_{\Delta 65-558}$ $yopP_{23}$ $yopM_{23}$ $yopB_{\Delta 89-217}$ $yopD_{\Delta 121-165}$, called ΔHOPEMBD, obtained by allelic exchange between ΔHOPEMB and pMSL19	Neyt and Cornelis, unpublished data
pCNK4005	Introduction of pMSL19 into $yopB_{\Delta 89-217}$ mutant bacteria, giving $yopB_{\Delta 89-217}$ $yopD_{\Delta 121-165}$	Neyt and Cornelis, unpublished data
Expression plasmids and vectors		
pBF15	pCNR26 p*yopE sycB*$^+$ *yspB*$^+$ *yspC*$^+$ (from A127/90)	This work
pBF17	pBF15 *sycB*$^+$ *yspB*$(His)_6{}^+$ *yspC*$^+$ (A127/90)	This work
pBF20	pGEX-6P-1 p*tac gst-yspC*$^+$ (A127/90)	This work
pBF21	pGEX-6P-1 p*tac gst-yspB*$^+$ (A127/90)	This work
pBF22	pGEX-6P-1 p*tac gst-sycB*$^+$ (A127/90)	This work
pBF23	pGEX-6P-1 p*tac gst-sycB*$^+$ *yspB*$(His)_6{}^+$ (A127/90)	This work
pBF24	pBF23 p*tac gst-sycB*$_{1-21}{}^+$ *yspB*$(His)_6{}^+$ (A127/90)	This work
pBF25	pBluescriptSK(−) p*lac sycB*$^+$ (A127/90)	This work
pBF26	pBluescriptSK(−) p*lac sycB*$^+$ *yspB*$^+$ (A127/90)	This work
pBF27	pBluescriptSK(−) p*lac sycB*$^+$ *yspB*$^+$ *yspC*$^+$ (A127/90)	This work
pBF29	pBluescriptSK(−) p*lac sycB*$^+$ *yspC*$^+$ (A127/90)	This work
pMM100	pACYC184 *lacI*$^+$	41
pPT14	pGEX-4T-3 *yspD*$^+$ (A127/90)	This work
pCNR26	Cloning vector with p*yopE* and optimized ribosome binding site	49
pGEX-4T-3	Cloning vector, for generation of fusion with GST	Pharmacia Biotech
pGEX-6P-1	Cloning vector, for generation of fusion with GST	Pharmacia Biotech
pBluescript SK(−)	Cloning vector	Stratagene
Suicide vectors and mutator plasmids		
pBF19	399 bp of *yspB* (A127/90) cloned into pKNG90A	This work
pFM2	402 bp of *ysaN* (A127/90) cloned into pKNG90A	This work
pPT15	510 bp of *yspD* (A127/90) cloned into pPT7	This work
pPT16	501 bp of *yspA* (A127/90) cloned into pPT7	This work
pKNG90A	Suicide vector	23
pMS154	*inv* mutator	53
pMSL19	pKNG101 $yopD^+{}_{\Delta 121-265}$	35
pPT7	Derivative of pKNG101 without *Pst*I sites and *sacBR*	This work

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% 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 for coimmunoprecipitation. For GST–SycB-YspC binding, GST-SycB production was induced with 0.5 mM IPTG for 80 min. Bacteria (5×10^8) 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-min centrifugation step (14,000 × *g*) at 4°C. Twenty micrograms of purified YspC 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-SycB + YspB-$(His)_6$ and GST-$SycB_{1–21}$ + YspB-$(His)_6$ 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 5×10^8 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% CO_2. At 20 h before infection, cells (4×10^5 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-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl 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 fluorescence was measured with a spectrofluorimeter (Perkin-Elmer HTS 7000+) with an excitation wavelength of 490 nm and a reading 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).

YopE 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 30 min, 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 μM 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 SDS–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 ng/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 (ZipTip C_{18}; Millipore), desalted by being washed with 1% acetic acid, and eluted in 4 to 6 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 scan time of 3.6 s. 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 *sycB-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 *syc-ysp* locus. The organization of the *syc-ysp* locus, which consists of the *sycB*, *yspB*, *yspC*, *yspD*, *yspA*, and *acpY* genes, suggests that it constitutes an independent operon, since *sycB* 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. *sycB* 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 *ysaN*::pFM2. A set of approximately 10 proteins called YspA to -K has been detected in the culture medium of *Y. enterocolitica*

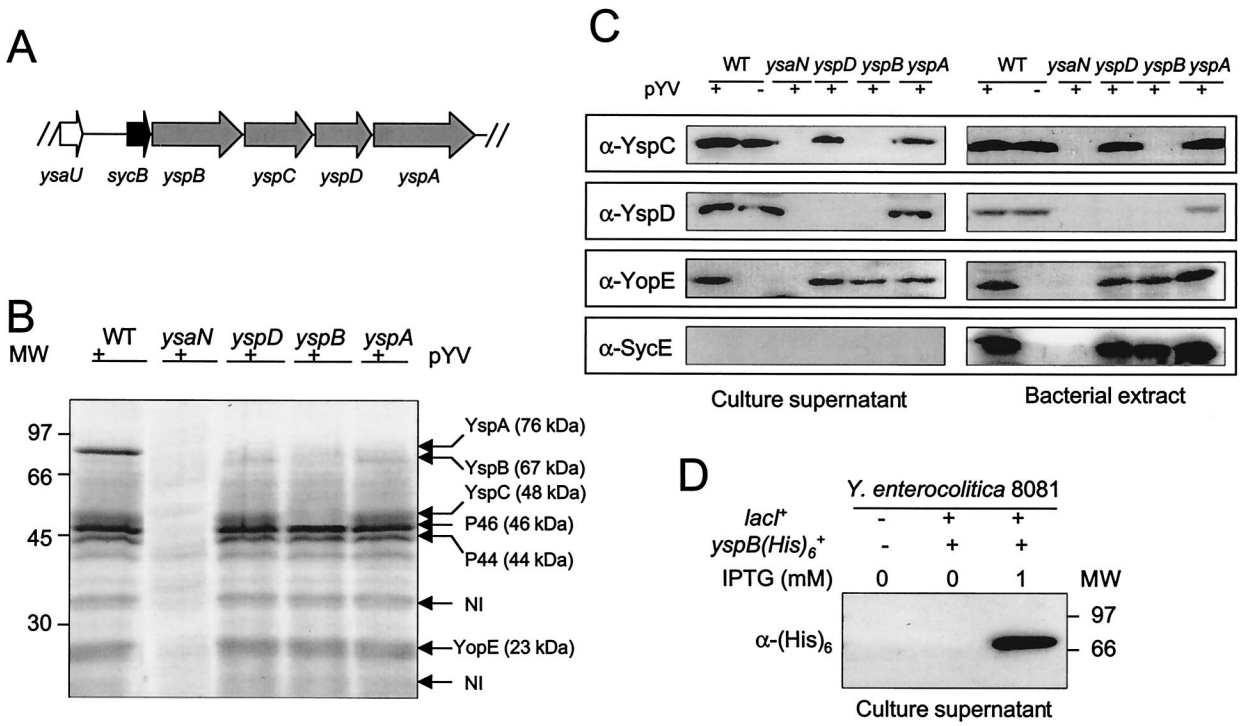

FIG. 1. Identification of proteins secreted by the Ysa TTSS. (A) Detail of the organization of the *sycB-yspB-yspC-yspD-yspA* operon located 98 bp downstream of *ysaU* (white arrow). (B) Culture supernatants of 1.5×10^8 *Y. enterocolitica* 8081 bacteria grown at 26°C for 18 h in LB containing 490 mM 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.

strain 8081 after growth at 28°C for 18 h in LB containing 490 mM NaCl (14) or at 26°C for 6 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/TQVI/LD, 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/LDA I/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 pBF20. The product was purified from the recombinant *E. coli* strain and used to immunize a rabbit. The resulting serum reacted specifically with the 48-kDa protein secreted by 8081 (Fig. 1C), confirming that this protein is YspC.

The sequences of two peptides were obtained from the analysis of the 23-kDa protein: NHDQFATGSGPLR and SGVDI/LTQAANEI/LK. 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 that had been cured of the pYV plasmid (Fig. 1C), confirming that the 23-kDa protein was indeed YopE. Under LTHS conditions, YopE was secreted through the Ysa TTS apparatus, since YopE was absent from the supernatant of the *ysaN* mutant carrying the pYV virulence plasmid (Fig. 1C). It is interesting that YspC, YopE, and even SycE are not produced by the *ysaN* mutant under LTHS conditions (Fig. 1C), indicating that there is a strong inhibition of their synthesis when the Ysa apparatus is not functional. This is particularly interesting in the case of the pYV-encoded YopE because it indicates that YopE belongs to two independent regulons.

The 46- and 44-kDa proteins were also sequenced, but the resulting peptides did not match any of the proteins encoded in the *ysp* locus. These proteins cannot be encoded by the pYV virulence plasmid because they were still present in the supernatant of *Y. enterocolitica* strain 8081 lacking the pYV plasmid (data not shown). Identification of the genes specifying these peptides is in progress.

Identification of YspD and YspB among secreted proteins. As YspD was not one of the five major proteins that were no longer secreted by the *ysaN* mutant, we investigated whether it could be detected by Western blot analysis of secreted proteins. YspD was purified from *E. coli* carrying pPT14, a recombinant plasmid encoding YspD, and used to raise a rabbit polyclonal serum. Proteins from the supernatant of a culture of *Y. enterocolitica* strain 8081 and of the *ysaN* mutant grown under LTHS conditions were analyzed by Western blotting. The anti-YspD antiserum reacted with a 38-kDa protein secreted by the wild type but not by the *ysaN* mutant (Fig. 1C). To confirm that this protein was encoded by *yspD*, we created a polar mutation by inserting the suicide plasmid pPT15 in *Y. enterocolitica* strain 8081. No signal was detected by Western blotting from the supernatant of this mutant (Fig. 1C). Moreover, YspA was no longer secreted by the *yspD* mutant, presumably as a result of the polar effect of the *yspD* mutation on transcription of the *yspA* gene (Fig. 1A and C). These results indicate that YspD was also secreted by the Ysa TTS apparatus.

Due to the instability and hydrophobicity of YspB, we could not purify this protein to raise an antibody. To investigate whether YspB was also secreted, we constructed an insertion mutation in *yspB*. Proteins identified as YspA (76 kDa), YspD (38 kDa), and YspC (48 kDa) were no longer secreted by the *yspB* mutant, presumably as a result of the polar effect of the insertion on transcription of downstream genes (Fig. 1A and B). In addition, a protein of 67 kDa was also missing in the culture supernatant of the *yspB* mutant, suggesting that the 67-kDa protein secreted by the wild-type strain is YspB. To further confirm this, we introduced a *yspB*$(His)_6$ gene into *Y. enterocolitica* strain 8081 and found that the product appeared indeed as a 67-kDa protein (Fig. 1D).

In previous reports, the letter code for the Ysp proteins had been assigned according to their molecular weight (14, 63). In the Foultier 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 Ipas 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*$(His)_6$ p*tac*-dependent operon. To serve as a control, we used plasmid pBF24 encoding truncated GST-$SycB_{1-21}$ 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-$SycB_{1-21}$. 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-$SycB_{1-21}$ (Fig. 4B).

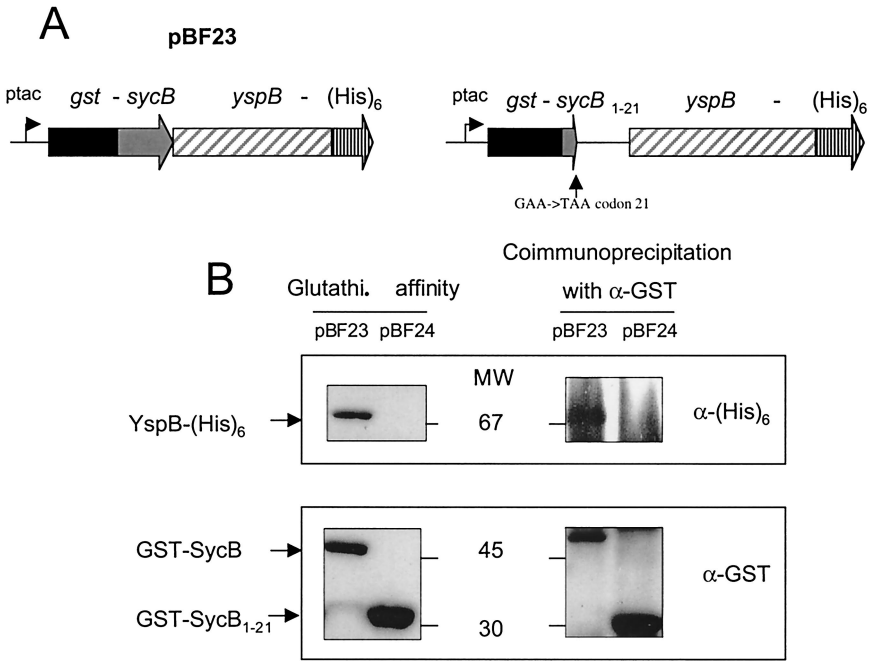

FIG. 2. SycB binds YspB. (A) Detail of the bicistronic constructs of pBF23 and pBF24. (B) Western blot analysis of proteins eluted from glutathione-Sepharose CL-4B or protein A–anti-GST–Sepharose CL-4B loaded with clear lysates of *E. coli* expressing GST-SycB and YspB-$(His)_6$ (lanes 1) or GST-$SycB_{1–21}$ and YspB-$(His)_6$ (lanes 2). MW, molecular weights in thousands; Glutathi., glutathione.

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 ΔHOPEMYscN strain. This strain lacks the YscN ATPase and is defective for Yop secretion. When secretion by the Ysc injectisome was induced,

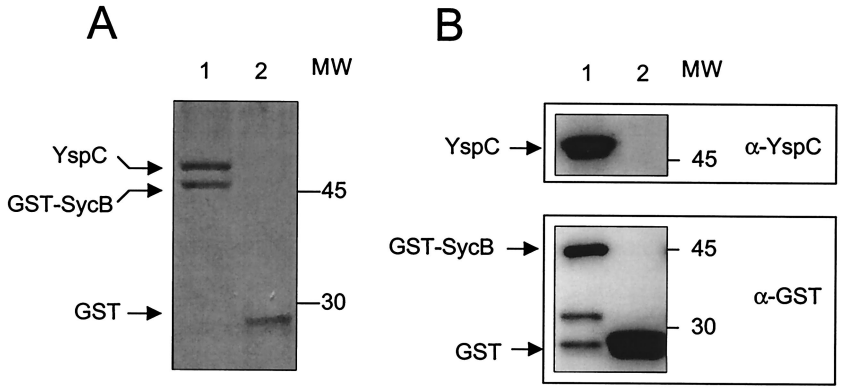

FIG. 3. SycB binds YspC. (A) Coomassie blue-stained SDS-polyacrylamide gel. (B) Western blot analysis of proteins eluted from a glutathione-Sepharose CL-4B column preincubated with extracts of *E. coli* producing GST alone (lane 2) or GST-SycB (lane 1) and loaded with 20 μg of purified YspC. The Western blot was revealed with an anti-YspC polyclonal antibody (upper part) or anti-GST polyclonal antibody (lower part). MW, molecular weights in thousands.

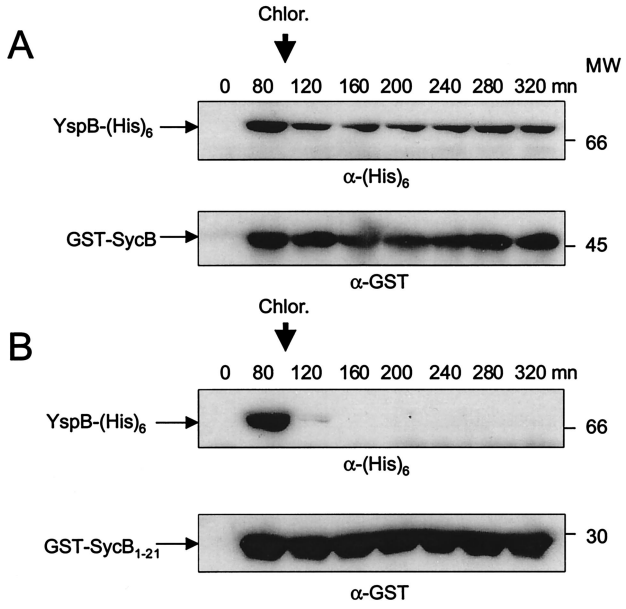

FIG. 4. SycB stabilizes YspB. Shown are results of Western blot analysis of crude extracts of *E. coli* XL-1 Blue(pBF23) (A) and XL-1 Blue(pBF24) (B) showing the steady-state level of YspB-$(His)_6$ and GST-SycB or GST-$SycB_{1–21}$ after inhibition of protein synthesis by addition of chloramphenicol (Chlor.) after 80 min of inoculation under LTHS conditions. YspB-$(His)_6$ was detected by an anti-His (C-terminal) monoclonal antibody, and GST-SycB or GST-$SycB_{1–21}$ was detected with an anti-GST polyclonal antibody. MW, molecular weights in thousands.

YspC and YspB-$(His)_6$ were produced (Fig. 5A) but they were not secreted (Fig. 5A). The multicopy character of the plasmid seemed to override the feedback inhibition of p*yopE* due to the lack of secretion. No signal was observed in both crude extract and supernatant of the ΔHOPEM host bacteria, proving that the antibodies did not cross-react with *Y. enterocolitica* ΔHOPEM proteins. This showed that YspC and YspB-$(His)_6$ are recognized as substrates for secretion by the Ysc injectisome.

YspB and YspC do not promote entry into HeLa cells. It was shown elsewhere that the IpaB/IpaC complex is sufficient to promote entry of latex beads into nonphagocytic cells (29). We wondered if YspB and YspC could have the same property when secreted upon contact with nonphagocytic cells. Since the known conditions of expression of the Ysa system are not compatible with eukaryotic cell culture conditions, we took advantage of plasmid pBF17 containing *sycB*, *yspB*$(His)_6$, and *yspC* downstream from the *yopE* promoter. We introduced pBF17 into the *Y. enterocolitica* ΔHOPEMT*inv*, a strain defective in entry due to its lack of invasin (N. Grosdent et al., unpublished data). We monitored entry of ΔHOPEMT*inv* carrying pBF17 into HeLa cells by both the gentamicin protection assay and the double immunofluorescence assay. In both cases, no entry of the bacteria into HeLa cells could be observed (data not shown). Although it was not possible in the experiment to determine whether YspB and YspC were released upon contact with HeLa cells, we assumed it was the case on the basis of our in vitro secretion experiment (Fig. 5A).

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. IpgC is required for the stability of IpaB in the cytoplasm of *S. flexneri* (31), and inactivation of *ipgC* results in a decreased amount of IpaB (Fig. 6A). To test whether SycB could replace IpgC 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 (*ipgC*). 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 *ipgC* mutant bacteria and the supernatant of *ipgC* p*lac-sycB*$^+$ bacteria (Fig. 6A). Production of SycB from pBF25 in the *ipgC* 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, IpgC 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,

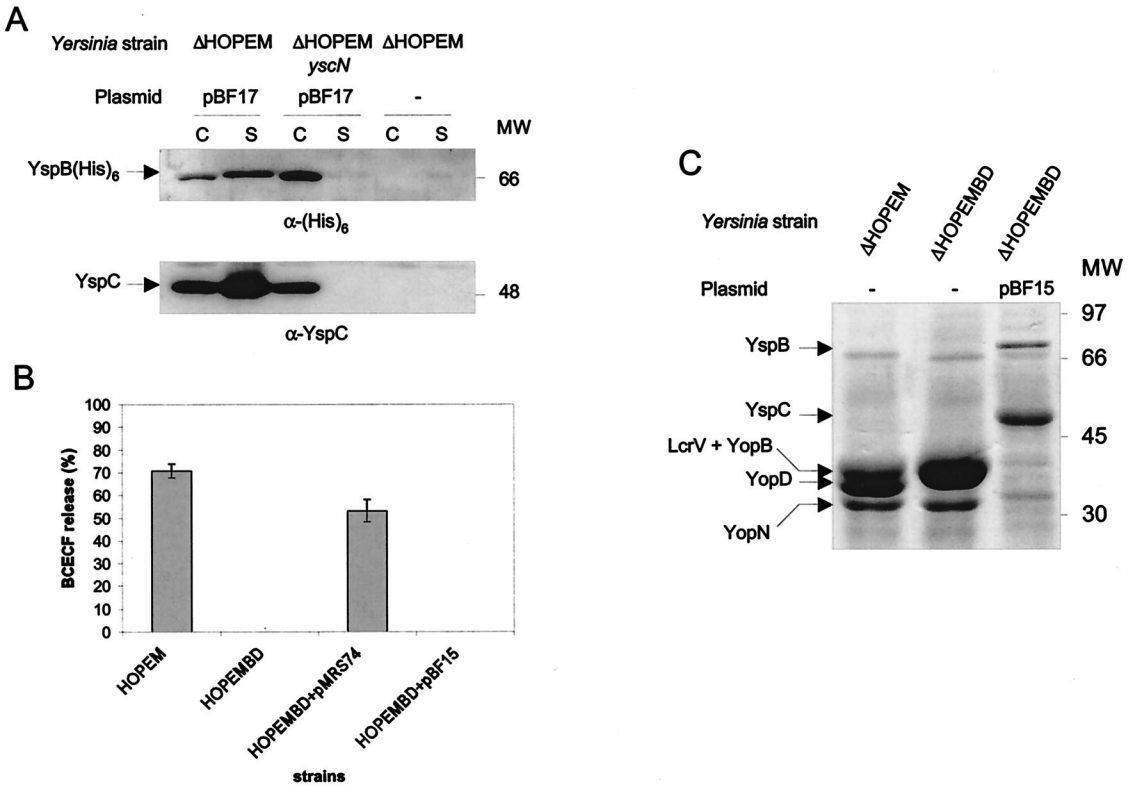

FIG. 5. Lack of activity of YspB-$(His)_6$ or YspC secreted by the Ysc injectisome of *Y. enterocolitica* ΔHOPEM. (A) Bacteria (2.5×10^7; C) or the culture supernatant of 5×10^9 bacteria (S) was loaded on an SDS-polyacrylamide gel and analyzed by Western blotting with an anti-His (C-terminal) monoclonal antibody or an anti-YspC polyclonal antibody. The detection was performed with Supersignal chemiluminescent substrate (Pierce). (B) BCECF release from preloaded macrophages upon infection with *Y. enterocolitica* ΔHOPEM, ΔHOPEMBD, ΔHOPEMBD (pMRS74), and ΔHOPEMBD(pBF15). Results are expressed as the percentages of Triton-lysed macrophages after subtraction of the value measured in the supernatants of uninfected cells. Data show the means and standard deviations of assays performed in triplicate. (C) SDS-polyacrylamide gel stained with Coomassie blue loaded with the culture supernatant of 5×10^9 *Y. enterocolitica* ΔHOPEM, ΔHOPEMBD, and ΔHOPEMBD(pBF15) bacteria expressing *sycB*, *yspB*, and *yspC* from the *yopE* promoter under in vitro conditions. MW, molecular weights in thousands.

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 coactivator 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 the *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, indicating that YspB was not functionally equivalent to IpaB for regulating 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 immunofluorescence 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 culture 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 proteins 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 invasion 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. However, one cannot rule out the possibility that YspC is not linked to cell invasion.

DISCUSSION

We had previously identified four open reading frames, *yspBCDA*, located within the *ysa* locus and close to genes specifying 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 machinery. An MS analysis after trypsin digestion of the five major

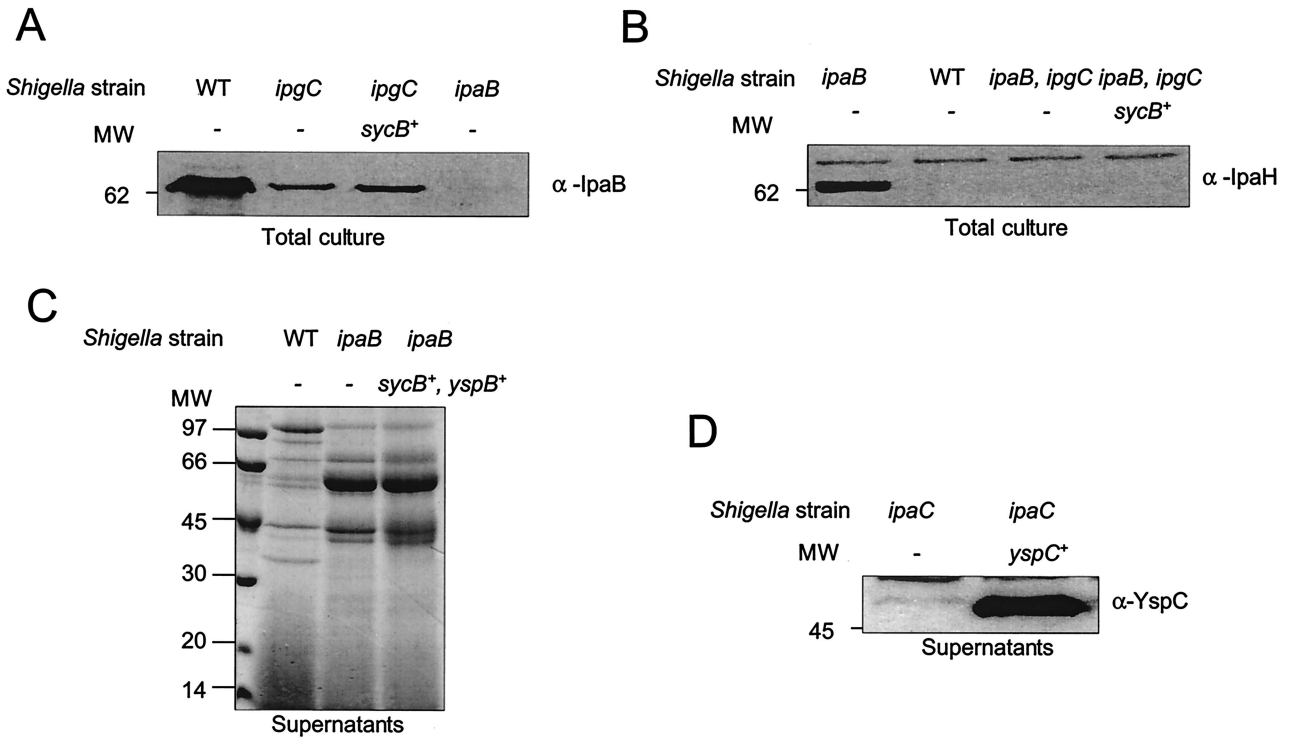

FIG. 6. Lack of complementation of *S. flexneri* mutations. (A) Protein contents of cultures of *S. flexneri* wild-type (M90T), *ipgC* (RM97), *ipgC* carrying p*lac sycB* (pBF15), and *ipaB* (RM221) strains grown for 4 h at 37°C were analyzed by Western blotting with monoclonal anti-IpaB antibody. (B) Protein contents of cultures of the *S. flexneri ipaB* strain, wild-type strain, *ipaB ipgC* (SF1068) strain, and *ipaB ipgC sycB*$^+$ 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 sycB*$^+$ *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* (RM81) strain and *ipaC sycB*$^+$ *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.

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*, which is encoded within a temperate bacteriophage located outside SPI-1 but secreted via the SPI-1 TTSS (36).

The sequence of peptides from the third secreted protein that is not encoded in the *ysa* locus exactly matched internal sequences of the pYV-encoded YopE protein, and an anti-YopE polyclonal antibody reacted with the protein. Moreover this protein was no longer present in the supernatant of bacteria lacking the pYV plasmid, demonstrating unambiguously that this protein is YopE, the pYV-encoded GTPase-activating protein which was first characterized as a substrate of the Ysc TTSS (34). However, under LTHS conditions YopE secretion did not occur through the Ysc machinery, since YopE was not present in the supernatant of *ysaN* mutant bacteria still possessing the pYV plasmid and grown under LTHS conditions. This result is in perfect agreement with the results of Young and Young that appeared while this paper was under revision showing that the Ysa system secretes Yop proteins (62). We speculate that, under in vivo infection, Ysa also recruits YopE. This is reminiscent of the *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-

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 invasin-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 *sycB-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 IpgC 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 IpgC, 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.

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