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Regulation of the Ysa Type III Secretion System of *Yersinia enterocolitica* by YsaE/SycB and YsrS/YsrR

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Yersinia enterocolitica **biovar 1B contains two type III secretion systems (TTSSs), the plasmid-encoded Ysc-Yop system and the chromosomally encoded Ysa-Ysp system. Proteins secreted from the Ysa TTSS (Ysps) have only been detected in vitro when cells are cultured at 26°C in a high-NaCl medium. However, the exact role of the Ysa TTSS is unclear. Thus, investigations into the regulation of this system may help elucidate the role of the Ysps during the life cycle of *Y. enterocolitica*. Here we present evidence that the AraC-like regulator YsaE acts together with the chaperone SycB to regulate transcription of the *sycByspBCDA* operon, a phenomenon similar to that seen in the closely related *Salmonella* SPI-1 and *Shigella flexneri* Mxi-Spa-Ipa TTSSs. Deletion of either *sycB* or *ysaE* results in a twofold reduction in the activity of a *sycB-lacZ* fusion compared to the wild type. In a reconstituted *Escherichia coli* system, transcription of *sycB* was activated sixfold only when both YsaE and SycB were present, demonstrating that they are necessary for activation. *ysrR* and *ysrS* are located near the *ysa* genes and encode a putative two-component regulatory system. Mutations in either gene indicated that both YsrR and YsrS were required for secretion of Ysps. In addition, transcription from *sycB-lacZ* and *ysaE-lacZ* fusions was decreased 6.5- and 25-fold, respectively, in the *ysrS* mutant compared to the wild type. Furthermore, in the absence of NaCl, the activity of *ysaE-lacZ* was reduced 25-fold in the wild-type and Δ*ysrS* strains, indicating that YsrS is probably required for the salt-dependent expression of the *ysa* locus. These results suggest that the putative two-component system YsrRS may be a key element in the regulatory cascade for the Ysa TTSS.**

The genus *Yersinia* has three species that are pathogenic to humans. *Y. pestis* is the causative agent of bubonic and pneumonic plagues, and *Y. enterocolitica* and *Y. pseudotuberculosis* primarily cause gastroenteritis. Most prevalent of the yersiniae in humans, the *Y. enterocolitica* infection is usually self-limiting to the gastrointestinal tract and mesenteric lymph node, causing gastroenteritis and lymphadenitis (12). However, in immunocompromised individuals, *Y. enterocolitica* can become systemic, and it has a 50% mortality rate in such cases (12).

Consumption of contaminated food or water is the primary source of *Y. enterocolitica* infection. Ingested bacteria are capable of surviving the gastric barrier and then migrate to the terminal ileum, where they attach to and subsequently invade the M cells that overlie the Peyer's patches (9, 25). Once inside the Peyer's patches, the bacteria replicate to high titers and can then disseminate to the mesenteric lymph nodes, spleen, and other organs, resulting in systemic disease (6, 8, 42, 51). The ability of *Yersinia* spp. to survive and replicate within the host is linked to the presence of a large virulence plasmid (43). This plasmid carries genes encoding the Ysc type III secretion apparatus as well as translocators, regulators, and effector proteins (Yops). At 26°C, the optimal growth temperature for *Yersinia* spp. outside the host, several copies of the secretion apparatus, called injectisomes, are detectable on the surface of the bacterium (37). At 37°C, in response to contact with target cells (in vivo) or to loss of Ca^{2+} ions (in vitro), the Yops are secreted. The functions of several Yops have been identified; these include translocation of effector Yops into host cells, impairment of phagocytosis, and downregulation of the host's inflammatory response (reviewed in reference 10).

While the virulence plasmid is necessary for full virulence, it is not sufficient, and several chromosomal genes have been identified as being important for the progression of disease. The genes encoding invasin, the primary invasion factor for *Y. enterocolitica* and *Y. pseudotuberculosis*, and its regulator, RovA, are located on the chromosome (26, 39, 40, 44). The highly virulent strains of biotype 1B have a high-pathogenicity island that contains genes involved in iron uptake (7). In addition, several genes have been identified through various means as having a role in virulence, but their functions are not yet understood (reviewed in reference 45).

Recently, a chromosomally encoded type III secretion system (TTSS) was discovered in *Y. enterocolitica* and designated Ysa, for *Yersinia* secretion apparatus (23). This system is only present in a subset of *Y. enterocolitica* strains, the highly virulent biotype 1B strains (serotypes O:4, O:8, O:13, and O:21) (19). A recent phylogenetic analysis of TTSSs revealed that the Ysa system is closely related to the *Salmonella* SPI-1 and *Shigella flexneri* Mxi/Spa TTSSs (19). Interestingly, there is a TTSS on the chromosome of *Y. pestis*, but it is more closely related to the *Salmonella* SPI-2 TTSS, indicating that the chromosomal TTSSs of *Y. enterocolitica* and *Y. pestis* were acquired after divergence of the species (19).

Several proteins, referred to as Ysps (*Yersinia* secreted proteins), can be detected in the supernatants of cultures grown at 26°C in the presence of high NaCl concentrations (20, 23). No proteins were detected when genes encoding putative Ysa ap-

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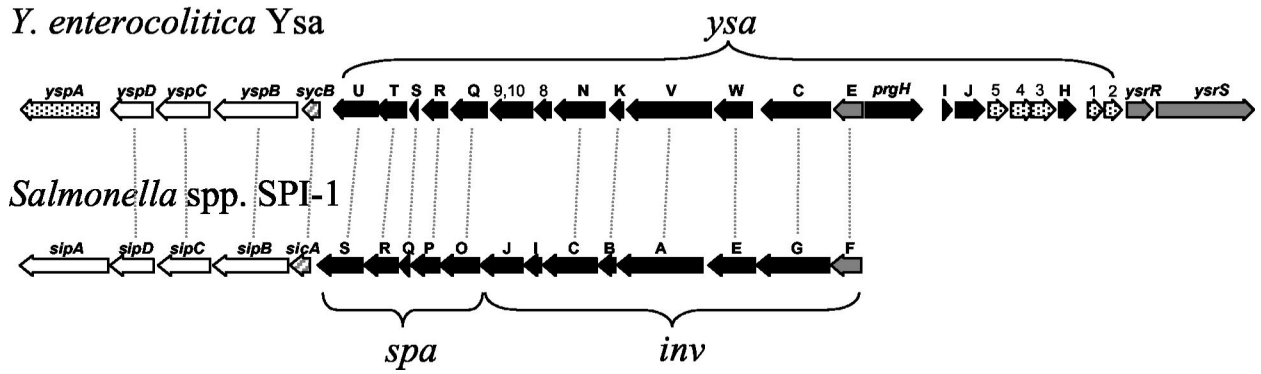

FIG. 1. Organization of the *ysa* operon. Black arrows indicate putative apparatus genes, gray arrows indicate genes encoding regulators, and white arrows indicate genes encoding secreted proteins. Speckled genes and those labeled with numbers are unique. Open reading frames 8 and 9/10 are believed to encode proteins that are part of the TTSS apparatus (19) and are therefore colored black. *sycB* and *sicA* have dual functions as chaperone and regulator. Dotted lines indicate homologous genes between the two systems (only a portion of SPI-1 is shown). The intergenic region between *ysaU* and *sycB* is 96 bp; the analogous region in SPI-1 (*spaS* to *sicA*) is 137 bp. No terminator structure was predicted to exist in a 300-bp region that includes the *ysaU-sycB* intergenic region with Mfold (http://www.bioinfo.rpi.edu/applications/mfold/).

paratus components were disrupted or when cultures were grown at 37°C (20, 23, 55). Analysis by 50% lethal dose of a strain carrying a mutation in a putative apparatus gene, *ysaV*, showed no attenuation by intraperitoneal injection. However, by an oral route it was attenuated 10-fold, suggesting that the Ysa TTSS played a role in the early stages of infection (23). Seven Ysps have been identified to date. Three were identified as YopE, YopN, and YopP, which are encoded by the virulence plasmid (20, 54). The amounts of these Yops secreted under Ysa secretion conditions are significantly less than under Yop secretion conditions, and the relevance of their secretion is not understood. However, YopP, secreted only through the Ysa TTSS, was able to suppress the production of tumor necrosis factor alpha by infected macrophages (54). The other identified Ysps are encoded by the *yspBCDA* genes just downstream of the *ysa* apparatus genes (20). YspB, YspC, and YspD are homologous to proteins involved in translocation, but YspA is a unique protein (20). In addition, SycB has been demonstrated to function as a chaperone for YspB (20).

While a number of the proteins secreted by the Ysa TTSS have been identified and the functions for many genes in the locus have been inferred based on homology, nothing is known about the regulation of this system other than a requirement for growth at 26°C in high NaCl concentrations (23). The closely related *Salmonella* SPI-1 and *S. flexneri* Mxi/Spa TTSSs have an interesting regulation system that has not been observed in other TTSSs: each utilizes an AraC-like regulator and a chaperone to regulate the transcription of genes encoding secreted effectors (14, 15, 34). In *Salmonella* spp., it has been shown that InvF (AraC-like regulator) and SicA (chaperone) interact, and this interaction is likely to be required for the transcriptional activation because InvF alone can bind DNA but not activate transcription (15). The *ysa* locus has homologs of InvF and SicA, designated YsaE and SycB, respectively, and the genetic organization of these and surrounding genes is very similar (Fig. 1). Transcription of the TTS apparatus genes is regulated by HilA in *Salmonella* spp. (3, 31) and by VirB in *S. flexneri* (4), both of which are themselves regulated by various environmental conditions (reviewed in references 17 and 32). It appears that HilA and VirB serve as the focal point for transmitting the environmental signals that lead to expression of these type III secretion systems and their effectors. These regulators do not show any homology to each other, and no homologue of either protein exists in *Y. enterocolitica*. Therefore, identifying regulators upstream of the Ysa system is of interest and may facilitate an understanding of the role of the Ysa TTSS.

In this work, we investigated the transcriptional regulation of the *ysa* and *ysp* genes. We show that the AraC-like protein, YsaE, and chaperone, SycB, are both required to activate transcription of the *sycByspBCDA* operon, a phenomenon similar to that seen in *Salmonella* spp. and *S. flexneri* (13–15, 27, 34). In addition, we show that YsrS, the putative sensor protein of a two-component system, is required for expression of the *ysaE* promoter and that this activation is NaCl dependent. These results indicate that the putative two-component system YsrRS may be a key component in the regulatory cascade for the Ysa secretion apparatus.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1 and described in detail below. Overnight cultures were typically grown in Luria broth (LB) (170 mM NaCl; Difco) at 26°C for *Y. enterocolitica* or 37°C for *Escherichia coli*, unless otherwise stated. For examination of secreted proteins, cultures were grown overnight in L-broth (1% tryptone, 0.5% yeast extract; referred to hereafter as LB-0) and subcultured into L-broth containing 290 mM NaCl (referred to hereafter as LB-290). Antibiotics were added as needed at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 100 μg/ml; nalidixic acid, 20 μg/ml; chloramphenicol, 12.5 μg/ml; spectinomycin, 50 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 7.5 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to a final concentration of 40 μg/ml.

Construction of plasmids. The plasmids used to generate in-frame deletion mutants of *sycB*, *ysrR*, *ysaE*, and *ysrS* were constructed as follows. Primers *sycB*-delA and -delB were used to amplify a ≈500-bp region containing a few N-terminal codons and upstream sequence. This fragment was digested with SalI and BamHI and cloned into the same sites of pSR47S. Primers *sycB*-delC and -delD were used to generate a similarly sized fragment containing a few C-terminal codons and additional downstream sequence. This product was digested with BamHI and NotI and cloned into those sites of pSR47S containing the upstream fragment, resulting in plasmid pKW10. Plasmid pKW16 was constructed in an identical fashion with primer pairs *ysrR*-delA/delB and *ysrR*-delC/delD.

The PCR products generated with *ysaE*-delA/delB and *ysaE*-delC/delD were

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype	Reference or source
E. coli		
DH5α	F^- ϕ 80Δ*lacZM15* Δ(*lacZYA-argF*)*U169 deoP recA1 endA1 hsdR17* (r_K^- m_K^-)	Invitrogen
S17-1λ*pir*	Tp^r Str^r *recA thi pro hsdR hsdM*$^+$ RP4::2-Tc::Mu::Km Tn*7* λ*pir* lysogen	39
CC118λ*pir*	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	24
VM1264	CC118λ*pir* carrying pRW50, pWKS130, pHG329	This work
VM1265	CC118λ*pir* carrying pKW21, pKW22, pKW23	This work
VM1266	CC118λ*pir* carrying pKW21, pWKS130, pKW23	This work
VM1267	CC118λ*pir* carrying pKW21, pKW22, pHG329	This work
VM1272	CC118λ*pir* carrying pKW21, pWKS130, pHG329	This work
Y. enterocolitica[a]		
JB580v	8081v (r^- m^+ Nal^r)	28
JB580c	JB580v cured of pYVe8081	Lab strain
YVM1063	JB580c carrying pKW22	This work
YVM1064	JB580c carrying pWKS130	This work
YVM886	JB580v *ysaC*::pEP185.2	This work
YVM932	JB580v Δ*ysaE*	This work
YVM981	JB580v Δ*sycB*	This work
YVM997	JB580v Δ*ysaE* Δ*sycB*	This work
YVM969	JB580v Δ*ysrS*	This work
YVM1006	JB580v Δ*ysrR*	This work
YVM917	YVM886c	This work
YVM971	YVM932c	This work
YVM1025	YVM971 carrying pKW22	This work
YVM1042	YVM971 carrying pWKS130	This work
YVM996	YVM981c	This work
YVM1035	YVM996 carrying pKW28	This work
YVM1052	YVM996 carrying pKW27	This work
YVM972	YVM969c	This work
YVM1081	YVM972 carrying pKW31	This work
YVM1043	YVM972 carrying pWKS130	This work
YVM1089	YVM1006c	This work
YVM1102	YVM1089 carrying pKW24	This work
YVM1103	YVM1089 carrying pWKS130	This work
YVM987	JB580v *sycB-lacZYA*	This work
YVM988	YVM981 *sycB-lacZYA*	This work
YVM989	YVM932 *sycB-lacZYA*	This work
YVM1002	YVM997 *sycB-lacZYA*	This work
YVM990	YVM969 *sycB-lacZYA*	This work
YVM1054	YVM988 carrying pKW28	This work
YVM1054	YVM988 carrying pKW27	This work
YVM1019	YVM989 carrying pKW22	This work
YVM1044	YVM989 carrying pWKS130	This work
YVM1093	YVM1002 carrying pKW28	This work
YVM1094	YVM1002 carrying pKW27	This work
YVM1020	YVM1002 carrying pKW22	This work
YVM1045	YVM1002 carrying pWKS130	This work
YVM1062	YVM1002 carrying pKW28 and pKW22	This work
YVM1087	YVM1002 carrying pKW27 and pWKS130	This work
YVM1073	YVM990 carrying pKW31	This work
YVM1056	YVM990 carrying pWKS130	This work
YVM1061	YVM990 carrying pKW28 and pKW22	This work
YVM1060	YVM990 carrying pKW27 and pWKS130	This work
YVM1074	YVM995 carrying pKW31	This work
YVM1057	YVM995 carrying pWKS130	This work
YVM925	JB580v *ysaE-lacZYA*	This work
YVM970	YVM932 *ysaE-lacZYA*	This work
YVM995	YVM969 *ysaE-lacZYA*	This work
Plasmids		
pRW50	Tet^r; low-copy-number transcriptional reporter vector	30
pSR47S	Kan^r; MobRP4 *ori*R6K, cloning vector	36
pWKS130	Kan^r; low-copy-number cloning vector	52
pHG329	Amp^r; medium-copy-number cloning vector	48
pEP185.2	Cm^r; MobRP4 *ori*R6K, cloning vector	28
pGEX-6P-1	Amp^r; cloning vector for generation of GST fusion proteins	Amersham
pKN8	Cm^r; MobRP4 *ori*R6K, transcriptional reporter vector	18
pKW27	Cm^r Str^r Sp^r; pACYC184 with Str/Sp cassette	This work

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype	Reference or source
pKW6	*ysrS* lacking codons for amino acids 196–589 cloned into pSR47S	This work
pKW7	*ysaE* lacking codons for amino acids 91–178 cloned into pSR47S	This work
pKW10	*sycB* lacking codons for amino acids 68–140 cloned into pSR47S	This work
pKW16	*ysrR* lacking codons for amino acids 25–207 cloned into pSR47S	This work
pSAH1	*ysaC* codons 240–447 cloned into pEP185.2	This work
pKW11	*sycB* promoter region cloned into pKN8	This work
pKW5	*ysaE* promoter region cloned into pKN8	This work
pKW21	*sycB* promoter region cloned into pRW50	This work
pKW22 (pYsaE)	*ysaE* coding sequence and promoter cloned into pWKS130	This work
pKW23	*sycB* coding sequence and promoter cloned into pHG329	This work
pKW24 (pYsrR)	*ysrR* coding sequence and promoter cloned into pWKS130	This work
pKW28 (pSycB)	*sycB* coding sequence and promoter cloned into pKW27	This work
pKW31 (pYsrS)	*ysrS* coding sequence cloned into pWKS130	This work
pYW2	*yspC* coding sequence cloned into pGEX-6P-1	This work

[a] The suffix v denotes strains carrying the pYVe8081 virulence plasmid, and the suffix c denotes strains that have been cured of this plasmid as described in the text.

digested with ClaI and BglII and with BglII and XbaI, respectively. They were sequentially cloned into pEP185.2, generating pKW4. The SalI-NotI fragment from pKW4, which contained the *ysaE* inserts, was subcloned into pSR47S to make pKW6. PCR products generated with *ysrS*-delA/delB and *ysrS*-delC/delD were digested with KpnI and XbaI and with XbaI and SacI, respectively, and sequentially cloned into pEP185.2 to make pKW3. Primers *ysrS*-delA and *ysrS*-delD were used to amplify the *ysrS* inserts from pKW3, and the product was cloned into pCR2.1 TOPO. The SalI-NotI fragment from this plasmid was then subcloned into pSR47S, generating pKW7. Following ligation, each plasmid was transformed into *E. coli* strain S17-1λ*pir* by electroporation. All constructs were confirmed by restriction digestion and sequenced to ensure that no errors were generated during amplification.

For generating a disruption in the *ysaC* gene, an internal region containing approximately 500 bp of the *ysaC* gene was amplified with primers *ysaC*-F1 and *ysaC*-R1 (Table 2) and cloned into the pCR2.1-TOPO vector (Invitrogen). The KpnI-XhoI fragment was cleaved out of that plasmid and cloned into the same sites of pEP185.2, resulting in pSAH1. This plasmid was transformed into *E. coli* strain S17-1λ*pir* by electroporation and confirmed by restriction digestion.

Transcriptional *lacZ* fusions were constructed by cloning putative promoter regions into pKN8 (18). For the *ysaE* and *sycB* promoters, approximately 300 bp of promoter sequence and 250 bp of coding sequence were amplified, digested with XbaI and BglII, and cloned into those sites of pKN8, resulting in plasmids pKW5 and pKW11, respectively. The plasmids were transformed into S17-1λ*pir* by electroporation, confirmed by restriction digestion, and sequenced to ensure that no errors were generated during amplification. Plasmid pKW21 was made by digesting a PCR-generated fragment of the *sycB* promoter region with EcoRI and BamHI and ligating it into the same sites of pRW50. The ligated plasmid was transformed into *E. coli* strain DH5α, confirmed by restriction digestion, and sequenced. The primer sequences and pairs used for these constructs are listed in Tables 2 and 3, respectively.

Two plasmids carrying the *sycB* coding region were constructed. In the first, primers *sycB*-FP3 and -RP2 were used to amplify the *sycB* gene and promoter region. The product was digested with EcoRI and HindIII, cloned into those sites of pHG329, and transformed into DH5α, giving pKW23. The second PCR product, generated with primers *sycB*-FP1 and -RP2, was digested with BglII and HindIII and cloned into those sites in pKW27, giving pKW28. Plasmid pKW27 is pACYC184 with a streptomycin-spectinomycin resistance cassette from p34-Sm (16) cloned into the BamHI site; the tetracycline resistance cassette was disrupted by this insertion. The *ysaE*-complementing clone was made by digesting a PCR product from primers *ysaE*-delA and -delD with SalI and NotI and cloning it into those sites of pWKS130 to give pKW22. The *ysrR*-complementing clone was made by digesting a PCR product from primers *ysrR*-delA and -RP1 with SalI and KpnI and cloning it into those sites of pWKS130 to give pKW24. For the *ysrS*-complementing clone, a PCR product generated by primers *ysrS*-OEF1 and -RP3 was digested with SalI and NotI and cloned into those sites of pWKS130 to give pKW31. All plasmids were transformed into DH5α by electroporation, confirmed by restriction digestion, and sequenced to ensure that no errors occurred during amplification. Primer sequences and pairs used for these constructs are given in Tables 2 and 3, respectively.

Primers *yspC* 1.1 and *yspC* 2.2 (Table 2) were used to amplify the entire coding sequence of YspC. The PCR product was digested with SalI and NotI and cloned into the same sites of pGEX-6P-1 (Amersham). The resulting plasmid, pYW2, encodes a glutathione *S*-transferase (GST)-YspC fusion protein.

Strain construction. (i) In-frame deletions. Strains YVM969, YVM932, YVM981, and YVM1006 containing chromosomal in-frame deletions in *ysrS*, *ysaE*, *sycB*, and *ysrR*, respectively, were made by conjugation as follows. Equal volumes of saturated cultures of *E. coli* carrying the desired plasmid (pKW6, -7, -10, or -16) and *Y. enterocolitica* JB580v were mixed, plated on LB agar, and allowed to incubate at 26°C overnight. The resulting lawn of cells was scraped into 1 ml of 1× PBS, diluted 1:100, and plated on LB agar plates containing

TABLE 2. Primers used in this work

Primer	Sequence[a] (5′→3′)
ysaC-F1	GGGTGAACCGACGATCGAA
ysaC-R1	CAAGTTTGCCCGAGTTGTCA
ysaE-delA	CCATCGATCGATTCGATGGCTACCCGCTTTGAG
ysaE-delB	GAAGATCTTGCAGCATCAATCGTTGCGAGAGTTTCG
ysaE-delC	GAAGATCTGGCGTCTCTGCGGCCTACTTCAGGC
ysaE-delD	GCTCTAGACGGCTTCTCCAGCCGTTCAGCGACG
ysaE-FP1	GCTCTAGACGATTCGATGGCTACCCGCTTTGAG
ysaE-FP4	CGGGATCCGATTCGATGGCTACCCGCTTTGAG
ysaE-RP2	CCCAAGCTTATGCAGCATCAATCGTTGCGAGAG
sycB-delA	ACGCGTCGACGGCTGGTACGCGTTGAGCTGG
sycB-delB	CGGGATCCGCGAAAGAACGTTTCGGCTTC
sycB-delC	CGGGATCCGGGAGTGATGATTTGGAGTTG
sycB-delD	ATAAGAATGCGGCCGCGCCAACGACCCCATCAACG ATG
sycB-FP1	GCTCTAGACCGGTAGCACGGCAGCTATGGCGG
sycB-RP1	GAAGATCTGCTGATAAACAGCTGCCAACCCC
sycB-FP2	CGGAATTCCCGGTAGCACGGCAGCTATGGCGG
sycB-FP3	CCCAAGCTTCCGGTAGCACGGCAGCTATGGCGG
ysrS-delA	GGGGTACCTCACCGCAAGAGCTGG
ysrS-delB	GCTCTAGACGCAGCTTCAGCCTGCCG
ysrS-delC	GCTCTAGACCTGCTGCGGCTCGTGGG
ysrS-delD	GCGAGCTCACGGGCGCGCTGCGCATC
ysrS-OEF1	GCGTCGACGGGCTTACTTCAAACACTGATTTC
ysrS-RP3	ATAAGAATGCGGCCGCTCAGTCATGTTCTTTTTCCTT AG
ysrR-de1A	ACGCGTCGACGCAGGATAATCCGATGAAATCTCG
ysrR-de1B	CGGGATCCCATCAGCGCAAGGCGACTGAAAGG
ysrR-de1C	CGGGATCCGTATCGAACACGAAAACGCGTGCC
ysrR-de1D	ATAAGAATGCGGCCGCGCTTGGTAAACCACTCAATC AGCG
ysrR-RP1	GGGGTACCTGGCCTCGGCAGCATAAACAGCCG
yspC-1.1	ACGCGTCGACTCATGACCACTATTCAACAAGCCACG CAC
yspC-2.2	ATATTGAATGCGGCCGCTTAACCCTTAACAATGGCC TGATTG
KW114	TAGCACGGCAGCTATGGC
KW115	AGCTGATAAACAGCTGCCAAC

[a] Restriction enzyme sites are underlined.

TABLE 3. Primer pairs used for transcriptional fusions and complementing clones

Gene	Plasmid	5′ primer	3′ primer	Region amplified (bp)[a]
ysaE	pKW5	*ysaE*-FP1	*ysaE*-de1B	−291 to +249
sycB	pKW11	*sycB*-FP1	*sycB*-RP1	−311 to +264
sycB	pKW21	*sycB*-FP2	*sycB*-de1B	−311 to +200
sycB	pKW23	*sycB*-FP3	*sycB*-RP2	−311 to +574
sycB	pKW28	*sycB*-FP1	*sycB*-RP2	−311 to +574
ysaE	pKW22	*ysaE*-de1A	*ysaE*-de1D	−291 to +963
ysrS	pKW31	*ysrS*-OEF1	*ysrS*-RP3	−31 to +2376
ysrR	pKW24	*ysrR*-de1A	*ysrR*-RP1	−476 to +772

[a] Base pairs amplified relative to the putative start codon (+1).

nalidixic acid to select against *E. coli* and kanamycin to select against *Y. enterocolitica* lacking the plasmid. Replication of pSR47S requires the *pir* protein; all *Y. enterocolitica* strains used lack *pir*, and thus survivors would have undergone site-specific recombination. Several transconjugants were streaked onto LB agar plates containing nalidixic acid and 5% sucrose for selection of colonies that had undergone a second recombination step and lost the vector. Cells retaining a functional *sacB* gene should not grow in the presence of sucrose. Several of these colonies were then screened for kanamycin sensitivity, of which 10 to 20 were picked for confirmation of the in-frame deletion by colony PCR (see below).

To ensure that recombination occurred in the proper location, strains selected for experiments were analyzed by Southern blotting. Strains used for analysis of Ysa-dependent secretion were subsequently cured of the virulence plasmid by inoculation on LB agar containing 20 mM $MgCl_2$ and 20 mM NaC_2O_4 and grown at 37°C. Loss of the virulence plasmid was verified by visualization of plasmid DNA preparations on 0.8% agarose gels. The test strains were always compared to plasmid preparations from JB580v and JB580c, which served as positive and negative controls, respectively. The cured strains of YVM969, YVM932, YVM981, and YVM1006 are designated YVM972, YVM971, YVM996, and YVM1089, respectively.

(ii) Plasmid integrations. Strain YVM886 was generated by conjugating pSAH1 into JB580v as described above but with selection on LB agar containing nalidixic acid and chloramphenicol. Proper insertion of the plasmid was confirmed by Southern blotting. YVM886 was then cured of pYVe8081 as described above to yield YVM917. Strains carrying chromosomal promoter-*lacZ* fusions were constructed by conjugating either pKW5 or pKW11 into the desired *Y. enterocolitica* strain, followed by selection on LB agar containing nalidixic acid and chloramphenicol, giving YVM925 and YVM987, respectively. Because of the region that was cloned, merodiploid strains were generated by the recombination event. Proper integration of the plasmid was confirmed by Southern blotting prior to analysis. These strains were not subsequently cured of pYVe8081.

(iii) Plasmid transformation. For *Y. enterocolitica*, the strain to be transformed was inoculated into LB broth containing 1% glucose and grown overnight. Approximately 500 μl of the saturated culture was washed twice with an equal volume of ice-cold distilled H_2O and resuspended in 40 μl of 10% glycerol; 1 to 2 μl of plasmid DNA was added to the cells and electroporated by standard procedures. Ten percent of the recovered culture was plated on LB agar with appropriate antibiotics. When two plasmids were needed, both were transformed simultaneously. For *E. coli* strains VM1265, VM1266, VM1267, and VM1272, electrocompetent CC118 λ*pir* cells were simultaneously transformed with all three desired plasmids. Ten percent of the recovered culture was plated on LB agar with appropriate antibiotics.

PCR and DNA sequencing. Standard methods for PCR were conducted under the conditions specified by the supplier with either *Pfu* polymerase (Stratagene, La Jolla, Calif.) or *Taq* polymerase (Qiagen, Valencia, Calif.). Colony PCR was performed in a standard reaction in 50 μl. A single colony was resuspended in 50 μl of distilled H_2O and vortexed, and 5 μl was used as the template. A 5-min incubation at 95°C preceded the cycling reactions to ensure cell lysis. DNA sequencing was performed with the Big Dye termination cycle sequencing ready reaction system under the conditions specified by the supplier (PE Applied Biosystems, Foster City, Calif.). Reactions were analyzed at the Protein and Nucleic Acid Chemistry Laboratory at the Washington University School of Medicine.

β-Galactosidase assays. Saturated cultures grown overnight in LB-0 were diluted into fresh LB-290 to an initial optical density at 600 nm (OD_{600}) of 0.2 and grown for 4 h at 26°C on a roller drum. Antibiotics were added as necessary to retain plasmids. Assays were performed as described before (38).

Total RNA extraction and RT-PCR. A saturated culture of JB580v grown overnight in LB-0 was diluted into fresh LB-290 to an initial OD_{600} of 0.2 and grown for 4 h at 26°C on a roller drum. RNAprotect bacterial reagent (Qiagen) was added to the cell sample as described by the manufacturer. The cells were collected by centrifugation, and total RNA was extracted with the MasterPure RNA extraction kit from Epicenter. DNA was removed from 20 μg of sample with DNA-free (Ambion) following the manufacturer's protocol. For cDNA synthesis, 2 μg of RNA was used as a template with 200 U of Superscript III as described by the supplier (Invitrogen). PCR was performed with the cDNA synthesis products as the template with primers KW114 and KW115 (Table 2) and *Taq* polymerase (Qiagen) in a 50-μl reaction volume. For controls, PCR was performed without cDNA template as well as with genomic DNA to show the expected size of the product generated with these primers. One fifth of the reaction was separated on a 1.2% agarose gel and stained with ethidium bromide.

Preparation of secreted proteins, SDS-PAGE, and Western blot analysis. Extracellular proteins were collected as described before (55). Briefly, saturated cultures grown in LB-0 were diluted into fresh LB-290 to an initial OD_{600} of 0.2 and grown for 6 h at 26°C on a roller drum. The longer culture time for protein preparations than for expression studies was chosen because the preparations were cleaner, making detection of individual bands easier. Antibiotics were added as necessary to retain plasmids. The cells were removed from 4.5 ml of culture by centrifugation in microcentrifuge tubes for 1 min at 13,000 rpm. The supernatant was centrifuged a second time, followed by passage through a 0.22-μm syringe filter. Ice-cold trichloroacetic acid was added to a final concentration of 10% (vol/vol) and incubated on ice for 10 to 20 min. The samples were centrifuged at 4°C for 10 min at 13,000 rpm, washed once with ice-cold acetone, and resuspended in 1 M Tris-HCl, pH 9.0. The proteins were boiled for 5 min in 1× sample buffer (46), and OD_{600} equivalents were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were visualized by staining with silver nitrate (Bio-Rad) or transferred to nitrocellulose for Western analysis with a Bio-Rad Trans-Blot SD semidry transfer apparatus as specified by the supplier. Blots were blocked in 1× phosphate-buffered saline (PBS) with 0.1% Tween 20 and 5% skim milk (PBST-milk) for 1 h at room temperature. Primary antibody directed against YspC was diluted 1:1,000 in PBST-milk and allowed to react overnight at 4°C. The membranes were washed several times with PBST-milk and then incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase at 1:25,000 in PBST-milk for 1 h at room temperature. The membranes were washed again in PBST-milk, and proteins were detected by chemiluminescence (ECL; Amersham).

To generate the anti-YspC antibody, a GST-YspC fusion protein was purified from *E. coli* carrying pYW2 with the bulk GST purification module as specified by the supplier (Amersham). Approximately 1 mg of GST-YspC was sent to Covance Research Products for immunization of a New Zealand White rabbit.

RESULTS

Regulation of the *sycB-yspBCDA* operon. The organization of the *ysa-syc-ysp* genes in *Yersinia* spp. is quite similar to that in *Salmonella*. SPI-1 and to a lesser degree to the *S. flexneri mxi-spa-ipa* genes. Given that both the *Salmonella* and *Shigella* systems employ an AraC-like regulator (InvF and MxiE, respectively) and a TTSS chaperone (SicA and IpgC, respectively) to regulate the expression of genes encoding secreted proteins, it was of interest to investigate if the analogous proteins, YsaE and SycB, functioned similarly in the Ysa system. To test this hypothesis, in-frame deletions of *ysaE* and *sycB* were constructed, and the strains were examined for their ability to secrete proteins after growth in L-broth with 290 mM NaCl (LB-290). Both strains showed a loss of most protein bands (Fig. 2A). This indicates that YsaE and SycB are required for wild-type levels of secretion.

Western blots probed with anti-YspC antibody, which recognizes the secreted protein YspC, showed that secretion was restored when the wild-type gene was provided in *trans*, demonstrating that the reduced secretion was due to the loss of the

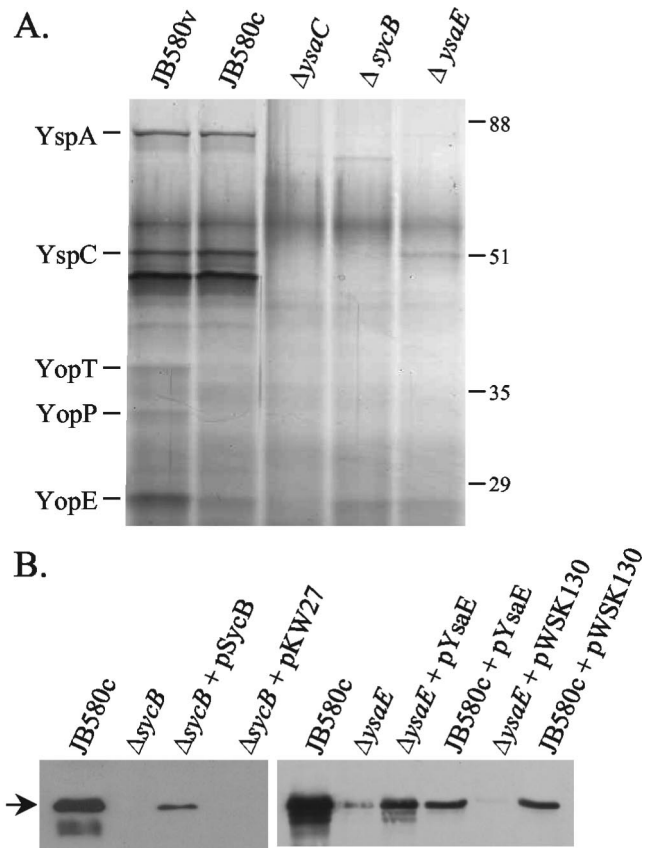

FIG. 2. Strains lacking *sycB* or *ysaE* do not secrete Ysps. Proteins were precipitated from culture supernatants and separated by SDS–10% polyacrylamide gel electrophoresis as described in the text. (A) Silver-stained gel showing loss of Ysps from the culture supernatants of Δ*sycB* and Δ*ysaE* strains. Δ*ysaC* carries a disruption in the *ysaC* gene and is thought to have a defective apparatus. The culture equivalent of 2 OD units was loaded in each lane. (B) Complementation of the mutant strains as determined by Western blotting with anti-YspC antibody. YspC is indicated by the arrow. The culture equivalent of 1 OD unit was loaded in each lane.

deleted gene (Fig. 2B). Curiously, the complemented Δ*ysaE* strain consistently appeared to secrete less YspC than the wild-type strain. The presence of kanamycin in the medium did not significantly impair the growth of the cultures (not shown). However, the antibiotic (or plasmid carriage) may somehow interfere with secretion or precipitation of Ysps, since the wild-type strain carrying pWKS130 or pKW22 secreted less YspC than the strain without either plasmid (Fig. 2B). A similar phenomenon was observed with the *sycB*-complementing clone (not shown).

In related TTSSs, InvF/SicA and MxiE/IpgC act by stimulating the transcription of genes encoding secreted proteins (13–15, 27, 34). To test the hypothesis that YsaE and SycB were acting as transcriptional regulators, a *sycB-lacZ* fusion was introduced into the Δ*sycB* and Δ*ysaE* strains, and β-galactosidase activities were determined. The activity of the *sycB* promoter decreased about twofold in the Δ*ysaE* and Δ*sycB* strains, suggesting that both proteins play a role in the transcription of the *sycByspBCDA* operon (Table 4). The *ysaE* and *sycB* mutants could be fully complemented for *sycB* expression

TABLE 4. Regulation of *sycB* promoter by SycB and YsaE

Relevant phenotype	Strain	Plasmid[a]	Mean β-galactosidase activity (Miller units)[b] ± SD
SycB$^+$, YsaE$^+$	YVM987	None	512 ± 58
SycB$^-$, YsaE$^+$	YVM988	None	282 ± 33
	YVM1054	pSycB	2,851 ± 426
	YVM1055	pKW27	295 ± 4
SycB$^+$, YsaE$^-$	YVM989	None	297 ± 13
	YVM1019	pYsaE	1,784 ± 286
	YVM1044	pWKS130	263 ± 9
SycB$^-$, YsaE$^-$	YVM1002	None	331 ± 26
	YVM1093	pSycB	267 ± 20
	YVM1094	pKW27	232 ± 12
	YVM1020	pYsaE	279 ± 3
	YVM1045	pWKS130	292 ± 23
	YVM1062	pSycB, pYsaE	3,867 ± 132
	YVM1087	pKW27, pWKS130	297 ± 52

[a] Plasmid pSycB is pKW28, and pYsaE is pKW22; pKW27 is the vector for pSycB, and pWSK130 is the vector for pYsaE.
[b] Miller units represent the mean of at least three independent assays with standard deviations.

when the respective wild-type gene was provided on a plasmid; strains carrying the vector alone showed no change in activity from the mutants. A similar twofold reduction in transcription was observed in the *ysaE sycB* double mutant, and activity of *sycB-lacZ* was only restored when both *ysaE* and *sycB* were provided in *trans*. Promoter activity in each of the complemented strains was much higher than in the wild type, indicating that YsaE or SycB may be limiting in the wild type under the conditions examined. Expression in the wild-type strain carrying plasmid pSycB, pYsaE, or both was also increased, but no difference was observed with the vectors (not shown). The observed effects of SycB and YsaE were independent of genes encoded by the virulence plasmid. Loss of the virulence plasmid had no effect on *sycB-lacZ* transcription in a wild-type strain (not shown).

The possibility existed that transcription initiating at the *ysaE* promoter may also transcribe the *sycByspBCDA* genes, as is thought to be the case in SPI-1 (13). If this is so, then the decrease in transcription of the *sycB* promoter observed in the Δ*ysaE* and Δ*sycB* strains may not be an accurate measure of the contribution of these two proteins on transcription initiating at the *sycB* promoter. To determine if a transcript existed that initiated upstream of *ysaU*, RT-PCR was used to amplify a region that encompassed the *ysaU-sycB* intergenic region. A product was detected only when genomic DNA or cDNA was added to the reaction, but not from reactions containing no template or template from a cDNA synthesis reaction lacking reverse transcriptase (Fig. 3). These data demonstrate that transcription of *sycByspBCDA* genes can indeed initiate at a promoter upstream of *ysaU* and contributes to the transcription of this operon.

In order to analyze the effects of SycB and YsaE on *sycB-lacZ* expression without the contributions from the upstream promoter, a plasmid-based system was reconstituted in *E. coli*. Transcription from *sycB-lacZ* was at background levels if only

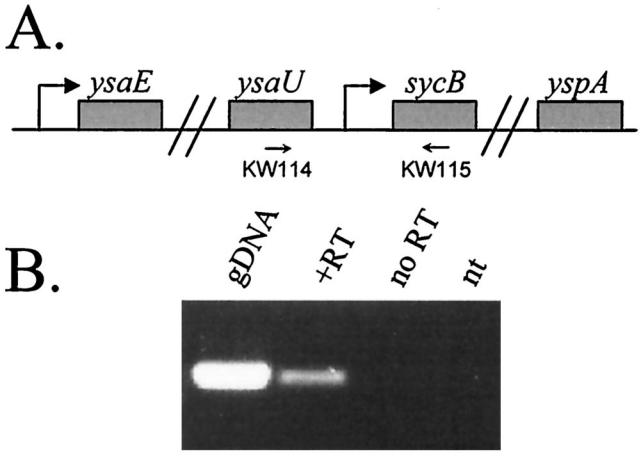

FIG. 3. Transcription of *sycB* originates at a promoter upstream of *ysaU*. (A) Schematic of the *ysa* locus encompassing the *sycB* promoter region. The approximate locations of primers KW114 and KW115 are indicated. (B) RT-PCR was performed with primers KW114 and KW115 and cDNA generated from 2 μg of total RNA that was isolated from JB580v as described in the text; 20% of the reaction was loaded on a 1.2% agarose gel and stained with ethidium bromide. Templates for the PCR are listed above each lane and were as follows: gDNA, genomic DNA; +RT, products from cDNA synthesis reaction with Superscript III added; no RT, products from cDNA synthesis reaction with no Superscript III; nt, no DNA or cDNA added.

SycB or YsaE was present (Table 5). However, when both YsaE and SycB were present, the *sycB* promoter was activated about sixfold. This suggests that both proteins are necessary and probably sufficient to stimulate transcription from the *sycB* promoter, although we cannot exclude the existence of additional regulators.

Furthermore, these data, combined with the RT-PCR data, indicate that at least two promoters are transcribing the *sycByspBCDA* genes.

Regulation of the *ysa* operon. Since *Y. enterocolitica* does not appear to have a homologue to known key regulators of the SPI-1 and Mxi-Spa TTSSs, there is no obvious regulator for the expression of the *ysa* genes. However, proximal to the *ysa* locus are two genes that encode a putative two-component regulatory system, *ysrR* and *ysrS* (23) (Fig. 1). These genes are closely related to the *rcsB* and *rcsC* genes, respectively, which encode a two-component regulatory system that regulates gene transcription in a number of enteric organisms, often in response to

TABLE 5. YsaE and SycB are sufficient to activate the *sycB* promoter in *E. coli* CC118 λ*pir*

Strain	Plasmid[a]			Mean β-galactosidase activity (Miller units)[b] ± SD
	sycB-lacZ	YsaE	SycB	
VM1265	+	+	+	57.8 ± 2
VM1266	+	−	+	9.9 ± 0
VM1267	+	+	−	9.1 ± 1
VM1272	+	−	−	10.0 ± 1

[a] Plasmid constructs are as follows: *sycB-lacZ*, pKW21; YsaE, pKW22; SycB, pKW23; +, presence of construct; −, vector only.
[b] Miller units represent the mean of at least three independent assays with standard deviations.

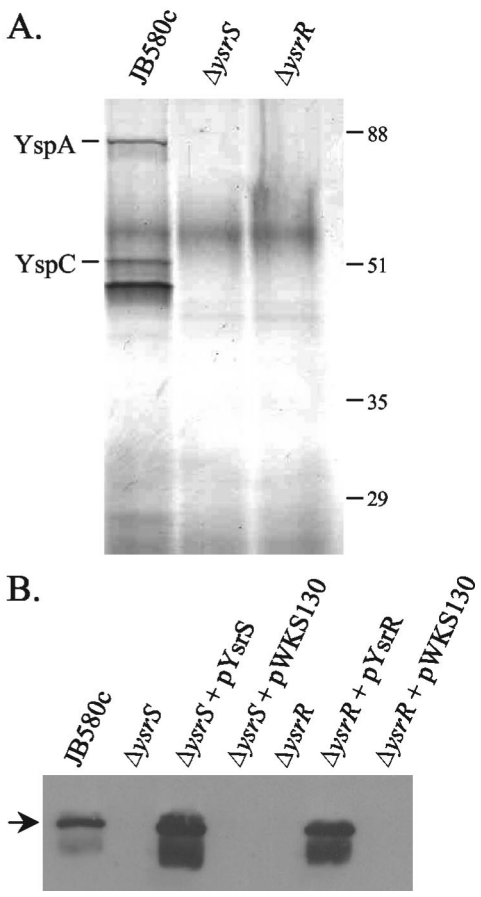

FIG. 4. Strains lacking *ysrS* or *ysrR* do not secrete Ysps. Proteins were precipitated from culture supernatants and separated by SDS–10% polyacrylamide gel electrophoresis as described in the text. (A) Silver-stained gel showing the loss of all Ysps from the culture supernatants of the Δ*ysrS* and Δ*ysrR* strains. The culture equivalent of 2 OD units was loaded in each lane. (B) Complementation of the mutant strains as determined by Western blotting with anti-YspC antibody. YspC is indicated by the arrow. The culture equivalent of 1 OD unit was loaded in each lane.

osmotic shock (2, 47). YsrS is the putative hybrid sensor component and has regions similar to the conserved His- and Asp-containing domains of sensor proteins but, like RcsC, lacks the conserved His residue in the phosphotransfer (HPt) domain. YsrR has regions similar to the Asp-containing receiver domain and a putative helix-turn-helix DNA binding domain. To see if YsrRS is involved in regulating the *ysa* genes, in-frame deletions were constructed in the *ysrS* and *ysrR* genes. Examination of proteins in culture supernatants after growth in LB-290 indicated that both the Δ*ysrS* and Δ*ysrR* strains were defective in the secretion of all of the Ysps (Fig. 4A). These mutant phenotypes could be complemented by providing the wild-type gene in *trans*, indicating that the loss of secretion was due to the absence of functional YsrR or YsrS (Fig. 4B).

To determine if the defect in Ysp secretion observed in the Δ*ysrS* and Δ*ysrR* strains was due to decreased transcription, the activity of the *ysaE-lacZ* and *sycB-lacZ* fusions was examined in the Δ*ysrS* strain. The β-galactosidase activities of the *sycB* and *ysaE* promoters were down 6.5- and 25-fold, respectively, com-

TABLE 6. Regulation by YsrS in *Y. enterocolitica*

Relevant phenotype	Plasmid[a]	Strain	β-Galactosidase activity with *sycB-lacZ*[b]	Strain	β-Galactosidase activity with *ysaE-lacZ*[b]
YsrS$^+$	None	YVM987	512 ± 58	YVM925	398 ± 6
YsrS$^-$	None	YVM990	78 ± 3	YVM995	16 ± 1
	pYsrS	YVM1073	3,770 ± 369	YVM1074	1,688 ± 70
	pWKS130	YVM1056	75 ± 5	YVM1057	16 ± 1
	pSycB, pYsaE	YVM1061	1,622 ± 251		ND
	pKW27, pWKS130	YVM1060	41 ± 2		ND

[a] Plasmid pYsrS is pKW31, pSycB is pKW28, and pYsaE is pKW22. pKW27 is the vector for pSycB, and pWSK130 is the vector for pYsaE and pYsrS.
[b] Values are expressed as Miller units and represent the mean of at least three independent assays with standard deviations. ND, not determined.

pared to the wild-type strain, indicating that YsrS is required for full activity of both promoters (Table 6). Addition of *ysrS* in *trans* restored promoter activity, demonstrating that the observed decrease was indeed due to loss of *ysrS*; no change in activity was observed in strains carrying the vector. However, the YsrS regulatory pathway could be acting indirectly at the *sycB* promoter by activating expression of the upstream *ysaE* promoter and thus production of YsaE and SycB. To determine if the reduced *sycB-lacZ* activity resulted from a direct loss of activation at the *sycB* promoter, the Δ*ysrS* strain was transformed with pSycB and pYsaE. If the *ysrS* defect can be overcome by expressing *ysaE* and *sycB* in *trans*, this would suggest that the YsrRS system acts indirectly at the *sycB* promoter. Indeed, the activity of the *sycB-lacZ* fusion was restored in the Δ*ysrS* strain carrying pYsaE and pSycB, indicating that the observed decrease in activity at this promoter in the Δ*ysrS* strain is probably a downstream effect from the loss of activation at the *ysaE* promoter.

Regulation by YsrS requires NaCl. Because the function of sensor proteins is generally to detect environmental cues and secretion of Ysps is only observed when cells are cultured in high NaCl, we hypothesized that YsrS responds to the NaCl in the growth medium. To test the idea that YsrS senses NaCl, wild-type and Δ*ysrS* strains containing the *ysaE-lacZ* fusion were grown in LB-0 and LB-290 and assayed for β-galactosidase activity (Table 7). In LB-0, β-galactosidase activity in the wild-type strain was the same as in the Δ*ysrS* strain. In addition, no induction by NaCl was observed in the Δ*ysrS* strain, yet the wild-type strain showed 25-fold induction. Furthermore, induction of *ysaE* by NaCl could be restored in the Δ*ysrS* strain by providing *ysrS* in *trans*. This revealed that, in the absence of YsrS or in the absence of NaCl, there is no activation of the *ysaE* promoter.

TABLE 7. Regulation of the *ysaE* promoter by YsrS requires NaCl

Relevant phenotype	Strain	Plasmid[a]	β-Galactosidase activity[b] (% of wild-type level) with:	
			290 mM NaCl	0 mM NaCl
YsrS$^+$	YVM925	None	398 ± 6 (100)	16 ± 1 (4)
YsrS$^-$	YVM995	None	16 ± 1 (4)	20 ± 2 (5)
	YVM1074	pYsrS	1,688 ± 70 (425)	30 ± 7 (7)
YsaE$^-$	YVM970	None	309 ± 13 (78)	16 ± 1 (4)

[a] pYsrS is pKW31.
[b] Values are expressed as Miller units and represent the mean of at least three independent assays with standard deviations. Percent activity for each promoter relative to the wild-type strain grown in 290 mM NaCl is shown in parentheses.

To confirm that YsrS was required for the NaCl-dependent activation, the *ysaE-lacZ* fusion was similarly tested in the Δ*ysaE* strain. Consistent with previous studies on InvF (22), loss of YsaE did not lead to a significant reduction in activity of its own promoter when cultured in LB-290. Loss of SycB also did not affect the expression of *ysaE-lacZ* (not shown). However, just as in the wild-type strain, activity from the *ysaE* promoter was reduced when it was cultured in LB-0. This indicates that the NaCl-dependent activation of the *ysa* operon requires YsrS but not YsaE. This effect could be a response to changes in osmolarity rather than a specific NaCl-dependent effect, but this has not been fully explored.

Intriguingly, the growth rate of the Δ*ysrS* and Δ*ysrR* strains was slightly but reproducibly faster than that of the wild-type strain when grown in LB-290. Doubling times for the Δ*ysrS* and Δ*ysrR* strains was typically about 10 min faster than for the wild type (≈83 min for the Δ*ysrS* and Δ*ysrR* strains and ≈93 min for the wild type). This increased doubling time was not observed for these strains grown in LB-0, nor was it observed for the Δ*ysaE* or Δ*ysaC* strains grown in LB-290, indicating that it is specific to the Δ*ysrS* and Δ*ysrR* strains when cultured in the presence of NaCl. This also suggests that it is not related to the secretion of Ysps.

DISCUSSION

In this work, we showed that the AraC-like regulator YsaE and the chaperone SycB are involved in the regulation of a subset of the Ysps. Further examination of this phenomenon showed that the *sycByspBCDA* operon is transcriptionally regulated by YsaE and SycB. Loss of either activator resulted in a reduction in *sycB-lacZ* activity in *Y. enterocolitica*. Similarly, a reconstituted system in *E. coli* showed a sixfold activation of *sycB-lacZ* only in the presence of both regulators. These data indicate that YsaE and SycB are necessary and likely sufficient to activate transcription from this promoter, although the existence of additional regulators cannot be excluded. The increased activation observed in the isolated *E. coli* system compared to that observed in *Y. enterocolitica* is probably a consequence of transcription of *sycBysp BCDA* initiating at the upstream promoter (*ysaE*) that is not subject to regulation by YsaE and SycB. This is also probably the case in *Salmonella* SPI-1, where transcription of the *sicAsipBCDA* genes can occur through initiation at an upstream promoter (possibly the *invF* promoter), as well as at the *sicA* promoter (13).

A similar mechanism of type III effector gene regulation by MxiE and IpgC exists in *S. flexneri*. Here, it is not the translo-

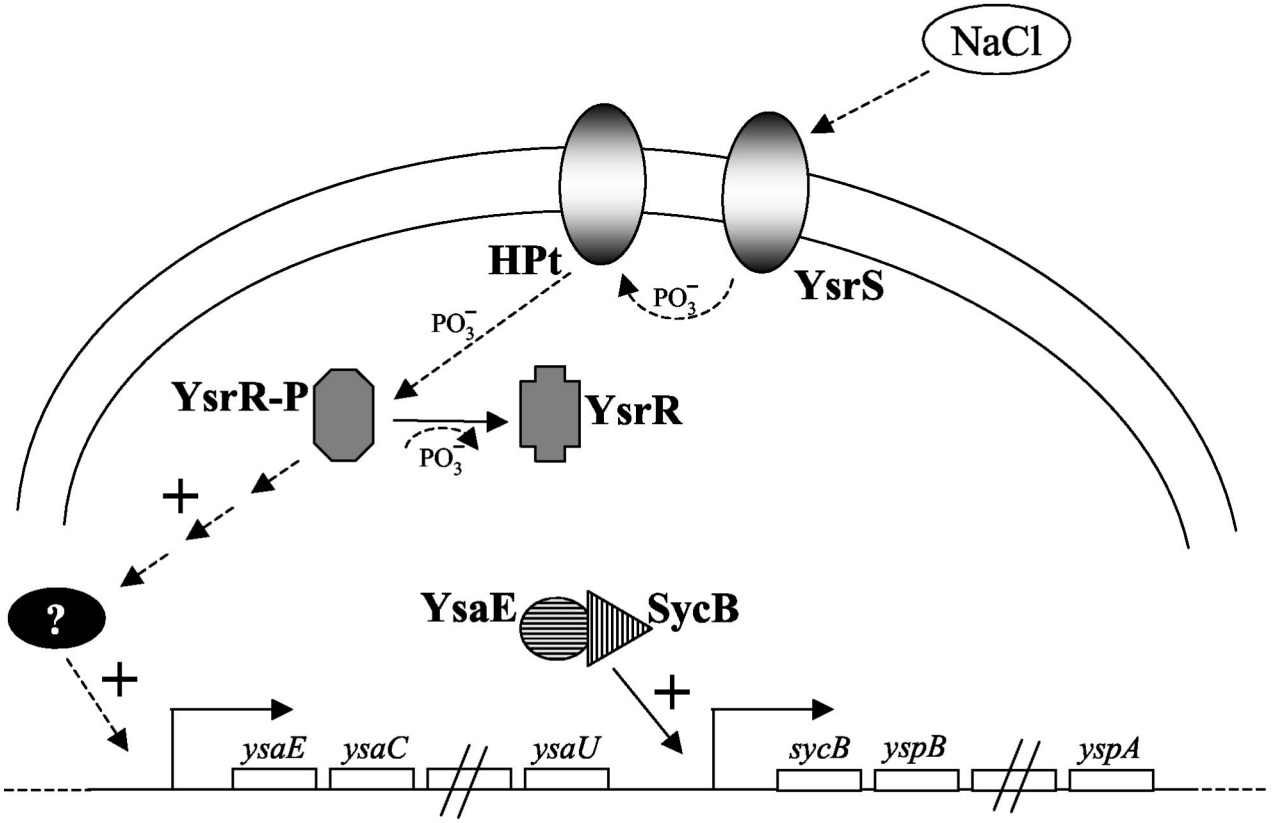

FIG. 5. Model for activation of the *ysaE* and *sycB* promoters. YsrS senses NaCl in the culture medium by an unknown mechanism and initiates a phosphorelay that leads to phosphorylation of YsrR. The activated YsrR then stimulates transcription of the *ysaE* promoter, either directly or indirectly. Once sufficient levels of YsaE and SycB have accumulated, they stimulate transcription of the *sycB* promoter.

cator operon that is affected but a set of proteins whose secretion is only observed under conditions of active secretion (27, 34). By incrementally overexpressing IpgC, Mavris et al. showed that the expression of these proteins increased as the concentration of IpgC increased (34). The authors concluded that the level of free IpgC, which would be found when its cognate proteins had been secreted, is the signal that leads to increased transcription of the secreted proteins. This report marks the third example of an AraC-like regulator acting with a type III chaperone to stimulate transcription of secreted proteins. Thus, it is likely that this is a conserved mechanism by which the cell monitors its secretion state and links it to transcriptional regulation.

The mechanism(s) behind this activation is not well understood. It has been shown that InvF can bind DNA in the absence of SicA, but SicA by itself does not bind DNA (15). The DNA binding sites for InvF and MxiE have been identified and are strikingly similar; in the center of each site, there is a T-rich region (15, 35), which may facilitate bending of the DNA by the protein. These promoters also lack an obvious −35 consensus sequence. In the region thought to contain the *sycB* promoter, a T-rich region is in roughly the same location as in the InvF- and MxiE-regulated genes, and there is no definable −35 region. However, much work remains to determine if this is indeed a YsaE binding site.

Many bacteria, both gram-negative and gram-positive, use two-component regulatory systems to regulate virulence genes. For example, RcsBC is required for the expression of capsule genes in *E. coli* (49), *Erwinia amylovora* (5), and *Klebsiella pneumoniae* (1) as well as Vi antigen expression in *Salmonella enterica serovar Typhi* (2). In *S. enterica serovar Typhimurium*, the type III secretion systems encoded on both SPI-1 and SPI-2 are regulated by multiple two-component systems (29, 32, 33, 41). In this work, we present data suggesting that expression of the *ysaE* promoter requires YsrS and most likely YsrR. YsrS and YsrR are encoded by genes adjacent to the *ysa* locus and comprise a putative two-component regulatory system (19, 23). Analysis of secreted proteins as well as the *lacZ* fusion to *ysaE* in the *ysrS* mutant revealed that YsrS is a key component in the expression of the *ysa* locus. Loss of YsrS also resulted in lower *sycB* promoter activity. However, providing *ysaE* and *sycB* in *trans* in the Δ*ysrS* strain complemented *sycB* activity. Together, these results indicate that the *ysaE* promoter is regulated by YsrS and also suggests that initiation at *ysaE* can lead to transcription of *sycByspBCDA*. YsrR is also a critical component, as an in-frame deletion of *ysrR* similarly resulted in complete loss of all secreted proteins in culture supernatants. Secretion of Ysps has not been observed in the absence of NaCl in the culture medium, and stimulation of *ysaE* transcription by YsrS requires NaCl. Thus, YsrS may be functioning as an environmental sensor of NaCl or osmolarity. Furthermore, since YsrS is probably a membrane-bound protein, it is more

likely that the actual transcriptional regulator is YsrR; however, this has yet to be experimentally tested.

BLAST searches with YsrS revealed homology to RcsC in *E. coli* and other enteric bacteria. While much of the similarity was limited to the conserved domains, YsrS is also similar to RcsC in that they both lack a histidine phosphotransferase (HPt) domain. This domain contains the second His site that transfers the phosphate to the Asp residue on the response regulator (53). In *E. coli*, the HPt-containing protein YojN has been identified as the intermediate between RcsC and RcsB (50). Interestingly, YojN lacks the other necessary His and Asp residues typically found in sensors, suggesting that the only functional domain is the HPt (50). Another example of a third partner in phosphorelay is with the LuxN-LuxU-LuxO system in *Vibrio harveyi*. LuxN lacks an HPt domain, while LuxU contains the appropriate His residue (21). There are several open reading frames that contain HPt domains within the *Y. enterocolitica* genome, suggesting that such an intermediate protein providing this domain for the YsrS-YsrR phosphorelay does indeed exist. YsrR has homology to a number of response regulators, including RcsB. However, most of the conserved residues are in the LuxR-type helix-turn-helix DNA-binding motif.

Although the activity of YsrR and YsrS appears to require NaCl, they probably do not have a role in osmoprotection, as is suggested for the RcsC-YojN-RcsB system (56). This is evidenced by the increased growth rate observed in the Δ*ysrS* and Δ*ysrR* strains in the presence of high NaCl concentrations. In fact, the *Y. enterocolitica* genome contains genes that are probably the true RcsBC orthologues, based on amino acid similarity and genetic organization (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). Thus, YsrRS represent a new and uncharacterized two-component system. In virulence plasmid-containing *Yersinia* strains, growth of the bacterium slows when Yop secretion is induced (11 and references therein). The Δ*ysaC* strain, which is defective in secretion but not transcription, does not display altered growth. Therefore, the faster growth of the Δ*ysrS* and Δ*ysrR* strains might be a consequence of not using metabolic resources for expressing the *ysa* and *ysp* genes.

Based on the results presented here, a schematic model for Ysa TTSS regulation is proposed in Fig. 5. We hypothesize that YsrS senses an environmental cue (NaCl), transfers phosphate to an HPt-containing intermediate (HPt), which then transfers the phosphate to YsrR. The activated phospho-YsrR then stimulates transcription of the *ysaE* promoter, either directly or indirectly. Once levels of YsaE and free SycB are sufficient, they stimulate transcription of the *sycB* promoter and possibly others. From the experiments conducted with InvF and MxiE (15, 35), we can infer that YsaE probably binds DNA in the absence of SycB. It will be of interest to investigate if SycB enhances the DNA binding affinity of YsaE, if SycB makes any contact with the DNA, and if either protein contacts RNA polymerase. Similar questions arise surrounding the regulation by YsrRS. It remains to be demonstrated that YsrR directly regulates promoter activity and if it is indeed involved in a phosphorylation cascade with YsrS. It also remains to be demonstrated whether or not YsrS directly senses NaCl and other environmental cues for activating this system.

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