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Synchronous Gene Expression of the *Yersinia enterocolitica* Ysa Type III Secretion System and Its Effectors[▽]

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Type III secretion systems (T3SSs) are complex units that consist of many proteins. Often the proteins are encoded as a cohesive unit on virulence plasmids, but several systems have their various components dispersed around the chromosome. The *Yersinia enterocolitica* Ysa T3SS is such a system, where the apparatus genes, some regulatory genes, and four genes encoding secreted proteins (*ysp* genes) are contained in a single locus. The remaining *ysp* genes and at least one additional regulator are found elsewhere on the chromosome. Expression of *ysa* genes requires conditions of high ionic strength, neutral/basic pH, and low temperatures (26°C) and is stimulated by exposure to solid surfaces. The AraC-like regulator YsaE and the dual-function chaperone/regulator SycB are required to stimulate the *sycB* promoter, which transcribes *sycB* and probably *yspBCDA* as well. The putative phosphorelay proteins YsrRS (located at the distal end of the *ysa* locus) and RcsB, the response regulator of the RcsBCD phosphorelay system, are required to initiate transcription at the *ysaE* promoter, which drives transcription of many apparatus genes. In this work, we sought to determine which *ysp* genes were coordinately regulated with the genes within the *ysa* locus. We found that six unlinked *ysp* genes responded to NaCl and required YsaE/SycB, YsrRS, and RcsB for expression. Three *ysp* genes had unique patterns, one of which was unaffected by all elements tested except NaCl. Thus, while the *ysp* genes were likely to have been acquired independently, most have acquired a synchronous regulatory pattern.

Yersinia enterocolitica is a gram-negative enteric pathogen with a tropism for lymphoid tissues. Typically ingested through contaminated food or water, it is usually self-limiting to the gastrointestinal tract and mesenteric lymph node, causing diseases that include gastroenteritis and lymphadenitis. In children or immunocompromised adults, a systemic infection can develop that is associated with high mortality (6).

Y. enterocolitica strains are separated into several biovars based on biochemical properties, and there is variability in the severity of diseases caused by the different biovars. Biovars 2 to 5, considered the Old World strains, are found mostly in Europe and Japan and are classified as having low pathogenicity (42). Biovar 1A, also an Old World strain, has lost the virulence plasmid and is nonpathogenic (3). New World strains, isolated mostly in North America, are those of biovar 1B and are the highly pathogenic strains (42). All the pathogenic strains carry an ~70-kb virulence plasmid, called pYV, that encodes the Ysc-Yop type III secretion system (T3SS), the adhesin YadA, as well as regulators of these important virulence genes (reviewed in reference 5).

The highly pathogenic biovar 1B strains contain a large region (~200 kb) of the chromosome that is not found in other *Yersinia* strains (33). It varies considerably among different *Y. enterocolitica* isolates and has been termed the plasticity zone. Several defined and predicted virulence determinants are contained in the plasticity zone, including a region similar to the *Yersinia pseudotuberculosis* adhesion pathogenicity island, the Yts1 type II secretion system, and the Ysa T3SS (33). The two secretion systems have been shown to be required for full virulence in oral infection models (17, 18, 23). Studies using a mouse model have indicated about a 10-fold increase in the 50% lethal dose of *ysa* mutants compared to that of wild-type strains, and *ysa* mutants are defective for colonization of the intestinal tract (17, 23). This defect is observed only for oral inoculations; no attenuation has been observed in mice infected by intraperitoneal injection (17).

The *ysa* locus contains genes encoding all the components of a type III secretion apparatus, a chaperone (*sycB*), four secreted proteins (*yspBCDA*), and at least four regulatory proteins (*ysaE*, *sycB*, *ysrR*, and *ysrS*) (15, 17, 23, 38) (Fig. 1). Additional *ysp* genes are found dispersed around the chromosome (23, 41). This T3SS is a member of the Inv/Mxi-Spa family of T3SSs and shares not only homology but gene organization with the systems in *Salmonella* SPI-1 and *Shigella* spp. (36). In addition, closely related systems are found in *Sodalis glossinidius*, a plant symbiont (7), and in *Chromobacterium violaceum*, which is found primarily in soil in tropical regions but can cause lethal infections in humans (4).

The regulatory protein YsaE is a member of the AraC-like family and is required, along with SycB, for expression of a promoter upstream of *sycB* (38). SycB has similarity to chaperone proteins, and deletion of *sycB* was shown to result in smaller amounts of YspB, suggesting it indeed functions as a chaperone (15). Thus, SycB has dual functionality: transcriptional regulator and chaperone. This mechanism of regulation has been observed in *Salmonella enterica* serovar Typhimurium

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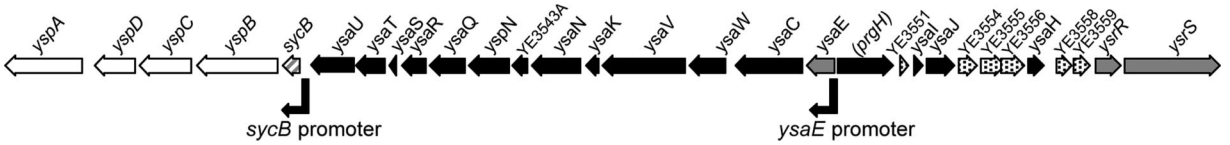

FIG. 1. Schematic representation of the *ysa* locus. The region presented is ~30 kb and is comprised of genes encoding the apparatus (designated *ysa*; black boxes), four secreted proteins (*ysp*; white boxes), and four regulatory proteins (gray boxes). SycB has dual function of chaperone and regulator (striped box). Stippled boxes indicate genes whose role/function is unknown. The *ysaE* and *sycB* promoters are indicated by arrows.

with InvF/SicA and in *Shigella flexneri* with MxiE/IpgC (9, 10, 24, 25). In both of these systems, direct interactions between the AraC-like protein and the chaperone have been observed (10, 31).

A promoter upstream of *ysaE* likely drives transcription of all genes from *ysaE* to *yspA* (38) (Fig. 1). High levels of NaCl in the growth medium induce secretion by the Ysa T3SS (17), and it was subsequently shown that the *ysaE* promoter was activated by NaCl (37, 38). The Ysa T3SS is regulated by the putative phosphorelay proteins YsrS and YsrR that are encoded by genes adjacent to the *ysa* locus (Fig. 1) and by the known phosphorelay protein RcsB (37, 38). YsrS, a putative hybrid sensor kinase, was shown to activate the *ysaE* promoter to the same level as NaCl and is hypothesized to sense the NaCl levels (38). YsrR is a putative response regulator that was shown to be required for Ysa-dependent secretion (38). RcsB is the response regulator of the RcsCDB system and is a DNA binding protein that can activate transcription alone or as a heterodimer with RcsA (reviewed in reference 22). This phosphorelay system has been implicated in the regulation of T3SS and other virulence determinants in several gram-negative bacterial species (2, 21, 34, 39). In addition, CRP has been implicated in the positive regulation of the *ysa* locus, but the mechanism remains unclear (30). Furthermore, while expression from the *ysaE* promoter is dependent on high levels of NaCl, it can be further stimulated by exposure to solid surfaces (27).

Of the four Ysp proteins encoded within the *ysa* locus, YspB, YspC, and YspD are homologous to "translocon" proteins that form a pore in host cells to allow for translocation of effector proteins (15, 17). YspA is unique, although the proteins encoded by genes in this relative position in the *Salmonella* (SipA) and *Shigella* (IpaA) systems have been defined and shown to affect actin polymerization (35, 44). In addition to these four proteins, Matsumoto and Young have identified seven other proteins secreted by this system (23). Two Ysp proteins showed homology to known proteins, and their biochemical activities were demonstrated in vitro; YspP is a phosphatase and YspK is a serine/threonine kinase (23). The remaining *ysp* genes showed no homology to known genes, and only *yspY* had a homolog in other bacterial species (*Yersinia pestis* and *Y. pseudotuberculosis*) (23). Since type III-secreted proteins are often coordinately regulated with the apparatus genes (9, 10, 20, 24, 25), the focus of this study was to examine the transcriptional expression and regulatory patterns of the newly identified *ysp* genes. To this end, we determined the expression pattern of *ysp* genes in a wild-type strain and examined their expression levels in several regulatory mutant strains. We found that most, but not all, *ysp* genes displayed a common regulatory expression pattern with the apparatus genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1 and described below. All cultures of *Escherichia coli* strains were grown in LB medium (170 mM NaCl; Difco) at 37°C. Cultures of *Y. enterocolitica* were grown at 26°C in LB medium, L broth (1% tryptone, 0.5% yeast extract, 0 mM NaCl), or L broth with 290 mM NaCl (referred to as LB-290). Antibiotics were added as needed at the following concentrations: kanamycin (Kan), 100 μg/ml; nalidixic acid, 20 μg/ml; chloramphenicol (Cm), 12.5 μg/ml; and spectinomycin (Sp), 100 μg/ml.

To examine transcript levels during the course of the growth curve, triplicate-saturated cultures of wild-type *Y. enterocolitica* JB580v grown in L broth were diluted to an initial optical density at 600 nm (OD_{600}) of 0.2 in 100 ml fresh L broth or LB-290 and grown at 26°C with aeration (in 250-ml flasks). At the specified times, a volume of cells was transferred to Oak Ridge tubes, snap-cooled in liquid nitrogen, and collected by centrifugation, and the pellets were stored at −80°C. For determination of regulation by YsaE, SycB, YsrR, YsrS, and RcsB, strains harboring in-frame deletions of each gene were similarly prepared but subcultured only into LB-290. These same culturing conditions were performed to determine if YsaE and SycB could complement a *ysrS* or *ysrR* deletion, but only the 2-h samples were collected for transcript analysis.

To ascertain how quickly gene expression responded to NaCl, saturated overnight cultures of JB580v grown in L broth were diluted to an OD_{600} of 0.2 in 250 ml fresh L broth and grown for 2 h at 26°C (in a 1-liter flask). The culture was split into two equal volumes, and the cells were collected by centrifugation. One pellet was resuspended in 125 ml fresh L broth and the other in 125 ml LB-290 (in 250-ml flasks), and the cultures were grown at 26°C with aeration. To avoid cold shock, the samples were kept at room temperature during all handling. Samples were taken from each set at 0, 20, 40, 60, 90, and 120 min after resuspension, snap-cooled, and collected by centrifugation, and the pellets were stored at −80°C.

Plasmid and strain construction. The plasmids and strains used in this study are listed in Table 1, and the primers used are listed in Table 2. A *ysrR-gfp* transcriptional fusion was constructed as follows. About 500 bp of the putative *ysrR* promoter region was amplified with primers ysrR-delA and ysrR-delB, digested with SalI and BamHI, ligated into the same sites of pPROBE-gfp[tagless], and transformed into *E. coli* DH5α. The resulting plasmid, pKW71, was confirmed by digest and sequencing and transformed into the desired *Y. enterocolitica* strains by electroporation. This same fragment was cloned into pCR2.1-TOPO (Invitrogen), from which an XbaI-BglII fragment was excised and cloned into pKN8 to generate pKW15. pKW15 was conjugated into JB580v as previously described (38), resulting in strain YVM1041. Plasmid pMWO-002 was generated for the overexpression of YsrR-His. Primers MWO-002 and MWO-003 were used to amplify the coding region of *ysrR*; the resulting product was cleaved with NheI and XhoI and cloned into those sites of pET24b.

RNA extraction and Northern blot analysis. Total cellular RNA was extracted from cell pellets that had been stored at −80°C using TRIzol LS reagent (Invitrogen). TRIzol was added to the frozen cell pellet to a density of 10 OD_{600} units/ml, and the remaining steps of the extraction were performed as described by the supplier. Total RNA was treated with DNase using DNA-free, following the manufacturer's protocol (Ambion). For Northern analysis gels, 10 μg RNA was separated in 1% formaldehyde agarose gels and transferred to nitrocellulose membranes by capillary action (32). For dot blots, 5 μg RNA was mixed with 50 μl of denaturing buffer (50% formamide, 7% formaldehyde, 1× SSC; 1× SSC is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and transferred to nitrocellulose membranes by vacuum suction using a dot blot apparatus (32).

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli		
DH5α	F^- ϕ80dΔ*lacZ*ΔM15 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 Tn7 λ*pir* lysogen	28
Y. enterocolitica		
JB580v	8081v (r^- m^+ Nal^r)	19
YVM932	JB580v Δ*ysaE*	38
YVM981	JB580v Δ*sycB*	38
YVM969	JB580v Δ*ysrS*	38
YVM1006	JB580v Δ*ysrR*	38
YVM1236	JB580v Δ*rcsB*	41
YVM1321	YVM969 carrying pKW22 and pKW28	This work
YVM1349	JB580v carrying pWKS130 and pKW27	This work
YVM1350	JB580v carrying pKW22 and pKW28	This work
YVM1351	YVM969 carrying pWKS130 and pKW27	This work
YVM1352	YVM1006 carrying pWKS130 and pKW27	This work
YVM1353	YVM1006 carrying pKW22 and pKW28	This work
YVM1041	JB580v *ysrR-lacZYA*	This work
Plasmids		
pPROBE-*gfp*[tagless]	Kan^r; *gfp* transcriptional fusion reporter	29
pET24b	Kan; overexpression vector with C-terminal His_6 tag	NEB
pSR47S	Kan^r; MobRP4 *ori*R6K, cloning vector	26
pWKS130	Kan^r; low-copy-number cloning vector	40
pKW27	Cm^r Str^r Sp^r; pACYC184 with Str/Sp cassette	38
pKN8	Cm^r; MobRP4 *ori*R6K, transcriptional reporter vector	14
pKW22 (pYsaE)	*ysaE* coding sequence and promoter cloned into pWKS130	38
pKW28 (pSycB)	*sycB* coding sequence and promoter cloned into pKW27	38
pKW53	*rcsB* lacking codons 7–211 cloned into pSR47S	41
pKW71	pPROBE-*gfp*[tagless] with *ysrR* promoter region	This work
pKW15	*ysrR* promoter region cloned into pKN8	This work
pMWO-002	*ysrR* coding region cloned into pET24b	This work

TABLE 2. Primers used in this work

Name[a]	Sequence[b] (5′ → 3′)
ysrR-delA[c]	ACGCGTCGACGCAGGATAATCCGATGAAATCTCG
ysrR-delB[c]	CGGGATCCCATCAGCGCAAGGCGACTGAAAGG
yspA F	GGAATTCCATATGCCTAATATCATGGCACCCAGTAC
yspA R	GCTCTAGAGTAGAATTCATTTGCTGCACCCATC
yspE F	CCGCTCGAGATGAGTAGGATAAGTCAAAGC
yspE R	GCTCAAGTTTATAACGGCCACC
yspF F	ATGACGCCAGCAAAAATTAGTTCGAG
yspF R	CTGAACTATTTCCTGTTGAATAGCAG
yspK F	CCGCTCGAGATGAAAATAACACCTACCATT
yspK R	GGAGTCCCGGGCACGCGTAGC
yspL F	ATGAATATTAAAGAGCGTATTAATTTC
yspL R	GCAATAATGCATATTCATCAGCAGAC
yspM F	CCGCTCGAGATGAGTATTAATTTTAACCACAAC
yspM R	GGTGAGGTCAGGAAATCAACCC
yspP F	ATGCTTACAAAAAACATCGCCCCAACACC
yspP R	ATTAGTATCACCAAGCGTACTGAG
yspY F	GGTTATTGTTATGGCCGATTATTTC
yspY R	CCTTTATTGGCTTGAGCTTCCCAC
ysaE F	GCCGGTAACGCCAGTGGCTCATTG
ysaE R	GTCGCATCATCTGGTGCATCAAGG
sycB F	GAACCAGAAACATGATGCGGCAG
sycB R	GCCAAAGAGTAAAGTTCCACTG
rcsB F	GACCATCCAATTGTGTTGTTTGGC
rcsB R	GCGTCGACCAGTTACCAGGAAACCTTCAGC
16S	CCGTCCGCCGCTCGCCGGCAA
MWO-002	CTACTAGCTAGCATGACACAAACGAAAACGC
MWO-003	GACTGCTCGAGTAGAGAAATTTCATGAGC

[a] F, forward primers; R, reverse primers.

[b] Restriction sites are underlined. Some primers used to generate products for Northern probes contain restriction sites but are not noted because the products were not digested.

[c] These primers were originally published in reference 38 and are included here for completeness.

Gene-specific PCR products were labeled with [α-^{32}P]dATP by random-primed labeling following the supplier's protocol (Roche), and blots were hybridized in 50% formamide buffer. To control for equivalent loading, the 16S RNA was probed. An oligonucleotide probe (KW157) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Hybridization and washes were conducted in sodium pyrophosphate buffer (hybridization buffer, 0.1% sodium pyrophosphate, 4× SSC, 5× Denhardt's reagent, 0.1% sodium dodecyl sulfate [SDS], 100 μg/ml salmon sperm DNA; and wash buffer, 0.1% sodium pyrophosphate, 4× SSC, 0.1% SDS) (Stephanie Strand, personal communication). Specific mRNA signals were quantified using a FLA-5000 phosphorimager and ImageGauge v4.22 software (Fuji Film Medical Systems, Stamford, CT). The gene-specific signal was normalized by dividing it by the 16S signal. Triplicate values were averaged and normalized to the peak of expression (for time course analysis) or to the wild-type levels (for regulation analysis). The expression differences relative to those of the wild type (set to 100) were determined by dividing the value obtained from the mutant strain by that from the wild-type strain. For the only exception to this protocol, see Fig. 4; the data were not normalized to 16S RNA and are presented as qualitative only.

Green fluorescent protein assays. Saturated cultures of *Y. enterocolitica* strains carrying pKW71 or pPROBE-gfp[tagless] grown in L broth were diluted to an OD of 0.2 in 100 ml LB-290 or L broth and cultured at 26°C with shaking. At various times, samples were removed for measurement of absorbance and fluorescence. Fluorescence (excitation at 485 nm, emission at 528 nm) was measured using a Synergy HT plate reader (BioTek, Winooski, VT), and absorbance at 600 nm was measured with a spectrophotometer. Fluorescence values in relative light units (RLU) were divided by the culture OD to generate RLU/OD and averaged. The average RLU/OD for the vector control was subtracted from those of strains carrying the promoter fusion.

β-Galactosidase assays. Saturated cultures grown overnight in L broth were diluted into fresh L broth or LB-290 to an initial OD_{600} of 0.2 and grown at 26°C with shaking. Assays were performed as described previously (32).

Western blot analysis. Saturated cultures of JB580v grown in L broth were subcultured into 100 ml fresh L broth or LB-290 and grown with aeration at 26°C. Samples were collected at 2, 4, 6, and 8 h and pelleted. Cell pellets were resuspended in 1× sample buffer (32), boiled, and loaded onto SDS-polyacrylamide gel electrophoresis gels. Proteins were transferred to nitrocellulose for Western blot analysis using a Bio-Rad Trans-Blot SD semidry transfer apparatus. Blots were blocked in phosphate-buffered saline with 0.1% Tween 20 (PBST) and 5% skim milk for 1 h at room temperature. Primary antibody directed against YsrR (1:500) was diluted in PBST-5% milk and allowed to react overnight at 4°C. The membranes were washed several times with PBST and then incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase at 1:30,000 in PBST-milk for 1 h at room temperature. The membranes were washed again in PBST and proteins were detected by chemiluminescence (Amersham).

RESULTS

Expression of the *ysp* genes is tightly growth phase regulated. In order to fully analyze the regulation of the individual *ysp* genes, it was first necessary to determine the time at which each gene was maximally expressed. Previous studies had indicated that several genes within the *ysa* locus were maximally expressed during early to mid-log phase using β-galactosidase reporters (37). However, since reporter data does not always agree with transcript levels, we chose to expand our analysis by examining expression of the *ysp* genes and related genes over the course of a growth curve by Northern analysis. All the *ysp* genes were quickly upregulated upon subculturing into LB-290 (L broth with 290 mM NaCl) and peaked in logarithmic phase, and levels were lower to undetectable by early stationary phase (Fig. 2). No mRNA from any gene probed could be detected from a 24-h culture, so this time point was omitted from the graphs. While nearly all the genes appeared to be very tightly growth phase regulated, nevertheless, two patterns could be observed. Several genes (*yspE*, *yspA*, *yspP*, and *yspM*) were maximally expressed at the first time point examined, 2 h (Fig. 2). Also peaking at this time point were the regulators *ysaE* and *sycB*. The second group, consisting of *yspK*, *yspL*, *yspI*, *yspF*, and *yspY*, was maximally expressed at 4 h. These data indicate that there are two clusters of temporal *ysp* expression, suggesting that there may be some regulatory hierarchy or independent mechanisms of gene activation.

Because several of the *ysp* genes were maximally expressed at the first time point examined, we decided to test how quickly they responded to NaCl. Cultures of wild-type cells were subcultured into L broth, grown for 2 h, and then pelleted and resuspended in LB-290. Samples were collected every 20 min, and gene expression was monitored by Northern blot analysis. The data showed that the genes that peaked at 2 h had a rapid response to NaCl, reaching maximal levels by 20 or 40 min (data not shown). As expected, the group that peaked at 4 h showed a slower response; it had elevated mRNA levels by 20 min but could not reach maximal levels until 40 or 60 min. The results of this experiment demonstrate that *ysp* expression responds immediately to NaCl but that other factors may control the time to maximum expression levels. The differences in the time to peak expression in this experiment are more subtle than in the experiment presented in Fig. 2 and are most likely a consequence of the different culturing methods. The culture density of the 20-min time point was much higher than the 2-h point in the first experiment (OD_{600}, >1.0 versus 0.4 to 0.5). Nonetheless, this experiment supports the previous analysis that the response to NaCl is rapid for all genes and that there are indeed two separate groups of temporal expression.

Effect of NaCl on *ysp* gene expression. Several previous studies showed that secretion of Ysp proteins and expression of selected *ysa* genes are induced by NaCl (17, 37, 38, 43). We therefore wanted to examine the impact of NaCl on *ysp* gene expression. With the exception of *yspM*, the expression profiles of the *ysp* genes in L broth (0 mM NaCl) did not appear to change in response to growth phase, and most showed little or no expression at all (Fig. 2). By comparing the relative expression between L broth and LB-290 at the time of peak expression in LB-290, we determined the fold change in expression upon growth in inducing conditions. Three distinct groups with respect to dependence on NaCl could be observed. Three genes, *yspY*, *yspI*, and *yspM*, were not induced or were only slightly induced by NaCl (1.7-, 2.0-, and 2.6-fold, respectively). The genes *yspA*, *yspK*, and *yspE* were moderately induced by NaCl 4-, 8.7-, and 12-fold, respectively. A third group contains genes whose expression is heavily dependent on NaCl. *yspP* and *yspF* were regulated 39- and 45-fold, respectively, and *yspL* absolutely required NaCl for expression; no signal for *yspL* could be detected from cultures grown in L broth. The regulatory gene *sycB* was also shown to be activated by NaCl 33-fold, confirming the results previously reported using β-galactosidase assays (37, 38). These results indicate that, like the apparatus genes, most *ysp* genes are at least partially dependent on NaCl for expression.

Effect of YsaE/SycB on *ysp* gene expression. Previous work from our lab showed that the *sycB* promoter was stimulated by YsaE and SycB during conditions of active secretion (i.e., high NaCl levels) (38). This regulatory mechanism is similar to that observed in the *Salmonella* SPI-1 regulation of the *sicA* promoter by InvF/SicA and the *Shigella* Mxi-Spa regulation of several *ipa* and *osp* genes by MxiE/IpgC (8–10, 24, 25). In both of these systems, genes encoding secreted proteins located outside the locus containing the apparatus genes are also coordinately regulated by InvF/SicA or MxiE/IpgC (10, 25), which implies that YsaE/SycB may regulate nonlinked *ysp* genes. To determine the influence of YsaE/SycB on *ysp* gene expression, we performed Northern analysis using RNA extracted from strains with an in-frame deletion for *ysaE* (YVM932) or *sycB* (YVM981). Six of the nine genes examined were dependent on both YsaE and SycB for full expression levels (Fig. 3). *yspP* and *yspE* were the most highly dependent, each being reduced over 100-fold in the Δ*ysaE* and Δ*sycB* strains. *yspF*, *yspL*, and *yspA* were reduced from 20- to 75-fold, while *yspM* was modestly reduced by about sixfold. The three genes that showed little or no dependence on YsaE or SycB for expression were *yspK*, *yspI*, and *yspY* (Fig. 3). Curiously, expression of *yspK* was actually somewhat elevated (less than threefold) in the absence of YsaE/SycB. These results indicate that YsaE and SycB are key regulators of many *ysp* genes. Furthermore, it draws another parallel with InvF/SicA and MxiE/IpgC, indicating that this regulatory mechanism may be a common theme for controlled expression of type III effector genes.

Effect of YsrRS and RcsB on *ysp* gene expression. YsrR, YsrS, and RcsB have been shown to be required for activation of the *ysaE* promoter (37, 38; K. A. Walker, S. Mildiner-Earley, and V. L. Miller, unpublished data), and we therefore wanted

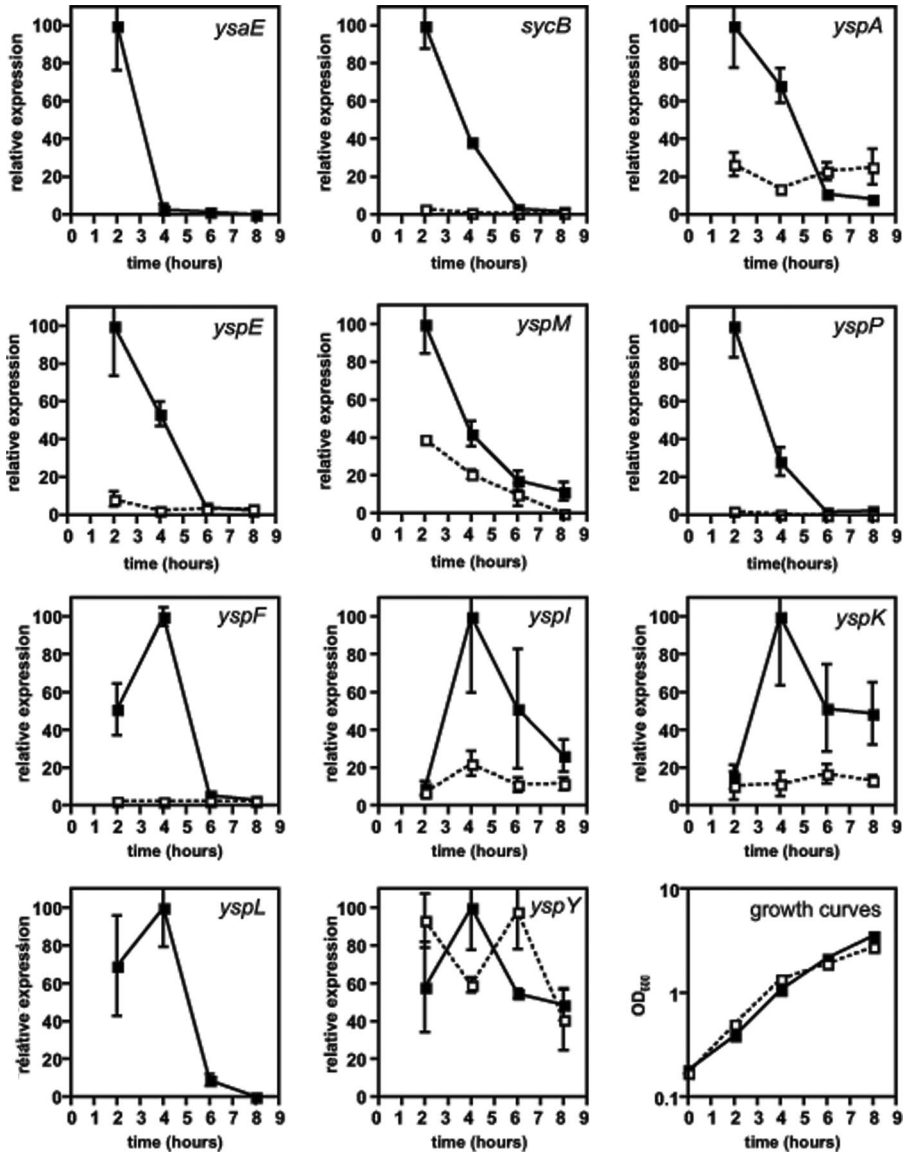

FIG. 2. Growth phase expression of the *ysa* and *ysp* genes. Triplicate saturated cultures of JB580v were diluted to a starting OD of 0.2 in L broth (open squares, dotted line) or LB290 (solid squares, solid line) and grown at 26°C. Total RNA was extracted from cells grown for the indicated times and used for Northern analysis. Individual genes were probed with ~300-bp fragments internal to the predicted coding region. The maximum signal intensity for each data set was assigned a value of 100, and all other signal intensities within each data set were normalized to this value. The panel in the bottom right corner shows the growth curves of the cultures from which the RNA was isolated. The data for *ysaE* are from the experiment presented in the legend to Fig. 3, which was conducted in identical fashion except that only LB-290 was used.

to examine if these proteins are required for expression of any *ysp* genes. Similar Northern blot analyses were performed as described above using strains with an in-frame deletion of *ysrR* (YVM1006), *ysrS* (YVM969), or *rcsB* (YVM1236). In agreement with previous studies using *lacZ* reporters (37, 38), *ysaE* and *sycB* were found to be dependent on YsrR, YsrS, and RcsB for full expression by Northern analysis. Levels of *ysaE* mRNA were decreased seven- to 13-fold relative to those of the wild-type strain, while those for *sycB* were decreased 65- to 165-fold (Fig. 3). The same *ysp* genes that are activated by YsaE/SycB also required all three phosphorelay proteins for expression: *yspP*, *yspE*, *yspL*, *yspF*, *yspA*, and *yspM* (Fig. 3). The genes *yspP*, *yspE*, *yspL*, and *yspF* were heavily dependent on these proteins, with expression reduced about 35- to 110-fold relative to that of the wild type. The genes *yspA* and *yspM* were somewhat less dependent, with expression decreased about 15- to 20-fold for *yspA* and about two- to fourfold for *yspM*.

As was observed in the Δ*ysaE* and Δ*sycB* strains, expression of *yspK* was slightly elevated in the Δ*ysrS*, Δ*ysrR*, and Δ*rcsB* strains (Fig. 3). Levels were increased by about twofold, indicating that its activation is fully independent of the known proteins regulating expression of the Ysa T3SS, even though its expression was dependent on NaCl. Curiously, the results obtained for *yspI* seem to indicate that YsrRS-RcsB do not always regulate *ysa* and *ysp* genes jointly (Fig. 3). YsrS and RcsB were required for full expression, but deletion of *ysrR* resulted in a

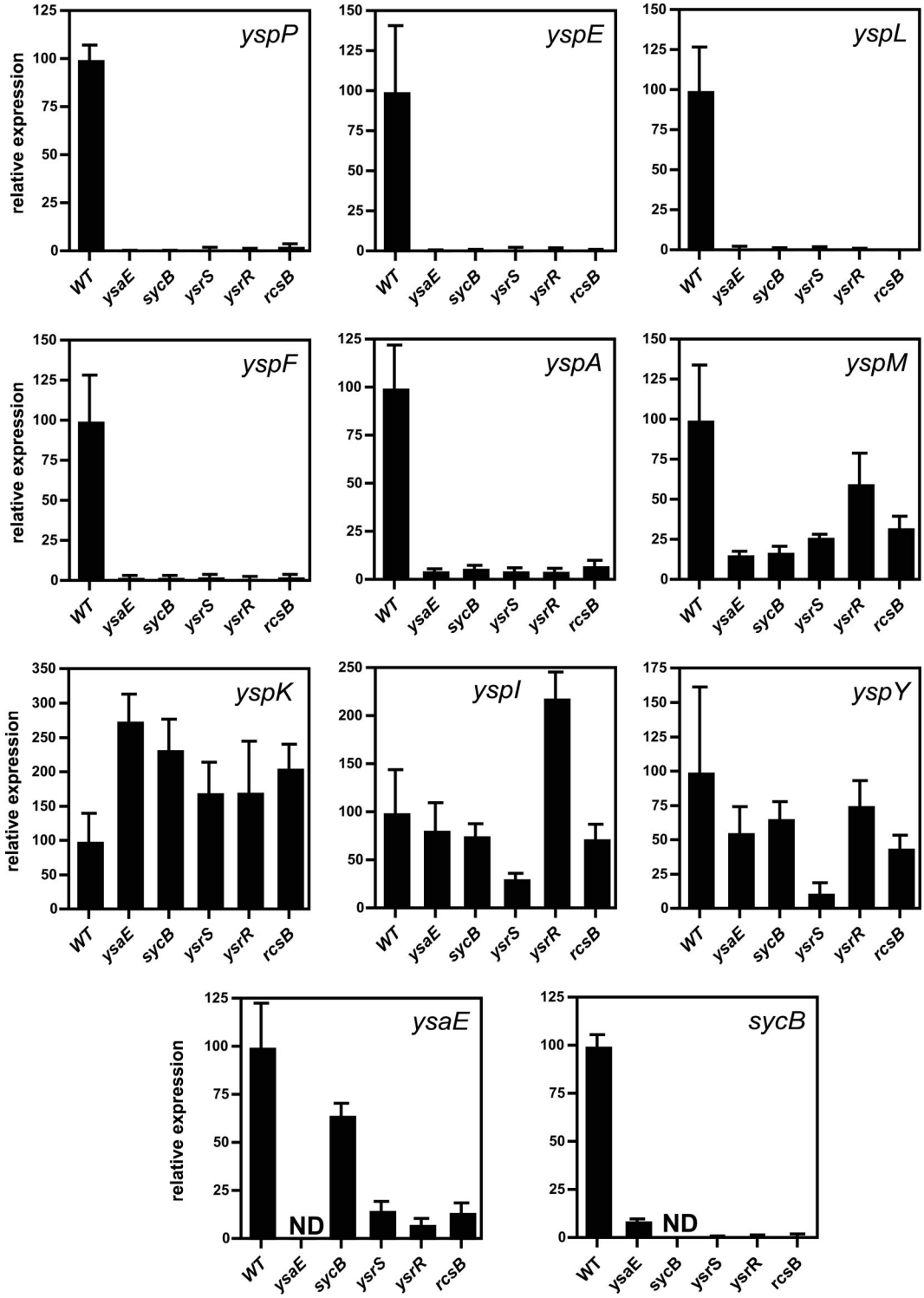

FIG. 3. Effect of YsaE, SycB, YsrR, YsrS, and RcsB on *ysp* gene expression. Triplicate-saturated cultures of the wild-type (WT; JB580v), Δ*ysaE* (YVM932), Δ*sycB* (YVM981), Δ*ysrS* (YVM969), Δ*ysrR* (YVM1006), and Δ*rcsB* (YVM1236) strains were diluted to a starting OD of 0.2 in LB-290 and grown at 26°C. Total RNA was extracted and used in Northern dot blot analysis. At the time of peak expression determined in the legend to Fig. 2, the signal intensity obtained in the mutant strains was divided by the signal intensity obtained for the wild-type strain. Wild-type expression levels were normalized to 100. The data for *yspM* have been presented elsewhere (41), and are included here for completeness. ND, not determined.

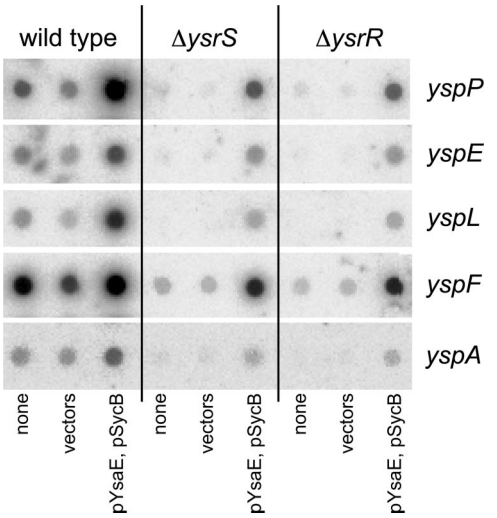

FIG. 4. Regulation of *ysp* expression by YsrRS is indirect. Triplicate-saturated cultures of the wild-type (WT; JB580v), Δ*ysrS* (YVM969), and Δ*ysrR* (YVM1006) strains alone, carrying plasmids pWKS130 and pKW27 (vectors), or pYsaE and pSycB were grown as described in the legend to Fig. 2 for 2 h in LB-290. Total RNA was extracted and used for qualitative Northern dot blot analysis as described in Materials and Methods. pYsaE is pKW22, and pSycB is pKW28.

subtle increase in *yspI* expression (2.2-fold). The role of these phosphorelay proteins also varies with *yspY* and *yspM*. With both of these *ysp* genes, the loss of YsrS reduced expression by more than threefold compared to the wild type, and the loss of RcsB reduced expression by two- to threefold. However, the loss of YsrR resulted in only a slight reduction in expression (less than twofold), similar to the trend observed for *yspI*. Using low-copy-number *gfp* reporter plasmids, similar trends of regulation have been observed for *yspK*, *yspI*, and *yspM* (data not shown). These regulatory patterns are somewhat surprising, as we would have predicted that YsrRS and RcsB were functioning together or in tandem to regulate *ysa* and *ysp* expression. These results suggest that there may be a third response regulator that is part of the YsrS phosphorelay cascade or that RcsB alone can partially compensate for the loss of YsrR.

Regulation of *ysp* genes by YsrRS/RcsB is probably not direct. All the genes that required YsrRS and RcsB were also dependent on YsaE/SycB. Because YsrRS and RcsB are required for expression of *ysaE*, and YsaE is required for expression of several *ysp* genes, it seemed likely that the impact observed by YsrRS/RcsB on *ysp* gene expression is not direct but a consequence of the loss of YsaE/SycB. To test this hypothesis, we transformed strains lacking *ysrR* or *ysrS* with plasmids carrying *ysaE* (pKW22) and *sycB* (pKW28) or vectors (pWKS130, pKW27) and then collected RNA from these strains grown for 2 h in LB-290. The results for this experiment are only qualitative but showed that for each gene tested, expression of *ysaE* and *sycB* together suppressed the deletions of *ysrS* and *ysrR*, whereas the vectors alone did not (Fig. 4). This is consistent with data previously obtained for *sycB*, where *sycB-lacZ* expression in a Δ*ysrS* strain was restored only if both pKW22 and pKW28 were present (38). These data indicate that the regulatory effect observed in the Δ*ysrS* and Δ*ysrR* strains is indirect at these promoters and is most likely a consequence of reduced levels of YsaE and SycB.

***ysrRS* and *rcsB* expression patterns do not appear to dictate *ysa* and *ysp* expression patterns.** Two-component regulator systems are thought to be constitutively expressed at least at low levels so that the proteins are readily available when their specific environmental signal is sensed. Many, like the PhoRB system (16), are autoupregulated when their specific signal is detected to produce higher protein levels to adequately respond to environmental conditions. To ascertain if changes in *ysrRS* or *rcsB* transcription dictated *ysp* gene expression, we examined the expression patterns of these putative phosphorelay genes. Northern analysis of *rcsB* indicated that it is expressed in the absence of NaCl (Fig. 5A) and that it is expressed at near-maximum levels over the course of a growth curve in both the presence and absence of NaCl. There does appear to be some growth phase-dependent regulation in the presence of NaCl, but the peak (at 4 h) was less than twofold higher than that in the absence of NaCl. Judging by the intensity of hybridization, *rcsB* mRNA levels are abundant throughout the course of growth even though there is fluctuation. Therefore, our data suggest that changes in expression of *rcsB* do not account for the salt dependence or growth phase regulation of the *ysp* genes.

Northern analysis could not be used to examine *ysrR* expression. A second putative two-component system located ~10 kb from the *ysa* locus has an exceptionally high degree of identity, making it difficult to design a *ysrR*-specific probe. Therefore, we constructed a *ysrR-gfp* reporter and examined its expression by fluorescence. The low-copy-number plasmid was transformed into JB580v, and the absorbance and fluorescence were measured over the course of a growth curve. The levels of *ysrR-gfp* gradually increased with time and were still elevated at the time points of 6 and 8 h, when most *ysp* genes showed notable decreases in mRNA levels (Fig. 5B). We did not observe a strong influence of NaCl, calculating a less than twofold difference between green fluorescent protein levels of cultures grown in L broth compared to those in LB-290 at 8 h. Since these data contradict what was published by Venecia and Young using a *ysrS-lacZ* reporter (37), we also examined expression using a *lacZ* reporter, with a transcriptional fusion to *ysrR* at amino acid 23. The β-galactosidase assay data are consistent with the fluorescence data, showing no dependence on NaCl (Fig. 5C). No growth phase-dependent regulation was observed using the *ysrR-lacZ* reporter, but the increases observed with the *ysrR-gfp* reporter in late log and early stationary phase are subtle (less than threefold higher than at the 4-h time point). Furthermore, preliminary Western blot analysis of YsrR protein levels under these same conditions showed that YsrR is not detectable until 8 h, a time when very few of the *ysp* genes had any detectible transcripts (not shown). These results suggest that *ysrRS* expression does not exhibit the same salt- and growth phase-dependent expression pattern as the *ysa* and *ysp* genes and therefore does not account for fluctuations in *ysa* and *ysp* expression.

Given that our data on *rcsB* and *ysrR* expression differed considerably from those of Venecia and Young (37), we continued our investigation by examining the impact of YsrR and RcsB regulating each other. By Northern blot analysis, tran-

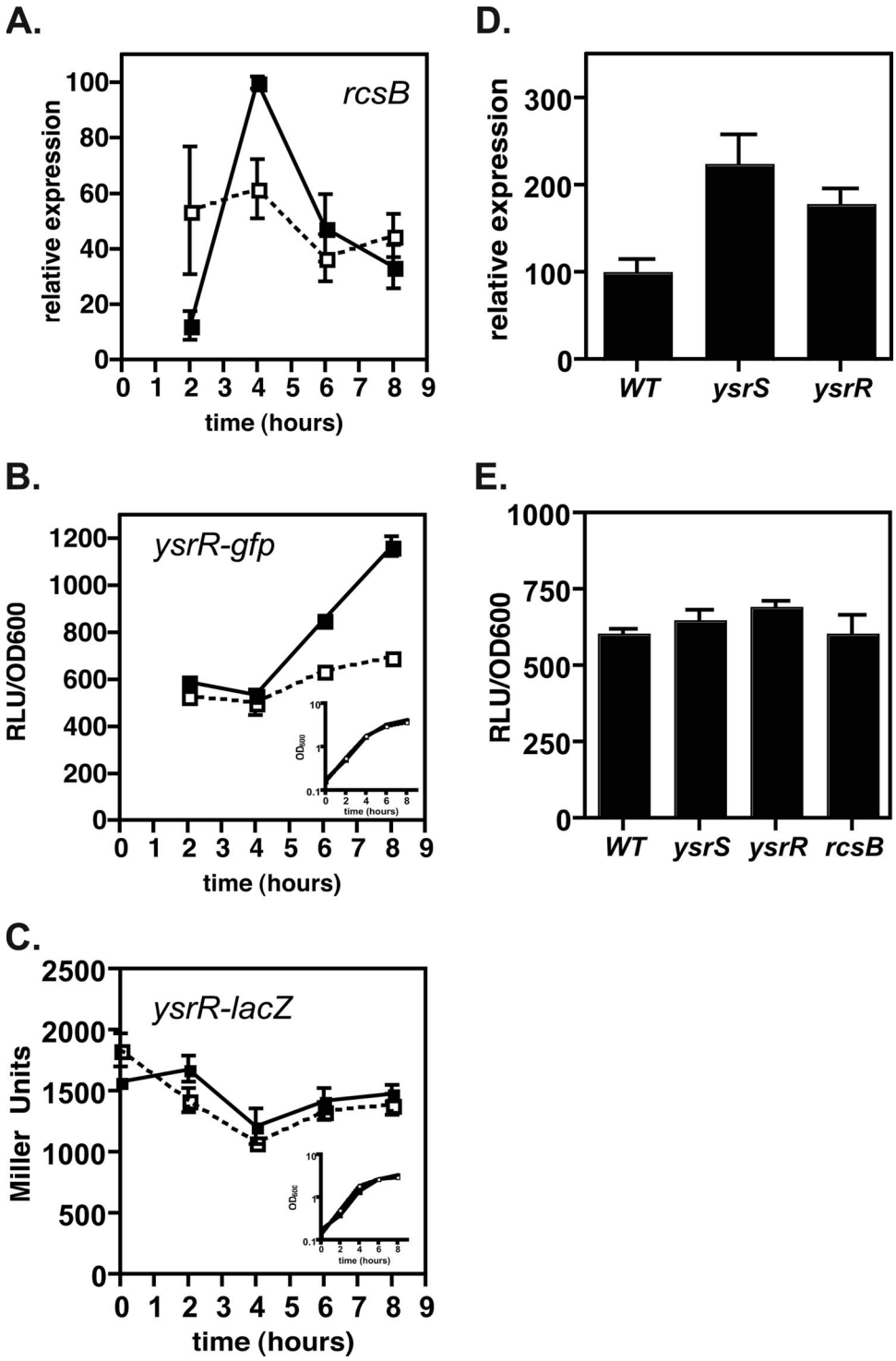

FIG. 5. Expression of *rcsB* and *ysrR*. (A) Growth phase expression of *rcsB*; data are from the experiment shown in Fig. 2. (B) Growth phase expression of *ysrR* monitored by fluorescence using a *ysrR-gfp* reporter. (C) Growth phase expression of *ysrR* monitored by a β-galactosidase assay using a *ysrR-lacZ* reporter (YVM1041). (D) Expression of *rcsB* in Δ*ysrS* and Δ*ysrR* strains; data were obtained as shown in Fig. 3. (E) Expression of *ysrR-gfp* in Δ*ysrS*, Δ*ysrR*, and Δ*rcsB* strains. All cultures were grown as described in the legend to Fig. 2, and data were obtained as described in Materials and Methods. For panels B and C, inset graphs show the growth curves for these experiments. For panels A, B, and C, expression in L broth is presented as a dotted line and expression in LB-290 as a solid line.

script levels of *rcsB* in the Δ*ysrR* and Δ*ysrS* strains were not significantly different than those of the wild type (less than a 2.5-fold change) (Fig. 5D). Using the *ysrR-gfp* reporter, no change in fluorescence was observed in Δ*rcsB*, Δ*ysrR*, or Δ*ysrS* strains (Fig. 5E). The data presented here showed that the loss of YsrRS did not result in a decrease in *rcsB* mRNA as was reported by Venecia and Young (37) and that the loss of RcsB did not result in a decrease in *ysrRS* mRNA. Furthermore,

there did not appear to be any feedback regulation of *ysrRS*. The data generated in this study suggest that the involvement of RcsB and YsrR in *ysa* and *ysp* gene activation is not due to a transcriptional regulatory cascade.

DISCUSSION

T3SSs are a well-established virulence determinant and are found in a wide variety of gram-negative bacterial plant and animal pathogens as well as symbionts (36). The genes encoding the secretion apparatus are usually found as a single cluster. The *Shigella* Mxi-Spa and *Yersinia* Ysc-Yop T3SSs are encoded on virulence plasmids which harbor not only the apparatus genes but also the secreted effectors and transcriptional regulators. However, in other T3SSs such as *Salmonella* SPI-1 and *E. coli* LEE, the genes encoding the apparatus, secreted effectors, and transcriptional regulators are scattered around the chromosome (1, 11, 12). In addition, transcriptional regulators encoded with or proximal to the apparatus genes have also been shown to be required for expression of unlinked effector genes (9, 10, 12, 13). In these situations, the system components are speculated to have been acquired in several independent transfer events and must have incorporated the careful coordination of expression during the evolution of the organism (1).

Synchronous regulation of Ysa T3SS components. Components of two phosphorelay systems, YsrRS and RcsB, are required for expression of *ysaE* as well as several *ysp* genes (37, 38). While the specific mechanism(s) by which they function has yet to be determined, it can safely be concluded that they are requisite factors in the expression of the Ysa T3SS. Activation of the *ysaE* promoter by the sensor YsrS requires elevated levels of NaCl; no expression is observed in the absence of NaCl or in the absence of YsrS (37, 38). The transcript initiated at the *ysaE* promoter likely results in expression of about 18 genes (*ysaE* through *yspA*) (Fig. 1). We previously showed that YsaE and SycB are both required for stimulation of the *sycB* promoter, which leads to upregulation of *sycB* and probably at least some of the downstream *ysp* genes (*yspB* to *yspA*) (38). This body of work sought to determine if any of these known regulators were also involved in the expression of the unlinked *ysp* genes that were recently reported by Matsumoto and Young (23) and Witowski et al. (41). A group of six unlinked *ysp* genes was determined to be regulated by all five regulatory proteins examined, as follows: *yspP*, *yspL*, *yspE*, *yspF*, *yspA*, and *yspM*. In addition, all of the genes required NaCl for activation. Thus, these six genes are all coordinately regulated with the apparatus itself and, as such, should be produced simultaneously. While *sycB* mRNA was determined to require all of these elements, we further showed that the effect of YsrRS on *sycB* mRNA levels was not directly at the *sycB* promoter. This is consistent with previous reports showing that transformation of a Δ*ysrS* strain with plasmids expressing YsaE and SycB resulted in high levels of *sycB* expression (38). Similarly, the *ysp* genes listed above were determined to be indirectly regulated by YsrRS, as their expression was also restored in Δ*ysrS* and Δ*ysrR* strains carrying pYsaE and pSycB. While it has not been tested, we would predict a similar trend in the Δ*rcsB* strain carrying pYsaE and pSycB. These data suggest that the regulation of this group of *ysp* genes is dictated by levels of YsaE/SycB, which presumably form a complex, as was shown for the analogous regulators InvF/SicA and MxiE/IpgC (10, 31). Several promoters regulated by InvF and MxiE have a conserved sequence that appears to be the DNA binding site for these regulators (10, 20, 25). Based on this sequence, we examined the region upstream of genes regulated by YsaE and have identified putative binding sites in four promoter regions (*sycB*, *yspF*, *yspL*, and *yspE*), and a weaker site was identified upstream of *yspP*. No putative YsaE binding site could be found upstream of *yspA*, but it may be transcribed as part of the *sycB-yspBCD* operon. These observations are based purely on promoter gazing and await formal testing. Nonetheless, they support the model that these promoters are directly regulated by YsaE/SycB.

Noncoordinately regulated genes. Three *ysp* genes showed regulatory patterns distinct from those described above. While *yspY* and *yspI* did display growth phase regulation, there appears to be no involvement of YsaE or SycB and only a slight dependence on YsrR and RcsB. However, both genes required YsrS for expression. These results suggest that the regulation by phosphorelay proteins is quite complex and probably involves more than the Ysr and Rcs systems. In addition, neither *yspY* nor *yspI* was strongly dependent on NaCl levels. While there are many models to explain these data, the simplest explanation is that a third response regulator works in concert with YsrS and that YsrS senses a signal other than NaCl (or general ionic strength) and phosphorylates this regulator in response to that signal. The study of *ysa* and *ysp* gene regulation could provide interesting insights into the complexity of phosphorelay regulatory circuits.

The levels of *yspK* mRNA were quite abundant in all experiments. While it displayed growth phase-dependent regulation and required high levels of NaCl, it does not require any of the protein regulators examined in this study. Furthermore, levels of *yspK* mRNA were somewhat elevated in the mutant strains examined (two- to threefold), suggesting that it may actually be slightly repressed by one or more of these factors. We previously observed that *ysaE-lacZ* expression was equally low when measured from wild-type cells grown in L broth (no salt) or from Δ*ysrS* cells grown in either L broth or LB-290 (38). Thus, we predicted that YsrS was sensing NaCl concentrations and initiating a phosphorelay event in response to high levels of NaCl. While this may still be the case for the genes regulated by YsrRS, *yspK* likely derives its salt dependence from a different mechanism. YspK may represent an example of an effector whose expression is such that it is producing protein to be ready for secretion at the first moment that the bacterium meets its target host cell. It has been shown to possess serine/threonine kinase activity and is speculated to play a role in altering the innate immune response (23). If this is indeed its function, it would be consistent with it being immediately available and one of the first proteins secreted through the Ysa T3SS.

An additional point of curiosity regarding the expression of two effectors not coordinately regulated with the apparatus is that *yspI* and *yspK* are nearby neighbors on the chromosome, encoded by open reading frames YE2444 and YE2447, respectively (23). These genes are transcribed in opposite directions and thus are not in an operon. However, their proximity to each other could indicate they were acquired during a single

DNA transfer event. It is tempting to speculate that they were acquired together and are still in the process of developing coordinated regulation; *yspI* has acquired dependence on YsrS and *yspK* has acquired dependence on NaCl.

Regulation of phosphorelay components. The transcriptional expression data reported here suggests that *ysrRS* is not strongly growth phase dependent and not affected by high NaCl levels as are many of the *ysp* genes. Expression of *rcsB* is somewhat growth phase dependent but not regulated by NaCl. In addition, neither YsrR nor RcsB appear to regulate each other, and *ysrRS* is not autoregulated. Venecia and Young reported that expression of *ysrRS* and *rcsB* was dependent on NaCl and that *rcsB* expression was dependent on YsrS (37). A plausible source of these differences is the use of different strains and methodology. Venecia and Young used an *rcsB-lacZYA* fusion generated by transposon mutagenesis in which the transposon insertion disrupts the gene, presumably rendering it nonfunctional. This can be problematic when studying transcriptional regulators because many are autoregulated. In this study, we examined *rcsB* mRNA levels, which can be a more direct measurement of transcriptional activity. In addition, all our mutant strains are constructed as in-frame deletions rather than insertional mutants as utilized by Venecia and Young. However, the observed differences in expression patterns beg for a more detailed analysis. While the regulation of these genes differs, our data agree that YsrRS and RcsB are required for *ysa* gene expression. A more complete set of strains, including deletions of *rcsC* and *rcsD*, and more direct probing of the involved promoters will be required to fully ascertain the regulatory properties of the genes encoding these phosphorelay proteins. Current research in our lab will provide the necessary strains for such an investigation. While the model built by Venecia and Young that implies that regulation of *ysrRS* largely dictates the expression of downstream genes, and ultimately the *ysa* genes, is a logical model based on their extensive experiments (37), our data do not support it. Based on the data we have obtained, we hypothesize that the Ysr and Rcs systems are not part of a regulatory cascade but rather are working together to function in an integrated mechanism to regulate expression of the *ysa* and *ysp* genes. The exact mechanism by which they function in this capacity is the focus of the current investigation.

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