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## Identification of YsrT and Evidence that YsrRST Constitute a Unique Phosphorelay System in *Yersinia enterocolitica*<sup>∇</sup>

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**Two-component systems (TCS) and phosphorelay systems are mechanisms used by bacteria and fungi to quickly adapt to environmental changes to produce proteins necessary for survival in new environments. Bacterial pathogens use TCS and phosphorelay systems to regulate genes necessary to establish infection within their hosts, including type III secretion systems (T3SS). The *Yersinia enterocolitica* ysa T3SS is activated in response to NaCl by YsrS and YsrR, a putative hybrid sensor kinase and a response regulator, respectively. Hybrid TCS consist of a sensor kinase that typically has three well-conserved sites of phosphorylation: autophosphorylation site H1, D1 within a receiver domain, and H2 in the histidine phosphotransferase (HPT) domain. From H2, the phosphoryl group is transferred to D2 on the response regulator. A curious feature of YsrS is that it lacks the terminal HPT domain. We report here the identification of the HPT-containing protein (YsrT) that provides this activity for the Ysr system. YsrT is an 82-residue protein predicted to be cytosolic and  $\alpha$ -helical in nature and is encoded by a gene adjacent to *ysrS*. To demonstrate predicted functions of YsrRST as a phosphorelay system, we introduced alanine substitutions at H1, D1, H2, and D2 and tested the mutant proteins for the ability to activate a *ysaE-lacZ* reporter. As expected, substitutions at H1, H2, and D2 resulted in a loss of activation of *ysaE* expression. This indicates an interruption of normal protein function, most likely from loss of phosphorylation. A similar result was expected for D1; however, an intriguing “constitutive on” phenotype was observed. In addition, the unusual feature of a separate HPT domain led us to compare the sequences surrounding the *ysrS-ysrT* junction in several *Yersinia* strains. In every strain examined, *ysrT* is a separate gene, leading to speculation that there is a functional advantage to YsrT being an independent protein.**

*Yersinia enterocolitica* is a human pathogen that causes several gastrointestinal conditions, with symptoms that include fever, vomiting, and diarrhea. In an otherwise healthy host, the infection is usually self-limiting, lasting 1 to 2 weeks (44). However, individuals with high blood iron levels or with compromised immune systems can develop a systemic *Y. enterocolitica* infection that is often fatal (8). Pigs are a major reservoir of some biotypes of pathogenic *Y. enterocolitica* (28), and while it is often isolated from the tonsils and intestinal tract, *Y. enterocolitica* does not cause obvious disease in swine (10). The bacteria are shed into the environment via feces or can contaminate pork products during processing, both of which can lead to contamination of human food and water supplies. In addition, *Y. enterocolitica* is frequently found during surveys of food-borne pathogens in milk supplies (22). The prevalence of *Y. enterocolitica* in contaminated meat and milk products may be enhanced by its ability to grow at cool temperatures, even as low as 0°C (3).

Based on biochemical properties, *Y. enterocolitica* strains

are subdivided into several biovars. Interestingly, there is a considerable range in the severity of disease caused by the different biovars. Biovar 1B strains are the most highly pathogenic for humans, and 1B is the only biovar that is lethal in a mouse model of yersiniosis (37, 38, and references therein). Not surprisingly, these isolates contain virulence factors not found in other biovars (19, 41). Among these is the chromosomally encoded Ysa type III secretion system (T3SS). In mouse infection studies, deletion of apparatus genes (*ysa*) or individual effector genes (*ysp*) results in a modest attenuation only at 24 h postinfection (27, 45) and a 10-fold increase in the 50% lethal dose, both following oral inoculation (17). Thus, the role of the Ysa T3SS in pathogenicity is subtle in the mouse model, leading to speculation that the secreted effectors (called Ysps) are important for gastroenteritis but not for systemic infection (45). *In vitro*, secretion of Ysps through the Ysa T3SS apparatus is only detected when the bacteria are cultured at 26°C and in the presence of a high concentration of salt (e.g., NaCl) (17, 50). Subsequent studies provided evidence that the genes encoding both the apparatus and effector proteins are up-regulated by growth in a high concentration of salt, indicating that the salt dependence is at the level of transcription (45–47).

As *Y. enterocolitica* exists as both a commensal and a pathogenic organism and has the capacity to grow in a wide range of temperatures, the expression of many genes must

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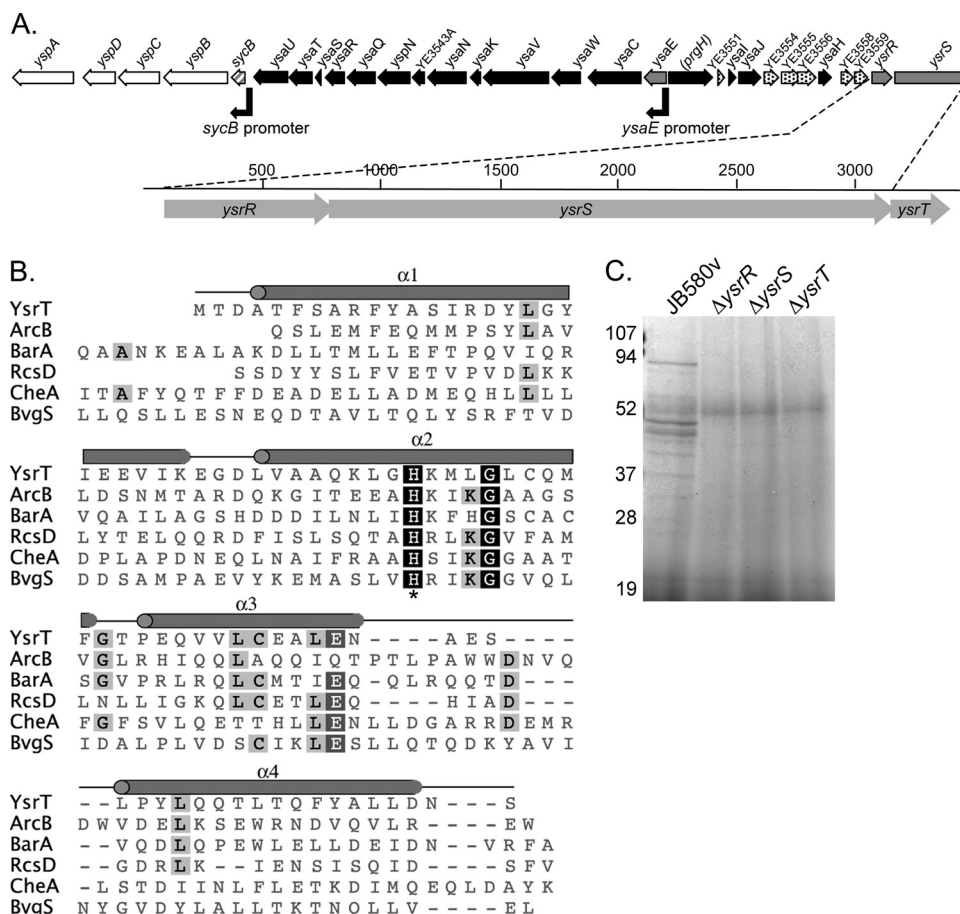


FIG. 1. Identification of YsrT. (A) Genomic organization of the *ysa* locus, zooming in on the *ysrRST* operon. Black boxes, apparatus genes; white boxes, secreted proteins; gray boxes, transcriptional regulators; hashed, chaperone/regulator; speckled, unknown. (B) Alignment of YsrT (YE3561a) with defined HPT domains. These domains were defined in other organisms, and the sequences given here are for the *Y. enterocolitica* homologs (accession numbers are given in Materials and Methods). The predicted  $\alpha$ -helices are designated with gray barrels, and the conserved histidine is marked with an asterisk. (C) Secretion of Ysps into culture supernatants at 26°C. Proteins were prepared as described in Materials and Methods. Supernatants equivalent to an OD of 2.5 were separated by SDS-PAGE and stained with Coomassie blue. Values to the left are the sizes of protein standards in kDa.

be tightly regulated to ensure survival under these often stressful conditions. One mechanism widely used by prokaryotes to respond to changes in their environment is signal transduction via two-component systems (TCS) (49). In the canonical TCS, a membrane-bound sensor histidine kinase (HK) autophosphorylates at a conserved histidine (His) residue in response to an environmental cue. The phosphoryl group is then transferred to a conserved aspartate (Asp) residue in the receiver domain of a response regulator (RR) protein. RR proteins also contain an effector domain, which is often a DNA-binding moiety; phosphorylation of the receiver domain activates the effector domain to bind DNA and alter gene transcription (13). A common variation of the canonical TCS is a phosphorelay (2). Phosphorelays involve two or more proteins and three phosphoryl group transfer events (His $\rightarrow$ Asp $\rightarrow$ His $\rightarrow$ Asp). In one subclass of the phosphorelay group, a hybrid sensor kinase contains not only an HK domain but also receiver (REC) and histidine phosphotransferase (HPT) domains, and the first two phosphotransfer events are within the sensor protein. The final phospho-

transfer event is to the RR protein. Additionally, variations on this theme have been reported where three or four proteins are required to provide the domains necessary for the phosphorelay (5, 12, 36, 39).

Studies conducted in our laboratory and others showed that the putative TCS proteins YsrR and YsrS are required for expression of the *ysa* apparatus genes and specifically of the *ysaE* promoter (Fig. 1A) (30, 45–47). Deletion of either gene results in a lack of NaCl-induced activation of the *ysa* genes. Furthermore, the level of *ysaE* expression in a  $\Delta$ ysrS mutant strain is the same as that in a wild-type strain grown in the absence of NaCl, thus implying that YsrS responds to the NaCl (46). One conundrum of this model is that YsrS appears to belong to the hybrid class of sensor kinases but lacks the terminal HPT domain presumably required to transfer the phosphate to YsrR. In this study, we report the identification of a gene encoding this HPT function and demonstrate genetically that the Ysr proteins do indeed conduct a phosphorelay cascade that leads to activation of the *ysaE* promoter and, hence, the *ysa* and *ysp* genes.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype	Source or reference
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoP recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> )	Invitrogen
S17-1 $\lambda$ <i>pir</i>	Tp <sup>r</sup> Str <sup>r</sup> <i>recA thi pro hsdR hsdM</i> <sup>+</sup> RP4::2-Tc::Mu::Km Tn7 $\lambda$ <i>pir</i> lysogen	33
<i>Y. enterocolitica</i>		
JB580v	8081v (r <sup>-</sup> m <sup>+</sup> Nal <sup>r</sup> ) serotype O:8	24
YVM925	JB580v <i>ysaE-lacZYA</i>	46
YVM1320	JB580v $\Delta$ <i>ysrS ysaE-lacZYA</i>	This work
YVM1323	JB580v $\Delta$ <i>ysrT ysaE-lacZYA</i>	This work
YVM1250	JB580v $\Delta$ <i>ysrR ysaE-lacZYA</i>	This work
YVM1239	JB580v $\Delta$ <i>rcsB ysaE-lacZYA</i>	This work
YVM1378	JB580v <i>ysrT-H38A ysaE-lacZYA</i>	This work
YVM1419	JB580v <i>ysrR-D75A ysaE-lacZYA</i>	This work
2440-87	<i>Y. enterocolitica</i> biovar 1B serotype O:8	32
9286-78	<i>Y. enterocolitica</i> biovar 1B serotype O:20	32
9287-78	<i>Y. enterocolitica</i> biovar 1B serotype O:20	32
634-83	<i>Y. enterocolitica</i> biovar 1B serotype O:4,32	32
655-83	<i>Y. enterocolitica</i> biovar 1B serotype O:18	32
657-83	<i>Y. enterocolitica</i> biovar 1B serotype O:20	32
658-83	<i>Y. enterocolitica</i> biovar 1B serotype O:21	32
Y295	<i>Y. enterocolitica</i> biovar 1B serotype O:8	32
Plasmids		
pSR47S	Kan <sup>r</sup> MobRP4 <i>oriR6K</i> cloning vector	29
pWKS130	Kan <sup>r</sup> low-copy-number cloning vector	48
pKW62	Kan <sup>r</sup> ; <i>ysrT</i> in-frame deletion (codons 8–78 deleted) in pSR47S	This work
pKW77	Kan <sup>r</sup> <i>ysrS</i> in-frame deletion (codons 28–768 deleted) in pSR47S	This work
pKW31/pYsr <sub>WT</sub>	Kan <sup>r</sup> <i>ysrS</i> coding sequence in pWKS130	46
pKW79/pYsr <sub>H320A</sub>	Kan <sup>r</sup> pKW31 with H320A substitution	This work
pKW80/pYsr <sub>D714A</sub>	Kan <sup>r</sup> pKW31 with D714A substitution	This work
pKW63/pYsr <sub>WT</sub>	Kan <sup>r</sup> <i>ysrT</i> coding sequence in pWKS130	This work
pKW76/pYsr <sub>H38A</sub>	Kan <sup>r</sup> pKW63 with H38A substitution	This work
pKW24/pYsr <sub>WT</sub>	Kan <sup>r</sup> <i>ysrR</i> coding sequence in pWKS130	46
pKW72/pYsr <sub>D75A</sub>	Kan <sup>r</sup> pKW24 with D75A substitution	This work
pKW78	Kan <sup>r</sup> <i>ysrT-H38A</i> in pSR47S	This work
pKW75	Kan <sup>r</sup> <i>ysrR-D75A</i> in pSR47S	This work

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The 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 (1% tryptone, 0.5% yeast extract, 170 mM NaCl; Difco) at 37°C. Cultures of *Y. enterocolitica* were grown at 26°C in LB, L broth (1% tryptone, 0.5% yeast extract, 0 mM NaCl), or L broth with 290 mM NaCl (referred to as LB-290). For preparation of secreted proteins, brain heart infusion (BHI; Difco) and BHI with 490 mM NaCl were used. Antibiotics were added as needed at the following concentrations: kanamycin, 100  $\mu$ g/ml; nalidixic acid, 20  $\mu$ g/ml; chloramphenicol, 12.5  $\mu$ g/ml.

**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. All of the plasmids made were confirmed by restriction digest patterns and sequencing.

In-frame deletions were constructed as described previously (46). Briefly, for *ysrS*, fragments of approximately 500 bp upstream and downstream were independently amplified using primers *ysrS*-delA2/delB2 (upstream) or *ysrS*-delC2/delD2 (downstream). These fragments were digested with SalI and BamHI (upstream) or BamHI and NotI (downstream), ligated into pSR47S cut with SalI and NotI, and transformed into S17-1  $\lambda$  *pir*. The resulting plasmid, pKW77, was introduced into the desired *Y. enterocolitica* strains by conjugation. Following counterselection, confirmation of the deleted gene was determined by diagnostic PCR using at least one primer outside the region cloned in pKW77. For *ysrT*, the same procedure was followed; the primer pairs used were *ysrT*-delA/delB and *ysrT*-delC/D, and the resulting plasmid was pKW62.

**Complementing clone for *ysrT*.** The complementing clone for *ysrT* was constructed by amplifying the gene with some additional upstream sequence to include the native ribosome binding site using primers KW197 and KW198. The resulting 300-bp product was digested with SalI and BamHI and ligated into those sites of pWKS130 to generate pYsr<sub>WT</sub> (pKW63).

**Point mutations made by overlap extension for *ysrS*.** Point mutations were introduced into the *ysrS* gene using overlap extension PCR (18). Overlapping forward and reverse primers were designed with the desired mutation. These primers were used in two separate PCRs with appropriate cognate primers. The two products were gel purified and used as template DNA for PCR with the flanking forward and reverse primers to amplify the intact full-length product. The product was digested with SalI and NotI and cloned into those sites of pWKS130 to yield pYsr<sub>H320A</sub> (pKW79) and pYsr<sub>D714A</sub> (pKW80). These plasmids are identical to pYsr<sub>WT</sub>, except that they contain the alanine substitutions. The primer pairs used were as follows. For H320A, the primers used to introduce the point mutation were H320A-R and H320A-F, used with cognate primers *ysrS*-OE F1 and *ysrS*-RP3, respectively. For D714A, the primers used to introduce the point mutation were D714A-R and D714A-F, used with cognate primers *ysrS*-OE F1 and *ysrS*-RP3, respectively.

**Point mutations by megaprimer PCR for *ysrT* and *ysrR*.** To generate an alanine substitution for His38 in *ysrT*, primer KW199 (containing the mutation) was used with KW198 to amplify an ~150-bp product that was gel purified and used as a reverse megaprimer in a second PCR with forward primer KW197. The resulting 300-bp fragment was digested with SalI and BamHI and ligated into those sites of pWKS130 to generate pYsr<sub>H38A</sub> (pKW76). A second construct was made for generation of the chromosomal copy of *ysrT*-H38A in a similar fashion. Primer KW199 was used with *ysrT*-delD to make a 370-bp megaprimer that was subsequently used in a second PCR with *ysrT*-delA. This product was digested with SalI and NotI and ligated into those sites of pSR47S to generate pKW78. The D75A mutation in *ysrR* was similarly constructed. Primers M13R and D75A-F were used with pYsr<sub>WT</sub> as template DNA to generate a megaprimer that was subsequently used with *ysrR*-delA to amplify the full coding sequence. This product was digested with SalI and KpnI and ligated into those same sites of pWKS130 to generate pYsr<sub>D75A</sub> (pKW72). To generate a chro-

TABLE 2. Primers used in this work

Name	Sequence <sup>a</sup> (5'→3')
ysrT-delA	ACGCGTCGACGCCCTATTACTCTGCCGCTG
ysrT-delB	CGGGATCCCTGAAGGTGGCATCAGTCATGTTTC
ysrT-delC	CGGGATCCCTAGATAACAGCTAAATAAATAGCCCCGGC
ysrT-delD	ATAAGAATGCGGCCGCCATAAAAACCGAAATAGTGGCCC
ysrS-delA2	GCGTCGACCTCGTAGCGGGTGAACAGATGAG
ysrS-delB2	CGGGATCCCTCGGCAGCATAAACAGCCGTCG
ysrS-delC2	CGGGATCCCGGTTGCACGCGAACAAATGGCAG
ysrS-delD2	ATAAGAATGCGGCCGCCCTAAATGTATCTCGAACCTGTAGG
KW197	GCGTCGACCAATGGCAGCAATTGATACG
KW198	CGGGATCCCTAGCTGTTATCTAGCAAGGC
KW199	GAAGCTCGGGCTAAAATGCTGGG
KW209	GCGTCGACGGCGTCGGGTTAATCAAGCGC
KW210	ATAAGAATGCGGCCGCCCTCTGGCAATTGTTAATGCAGAG
D75A-R	CGCTACGAGGAATATG <b>CC</b> CAGTAATAGTACTTCAATCTCAGGGTTC
D75A-F	GTACTATTACTGGC <b>AT</b> TATCCCTCGTAGCGGGTGAACAGATGAGAACCAG
H320A-R	GCGCAATTCGGCCGCAATAACCGACAGCTCCAGATTGTTTTGAACCC
H320A-F	GTTATTGCGGGCCGAATTGCGCACGCCGCTGATTGGTATTTTAACC
D714A-R	AAGCCGACAGGGCGTCCACAATAATAGTATGGTGGTGTGTAACCAGC
D714A-F	ATTATTGTGACCGCTGTCGGCTTGATGAGAGCGCAGCGCTTTGAAC
YsrS OE-F1 <sup>b</sup>	GCGTCGACGGCTTACTTCAACACTGATTC
YsrS RP3 <sup>b</sup>	ATAAGAATGCGGCCGCCCTCAGTCATGTTCTTTTTCCTTAG
YsrR delA <sup>b</sup>	ACGCGTCGACGCAGGATAATCCGATGAAATCTCG
YsrR delD <sup>b</sup>	ATAAGAATGCGGCCGCCCTTGGTAAACCCTCAATCAGCG

<sup>a</sup> Restriction sites are underlined (six- to eight-base sequences). In primers used to introduce alanine substitutions, the mutated codons are italicized and the bases that were substituted to code for alanine are bolded.

<sup>b</sup> Published elsewhere (46) and included here for completeness.

mosomal copy of *ysrR*-D75A, the megaprimer was synthesized using genomic DNA for the template and primers *ysrR*-delA and D75A-R. The full-length product was amplified using the megaprimer and *ysrR*-delD. This product was digested with SalI and NotI and cloned into those same sites of pSR47S to generate pKW75.

**Introduction of chromosomal point mutations for *ysrR* and *ysrT*.** Chromosomal copies of genes encoding point mutations were constructed using the same methods employed to delete genes. Plasmids pKW78 (*ysrT*) and pKW75 (*ysrR*) were conjugated into the *Y. enterocolitica* strain bearing a deletion of the targeted gene. Following counterselection, individual colonies were screened by PCR for the presence of the full-length gene. The regions surrounding the engineered point mutations were amplified and sequenced to verify that the desired strain had been constructed without errors. The resulting strains are referred to as *ysrT*-H38A (YVM1378) and *ysrR*-D75A (YVM1419).

**β-Galactosidase assays.** Saturated cultures grown overnight in L broth were diluted into fresh L broth or LB-290 to an initial optical density at 600 nm (OD<sub>600</sub>) of 0.2 and grown for 2 h at 26°C with aeration. Antibiotics were added as necessary to retain plasmids and chromosomal integrations. Assays were performed as described previously (31). Individual assays were conducted with at least three independent cultures for each strain tested, and the assays were repeated at least three times to ensure reproducibility. Representative assays are shown.

**Preparation of secreted proteins.** Proteins secreted into culture supernatants were collected essentially as described previously (46), except that the base growth medium was BHI or BHI with 490 mM NaCl. Briefly, overnight cultures grown in LB at 26°C were subcultured to an OD of 0.2 into BHI or BHI-490 mM NaCl and grown for 5 to 6 h at 26°C on a roller drum. The cells were pelleted and discarded. Supernatants were filtered and precipitated with 10% trichloroacetic acid at 4°C overnight. Protein pellets were resuspended in sample buffer at a concentration of 1 OD equivalent per 10 μl. Approximately 2.5 OD equivalents was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue.

**DNA sequencing and analysis.** All DNA sequencing was performed by Eton Bioscience, Inc., at their North Carolina facility. For sequencing of the *ysrS*-*ysrT* region of the biovar 1B isolates (listed in Table 1), genomic DNA was prepared and used as templates for PCR using primers *ysrS*-delC and *ysrT*-delD. The PCR products were gel purified and sequenced with primer *ysrS*-delC2. The sequences for *Y. mollaretii* (ATCC 43969, accession no. AALD00000000) (6), *Y. aldovae* (ATCC 35236, accession no. ACCB00000000) (6), JB580v (*Y. enterocolitica* 8081, accession no. NC008800) (41), and *Y. enterocolitica* WA-C (WA-314, accession no. AJ344214) (21) were obtained from the NCBI repository. Alignments were

conducted using the CLUSTAL W algorithm (40) in the Geneious Pro version 5 software package (BioMatters, Ltd., New Zealand).

**Secondary structural analysis and modeling.** Analysis of the secondary structure prediction for YsrT was performed using the Phyre server (<http://www.sbg.bio.ic.ac.uk/phyre>) (23). The sequences for the designated HPT-containing proteins in *Y. enterocolitica* were obtained using the following accession numbers: ArcB (YE3733), YP\_001007887.1; BarA (YE0742), YP\_001005086.1; ResD (YE1398), YP\_001005711.1; CheA (YE2577), YP\_001006780.1; BvgS (YE2663), YP\_001008383.1. The residues that make up the HPT domain were obtained from the MiST database (43), and the alignment was generated using CLUSTAL W as described above. The YsrS receiver domain was modeled using SWISS-MODEL (16) and generated using PyMOL (The PyMOL Molecular Graphics System, version 1.2; Schrödinger, LLC). Domain sequences from YsrR, YsrS, and YsrT were identified with the simple modular architecture research tool (SMART) and used to perform a BLASTp search (1). A subset of homologous proteins whose domains had been defined either genetically or biochemically were aligned using the CLUSTAL W algorithm (40) in the Geneious Pro version 5 software package (BioMatters, Ltd., New Zealand).

## RESULTS

**Identification of YsrT.** We and others previously demonstrated that YsrS and YsrR are required to activate expression of the *ysaE* promoter (30, 45, 46). While these proteins were predicted to be a hybrid TCS, we noticed that YsrS lacks an HPT domain. Hybrid TCS have four sites of phosphorylation in a His(H1)→Asp(D1)→His(H2)→Asp(D2) phosphorelay. Typically, H1, D1, and H2 are contained on the sensor and D2 is found on the RR but examples exist where this is not the case (49). Initial attempts to identify the protein containing the HPT domain for the Ysr phosphorelay were unsuccessful. All of the genes that could be identified at the time as encoding HPT proteins in *Y. enterocolitica* (YE2577 [*cheA*], YE0724 [*barA*], YE3733 [*arcB*], and YE1398 [*rcsD*]) were inactivated by plasmid insertion within the gene and the resulting mutants were tested for the ability to secrete Ysps into culture supernatants. None of these proteins appeared to be the intermediate be-

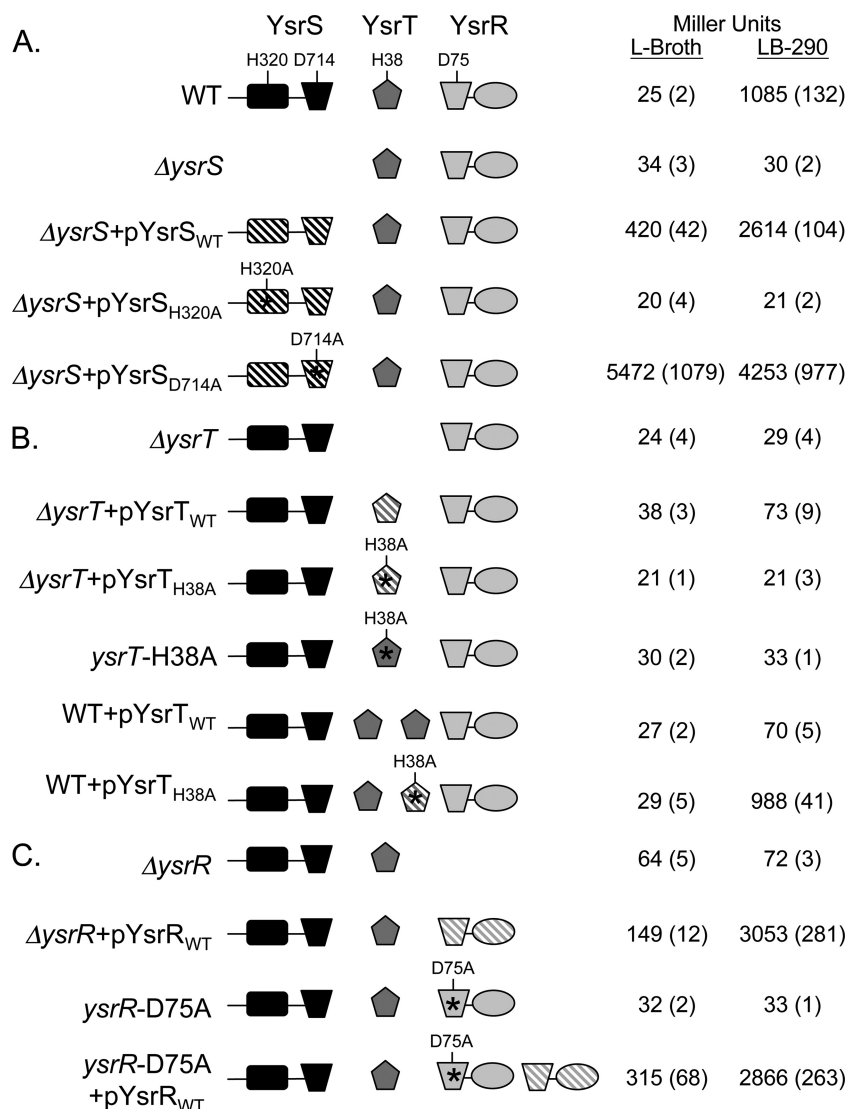


FIG. 2. Genetic analysis of the YsrS-YsrT-YsrR phosphorelay. Assays of  $\beta$ -galactosidase activity in strains harboring a *ysaE-lacZ* reporter were used to evaluate the role of each protein. Saturated cultures grown in L broth were subcultured into fresh L broth (noninducing) or LB-290 (inducing) and grown for 2 h at 26°C on a roller drum. Each protein is represented in cartoon form, with a different shape for each domain type. Solid, gene is single copy (chromosomal); hatched, gene is carried on a multicopy plasmid. Point mutations are indicated above the respective domains. The relevant genotype is given on the left, and the relative promoter activity is given on the right in Miller units with the standard deviation in parentheses. The strains and plasmids used to evaluate (A) YsrS, (B) YsrT, and (C) YsrR are listed in Table 1. WT, wild type.

tween YsrS and YsrR, as Ysp secretion was unaffected by these mutations (data not shown). Upon release of the annotated *Y. enterocolitica* genome, we noticed that a small open reading frame (ORF), designated YE3561a, was located adjacent to *ysrS* (Fig. 1A). BLASTp results revealed that this hypothetical protein had homology to HPT domains, and this is supported by aligning YE3561a with all of the known HPT domains found in *Y. enterocolitica* (Fig. 1B). YE3561a encodes an 82-amino-acid protein that contains a single histidine residue (H38) located within a short stretch of somewhat conserved residues found in HPT domains (13). Protein structure prediction using the Phyre server (23) predicted a high helical content for YE3561a, with the conserved H located on an exposed region of an alpha helix. These findings are consistent with the notion that YE3561a serves as an HPT protein.

To investigate if YE3561a encodes a protein important for the function of the *Ysa* T3SS, we constructed an in-frame deletion of this gene and tested the resulting strain for the presence of Ysps in culture supernatants. We found that no secreted proteins could be detected from cultures grown under inducing conditions when YE3561a was deleted (Fig. 1C). This result is the same as that observed in strains lacking *ysrR* and *ysrS* (45–47). We further tested this mutant in a strain carrying a chromosomal *ysaE-lacZ* reporter and found that *ysaE* expression was not activated when the bacteria were grown under inducing conditions. These data are described in detail in the following sections and shown in Fig. 2. Because of the critical role in *ysa* expression and its putative function as part of a phosphorelay, we have renamed YE3561a *ysrT* and refer to it as such here.

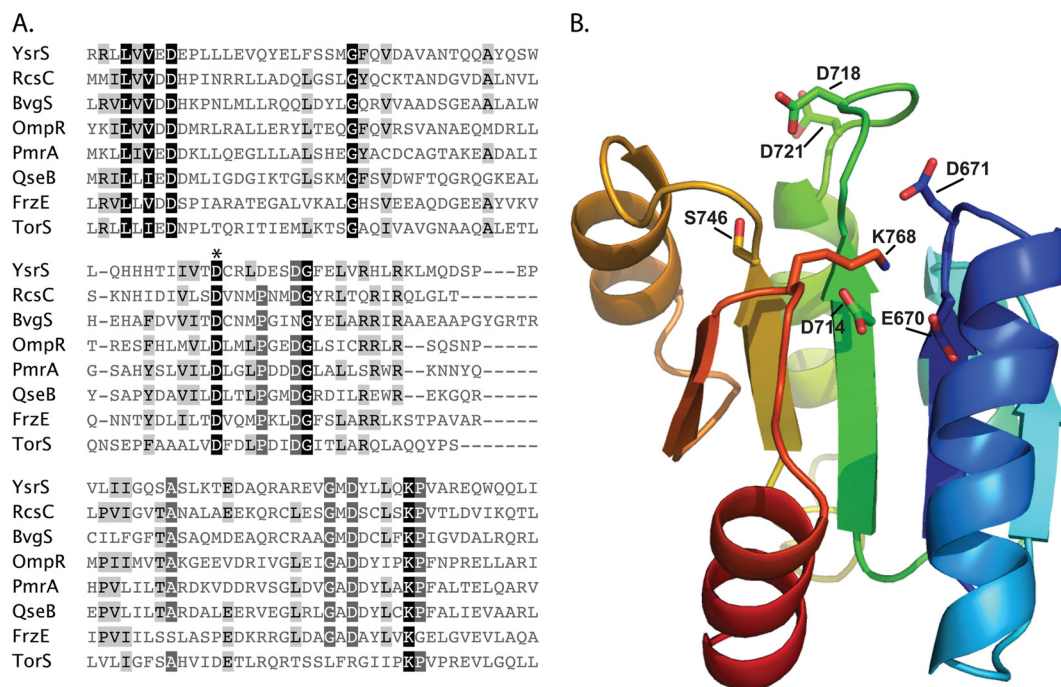


FIG. 3. Analysis of domains and critical residues in the YsrS receiver domain. (A) Sequences of selected REC domains are aligned with the predicted REC domain of YsrS. Conserved residues are shaded such that the darker boxes are more highly conserved. The phosphorylation site (D1) is designated with an asterisk (D714 for YsrS). The accession numbers for the sequences used are as follows: BvgS of *Bordetella bronchiseptica*, P26762; FrzE of *Myxococcus xanthus*, P18769; OmpR of *E. coli*, P0AA16; PmrA of *Pectobacterium carotovorum*, Q70FH0; QseB of *E. coli*, Q8XBS3; RcsC of *E. coli*, P14276; TorS of *E. coli*, P39453; YsrS, A1JQC5. (B) Model of the YsrS REC domain generated using SWISS-MODEL and edited using PyMOL. Highly conserved residues important for phosphorylation, as well as unusual residue D718, are shown as sticks.

**YsrS, YsrT, and YsrR are phosphorelay proteins.** With all of the components of a hybrid two-component system identified and shown to be requisite factors in transcriptional activation of the *ysaE* promoter, we wanted to determine if YsrSTR were indeed functioning as phosphorelay proteins. To this end, alanine substitutions were made at the histidine and aspartate residues predicted to participate in the phosphorelay. These residues were chosen based on alignments with other phosphorelay proteins in which the key residues had been defined (Fig. 1B; see also Fig. 3 and 4) (9). Strains carrying either plasmid or chromosomal copies of the mutated genes were used to evaluate their ability to activate *ysaE* expression.

**Analysis of YsrS.** In the wild-type strain (YVM925), *ysaE-lacZ* expression levels are very low in the absence of NaCl (L broth), resulting in about 25 Miller units (MU). In the presence of 290 mM NaCl (LB-290), *ysaE-lacZ* expression levels reach about 1,085 MU, a 43-fold activation (Fig. 2A). Consistent with previous observations with different *ysrS* mutant strains, no activation of *ysaE-lacZ* is observed in LB-290 in the  $\Delta$ *ysrS* mutant strain (YVM1320), indicating that YsrS is required for the salt-dependent activation of *ysaE* expression (45–47). This phenotype can be complemented by expressing *ysrS* on a low-copy-number plasmid (pYsrS<sub>WT</sub>), resulting in 420 MU in L broth and 2,614 MU in LB-290 (Fig. 2A). The elevated levels are likely the consequence of the multicopy plasmid. Plasmid pYsrS<sub>H320A</sub> carries *ysrS* with an alanine substitution at H320, the predicted site of autophosphorylation (H1). When pYsrS<sub>H320A</sub> is transformed into YVM1320 ( $\Delta$ *ysrS*), *ysaE* expression is the same as in a strain with no

plasmid or the vector only, with about 20 MU in L broth or LB-290 (Fig. 2A). This indicates that H320 is a critical residue for the function of YsrS in its role in activating *ysaE* expression in response to NaCl. A similar result was expected with pYsrS<sub>D714A</sub>; D714 is the predicted site of phosphorylation in the receiver domain of YsrS (D1) (Fig. 3A). However, a very different result was observed. This mutation caused levels of *ysaE* to be very high both in LB-290 and in L broth, with 4,253 and 5,472 MU, respectively (Fig. 2A). These results indicate that both H320 and D714 are required for the normal function of YsrS, but only H320A interferes with the ability of YsrS to activate *ysaE* expression.

The “constitutive on” phenotype from pYsrS<sub>D714A</sub> is seemingly contradictory to the assumption that YsrS is a hybrid sensor kinase, as one would have predicted a loss of function. We had noticed that there are two neighboring aspartate residues: D718 and D721 (Fig. 3). Curiously, the residue located four amino acids from the phosphorylated D is typically a proline; in the case of YsrS, this is D718. One hypothesis to explain the unusual phenotype associated with D714A is that one of these other sites could be the actual site of phosphorylation or could be phosphorylated if D714 were unavailable. To test this notion, a plasmid was generated that contained alanine substitutions at all three aspartate residues. When transformed into the  $\Delta$ *ysrS* mutant strain, this plasmid had the same impact on *ysaE* expression as pYsrS<sub>D714A</sub>: very high expression, independent of the NaCl concentration (data not shown). In addition, the YsrS receiver domain was modeled (Fig. 3B). D714 is located at the end of predicted  $\beta$ -strand 3, as is com-



mon for D1 phosphorylation sites, whereas D718 and D721 are located in the loop between  $\beta$ -strand 3 and  $\alpha$ -helix 3. Other highly conserved residues are in the proper positions, such as magnesium ion coordinating residues E670 and D671, as well as S746 and K768, which are important for signal transduction (4). Taking all of these data together, we conclude that YsrS is indeed a hybrid sensor kinase, that D714 is the D1 phosphorylation site, and that neither of the neighboring aspartate residues can substitute for D714 as a site for phosphorylation.

**Analysis of YsrT.** A similar analysis was conducted with YsrT. As mentioned above, deletion of *ysrT* prevented activation of *ysaE* expression, yielding 24 MU in L broth and 29 MU in LB-290 (Fig. 2B). When wild-type *ysrT* was provided in *trans* on pYsrT<sub>WT</sub>, *ysaE* levels were slightly activated in LB-290, with 73 MU. However, with pYsrT<sub>H38A</sub>, which expresses *ysrT* with an alanine substitution at H38, no activation of *ysaE* expression was observed (21 MU in L broth and LB-290). While the activation with the wild-type plasmid is subtle, the absence of this activation with H38A supports the hypothesis that YsrT functions as an HPT.

The lack of full complementation by pYsrT<sub>WT</sub> could be the result of the HPT domain playing a role in dephosphorylation of YsrR, which is a known function of these domains (14, 35). To test this notion, pYsrT<sub>WT</sub> was transformed into YVM925 (wild type). Expression of *ysaE* was markedly decreased, yielding only 70 MU in LB-290 (Fig. 2B). Transformation of pYsrT<sub>H38A</sub> into YVM925 had no negative impact on *ysaE* levels, which measured 29 and 988 MU in L broth and LB-290, respectively. Taken together, these data imply that YsrT possesses both kinase and phosphatase activities and that H38 is critical for both activities.

Because of the concerns of plasmid copy number, we constructed a strain with the *ysrT*-H38A mutation on the chromosome, designated *ysrT*-H38A (YVM1378).  $\beta$ -Galactosidase activity from the *ysaE* reporter in this strain is similarly low as with the  $\Delta$ *ysrT* mutant strain, further supporting the idea that H38 is required for YsrT to function properly (Fig. 2B). As was also observed with the  $\Delta$ *ysrT* mutant strain, addition of pYsrT<sub>WT</sub> to *ysrT*-H38A results in about a 2-fold increase in *ysaE* expression (not shown).

**Analysis of YsrR.** YsrR has two key domains, a C-terminal DNA-binding domain of the LuxR family of helix-turn-helix proteins and an N-terminal receiver domain. Compared to other RRs, YsrR appears to be somewhat unusual in that it has two stretches of sequence not found in other RR proteins (Fig. 4). Secondary structural analysis predicts that these regions of 15 and 12 amino acids are  $\alpha$ -helical and indicates that they form additional helices. It is possible, however, that they may form extensions of conserved helix 1 and helix 3, respectively. The receiver domain contains the probable site of phosphorylation (D2), which was predicted to be D75 in YsrR by alignment with other RRs (Fig. 4). In the  $\Delta$ *ysrR* mutant strain (YVM1250), the activity of *ysaE-lacZ* is very low, with 64 and 72 MU in L broth and LB-290, respectively (Fig. 2C). The  $\Delta$ *ysrR* deletion can be complemented by expressing *ysrR* in *trans* (pYsrR<sub>WT</sub>), resulting in 149 and 3,053 MU in L broth and LB-290, respectively. When the  $\Delta$ *ysrR* mutant strain is transformed with pYsrR<sub>D75A</sub>, the activation of *ysaE* in LB-290 is markedly less than with pYsrR<sub>WT</sub>, producing 733 MU in LB-

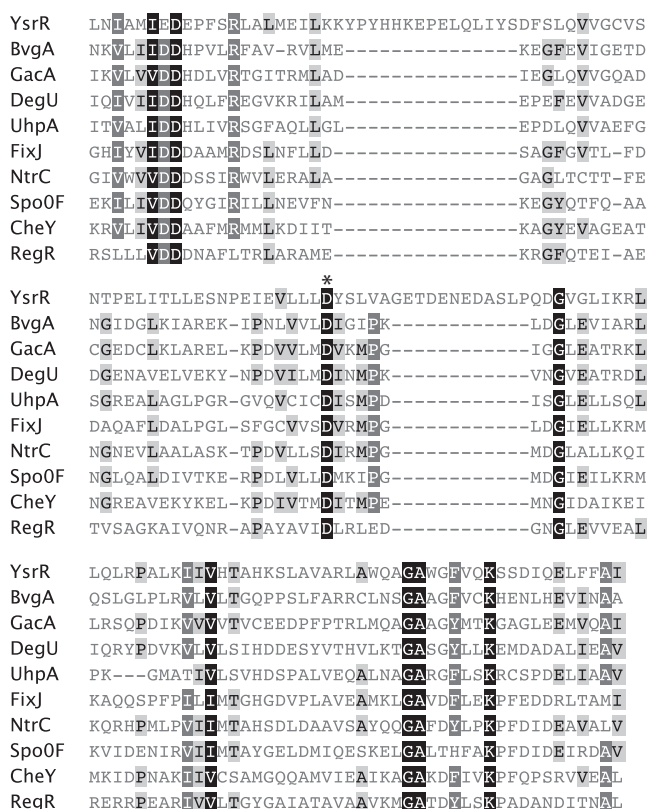


FIG. 4. Analysis of the YsrR receiver domain. Sequences of selected REC domains are aligned with the predicted REC domain of YsrR. Conserved residues are shaded such that the darker boxes are more highly conserved. The phosphorylation site (D2) is designated with an asterisk (D75 for YsrR). The accession numbers for the sequences used are as follows: BvgA of *Bordetella pertussis*, P0A4H2; CheY of *Thermotoga maritima*, Q56312; DegU of *Brevibacillus brevis*, P54662; FixJ of *Bradyrhizobium japonicum*, P23221; GacA of *Pseudomonas aeruginosa*, Q51373; NtrC of *Salmonella typhimurium*, P41789; RegR of *Rhodobacter capsulatus*, P42508; SpoOF of *Bacillus subtilis*, P06628; UhpA of *Salmonella typhimurium*, P27667; YsrR, A1JQC4.

290 (data not shown). This suggests that D75 is important for activation of *ysaE* expression.

As with *ysrT*, there were concerns of pleiotropic effects resulting from overexpression of transcriptional regulators. We therefore introduced *ysrR* with D75A into the chromosome of YVM1250 to generate a single copy of the mutant gene, designated *ysrR*-D75A (YVM1419). The levels of *ysaE* expression in this strain were measured at 32 and 33 MU in L broth and LB-290, respectively. Transformation of this strain with pYsrR<sub>WT</sub> restores *ysaE* promoter activity, yielding 315 and 2,866 MU in L broth and LB-290, respectively. This level of activity is similar to that seen when the  $\Delta$ *ysrR* mutant strain was complemented. The inability of the *ysrR*-D75A mutant strain to show activation of *ysaE* under inducing conditions supports the hypotheses that this protein is an RR and that phosphorylation of D75 is critical to its function as a transcriptional regulator.

**Effect of pYsrS<sub>D714A</sub> requires all other phosphorelay proteins.** The curious result of high *ysaE-lacZ* levels in the strain overexpressing pYsrS<sub>D714A</sub> (Fig. 2A) begs for further analysis. There are reports in the literature of a hybrid two-component system that has a phosphorylation event from H1 to D2, by-

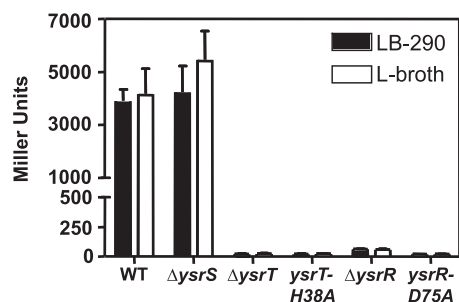


FIG. 5. The peculiar phenotype produced by pYsr<sub>D714A</sub> does not bypass any step of the phosphorelay. pYsr<sub>D714A</sub> was transformed into the specified strain and evaluated for the ability to activate *ysaE* expression, as measured by  $\beta$ -galactosidase activity. WT, wild type.

passing the D1 and H2 residues (15, 42). Although this was subsequently found to occur only when the respective proteins were overexpressed (25), we wanted to address if YsrS<sub>D714A</sub> acts independently of YsrT. The  $\Delta$ ysrT mutant strain was transformed with pYsr<sub>D714A</sub>, and *ysaE-lacZ* levels in this strain were the same as with the vector, indicating that YsrT is required for the constitutive on phenotype of pYsr<sub>D714A</sub> (Fig. 5). To determine if YsrR is also required and if phosphorylation of the conserved residues is an important part of this phenotype, we transformed pYsr<sub>D714A</sub> into the *ysrT*-H38A,  $\Delta$ ysrR, and *ysrR*-D75A mutant strains (Fig. 5). We observed that YsrT and YsrR must be present and phosphorylatable. If either gene is deleted or if the phosphorylation sites are mutated, *ysaE* levels are the same as the background (~25 to 60 MU). These results indicate that the peculiar phenotype observed with pYsr<sub>D714A</sub> requires wild-type copies of YsrT and YsrR, indicating that no step in the phosphorelay is bypassed.

#### The genetic structure of YsrRST is conserved in yersiniae.

In JB580v, the *ysrT* coding region begins within the *ysrS* coding region such that there is a 7-bp overlap. One theory for YsrT being a separate protein from YsrS is that a frameshift mutation was acquired at some point in our laboratory strain. To address if this is a likely explanation, we examined the genetic arrangement of this region in several *Yersinia* strains. With the knowledge that several *Y. enterocolitica* biotype 1B isolates

contain genes encoding the Ysa T3SS apparatus and effector genes (19, 41), we sequenced the region surrounding *ysrT* to determine if it is a separate gene in these strains. In every strain examined, *ysrT* was a separate gene (Fig. 6). A recent bioinformatic study of some of the nonpathogenic *Yersinia* strains has provided whole genome sequences for *Yersinia mollaretii*, *Yersinia aldovae*, and *Yersinia ruckerii*, among others (6). In these three species, genes with homology to TCS are located adjacent to remnants of what is likely the *ysa* locus. Closer examination revealed that *Y. aldovae* and *Y. mollaretii* have genes with reasonable similarity to *ysrR*, *ysrS*, and *ysrT*, and in these *Yersinia* strains, *ysrT* is encoded as a separate gene (Fig. 6). These results indicate that *ysrT* has been separate from *ysrS* since before the evolutionary divergence of these strains from the progenitor, indicating that there may be a functional purpose to YsrT being an isolated HPT protein.

## DISCUSSION

In this report, we present genetic evidence that the predicted two-component proteins YsrR and YsrS do indeed conduct a phosphorelay and that YsrT encodes a small protein providing the previously unidentified HPT domain that is necessary to shuttle the phosphoryl group from the sensor to the RR. This phosphorelay leads to the activation of genes encoding the Ysa type III secretion apparatus and its effectors. These, however, are not the only genes playing a regulatory role in *ysa* expression, but we believe they are at or near the top of the regulatory cascade. In addition to YsrRST, RcsB is also required for activation of the *ysaE* promoter (45, 47). RcsB is the RR of the RcsCDB phosphorelay system (discussed below), and the mechanism by which it acts at the *ysaE* promoter is the current focus of research in our laboratory. YsaE itself is a transcriptional regulator that acts in conjunction with the chaperone/regulator SycB to stimulate the expression of six of the nine *ysp* genes (46, 47). Thus, YsrRST and RcsB are requisite factors in the transcription of the *ysa* and *ysp* genes.

While YsrR and YsrS have been predicted to conduct a phosphorelay, we sought to demonstrate this phenomenon and to understand how the phosphate was transferred from YsrS to YsrR. The identification of YsrT solved this mystery, estab-

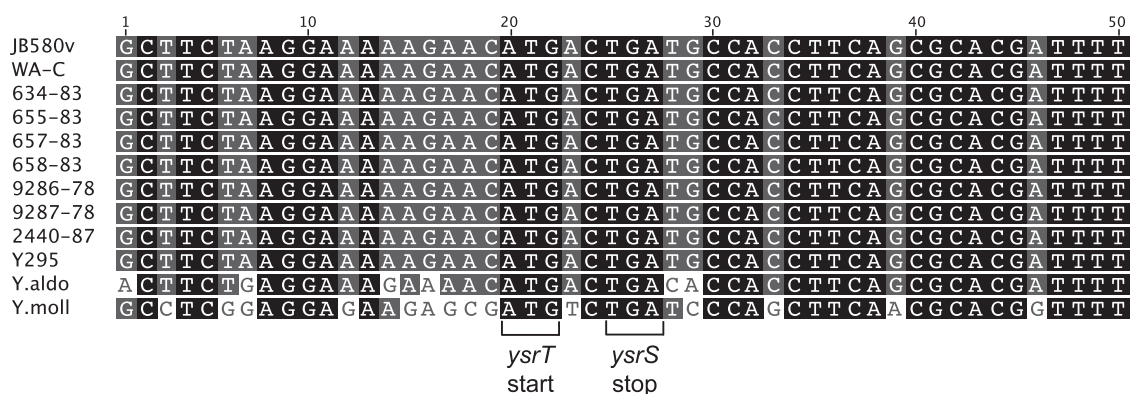


FIG. 6. The genetic structure of the *ysrS-ysrT* region is conserved in multiple *Yersinia* strains. Sequences for *Y. mollaretii*, *Y. aldovae*, and *Y. enterocolitica* strains JB580v and WA-C were obtained from NCBI (accession numbers are given in Materials and Methods), and the others were sequenced from PCR products generated in our laboratory as described in Materials and Methods. Alignments were generated using the CLUSTAL W algorithm in the Geneious Pro software package. The start codon for YsrT and the stop codon for YsrS are indicated by brackets.

lishing that YsrRST contain all of the predicted domains known to be required for phosphorelay. To determine if YsrRST are indeed phosphorelay proteins, we mutated all four predicted phosphorylation sites. Each mutation ablated the normal function of the proteins, and all but YsrS<sub>D714A</sub> (D2) produced the expected phenotype, with an inability to activate *ysaE* expression. While the phenotype produced by the YsrR D56A mutation was exactly as predicted, there are two structural deviations that indicate that the mechanism of how YsrR responds to phosphorylation may be atypical. First, there are two insertions of 12 and 15 amino acids, respectively, and second, a highly conserved proline residue located four residues from the phosphorylation site is absent. Both features may contribute to an altered conformation that could confer a unique function(s).

The constitutive on phenotype of the YsrS<sub>D714A</sub> mutant is not easily understood but is very intriguing. It has been proposed that the evolutionary advantage of hybrid sensors is the increased number of regulatory checkpoints to minimize phosphorylation of the REC under noninducing conditions (13, 20, 34). In addition to kinase activity, some HKs also appear to possess phosphatase activities (7, 14). One plausible explanation is that this mutation may have impaired the phosphatase function of YsrS without impairing the kinase activity. However, one would have to assume that a neighboring residue could substitute for D714 for the kinase activity but not for the phosphatase activity. YsrS has D residues at positions 718 and 721, but these are unlikely to become phosphorylated due to their locations just outside the active site. A curious feature of this region of YsrS is the absence of a highly conserved proline four residues from D714. It is tempting to speculate that the architecture of this loop may be altered and confer an atypical function. We are continuing experimentation with this mutant in an effort to understand this unusual phenotype. However, biochemical analyses often used to complement the genetic experiments and more definitively show transfer of phosphoryl groups have proven to be challenging for YsrS.

Two-component and phosphorelay systems are common mechanisms by which prokaryotes and fungi can rapidly adapt to their environments, and many bacterial strains contain 20 to 30 such systems (MiST2 database; 43). The majority of these systems are composed of two proteins, a sensor kinase and an RR, but in a few systems, multiple proteins are involved in a phosphorelay cascade. The first phosphorelay system described consisted of the sporulation regulators KinABC, Spo0F, Spo0B, and Spo0A of *Bacillus subtilis*, which have each domain encoded in a separate protein (5). More common among phosphorelay systems are the hybrid two-component systems which are composed of two proteins. However, many examples exist where the phosphorelay requires three proteins: a sensor kinase containing the HK (H1) and REC (D1) domains, an HPT (H2) protein, and an RR (D2). One such system is the Rcs system. In this system, RcsC is the sensor, RcsD has the HPT domain, and RcsB is the DNA-binding RR (reviewed in reference 26). In this case, the HPT protein is a large membrane-bound protein that has features suggesting that at one time it may have been a complete hybrid sensor (39). In the *Vibrio harveyi* Lux system, the HPT-containing protein, LuxU, is encoded as a small cytosolic protein (11). Intriguingly, LuxU serves as the HPT for two sensors (LuxN and LuxQ), allowing

cross communication and versatility in these important quorum-sensing regulators. While these two examples are cases of independent HPT domains, this is a relatively rare situation. Examination of the MiST2 database (43) shows that very few organisms have proteins that contain only an HPT domain. *Pseudomonas aeruginosa* PAO1 is a rare example of an organism that has several such free HPT proteins, with four. *Y. enterocolitica* has an average number of HPT-containing proteins (six) compared to other bacteria, and all are part of hybrid HKs. This database does not identify YsrT as an HPT protein (search last performed on 24/6/2010), perhaps because it is such a small ORF. This suggests that there may indeed be more such proteins, but since the similarity between the known HPT domains is weak, they may be difficult to identify *in silico*.

The location of *ysrT* led us to question if a frameshift mutation may have occurred in our strain, resulting in YsrT as a protein independent of YsrS. Sequence analysis indicates that this genetic organization is conserved not only in *Y. enterocolitica* 1B isolates but in more distantly related nonpathogenic *Yersinia* strains. Whole-genome sequencing has recently been performed for several nonpathogenic *Yersinia* strains, and *Y. ruckerii* and *Y. mollaretii* have reasonably well-conserved *ysrR* and *ysrS* sequences and *Y. mollaretii* has a gene that is likely *ysrT* (6). The region downstream of *ysrS* is more divergent in *Y. ruckerii*, and the protein sequence for the ORF downstream of *ysrS* is quite different from YsrT but still has homology to HPT domains (not shown). *Y. aldovae* appears to contain this locus, but the sequence suggests that a number of frameshift mutations have been acquired; the DNA sequence is well conserved, however, and indicates that *ysrT* would have encoded a separate protein. Thus, the conservation of the genetic structure of the *ysrRST* genes among not only the pathogenic 1B isolates but also environmental isolates implies that there is some functional importance to YsrT being produced as a separate protein. However, one should be careful about assuming that the presence of a *ysrRST* locus, as well as the *ysa* and *ysp* genes, will result in secretion of Ysps. Although we have shown here that biotype 1B isolates of *Y. enterocolitica* have *ysrT* and Howard et al. previously demonstrated by microarray analysis that these biotype 1B isolates have the *ysa* and *ysp* genes (19), we have found significant differences in the abilities of these same strains to secrete Ysps under laboratory conditions (K. A. Walker, S. E. Witowski, and V. L. Miller, unpublished results).

This body of research serves to define genetically that the YsrRST system comprises a phosphorelay system and that phosphorylation is a requisite element in the normal function of these proteins in their role as activators of the *ysa* type III secretion genes. Several elements of this system are unusual in that (i) the hybrid sensor lacks an HPT domain, (ii) the HPT domain is provided on the small cytosolic protein YsrT, (iii) *ysrT* is encoded immediately downstream of *ysrS*, and (iv) YsrT is unique to *Y. enterocolitica* and nonpathogenic *Yersinia* species. In addition, YsrR contains stretches of amino acids that appear to be insertions not found in similar RRs, indicating that it may have some unique properties/functions as well. Like many Gram-negative bacteria, *Y. enterocolitica* has about 30 TCS, with 24 HKs, 6 of which are hybrid HKs, and 31 RRs. Of the six hybrid HKs, two are unique to yersiniae: YsrRST and YE3578-YE3579. Curiously, YE3578 and YE3579 are nearly identical to YsrR and YsrS, respectively, on both the amino

acid and DNA levels. Adding to the intrigue of this second system is that there is no Hpt-containing protein associated with YE3578-YE3579. In analogy to the LuxNUO and LusQUO systems, it is tempting to speculate that YsrT could function as the Hpt for both YsrS and YE3579, thus explaining the significance of *ysrT* encoding a protein detached from its cognate sensor. Efforts to elucidate whether YE3578-YE3579 utilizes YsrT for regulating *ysaE* or other promoters are ongoing in our laboratory.

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