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The YsrS Paralog DygS Has the Capacity To Activate Expression of the *Yersinia enterocolitica* Ysa Type III Secretion System

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ABSTRACT

The *Yersinia enterocolitica* Ysa type III secretion system (T3SS) is associated with intracellular survival, and, like other characterized T3SSs, it is tightly controlled. Expression of the *ysa* genes is only detected following growth at low temperatures (26°C) and in high concentrations of sodium chloride (290 mM) in the medium. The YsrSTR phosphorelay (PR) system is required for *ysa* expression and likely responds to NaCl. During our investigations into the Ysr PR system, we discovered that genes YE3578 and YE3579 are remarkably similar to *ysrR* and *ysrS*, respectively, and are probably a consequence of a gene duplication event. The amino acid differences between YE3578 and *ysrR* are primarily clustered into two short regions. The differences between YE3579 and *ysrS* are nearly all located in the periplasmic sensing domain; the cytoplasmic domains are 98% identical. We investigated whether these paralogs were capable of activating *ysa* gene expression. We found that the sensor paralog, named DygS, is capable of compensating for loss of *ysrS*, but the response regulator paralog, DygR, cannot complement a *ysrR* gene deletion. In addition, YsrR, but not DygR, interacts with the histidine phosphorelay protein YsrT. Thus, DygS likely activates *ysa* gene expression in response to a signal other than NaCl and provides an example of a phosphorelay system in which two sensor kinases feed into the same regulatory pathway.

IMPORTANCE

All organisms need mechanisms to promote survival in changing environments. Prokaryotic phosphorelay systems are minimally comprised of a histidine kinase (HK) that senses an extracellular stimulus and a response regulator (RR) but can contain three or more proteins. Through gene duplication, a unique hybrid HK was created. We show that, while the hybrid appears to retain all of the phosphorelay functions, it responds to a different signal than the original. Both HKs transmit the signal to the same RR, which activates a promoter that transcribes a set of genes encoding a type III secretion system (T3SS) whose function is not yet evident. The significance of this work lies in finding that two HKs regulate this T3SS, highlighting its importance.

Yersinia enterocolitica is a foodborne pathogen known to cause a variety of gastrointestinal disorders, ranging from mild to severe (1). Most healthy individuals only experience fever, vomiting, and diarrhea, lasting just a few days. However, in young children and those with weak or compromised immune systems, *Y. enterocolitica* can spread systemically, resulting in a 50% mortality rate (2). In addition, postinfection sequelae can be problematic, with the development of reactive arthritis and thyroid disorders (1). More recently, development of inflammatory bowel disease (IBD) has been linked to gastrointestinal infections. While the number of patients developing IBD following *Y. enterocolitica* infection is comparatively low, the rate is much higher than in patients who were infected with other common enteric pathogens (3, 4). Diagnosis of *Y. enterocolitica* infection is relatively low compared to other gastrointestinal pathogens, and this is largely because the symptoms are often mild enough that patients do not seek medical attention and because detection of *Y. enterocolitica* in clinical samples is challenging (5, 6).

Y. enterocolitica is classified into several biotypes and serotypes that vary in the severity of disease symptoms. Biotype 4, serotype O:3, is one of the most common pathogenic biotypes isolated from humans (7–9). This biotype is the most prevalent in pig samples from European slaughterhouses, and consumption of undercooked pork is a well-known source of *Y. enterocolitica* infection (10, 11). However, the most pathogenic biotype is biotype 1B. *Y. enterocolitica* strains contain a plasticity zone, which is a large chromosomal region that is highly variable among the different biotypes (12). The plasticity zone of biotype 1B strains contains a large number of genes not found in biotypes 2 to 4, nor in *Yersinia pestis* or *Yersinia pseudotuberculosis*, and many of these genes contribute to virulence and broadened metabolic capacities that presumably enhance fitness in a wider variety of environments (12). Encoded within this plasticity zone of 1B isolates is the Ysa type III secretion system (T3SS). This T3SS varies considerably from the well-characterized Ysc/Yop T3SS encoded on the virulence plasmid in function (13, 14), expression (15), and phylogenetic class (16), but the exact role of this system in the *Y. enterocolitica* life cycle is still the subject of investigation. Ysa mutant strains were attenuated in mouse infection studies, but only at early time points postinoculation (17). This early-infection phenotype leads to the notion that the Ysa system is important during

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the gastroenteritis phase of disease, which is not well recapitulated in mouse models. While generally viewed as an extracellular pathogen, the Ysa T3SS is required for intracellular survival in a *Drosophila melanogaster* S2 tissue culture model, suggesting a role for this system during a potential intracellular phase of infection (14). In addition, this T3SS may also provide a survival benefit in a mammalian host environment. During mouse infections, activation of *ysa* expression was evident in intestinal and lymphatic tissue by 48 h postinfection (18). Upregulation of *ysa* genes was also detected from intracellular *Y. enterocolitica* during mouse macrophage tissue culture infection (19). Thus, the Ysa system may promote survival of *Y. enterocolitica* strains that fail to subvert phagocytosis.

While the exact purpose of the Ysa T3SS is still a mystery, several lines of evidence suggest that it is a critical element in the life cycle of this pathogen. First, the apparatus and effector genes occupy over 40 kb of DNA that appear to have been under selective pressure to maintain function. Second, most of the effector genes are unlinked with the apparatus locus and likely were acquired by multiple horizontal transfer events (17). Third, many of the effector genes are coordinately regulated with apparatus gene transcription, and this coordinated regulatory mechanism would likely have evolved after acquisition (20). Finally, transcription of a primary promoter driving expression of the *ysa* genes is tightly regulated by environmental factors (temperature and salt), by a cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP), and by a complex phosphorelay system (13, 15, 21–23). Collectively, these observations indicate that there has been strong selective pressure to maintain a functional Ysa T3SS and to tightly regulate it, such that it is fully available under the necessary conditions and only under those conditions to avoid wasting cellular resources.

The Ysr phosphorelay system is part of the regulatory mechanism leading to expression of the *ysa* genes and is comprised of YsrS, YsrT, and YsrR (20). YsrS is a hybrid-type sensor kinase that contains a histidine kinase domain where autophosphorylation occurs and a receiver domain with an aspartate that gets phosphorylated. YsrT is a small protein that functions as a histidine phosphotransferase, shuttling the phosphoryl group from the receiver of YsrS to the receiver domain of YsrR. YsrR has a DNA-binding domain (DBD) and is a member of the LuxR class of response regulators. During our investigations into the genetics of this phosphorelay system, we discovered that genes encoding paralogs of YsrR and YsrS were located about 10 kb downstream of the *ysrRST* genes. These paralogs, YE3578 and YE3579, share 81% and 87% amino acid identity to YsrR and YsrS, respectively, but there is no YsrT counterpart. Because of the strikingly high similarity to YsrR and YsrS, we investigated whether these paralogs were capable of participating in the transcriptional regulation of the *ysa* operon. We report here that the sensor kinase, named DygS (duplication of *ysr* gene), has the capacity to participate in the phosphorelay, but the response regulator, named DygR, does not. Protein alignments identify specific regions that are quite different between the homologs and provide some insight into the phenotypes observed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1 and described below. *Escherichia coli* strains were cultured in LB (1% tryptone, 0.5% yeast extract, 170 mM NaCl; Difco) at 37°C, except *E. coli* BTH101, which was maintained at either 26°C or 30°C. *Y. enterocolitica* strains were cultured 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). Antibiotics were included as needed at the following concentrations unless otherwise stated: kanamycin (Kan), 100 μg/ml; nalidixic acid (Nal), 20 μg/ml; chloramphenicol (Cam), 12.5 μg/ml; and carbenicillin or ampicillin (Amp), 100 μg/ml.

Plasmid and strain construction. Table 1 lists the plasmids and strains used in this study, and Table 2 lists all of the primers used for PCR. Construction of all strains and plasmids is described in the following sections. Unless stated otherwise, wild-type *Y. enterocolitica* genomic DNA was used as a template for PCR. The *ysaE-lacZ* reporter was introduced into desired *Y. enterocolitica* strains by conjugation with *E. coli* S17-1λ*pir* carrying pKW5, as described previously (22).

(i) In-frame deletions and *dygR-lacZ* fusion. In-frame deletions and the *dygR-lacZ* fusion were constructed as described previously (22). Briefly, for *dygR*, fragments of approximately 500 bp upstream and downstream were independently amplified using primers MWO-019/020 (upstream) and MWO-106/107 (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 S17-1λ*pir*. The resulting plasmid, pMWO-057, was introduced into *Y. enterocolitica* by conjugation. Following counterselection, confirmation of the deleted gene was determined by diagnostic PCR. Each strain was subsequently conjugated with S17-1λ*pir* carrying pKW5, which introduced a chromosomal *ysaE-lacZ* fusion via homologous recombination at the native site (22). In-frame deletion of *dygS* was similarly constructed using the primer pairs MWO-108/109 and MWO-021/022 for plasmid pMWO-056.

The *dygR-lacZ* fusion strain was constructed by amplifying the putative promoter region with primers MWO-056 and MWO-057, digesting with XbaI and BamHI, and ligating into those same sites of pKN8 (30). The resulting plasmid was confirmed by sequencing and then introduced into *Y. enterocolitica* via conjugation with selection on nalidixic acid and chloramphenicol, as described previously (22).

(ii) Complementing clones. The complementing clone for *dygR* (pRPS1/pDygR) was constructed by amplifying the *dygR* region with primers MWO-104 and MWO-105, digesting it with SalI and BamHI, and ligating it into those same sites of pWKS130. pRPS2/pDygS, expressing *dygS*, was similarly constructed using primers MWO-102 and MWO-103. pRPS3/pChimera contains a chimeric protein with the periplasmic and transmembrane regions of YsrS and the cytoplasmic region of DygS in pWKS130. The insert was constructed by overlap PCR. In the first step, primers MWO-110 and MWO-111 were used to amplify the region encoding residues 1 to 294 of YsrS, and primers MWO-112 and MWO-113 were used to amplify the region encoding residues 293 to 785 of DygS. These two products were gel purified and used in a second reaction with MWO-110 and MWO-113. This product was digested with HindIII and PstI and ligated into pWKS130, generating pRPS3. pMWO-034 is a low-copy-number vector containing the *tetR* gene, encoding the TetR repressor, and the *tet* operator, to drive expression of a cloned gene of interest (29). The *ysrS* gene encoding a D-to-A substitution at residue 714 ($ysrS_{D714A}$) was subcloned from pKW80 into pMWO-034, creating pMWO-049/p*i*$YsrS_{D714A}$.

(iii) Constructs for bacterial two-hybrid system. To construct *cya* fusion proteins for the bacterial two-hybrid analysis, standard cloning methods were used. Genes of interest were amplified by PCR, digested, gel purified, and ligated into the desired vectors cut with the same restriction enzymes. Plasmid names and primer pairs are as follows: pAB3 (*ysrS*-T25), KW251 and KW252; pAB2 (*dygS*-T25), KW253 and KW259; pKW143 (*ysrT*-T25), KW263 and KW262; pAB1 (*ysrT*-T18), KW254 and KW255; pKW139 (*dygR*-T25), KW256 and KW265; pKW144 (*dygR*-T18), KW257 and KW258; pKW120 (*ysrR*-T25), KW223 and KW224; and pKW107 (*ysrR*-T18), KW227 and KW228. For *dygS* and *ysrS*, only the region encoding the cytoplasmic domains was cloned. This comprised the

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or description	Reference or source
E. coli		
DH5α	F^- ϕ80*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 Tn*7* λ *pir* (lysogen)	24
BTH101	F^- *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	25
Y. enterocolitica		
JB580v	8081v [(r^- m^+) Nal^r]; serotype O:8	26
YVM925	JB580v *ysaE-lacZYA*	22
YVM1250	JB580v Δ*ysrR ysaE-lacZYA*	22
YVM1320	JB580v Δ*ysrS ysaE-lacZYA*	22
YVM1250	JB580v Δ*ysrT ysaE-lacZYA*	23
YVM1562	JB580v Δ*dygR ysaE-lacZYA*	This work
YVM1559	JB580v Δ*dygS ysaE-lacZYA*	This work
YVM1561	JB580v Δ*ysrS* Δ*dygS ysaE-lacZYA*	This work
YVM1563	JB580v Δ*ysrR* Δ*dygS ysaE-lacZYA*	This work
YVM1429	JB580v *dygR-lacZYA*	This work
Plasmids		
pSR47S	Kan^r; MobRP4 *ori*R6K cloning vector	27
pWKS130	Kan^r; pSC101 *ori* cloning vector	28
pMWO-034	Kan^r; pSC101 *ori* cloning vector; *tetO*	29
pT18	Amp^r; vector for C-terminal fusion to the AC T18 domain	25
pT25	Cm^r; vector for N-terminal fusion to the AC T25 domain	25
pKW5	*ysaE-lacZYA* fusion	22
pMWO-018	*dygR-lacZYA* fusion	This work
pMWO-049	*ysrS* encoding D-to-A substitution at residue 714 in pMWO-034	This work
pMWO-056	*dygS* with in-frame deletion (deletion of codons 4–782) in pSR47S	This work
pMWO-057	*dygR* with in-frame deletion (deletion of codons 5–211) in pSR47S	This work
pRPS2	*dygS* coding sequence in pWKS130	This work
pRPS1	*dygR* coding sequence in pWKS130	This work
pRPS3	*ysrS-dygS* chimera in pWKS130	This work
pKW31	*ysrS* complementing clone in pWKS130	22
pKW80	*ysrS* encoding D-to-A substitution at residue 714 in pWKS130	23
pT18-zip	pT18 with leucine zipper	25
pT25-zip	pT25 with leucine zipper	25
pAB1	pT18 with coding region for YsrT	This work
pAB2	pT25 with coding region for residues 293–785 of DygS	This work
pAB3	pT25 with coding region for residues 295–791 of YsrS	This work
pKW107	pT18 with coding region for YsrR	This work
pKW120	pT25 with coding region for YsrR	This work
pKW139	pT25 with coding region for DygR	This work
pKW143	pT25 with coding region for YsrT	This work
pKW144	pT18 with coding region for DygR	This work

region encoding residues 293 to 785 for *dygS* and 295 to 791 for *ysrS*. All inserts were verified by restriction digestion and sequencing.

Protein alignments. The primary sequences for the Dyg and Ysr proteins were aligned using the BLOSUM62 algorithm provided within the Geneious v. 5.3 software package (31). Percent identity values were taken from the BLOSUM62-generated analysis.

β-Galactosidase assays. Cultures grown overnight to saturation in L-broth were diluted into fresh L-broth or LB-290 to an initial optical density at 600 nm (OD_{600}) 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 (32). For experiments with pMWO-049, expression of $ysrS_{D714A}$ was induced by the addition of 5 ng/μl anhydrotetracycline (ATc) at the time of subculture. Individual assays were conducted with at least three independent cultures for each strain tested, and the assays were repeated at least three times with freshly transformed strains to ensure reproducibility. Representative assays are shown.

Bacterial two-hybrid analysis. Combinations of plasmids, one pT25 and one pT18 derivative, were cotransformed into *E. coli* strain BTH101 and plated onto MacConkey agar supplemented with 1% maltose, 100 μg/ml carbenicillin, and 25 μg/ml chloramphenicol, as described previously (33). Plates were incubated at 30°C for several days, during which the colony color was monitored. After 3 days of incubation, 3 to 5 colonies from each plate were inoculated into LB and grown overnight at 26°C with aeration. These cultures were diluted to an OD_{600} of 0.2 and grown at 37°C for 30 min. Samples were chilled on ice and subjected to β-galactosidase (β-gal) activity, as described above. Transformations and β-gal assays were performed at least 3 times for each plasmid pair; representative assay results are shown.

RESULTS

Response regulator alignment. DNA-binding response regulators are classified based on the type of DNA-binding domain they

TABLE 2 Primers used in this work

Name	Sequence[a] (5′→3′)
MWO-019	CATATCGTCGACCTGATCCGCTGGGGCATTGATGC
MWO-020	CTGTGGATCCCGTTTCTATCATAATTCCGAC
MWO-021	AGACGGATCCGCCTCAGGAAAATAACAACAAG
MWO-022	CAGTAGCGGCCGCCTGTTCAGAATCGGAAAAACC
MWO-056	GGGGAATCTAGAGGGTTATTGCATGTGGTGGCG
MWO-057	GGCAAGTAGATCTGCGCAAGGCGACTGAACGG
MWO-102	GCGTCGACGCGCTTTCAAAAAACTGGGG
MWO-103	CGGAATTCTTATTTTCCTGAGGCGAAATAGTC
MWO-104	GCGTCGACCCCACCACTCCTCAATGCCAC
MWO-105	CGGGATCCGGTTTTATAAAGAAATTTCATGGGC
MWO-106	AGACGGATCCCGCGCTTTCAAAAAACTGGGG
MWO-107	CAGTAGCGGCCGCCGACGGCAGCTTTTGCTCGC
MWO-108	CATATCGTCGACGCTGATTTACAGCGCCTTTTC
MWO-109	CTGTGGATCCTTTATAAAGAAATTTCATGGGCATAATGG
MWO-110	CATATCTAAGCTTGTGCCTTCAAAAAACTGGGG
MWO-111	CCATTTTTAGCTGCCGGTTACGATAGAAAACGAATAACAGCAAGC
MWO-112	GCTTGCTGTTATTCGTTTTCTATCGTAACCGGCAGCTAAAAATGG
MWO-113	CATATCCTGCAGTTATTTTCCTGAGGCGAAATAGTC
KW223	AAGGATCCGATGACACAAACGAAAACGCTCAATATAG
KW224	CGGGTACCTTATAGAGAAATTTCATGAGCATATTTAAAG
KW227	CCGCTCGAGGATGACACAAACGAAAACGCTCAATATAG
KW228	CCCCCAAGCTTCCTAGAGAAATTTCATGAGCATATTT
KW251	AACTGCAGGGTATCGCTACCGGCGATTAAAAATGG
KW252	GGGGTACCTCAGTCATGTTCTTTTTCCTTAG
KW253	AACTGCAGGGTATCGTAACCGGCAGCTAAAAATGGC
KW259	GGGGTACCTTATTTTCCTGAGGCGAAATAGTC
KW254	CCGCTCGAGCATGACTGATGCCACCTTCAGCGCAC
KW255	GCGTCGACGCGCTGTTATCTAGCAAGGCATAAAATTGC
KW263	AACTGCAGGGATGACTGATGCCACCTTCAGCGCAC
KW264	CGGGATCCTTAGCTGTTATCTAGCAAGGCATAAAATTGC
KW256	AACTGCAGGGATGATAGAAACGAAAATGTTAAATATAGCC
KW257	CCGCTCGAGCATGATAGAAACGAAAATGTTAAATATAGCC
KW265	CCCAAGCTTGCTAAAGAAATTTCATGGGCATAATGG

[a] Restriction sites are underlined.

contain (34). YsrR and DygR belong to the LuxR class and contain a helix-turn-helix DBD. These proteins are 81% identical, but the amino acid differences are primarily clustered into two regions (Fig. 1A). In the receiver domain, YsrR and DygR contain two short regions that appear to be insertions compared to other proteins of the same class (23). One of these insertion regions harbors one of the clusters of differences, and the second cluster is located just at the beginning of the DBD. The putative phosphorylation site (D75) and other residues important for the phosphorylation and signal transduction (E13, D14, T115, and K137) are conserved, as are other key structural residues (P95, G95, and A116) (35). The conservation of these important residues suggests that the receiver domain of DygR is functional.

Sensor kinase alignment. The alignment of YsrS and DygS shows a strikingly high identity between the two proteins (Fig. 1B). Within the cytoplasmic portion of these proteins, they are 97.8% identical, differing only in five amino acids just C terminal of the second transmembrane domain, three residues in the midsection, and the very C terminus. The DNA sequences encoding these cytoplasmic domains are 98.3% identical; there are only five synonymous changes, and these are located adjacent to the regions where amino acids are different. In contrast, the periplasmic regions, including the two transmembrane domains, are only 68.2% identical. While there are ~20-residue stretches of identity, the differences are evenly distributed within this domain. Based on examination of the DygS amino acid sequence, it can be interpreted that DygS has similar phosphorelay capabilities but responds to a signal different from the signal that YsrS responds to (NaCl).

Genomic context. The ~30-kb *ysa* locus contains a long operon with genes encoding the secretion apparatus, translocon proteins, and one effector protein. Adjacent to this operon, located on the opposite strand, are a few open reading frames encoding the recently identified YsaP pilotin protein (36) and other proteins that are likely part of the secretion apparatus. The *ysrRST* genes are just downstream of this region, and it was their proximity to the *ysa* genes that led us to investigate their role in *ysa* gene expression (22). The *dygRS* genes are approximately 10 kb downstream of *ysrRST*. The region duplicated seems only to include a few base pairs upstream of the YsrR/DygR start codon through a few base pairs upstream of the stop codon of YsrS/DygS. The promoter region for *ysrRST* shares very little with the region upstream of *dygR*, and there is no gene encoding a histidine phosphotransferase (HPt)-containing protein downstream of *dygS*. Between the *ysrRST* genes and the *dygRS* genes are genes encoding a type II secretion system (T2SS) that is important for virulence (37) and one of the three T2SS-secreted proteins (38). It is somewhat curious that there is a sizable distance between the *ysr* and *dyg* genes, as

A.

```
          1                                                                                                100
1. DygR   MIETKMLNIAMIEDEPFSRLALMEILKKYPYHHKKPERQLIYSAFSLQIAGCVSNAPELIHLLKNTPAIEVLLLDYSLT--DDEKKADTSQPQDGVGLIK
2. YsrR   MTQTKTLNIAMIEDEPFSRLALMEILKKYPYHHKEPELQLIYSDFSLQVVGCVSNTPELITLLESNPEIEVLLLDYSLVAGETDENEDASLPQDGVGLIK
          110                                                                                              200
1. DygR   RLLQLYPALKIIVHTAHKNLMVARLAWQAGAWGFVQKSLDTQELFFAISYVARGKKFFPIELAALPPSTTKNAQHALTERQTEVLRMLLNGVKQKEISMH
2. YsrR   RLLQLRPALKIIVHTAHKSLAVARLAWQAGAWGFVQKSSDIQELFFAISYVARGKKFFPIELASLSQSAQKNTHHTLTGRETEVLRMLLNGLKQKEISVR
          210                                237
1. DygR   LNISFKTVSNTKNRAFKKLGLTSNTDFFHYAHEISL*
2. YsrR   LNISFKTVSNTKTRAFKKLGLTSNTDFFKYAHEISL*
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B.

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          1                                                                                                100
1. DygS   MPMKFLYKIYLTSLFFLLLLTNGYAV-AITFSPQEQHYINTHPVVKYGISPHFYPIENFNSTGEHIGLTRDYIDIISAATGIKFQPVFSNNSIRPFVNLQ
2. YsrS   MLMKFLYKIYLTWALTLLSSTAVYAAEAIVFSPQELDYIKTHPVVNYGIFPNSYPIEKFNSIGEHIGLTRDYIDIISTVTGIKFKPVFSNNDRESVTNLQ
          110                                                                                              200
1. DygS   SGHVSLLTSTSSAFAQAKGLASSIPMFSTWPVTVTRKATRHIVTPDDLQEGYVSITDYLSLIEWFTEQYPNINYKISRSPEEAIGEVIHGRAKAAVVLSP
2. YsrS   SGKISLLTSTSISFAQTHGLISSVPIFTTWPLTVTRKATRHIATPDDLMEGYVSITDYSSLIEWFTKQFPGVNYKIVYSPEETIAEVINRQAEAAVVLSP
          210                                                                                              300
1. DygS   TALYYMNVVHPGQLKMSNPHKVKIPRVMSARPEDQILIDIINKVIASVSAKQQSELMAKWMLSDT-LSQPDNTQPNWYYYSLVTILLCLSCFVFYRNRQL
2. YsrS   TALYYMNVKYPGQLKISRPHSAKISLAMAAHPEDQILINIINKAIASISAKQQAELTAKWIISDTNIPLPKDYELTFYVGGLATTLLCLLLFVFYRYRRL
          310                                                                                              400
1. DygS   KMAFIRLGSKNNLELSVIAHELRTPLIGILTACEGLVDKIVSASQRERLANVIHVTRELLDNLDLSLDNAKINAGSVTQNPQPQLLAELCDTTVKLFTSF
2. YsrS   KMELVRLGSKNNLELSVIAHELRTPLIGILTACEGLVDKIVSASQRERLANVIHVTRELLDNLDLSLDNAKINAGSVTQNPQPQLLAELCDTTVKLFISF
          410                                                                                              500
1. DygS   AETHGTTIQVRYLSKQFFLPHLFDGTLVSQALNNIVSNAIKHTHDGMVLIECSLLQVDGKNMFSIEVIDTGTGIPSKVLARLSEPFYQGKFSRTDSDTPH
2. YsrS   AETHGTTLQVRYQSKQFFLPHLFDGTLVSQALNNIVSNAIKHTHDGMVLIECSLLQVDGKNMFSIEVIDTGTGIPSKVLARLSEPFYQGKFSRTDSDTPH
          510                                                                                              600
1. DygS   PKGTGLGLFVAKKNMHLTGGHLAIVSQPGVGSRVTIALPAIAAHYAIENPLPEGLHIIMPTEIPSSLSGEITQILDGCELPYYSAAEPLPAAARGPEIDL
2. YsrS   PKGTGLGLFVAKKNMHLTGGHLAIVSQPGVGSRVTIALPAIAAHYAIENPLPEGLHIIMPTEIPSSLSGEITQILDGCELPYYSAAEPLPAAARGPEIDL
          610                                                                                              700
1. DygS   QLDVAQKHWQLHNKQGDSVIIPRPVYASALYLAITDLCNEEQPLESSSDSPELHLPTTIITESRRLLVVEDEPLLLEVQYELFSSMGFQVDAVANTQQAY
2. YsrS   QLDVAQKHWQLHNKQGDSVIIPRPVYASALYLAITDLCNEEQPLESSSDSPELHLPTTIITESRRLLVVEDEPLLLEVQYELFSSMGFQVDAVANTQQAY
          710                                                                                  792
1. DygS   QSWLQHHHTIIVTDCRLDESDGFELVRHLRKLMQDSPEPVLIIGQSASLKTEDAQRAREVGMDYLLQKPVAREQWQQLIRDYFASGK*
2. YsrS   QSWLQHHHTIIVTDCRLDESDGFELVRHLRKLMQDSPEPVLIIGQSASLKTEDAQRAREVGMDYLLQKPVAREQWQQLIRDYFASKEKEHD*
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FIG 1 Alignment of the amino acid sequences comprising the response regulators (A) and sensor kinases (B). Red boxes in panel A indicate the insertion regions relative to other RR proteins (23); in panel B, the red boxes outline the two predicted transmembrane regions designated by the MiST2 database (45). The DNA-binding domain of YsrR/DygR is indicated with a green line. Residues that become phosphorylated are indicated with blue boxes.

duplication events typically generate tandem genes (39). One possible explanation is that, following the duplication event, this segment of DNA encoding the T2SS inserted itself between the tandem repeats.

Given the pronounced differences in DNA sequence immediately upstream of the *dygR* start codon, we investigated if there was a functional promoter by constructing a chromosomal *lacZ* reporter in the region upstream of *dygR* in our wild-type *Y. enterocolitica* strain. When grown at 26°C in L-broth and LB-290, β-gal activity was measured at 755 ± 103 and 1,841 ± 117 Miller units (MU; averages ± standard deviations), respectively. We know this is an indication of transcriptional activity, as the parent strain lacking any reporter produces less than 50 MU (data not shown); this low-level activity is likely derived from the intact native *lac* operon. It is curious to note the ~2-fold increase in the presence of 290 mM NaCl. We also tested the *dygR-lacZ* reporter strain following growth under several other conditions, such as LB with high sucrose, iron depletion, acidic and basic pHs, and growth at 37°C, and we found that it was always expressed and with little variation in levels (data not shown). If this DNA region upstream of *dygR* was part of the T2SS-containing insertion event mentioned above, it is perhaps somewhat serendipitous that it included a functional promoter.

DygS can compensate for loss of YsrS. In order to determine if DygS plays a role in regulating the *ysa* T3SS genes, we examined expression of the Ysr-dependent *ysaE* promoter, a key promoter that drives transcription of a long operon that contains many of the *ysa* apparatus genes (13, 22). An in-frame deletion of *dygS* was constructed, and β-gal activity was measured from a chromosomally encoded *ysaE-lacZ* fusion following growth under the known Ysa-inducing condition, LB with 290 mM NaCl (LB-290). We found that deleting the *dygS* gene had no impact on *ysaE* expression (Fig. 2A). Given that the putative promoter region is active under these growth conditions, this suggests that DygS plays no role in regulating *ysaE* transcription when cultured in LB-290. A preliminary screen of easily tested growth conditions, including pH, 1% sucrose, magnesium, and iron levels (supplemented and depleted), failed to reveal any role for DygS in *ysaE-lacZ* expression (data not shown). However, because it was still possible that there were conditions under which DygS could regulate *ysaE* expression, we constructed a plasmid constitutively expressing *dygS* (pDygS) to assess if overproduction of DygS could modulate *ysaE-lacZ* expression. pDygS was transformed into the wild-type and Δ*ysrS*, Δ*dygS*, and Δ*ysrS* Δ*dygS* mutant strains carrying the *ysaE-lacZ* reporter, and the resulting strains were assayed for β-gal activity following growth in LB-290. As controls, the vector pWKS130 and the *ysrS* complementing clone, pYsrS, were included. When overproduced in *trans*, strains carrying pDygS yielded between 1,300 and 1,950 MU, comparable to the activity in the wild-type strain carrying the vector pWKS130 (Fig. 2A). Thus, pDygS restored expression in the Δ*ysrS* and Δ*ysrS* Δ*dygS* mutant strains, indicating that DygS has the capacity to compensate for loss of YsrS.

Because the cytoplasmic domains of YsrS and DygS are so highly conserved, we hypothesized that the cytoplasmic domain of DygS, which is nearly identical to YsrS, could participate in the Ysr phosphorelay. To test this, we constructed a plasmid with a chimeric gene encoding the YsrS sensing (periplasmic) domain with the DygS cytoplasmic domains, called pChimera. As predicted, this plasmid complemented the *ysrS* gene deletion, yielding 2,208

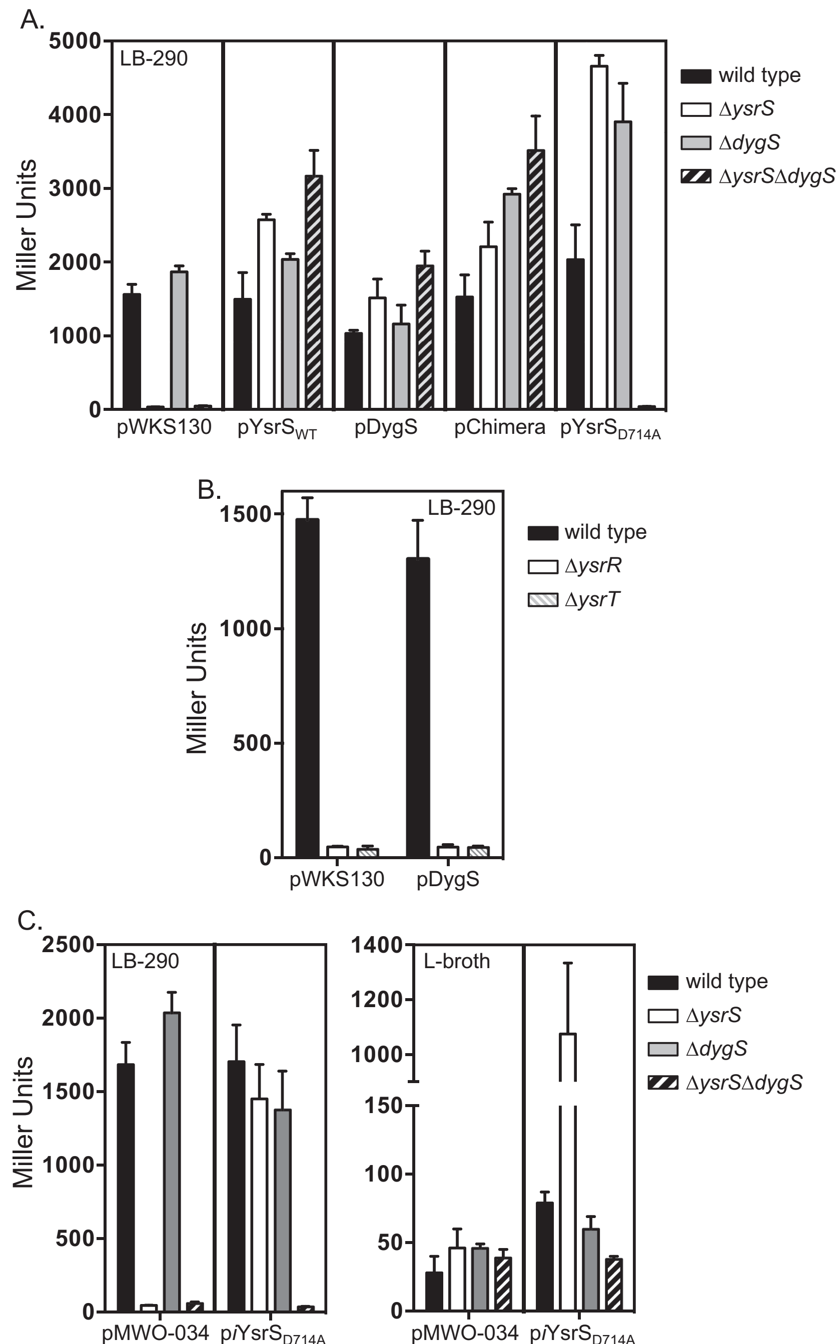

FIG 2 DygS compensates for loss of YsrS. (A) β-Galactosidase assay results for wild-type, Δ*ysrS*, Δ*dygS*, and Δ*ysrS* Δ*dygS* strains carrying a *ysaE-lacZ* fusion. Each strain was transformed with a plasmid expressing *ysrS* (pYsrS), *dygS* (pDygS), the *ysrS-dygS* chimera (pChimera), or *ysrS*$_{D714A}$ (pYsrS$_{D714A}$) or with the vector (pWKS130). (B) pDygS was transformed into the Δ*ysrR* and Δ*ysrT* strains to test the roles of YsrR and YsrT in the phosphorelay initiating with DygS. (C) *ysrS*$_{D714A}$ was cloned into pMWO-034 with an inducible promoter (29). For all assays, saturated cultures grown in L-broth were diluted into L-broth or LB-290 as indicated and grown for 2 h at 26°C. Bars represent averages for at least three independent cultures, with standard deviations. Cultures represented in panel C contained 5 ng/μl of ATc to allow low-level expression of the *ysrS* gene.

and 3,513 MU in the Δ*ysrS* and Δ*ysrS* Δ*dygS* strains, respectively (Fig. 2A). These expression levels were higher than those obtained when pDygS was transformed into these same strains but similar to those obtained with pYsrS; this may be due to the presence of the salt-responsive sensor domain on pChimera and pYsrS.

The results with pDygS and pChimera suggest that DygS is capable of transferring the phosphoryl group to YsrT, which then transfers to YsrR, resulting in *ysaE* transcription activation. To verify that these phenotypes are from DygS substituting for YsrS and not some other indirect effect, we transformed pDygS into the Δ*ysrR* and Δ*ysrT* strains and tested for *ysaE-lacZ* expression (Fig. 2B). The presence of pDygS in these strains could not restore *ysaE*

expression, further supporting our hypothesis that the observed complementation of the Δ*ysrS* mutant by pDygS and pChimera is due to DygS participating in the Ysr phosphorelay that leads to activation of *ysa* gene expression.

Further evidence of a putative role for DygS in regulation of *ysaE* expression came as we pursued a peculiar result from our genetic analysis of the Ysr phosphorelay. In a previous study, we made alanine substitutions in each of the aspartate and histidine residues predicted to be phosphorylated in YsrS, YsrT, and YsrR (23). All of the mutants behaved as expected, in that *ysaE* expression was not activated, with one striking exception. Mutation of the conserved aspartate residue in the receiver domain of YsrS to alanine (D714A) actually resulted in constitutive *ysaE-lacZ* expression, and this was dependent on the presence of wild-type YsrT and YsrR (23). Wild-type and Δ*ysrS* strains transformed with a plasmid expressing the mutant *ysrS* gene (pYsrS_{D714A}) yielded equally high levels of β-gal activity after growth in L-broth and LB-290 (23). After obtaining the results above that suggested that DygS had the capacity to participate in the Ysr phosphorelay, we transformed pYsrS_{D714A} into the Δ*dygS* and Δ*ysrS* Δ*dygS* strains. In the Δ*dygS* strain with pYsrS_{D714A}, *ysaE-lacZ* expression was exceptionally high, as observed in the Δ*ysrS* strain, yielding 3,905 and 4,659 MU, respectively (Fig. 2A). However, in the Δ*ysrS* Δ*dygS* strain with pYsrS_{D714A}, the *ysaE-lacZ* expression level was 41 MU, approximately the same as the level with pWKS130 (49 MU). This is consistent with the activity measured from strains with no *ysaE* expression and is what would normally be expected from the D714A mutation. To ascertain if the unusual phenotypes with pYsrS_{D714A} were a consequence of constitutive expression of the mutant gene, we subcloned the insert into a vector with an inducible promoter, creating p*i*YsrS_{D714A}. We then examined *ysaE-lacZ* levels when strains carrying this plasmid were cultured with a very low concentration of inducer (5 ng/μl of ATc). In this situation, we still saw that YsrS_{D714A} was capable of complementing the *ysrS* deletion, yielding 1,450 MU (roughly equivalent to the wild-type strain), and was still constitutive, in that it resulted in high levels of *ysaE* expression (1,075 MU) in the Δ*ysrS* strain in the absence of NaCl (Fig. 2C). Thus, it appears that, in a strain with a chromosomal copy of either *ysrS* or *dygS*, YsrS_{D714A} may form a dimer with YsrS or DygS, and the phosphorelay travels from H320 of YsrS_{D714A} to D714 (YsrS) or D712 (DygS). In the absence of a chromosomal copy of *ysrS* or *dygS*, there is no aspartate residue in the sensor receiver domain and, thus, no way to complete the phosphorelay. Why this creates a constitutively active phosphorelay in Δ*ysrS* is still a mystery. The high levels of expression under noninducing conditions are only evident when *dygS* is present and *ysrS* is absent, and it is possible that the backward movement of the phosphoryl group, a carefully controlled step in phosphorelay systems, is disrupted. Taken together, these data indicate that YsrS and DygS are similar enough to play nearly identical roles and suggest that, under certain conditions, DygS has the capacity to activate *ysaE* expression and, thus, production of the Ysa T3SS.

DygR does not compensate for loss of YsrR. Having determined that DygS can participate in the pathway leading to *ysaE* activation, we set out to examine if DygR could also activate *ysaE-lacZ* expression. We performed a similar set of experiments using a *dygR* in-frame deletion strain (Δ*dygR*) and *dygR*-overexpressing plasmid (pDygR). As was the case with Δ*dygS*, we found that deleting the *dygR* gene had no impact on *ysaE* expression (770 MU with pWKS130), suggesting that DygR plays no role in regulating

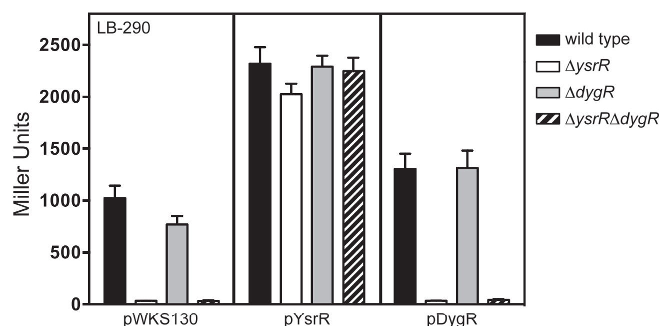

FIG 3 β-Galactosidase assay results for wild-type, Δ*ysrR*, Δ*dygR*, and Δ*ysrR* Δ*dygR* strains carrying a *ysaE-lacZ* fusion. Each strain was transformed with a plasmid expressing *ysrR* (pYsrR) or *dygR* (pDygR) or with the vector (pWKS130). For all assays, saturated cultures grown in L-broth were diluted into LB-290 and grown for 2 h at 26°C. Bars represent averages for at least three independent cultures, with standard deviations.

ysaE transcription in LB-290 (Fig. 3). However, the results from overexpressing DygR differed from DygS. In this case, we found that transformation with pDygR had no impact on *ysaE* expression in any strain background examined, yielding about 1,300 MU in strains with *ysrR* and <50 MU in strains lacking *ysrR*. Thus, activation of *ysaE-lacZ* by DygS most likely occurs via a mechanism other than the canonical sensor-response regulator pathway.

DygS, but not DygR, interacts with YsrT. The data presented in Figure 2B show that YsrT and YsrR are necessary for pDygS to enable *ysaE* activation. Presumably, for DygS to facilitate activation of *ysaE*, it must transfer the phosphoryl group to an HPt domain. There does not appear to be a gene encoding an HPt-containing protein in the *dyg* locus, and our data implicate YsrT in this role. Thus, we turned to the Ladant bacterial 2-hybrid system (B2H) (25) to examine protein-protein interactions that phosphoryl group transfer would necessitate. In this system, two vectors, each encoding a domain of the *Bordetella pertussis* adenylate cyclase (AC), are used to make AC fusions to proteins of interest and are cotransformed into *E. coli* BTH101 cells. If the fusion proteins interact, it brings the two AC domains together, allowing synthesis of cAMP. Then, cAMP binds to CRP, leading to the activation of maltose and lactose operons. BTH101 has a *cya* mutation that produces only very small amounts of cAMP, thus making the increased cAMP due to interaction of the two fusion proteins readily visible through enzymatic analysis of the maltose or lactose fermentation and β-gal activity. While we had genetic evidence of phosphoryl transfer from YsrS to YsrT and then to YsrR (23), we had yet to examine if these proteins interacted. Because YsrS and DygS are most likely anchored in the inner membrane, we cloned only sequences encoding their cytoplasmic domains into pT25, assuming that the N-terminal AC fusion would not interfere with the interaction regions. The genes *ysrR*, *ysrT*, and *dygR* were cloned into both pT18 and pT25 vectors for analysis of homodimerization as well as interactions with YsrS and DygS. *E. coli* strain BHT101 was cotransformed with one pT18-based plasmid and one pT25-based plasmid, plated on MacConkey agar plates containing 5% maltose (MacMal), and incubated at 30°C for a qualitative assessment of interactions (positive interactions yielded red-colored colonies). Quantitative assessment via β-gal assays was performed after 3 days on MacMal plates. Our positive-control strain contained pT18-zip and pT25-zip, each containing

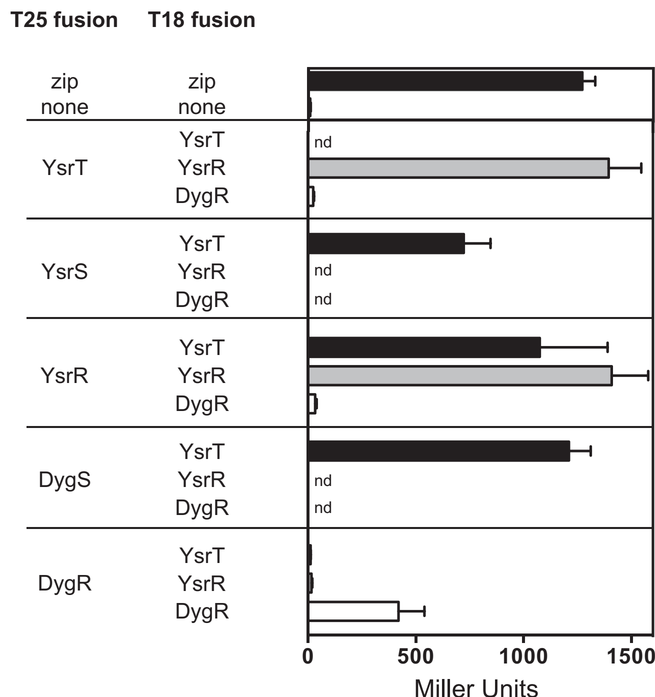

FIG 4 DygS, but not DygR, interacts with YsrT. Various components of the Dyg and Ysr phosphorelay systems were cloned into pT18 and pT25, generating adenylate cyclase fusion proteins for bacterial two-hybrid analysis. The indicated pairs of plasmids were cotransformed into *E. coli* BHT101 cells. Saturated cultures grown in LB at 26°C were diluted into fresh LB and grown for 30 min at 37°C. Bars represent averages for at least three independent cultures, with standard deviations. Vector-fusion plasmid controls were performed for each plasmid; the β-gal activities for these controls all fell below 12 MU. ND, not done.

a leucine zipper domain (25); the negative-control strain contained the vectors only. This assay revealed that both YsrS and DygS interact with YsrT, as strains carrying these pairs of plasmids produced 722 and 1,210 MU, respectively (Fig. 4). The β-gal activity from the YsrT-DygS strain was slightly higher than from the YsrT-YsrS strain; this can be interpreted to mean that the YsrT-DygS interaction was strong enough to facilitate phosphoryl transfer. Many response regulators form homodimers in their active state (reviewed in reference 40). As predicted, BHT101 transformed with each plasmid carrying *ysrR* yielded 1,408 MU, demonstrating that YsrR forms homodimers.

We included DygR in the B2H analysis to address (i) whether it could interact with YsrT, which could mean that it can participate in a phosphorelay with DygS-YsrT and regulate other genes, and (ii) whether the level of similarity with YsrR was sufficient for heterodimer formation. β-gal assays following cotransformation with DygR constructs indicate that DygR does not interact with YsrT or with YsrR; the measured activity was just slightly above the negative control at 11 and 16 MU, respectively (Fig. 4). However, the β-gal activity from the DygR-DygR strain (420 MU) indicates that it does homodimerize. This last result also indicates that these DygR constructs are functional, strengthening the interpretation that it does not interact with YsrT or YsrR.

DISCUSSION

DygS and DygR are putative phosphorelay proteins that share a strikingly high similarity to YsrS and YsrR. The Ysr phosphorelay system, comprised of the sensor kinase YsrS, the histidine phosphotransferase (HPt) YsrT, and the response regulator YsrR, is required for activation of the *ysaE* promoter that drives transcription of the chromosomally encoded Ysa type III secretion system (T3SS) (13, 22). The *dygS* and *dygR* genes (YE3579 and YE3578, respectively [12]) are located about 10 kb downstream of the *ysa/ysr* locus and most likely were acquired by a gene duplication event. Because of the similarity to YsrS and YsrR, we investigated whether DygS and DygR could also activate *ysaE* expression. We considered several hypotheses. First, the differences within DygR and the periplasmic region of DygS could have rendered them nonfunctional and the Dyg phosphorelay system a dying remnant of duplication. However, the nearly 100% conservation of the cytoplasmic region of DygS suggested that it likely was at least a partially functional histidine kinase. With the assumption that DygR and DygS both retain function, a second hypothesis we considered is that DygS autophosphorylates in response to a signal (other than NaCl) and transfers the phosphoryl group first to YsrT and then to YsrR, which could then activate *ysaE* expression. A third possibility is that the DygS-YsrT-DygR phosphorelay comprises a completely separate regulon that may or may not include *ysaE*. Our data suggest that what occurs is a combination of the first and second hypotheses: that DygS can initiate a phosphorelay with YsrT and YsrR to activate *ysaE* expression, but that DygR may have acquired enough deleterious mutations to lose function.

The data we obtained for DygR showed that it does not interact with YsrT and cannot promote *ysaE* transcription, even when overexpressed. We conclude that the differences between YsrR and DygR are mutations that have altered the function of DygR such that it can no longer become phosphorylated by YsrT, can no longer bind the *ysaE* promoter, or both. One cluster of differences lies near the DNA-binding domain. These differences could lead to changes in structure or binding specificity, such that it cannot bind the *ysaE* promoter. The second cluster of differences is in one of the two insertion sequences; these residues could be in the region that facilitates phosphotransfer from YsrT. The lack of interaction between DygR and YsrT suggests that, if it does participate in a phosphorelay with DygS, it does so with another HPt protein. Given that *Y. enterocolitica* contains only six HPt-containing proteins and that they are all part of characterized phosphorelay systems, we suspect that the amino acid changes acquired by DygR have caused a loss of function.

Through the use of gene deletions and *trans* complementation with a variety of plasmids expressing different forms of *ysrS* and *dygS*, we provide several lines of evidence indicating that DygS is capable of facilitating transcription of the *ysaE* promoter by participating in a phosphorelay with YsrT and YsrR. First, while loss of the *dygS* gene had no impact on *ysaE* expression, introducing a plasmid expressing either the wild-type *dygS* gene or the *ysrS-dygS* chimeric gene could compensate for loss of the *ysrS* gene. Second, the bacterial two-hybrid experiment demonstrated an interaction between DygS and YsrT with strength similar to that of the YsrS-YsrT interaction. Third, activation by DygS requires the *ysrT* and *ysrR* genes. Finally, the constitutive-on phenotype observed when complementing the Δ*ysrS* strain with pYsrS$_{D714A}$ requires the *dygS* (or *ysrS*) gene, providing strong genetic evidence that YsrS and DygS can heterodimerize. This is not surprising, given the 100% identity conservation of the HisKA domain (residues 313 to 366), which is known to promote dimerization (41).

If DygS does indeed activate *ysa* gene expression in response to

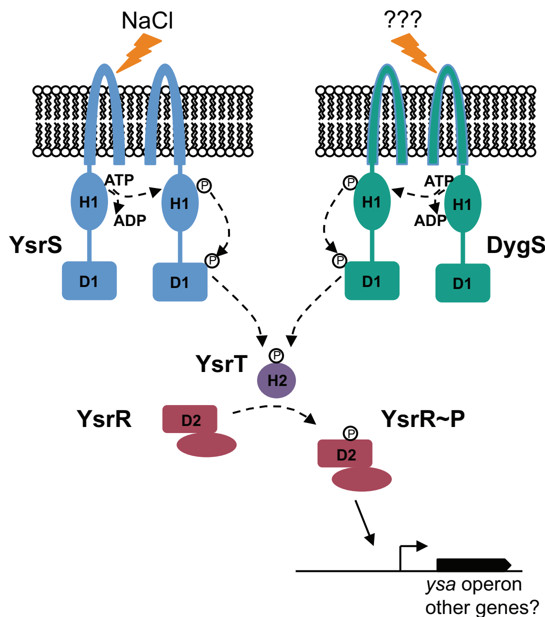

FIG 5 Model of potential phosphorelay events leading to the activation of *ysa* gene expression. Under growth in high-NaCl conditions, activation can occur via YsrS-to-YsrT-to-YsrR transfer. Under other conditions (yet to be identified), a signal is perceived by DygS, which can then transfer the phosphoryl group to YsrT and thus to YsrR.

a signal other than NaCl, it indicates that the *ysa* system has two sensors that can independently lead to expression (Fig. 5). This is not unlike the *Vibrio* quorum sensing phosphorelay systems responding to autoinducers. In this case, there are two sensors (LuxN and LuxQ), each responding to a different autoinducer, each relaying the phosphoryl group to the HPt-containing LuxU protein, which leads to phosphorylation of LuxO that regulates the transcription of downstream *lux* genes (reviewed in reference 42). In *Bacillus subtilis*, five separate sensor kinases (KinA through KinE) all lead to the phosphorylation of the response regulator Spo0A. These kinases respond to different signals and result in different cellular fates: sporulation, matrix production, and biofilm production (reviewed in reference 43). Thus, it is plausible that, under conditions yet to be identified, DygS initiates a phosphorelay signal that leads to activation of *ysa* gene expression and possibly other genes as well.

The data provided herein suggest that DygS has undergone evolution by the innovation-amplification-divergence model (44). In this model, there is an assumption that a gene that is duplicated has a side function (innovation). In this situation, it could be a trigger other than NaCl that initiates the Ysr phosphorelay. This side function is advantageous enough that the gene is duplicated to generate a larger dose of this function (amplification). In the duplicated gene, this side function is selected for through the acquisition of mutations that enhance this function (divergence). Evidence exists that other salts can activate *ysaE* expression (13) (K. A. Walker and V. L. Miller, unpublished data). While it has not been determined if these other salts initiate a phosphorelay through YsrS or DygS, it is tempting to speculate that one side function of YsrS was response to a secondary signal that became the primary signal for DygS. Because we have yet to find an environmental signal that triggers DygS-dependent activation of *ysaE* expression, we cannot rule out that the sensing periplasmic region acquired deleterious mutations that render it nonfunctional. However, the preservation of the cytoplasmic region at not just the amino acid sequence level but also the DNA sequence level suggests that DygS provides a vital function during some phase of the *Y. enterocolitica* life cycle and further supports the notion that the Ysa T3SS is a critical component for the survival of this pathogen.

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