In Salmonella enterica, the pathogenicity island 2 (SPI-2) regulator PagR regulates its own expression and the expression of a five-gene operon that encodes transketolase C

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Abstract

The enteropathogen *Salmonella enterica* subsp. *enterica* sv. Typhimurium str. LT2 (hereafter *S.* Typhimurium) utilizes a cluster of genes encoded within the pathogenicity island 2 (SPI-2) of its genome to proliferate inside macrophages. The expression of SPI-2 is controlled by a complex network of transcriptional regulators and environmental cues, which now include a recently characterized DNA-binding protein named PagR. Growth of *S.* Typhimurium in low phosphate low magnesium medium mimics conditions inside macrophages. Under such conditions, PagR ensures SPI-2 induction by upregulating the transcription of *slyA*, a known activator of SPI-2. Here we report that PagR represses the expression of a divergently transcribed polycistronic operon that encodes the two subunits of transketolase TktC (*i.e., tktD, tktE*) of this bacterium. Transketolases contribute to the non-redox rearrangements of phosphorylated sugars of the pentose phosphate pathway, which provide building blocks for amino acids, nucleotides, cofactors, etc. We also demonstrate that PagR represses the expression of its own gene and define two PagR binding sites between *stm2344* and *pagR*.

In *Salmonella enterica,* the pathogenicity island 2 (SPI-2) regulator PagR regulates its own expression and the expression of a five-gene operon that encodes transketolase C

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Running title: PagR regulates the synthesis of transketolase C

AUTHOR CONTRIBUTIONS

A.R.P., R.D.M., and J.C.E.-S. conceptualized the project, designed the research, analyzed data, wrote the first draft of the paper; A.R.P. performed most of the experiments. J.A.B. assisted in the performance of experiments and edited the paper. J.C.E.-S obtained funding for the performance of the work.

Summary

The enteropathogen *Salmonella enterica* subsp. *enterica* sv. Typhimurium str. LT2 (hereafter *S.* Typhimurium) utilizes a cluster of genes encoded within the pathogenicity island 2 (SPI-2) of its genome to proliferate inside macrophages. The expression of SPI-2 is controlled by a complex network of transcriptional regulators and environmental cues, which now include a recently characterized DNA-binding protein named PagR. Growth of *S.* Typhimurium in low phosphate low magnesium medium mimics conditions inside macrophages. Under such conditions, PagR ensures SPI-2 induction by upregulating the transcription of *slyA* , a known activator of SPI-2. Here we report that PagR represses the expression of a divergently transcribed polycistronic operon that encodes the two subunits of transketolase TktC (*i.e., tktD, tktE*) of this bacterium. Transketolases contribute to the non-redox rearrangements of phosphorylated sugars of the pentose phosphate pathway, which provide building blocks for amino acids, nucleotides, cofactors, etc. We also demonstrate that PagR represses the expression of its own gene and define two PagR binding sites between*stm2344* and *pagR* .

Keywords . PagR protein, pentose phosphate pathway, *tktDE*expression, transketolase, transcriptional regulation, *pagR* gene expression, *Salmonella* pathogenesis

Introduction

Transketolases are well-characterized enzymes that catalyze the non-oxidative transfer of a C2 unit from a ketose to an aldose, shortening the ketose by two carbons and lengthening the aldose by two carbons (Schneider and Lindqvist, 1998). Transketolases use thiamin pyrophosphate (TPP) as cofactor, whose ylid carbanion triggers a nucleophilic attack on the carbonyl group of the ketose. The addition product cleaves in a retro-aldol like reaction and the resulting enamine adds to the carbonyl group of the aldose. Elimination of the TPP ylid carbanion yields the final ketose product. Transketolase-catalyzed reactions are critical in processes such as the non-oxidative branch of the pentose phosphate pathway (non-oxPPP) (Stincone et al., 2015) and the Calvin-Benson-Bassham (CBB) cycle (Raines, 2003). Up until recently, the genome of *S.* Typhimurium was thought to encode only two transketolases.

TktC was recently characterized as an isozyme of transketolase A and transketolase B (Shaw et al., 2018). The TktA and TktB transketolases are housekeeping enzymes involved in the non-oxPPP of this bacterium. The reversibility of reactions catalyzed by transketolase enzymes involved in the non-oxPPP allows the production of intermediate compounds to meet metabolic demands of the cell through the diversion of metabolites to other pathways such as replenishment of glycolysis, or to provide building blocks for the biosynthesis of aromatic amino acids, nucleotides, or lipopolysaccharides (Stincone et al., 2015). PagR is a transcriptional regulator that indirectly induces the expression of SPI-2 through *slyA* , which is important for *S.* Typhimurium replication in macrophages and pathogenicity in mice (Jiang et al., 2020). During the analysis of the genomic context of the *pagR*gene (locus tag *stm2345*), we noticed that *pagR* was transcribed divergently from a set of genes encoding a putative sugar transporter (*stm2342* , *stm2343* , *stm2344*) and two genes (*tktD = stm2341* and *tktE = stm2340*) encoding the subunits of transketolase C (TktC, Fig. 1). In this paper we show that i) PagR regulates the expression of the divergently transcribed*tktDE* genes that encode the subunits of the TktC transketolase of*S.* yphimurium; ii); PagR represses the expression of its own gene; and iii); PagR directly binds the promoter region between*stm2344* and *pagR* , protecting two binding sites.

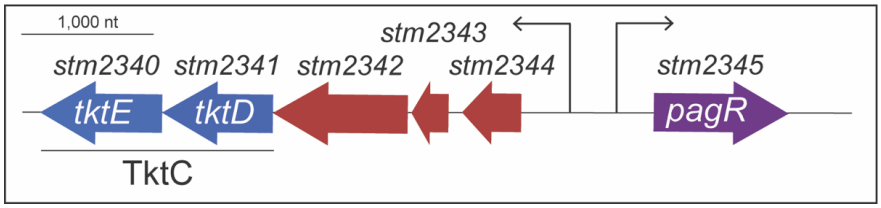

Figure 1. Genomic context of the *pagR* and *tktD, tktE* genes . PagR activates the transcription of the five-gene operon shown in the figure. The genes encoding the TktC transketolase are the most promoter-distal genes of the operon. *tktD* = 953 nt,*tktE* = 830 nt, *stm2342* = 1391 nt, *stm2343* = 272 nt,*stm2344* = 443 nt, *pagR* = 1019 nt. At present, the location of the promoters is hypothetical. The operon spans the region from 2,257,129 to 2,454, 101 nt (*i.e.,* 3,028 nt) of the *S.*Typhimurium chromosome. *pagR* spans the region from 2,457,382 to 2,458401 nt. All genes are at scale.

Results and Discussion

A *S.* Typhimurium [?]*pagR* strain grows poorly with succinate as a sole carbon and energy source . We probed for areas of*S.* Typhimurium metabolism that might be regulated by PagR. To this end, we tested the ability of a *S.* Typhimurium*pagR1::kan* $^{+}$ strain to catabolize various carbon sources. During this screen, we observed that a*pagR1::kan* $^{+}$ strain (JE21566) struggled to grow when succinate was the sole carbon and energy source (Fig. 2A, gray triangles *vs* red circles). The observed phenotype was solely due to the lack of PagR, since we restored growth to the rate and cell density of a culture of the *pagR* $^{+}$ strain by ectopically expressing the *pagR* $^{+}$ allele (Fig. 2A, dark green triangles). Surprisingly, we also corrected the growth phenotype by deleting the *tktD* and *tktE* genes encoding the two subunits of the TktC transketolase (*tktDE1* ::cat^{+} , Fig. 2A, blue squares). The latter was a notable result because the correction of the phenotype was as efficient as the ectopic expression of*pagR* $^{+}$. Conversely, the ectopic expression of the *tktDE* $^{+}$ genes repressed growth (Fig. 2B, magenta squares), mimicking the growth behavior of a*pagR1* ::kan^{+} strain (Fig. 2B, gray triangles). We wanted to learn whether the phenotype observed when the*tktDE* $^{+}$ genes were overexpressed was a result of having excess transketolase activity or whether TktC, was somehow different to TktA and TktB. For this purpose we expressed the other two*S.* Typhimurium transketolases in the*pagR1* ::kan^{+}*tktDE1* ::cat^{+} strain and monitored cell growth. Neither the ectopic expression of $tktA^{+}$ nor$tktB^{+}$ repressed the growth of the*pagR1* ::kan^{+}*tktDE1* ::cat^{+} strain (Fig. 2B, light green squares and yellow squares *vs* magenta squares). Rather, expression of $tktA^{+}$ and$tktB^{+}$ improved growth relative to the vector control. From these results we learned that the cell could not overcome whatever the deleterious effect of having TktC in excess was when growing on succinate.

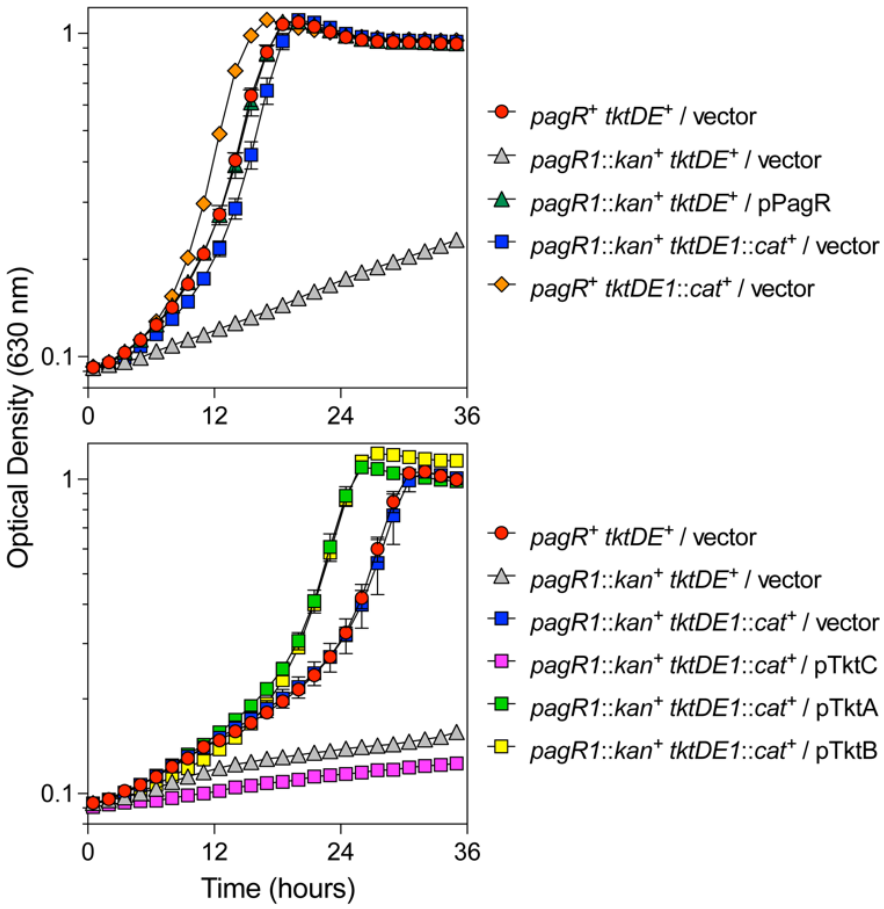

Figure 2. Growth of *S.* Typhimurium on succinate as a function of *pagR* and *tktDE*. *S.* Typhimurium was grown on NCE (no-carbon essential) minimal medium (Berkowitz et al., 1968) supplemented with sodium succinate (30 mM) as the sole source of carbon and energy. **A.** The*pagR1* ::*kan*$^{+}$ strain failed to grow after 36 h of incubation at 37^{o}C on succinate as the sole carbon source, and ectopic expression of *pagR* restores growth to wild-type. Removal of *tktDE* in the *pagR1* ::*kan*$^{+}$background restores growth as well. Growth of the*pagR*$^{+}$*tktDE* ::*cat*$^{+}$ strain doesn't significantly differed from the parent strain. Ectopic gene expression was induced with L-(+)-arabinose (100 µM). "pPagR" refers to plasmid pPagR-7.**B.** Expression of TktC, but not TktA or TktB, repressed growth of the *pagR1* ::*kan*$^{+}$*tktDE* ::*cat*$^{+}$ strain. Ectopic gene expression was induced with L-(+)-arabinose (1 mM). The strains used were*pagR* $^{+}$ *tktDE*$^{+}$ / vector (JE22070, red circles), *pagR1* ::*kan* $^{+}$*tktDE*$^{+}$ / vector (JE21566, grey triangles),*pagR1* ::*kan* $^{+}$*tktDE*$^{+}$ / pPagR (JE21577, dark green triangles),*pagR* $^{+}$*tktDE1* ::*cat*$^{+}$ / vector (JE25521, orange diamonds), *pagR1* ::*kan* $^{+}$*tktDE1* ::*cat*$^{+}$ / vector (JE25523, blue squares), *pagR1* ::*kan* $^{+}$*tktDE1* ::*cat*$^{+}$ / pTktC (JE25524, magenta squares), *pagR1* ::*kan* $^{+}$*tktDE1* ::*cat*$^{+}$ / pTktA (JE257201, light green squares), *pagR1* ::*kan* $^{+}$*tktDE1* ::*cat*$^{+}$ / pTktB (JE27202, yellow squares); 'vector' stands for the empty cloning vector pCV1 that contains an arabinose-inducible promoter (VanDrisse and Escalante-Semerena, 2016). This experiment was conducted in technical triplicate of biological duplicates three independent times. Error bars represent one standard deviation from the mean. Error bars that are not visible are smaller than the symbol. The statistical analysis was performed with Prism v9 (GraphPad)

The *tktD and tktE* genes are part of a five-gene operon . Bioinformatics analysis suggested that genes *stm2340 (tktE),stm2341 (tktD)* , *stm2342* , *stm2343* , and*stm2344* comprised a polycistronic operon (Biocyc.org). To test this possibility, we performed operon PCR with RNA isolated from a*pagR1* ::*kan*$^{+}$

strain. The RNA was treated with DNase, and cDNA was generated from purified total RNA. Genomic DNA, cDNA, and RNA were PCR amplified with four primer sets to generate products of four potential regions that overlap in neighboring putative genes (Fig. 3A). As shown in figure 3B, the use of such primers generated amplicons for the cDNA and the gDNA, but none amplified for the RNA, which served as the negative control. PCR amplification of all overlapping regions between genes *stm2340* and *stm2344* suggested these genes were part of a polycistronic unit.

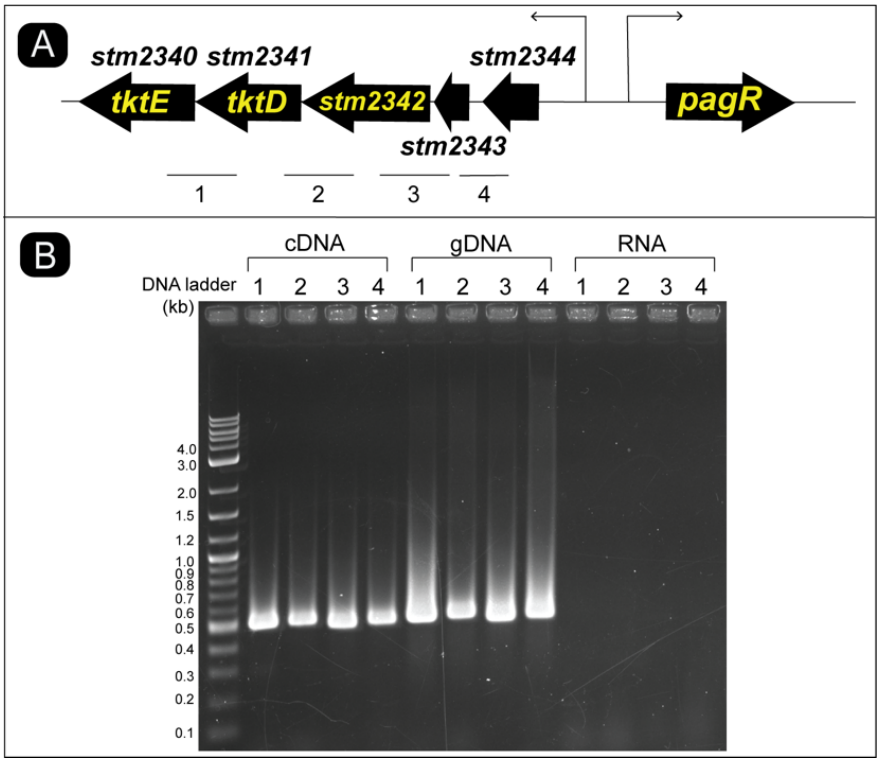

Figure 3. Genes encoding TktD and TktE are promoter-distal in a five-gene operon. A. The *pagR* gene is divergently transcribed from the operon containing the genes encoding the two subunits of TktC (*tktD* and *tktE*). The first three genes of the operon are annotated as putative phosphotransferase system (PTS)-type transporters.**B.** Operon PCR of the putative polycistronic operon containing the transketolase genes *tktD* and *tktE* encoding the subunits of TktC. RNA, cDNA and gDNA were PCR amplified using primers specific to overlapping regions between genes of the putative operon designated 1-4 in panel A. Positive controls are amplification of gDNA; negative controls are no amplification of RNA samples; experimental samples were the cDNA amplifications. Ladder on left shows corresponding amplification size (2-Log DNA Ladder, NEB).

PagR binds directly to the promoter region for its own gene and the polycistronic mRNA encoding *tktD* and *tktE*. To determine whether PagR directly bound to the promoter driving the expression of the *tktD and tktE* genes or was acting indirectly to repress the expression of the operon, PagR protein was purified to >95% homogeneity (Fig. S1) and tested for its ability to bind to the putative promoter region between *stm2344* and*pagR* (Fig. 3A). The putative promoter fragment of *pagR* and the polycistronic operon comprised of the *stm2340 -stm2344* genes was incubated with increasing concentrations of PagR protein then separated by native polyacrylamide electrophoresis (Fig. 4). The mobility of the DNA fragment (2,457,048 to 2,457,454 nt of the chromosome; 406 nt) that included the putative promoter region decreased as a function of increasing concentrations of PagR protein as compared to the no-PagR-protein-added control. This suggested that the oligomerization state of PagR changed as its concentration increased or that multiple

PagR-binding sites were present in the DNA fragment.

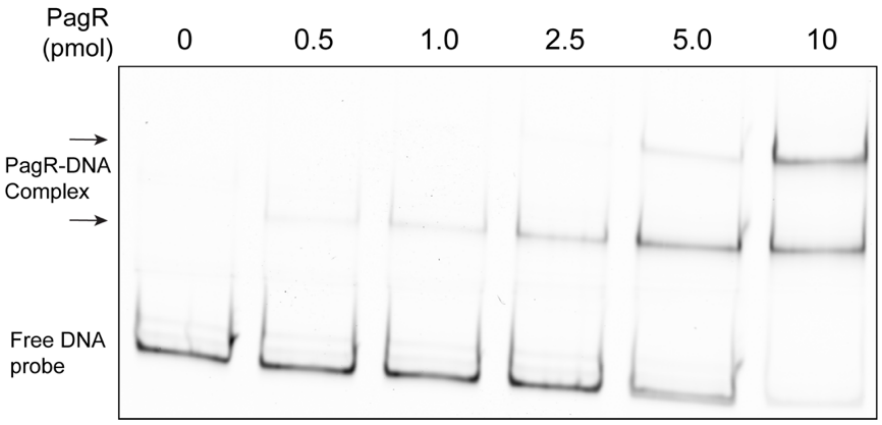

Figure 4. PagR binds the region between *pagR* and the five-gene operon that includes *tktD and tktE.* Representative results of an experiment designed to determine whether PagR could bind to the intergenic region between *pagR* and the five-gene operon containing genes encoding TktC. In this experiment we used 0.5 pmol of probe. The experiment was repeated three times and the amount of free probe (406 nt) decreased as a function of the amount of PagR added to the reaction mixture. More details about how the experiment was performed can be found under *Experimental procedures.*

PagR represses the expression of the operon containing*tktDE,* and its own gene . Given the proximity of *pagR* to the operon containing the *tktD and tktE* genes (Fig. 3A) and the data presented in figure 2, we investigated the possibility that PagR repressed the expression of the operon containing *tktD* and*tktE* . We isolated RNA from three strains, namely*pagR* $^{+}$ / vector,*pagR1* ::kan^{+} / vector, and*pagR1* ::kan^{+} / pPagR, and quantified the amount of *stm2343* , *tktD,* and *tktE* mRNA using RT-qPCR. For this purpose, cells were grown on lysogeny broth (LB) plus ampicillin and L-(+)-arabinose (100 μM), and RNA was isolated when cultures reached an OD_{600} of 0.5. As shown in figure 5A, expression of *stm2343* , *tktD* and *tktE* increased ˜10 fold in the*pagR1* ::kan^{+} / vector strain (red bars) when compared to the *pagR*$^{+}$ / vector and the*pagR1* ::kan^{+} / pPagR strains (black and blue bars, respectively). To assess whether PagR regulated its own expression, we constructed a *lacZY* transcriptional fusion with the chromosomal *pagR* gene and assayed β-galactosidase activity with and without ectopic expression of *pagR* . Cells were grown to an OD_{600} of ˜0.5 in LB plus ampicillin and L-(+)-arabinose (100 μM), then quantified β-galactosidase activity following a described protocol (Miller and Hershberger, 1984). The β-galactosidase activity of the*pagR3* ::$lacZY^{+}$ strain was significantly lower in the strain expressing *pagR* than in the vector control (Fig. 5B, blue vs red bars). Together, these data suggested that PagR repressed expression of the operon containing *tktDE,* and its own gene. This conclusion was consistent with its annotated homology to the LacI family of repressors.

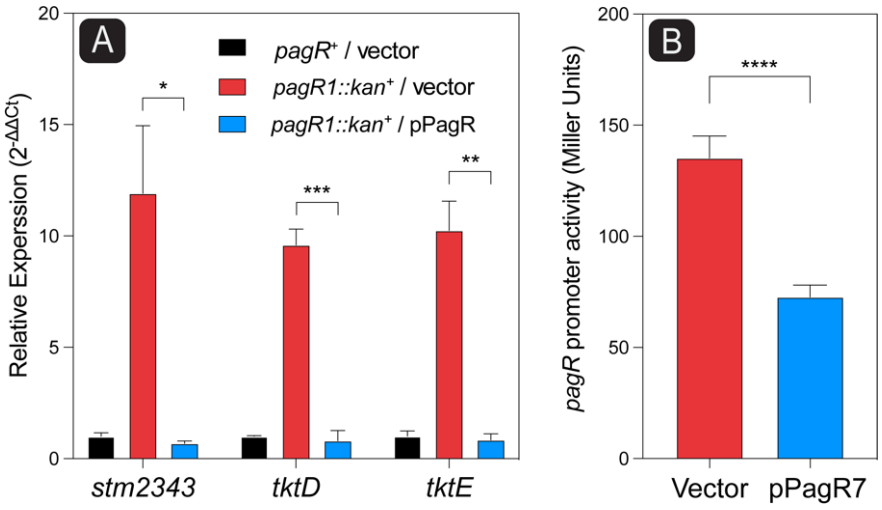

Figure 5. Expression analysis of the operon containing *tktDE* and of *pagR*, as a function of PagR. A. Effect of PagR on the expression of *stm2343* , *tktD* , and *tktE* as shown by RT-qPCR. The experiment was performed in biological and technical triplicates. **B.** Activity of the *pagR* promoter (in Miller Units) without and with *pagR* $^{+}$ expressed *in trans* (red vs blue bars, respectively). * = p [?] 0.05; ** = p < 0.01, ***, p [?] 0.001, **** = p [?] 0.0001. Cultures were grown in LB, and details of the RT-qPCR and β-galactosidase assay conditions used can be found in the *Experimental procedures* section. Vector = pCV1 (VanDrisse and Escalante-Semerena, 2016).

PagR protects two binding sites in the *stm2344* −*pagR* promoter region. DNase I footprinting was performed using a 291-nt DNA fragment spanning from 2,457,048 to 2,457,339 nt of the chromosome to define the DNA sequence bound by PagR. Results of an EMSA comparing PagR binding to this fragment with the one used in figure 4 suggested that the shorter probe used here contained all the relevant PagR binding sites (Fig. S1). Figure 6 shows the digestion results for the coding strand of the operon containing *tktD* and *tktE* (left to right going towards *stm2344*). The presence of PagR (Fig. 6, black trace) clearly protected two 21-bp regions of the *stm2344* - *pagR* promoter region that were not protected without PagR (Fig. 6, orange trace). A nearly identical sequence was observed in both (Fig. 6, red underlines). The underlined sequence in the protected region on the left is a perfect 14 base pair palindrome, 5'-TGATAGCGCTATCA-3', and the protected region on the right differs at only 2 bases (5'-TG*G* TAGCGCTATC*T* -3', differences underlined). These data supported our conclusion that PagR was a LacI family regulator, since LacI family repressors typically bind palindromic or inverted repeat DNA (Weickert and Adhya, 1992).

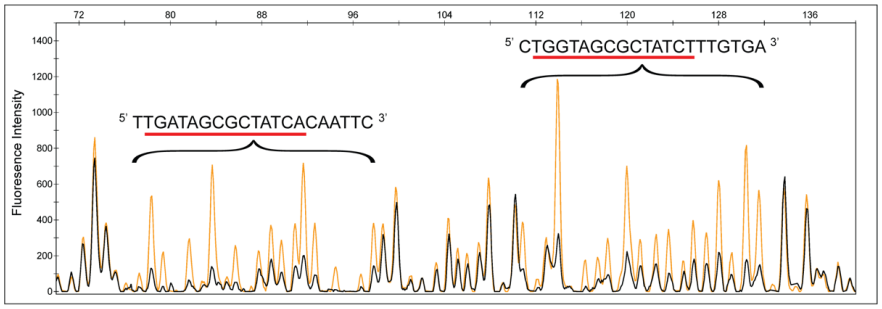

Figure 6. PagR binds two regions of the *stm2344* promoter. DNase 1 footprinting electropherograms from the coding strand of $P_{stm2344}$. Digestions were performed with 75 pmol PagR (black trace) and with no PagR (orange trace). This image is representative of two independent experiments.

PagR is likely a member of the LacI/GalR family of transcriptional regulators. Herein, we have presented data showing that PagR regulates gene expression in a manner like other proteins that belong to the LacI/GalR family of regulators. That is, we hypothesize PagR binds an as-yet-unidentified co-effector, dimerizes and then binds to the identified sequences within the region between *pagR* and the *tktDE* operon with the concomitant repression of its own expression, and the expression of adjacent genes encoding a putative transport system and subunits of a metabolic enzyme (Fig. 7).

Figure 7. Working model of PagR regulation of the *tktDE* operon. We hypothesize PagR requires a co-effector to dimerize, bind to the identified sites within the intergenic region between *pagR* and the 5-gene operon whose distal genes encode the subunits of transketolase C (TktC). Roman numerals represent: I, *stm2344* (putative PTS ascorbate transporter subunit IIA), II, *stm2343* (putative PTS mannitol subunit IIB), III, *stm2342* (putative PTS ascorbate transporter subunit IIC). No experimental evidence has been reported for the putative transporter. The red polygon represents the as-yet-unidentified PagR co-effector.

Moreover, PagR binding to the promoter region between its gene and *stm2344* protects a palindromic DNA sequence (Fig. 6). The repressor activity of PagR presented here contrasts with previous data reported by others, who showed that PagR is an activator of the *slyA* gene (Jiang et al., 2020). This dual activity, however, is not unprecedented for LacI/GalR family proteins (Swint-Kruse and Matthews, 2009). It is currently unknown what causes the switch between these functions for PagR. It is possible that the target of the uncharacterized, putative phosphotransferase system (PTS) (*stm2342-4*) adjacent to *pagR* may provide the signal, like galactose derepressing GalR (Swint-Kruse and Matthews, 2009). Expression of *pagR* increases under low phosphate and low magnesium conditions (Jiang et al., 2020), and it could be that PagR is regulated by phosphorylation or as a function of magnesium ions. All these interesting questions are currently being investigated.

Conclusions

Previous work demonstrated that PagR is necessary for full induction of the SPI-2 type-3 secretion system (Jiang et al., 2020). Our data show that PagR also regulates expression of the genes encoding the subunits of the TktC transketolase (*i.e., tktD, tktE*), the third transketolase enzyme identified in *S.* Typhimurium (Shaw et al., 2018). Transketolases are essential for *S.* Typhimurium virulence (Shaw et al., 2018), and PagR appears to be a link between genetic regulation of the pathogenic machinery and a metabolic function essential for the pathogenic lifestyle of this bacterium. Moreover, the data presented here indicate that TktC has a function in the cell that differs from those of TktA and TktB. Future research into the identity of the co-efector of PagR and into the function of TktC, will illuminate the connections between central metabolism and the pathogenic machinery in *S.* Typhimurium.

Experimental procedures

Bacterial strains. All strains were derivatives of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (hereafter *S* . Typhimurium) (Table 1). Gene deletions were constructed using the protocol that employs λ Red recombinase to engineer in-frame deletions as described elsewhere (Datsenko and Wanner, 2000).

Table 1. Strains and plasmids used in this study.

Strain	**Relevant genotype[1]**	**Reference/source[2]**
***S.* Typhimurium[3] strains**		
JE6583	*metE205* [?]*araB9*	K. Sanderson
Derivatives of JE6583		
JE22070	/ pCV1 (cloning vector) bla^+	(VanDrisse and Escalante-Semerena, 2016)
JE21107	*pagR1*::kan^+	
JE24887	*[?]pagR2* resolved	

JE27072	*pagR3*::*lacZY*$^{+}$ *kan*$^{+}$	
JE25373	*tktDE1*::c*at*$^{+}$	
JE25443	*pagR1*::*kan*$^{+}$ Δ*tktDE1*::c*at*$^{+}$	
JE21566	*pagR1*::*kan*$^{+}$ / pCV1 *bla*$^{+}$	
JE21577	*pagR1*::*kan*$^{+}$ / pPagR-7 *bla*$^{+}$	
JE25521	*pagR*$^{+}$ *tktDE1*::*cat*$^{+}$ / pCV1 *bla*$^{+}$	
JE25523	*pagR1*::*kan*$^{+}$ *tktDE1*::c*at*$^{+}$ /pCV1 *bla*$^{+}$	
JE25524	*pagR1*::*kan*$^{+}$ *tktDE1*::c*at*$^{+}$ / pTktC-1 *bla*$^{+}$	
JE27201	*pagR1*::*kan*$^{+}$ *tktDE1*::c*at*$^{+}$ / pTktA-1 *bla*$^{+}$	
JE27202	*pagR1*::*kan*$^{+}$ *tktDE1*::c*at*$^{+}$ / pTktB-1 *bla*$^{+}$	
JE27091	*pagR3*::*lacZY*$^{+}$ *kan*$^{+}$ / pCV1 *bla*$^{+}$	
JE27092	*pagR3*::*lacZY*$^{+}$ *kan*$^{+}$ / pPagR-7 *bla*$^{+}$	
JE6692	/ pKD46 *bla*$^{+}$	Laboratory collection
***E. coli* strains**		
E. coli C41 (λDE3)	*pka12*::*kan+ ompT hsdS* ($r_B m_B$) *gal* (λDE3)	Laboratory collection
E. coli DH5α	[?]*(argF-lacZ)169 [?]phoA8* l^{-}f80d*lacZ58*(M15) *thiE1* *glnX44*(AS) *deoR481 hsdR17* *gyrA96*(Nal^R) *recA1 endA1*	Laboratory collection

[1]In strains JE22070 and JE6692 the genotype preceding the forward slash is: *metE205* [?]*araB9.*

[2]Unless otherwise indicated, all strains used in this study were generated during the work.

[3]*S.* Typhimurium = *Salmonella enterica*subsp. *enterica* sv. Typhimurium strain LT2.

Deletion strain construction. Primers used in this study were synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA), and are listed in Table 2. The*pagR1* ::*kan* $^{+}$ and*tktDE1* ::*cat* $^{+}$ markers were engineered as follows: PFU Ultra II Fusion DNA polymerase (Stratagene) was used to amplify flanking regions of plasmids pKD3 (*cat* $^{+}$marker) or pKD4 (*kan* $^{+}$ marker) using primers designed with 36 bp of overlapping region to the beginning or the end of*pagR* or *tktD-tktE* genes. Polymerase chain reaction (PCR) amplicons were visualized by post-staining with ethidium bromide (0.5 mg /mL) for 10 min. Products were cleaned with the Wizard? SV gel and PCR clean up kit (Promega), and ˜200 ng of PCR product was electroporated into *S.* Typhimurium strain JE6692 (*metE205*[?]*araB9* / pKD46 *bla* $^{+}$) using a 0.2-cm electroporation cuvette (MidiSci) and a microPulser electroporator (Bio-Rad Laboratories) on Ec2 setting. Cells were recovered by incubation at 37 ^{o}C with shaking and plated on lysogeny broth (LB, Difco) agar supplemented with either 12.5 μg/mL of chloramphenicol for the *cat* $^{+}$ marker or 25 μg/mL of kanamycin for the *kan* $^{+}$ marker. Correct antibiotic insertions were PCR verified then moved by P22-mediated transduction into strain JE6583 as described elsewhere (Davis et al., 1980). Colonies were streaked to isolation and the genomes of isolated colonies were sequenced using Sanger sequencing technology to verify correct insertion location and sequence using primers flanking genes of interest.

Construction of *pagR3::lacZY* chromosomal fusion strain.The kanamycin resistance cassette was resolved from strain JE21107 (*pagR1* ::*kan* $^{+}$, yielding Δ*pagR2*) using pCP20 and a method described elsewhere (Datsenko and Wanner, 2000). The *lacZY*$^{+}$ fusion was created by FLP mediated integration of

pKG136 (Ellermeier et al., 2002). Briefly, pCP20 was transformed into the resolved Δ*pagR* strain (JE24887). Plasmid pKG136 was transformed into the resulting strain and cells were recovered overnight in LB at 37 ºC. The next morning cells were plated on LB agar with 50 μg/mL kanamycin to select for integration. Correct*lacZY*$^{+}$ *kan*$^{+}$ insertions were PCR verified then moved by P22-mediated transduction into strain JE6583 using a described protocol (Davis et al., 1980).

Table 2. Primers and DNA probes used in this study[1].

Primer Name	**Primer Sequence 5' 3'**
EMSA primers	**EMSA primers**
5'_*pagR*_EMSA (5' 6-FAM label)	CGCAAATTTCCAGCGCCTGCG
3'_*pagR*_EMSA (5' HEX label)	TACACCGGCAAGTTGGGCTAC
Deletion, Cloning, and RT-qPCR primers	
5'_*pagR*_wanner	ATGTCGATACCCCGTAAACGGCGCAGTA CCGGTAAAGTGTAGGCTGGAGCTGCTTC
3'_*pagR*_wanner	TTAGCAGCTTTCCCGCATACATAAACTG GAGGCGATCATATGAATATCCTCCTTAG
5'_*pagR*_BspQI	NNGCTCTTCNTTCATGTCGATACCCCGTAAACG
3'_*pagR*_BspQI	NNGCTCTTCNTTATTAGCAGCTTTCCCGCATAC
5'_*tktD*_BspQI	NNGCTCTTCNTTCATGAACGTAACCGAAA
3'_*tktE*_BspQI	NNGCTCTTCNTTATTAAAGCAGTGATTTCG
5'_*tktA*_BspQI	NNGCTCTTCNTTCATGTCCTCACGTAAAGAGCTTG
3'_*tktA*_BspQI	NNGCTCTTCNTTATTACAGCAGTGCTTTCGCTT
5'_*tktB*_BspQI	NNGCTCTTCNTTCATGTCCCGTAAAGACCTTGC
3'_*tktB*_BspQI	NNGCTCTTCNTTATTAGCCTTTGATGTTTAGCACC
5'_*tktD*_wanner	TCACCCAACTGGCGCGCGATATTCGCGT CGCCACGCGTGTAGGCTGGAGCTGCTTC
3'_*tktE*_wanner	TTTCGCGGCTTCTACAATCGCTTCGGCGG TGAGTCCCATATGAATATCCTCCTTAG
5'_*stm2343*_qPCR	GTTCCGGCCTGGGTAGTAGTT
3'_*stm2343*_qPCR	CCGATGAAAGATCGGAGTGT
5'_*tktD*_qPCR	CGATGATATCGCCGGACTAC
3'_*tktD*_qPCR	CCTCATTGAGCGTCTGCTTC
5'_*tktE*_qPCR	CTATCGATCGCATGTTGGTG
3'_*tktE*_qPCR	CTGCCCATAACGCTCTTTGA
Operon PCR Primers	
5'_operon PCR_1	AGCGCCATAAAGAGCTGA
3'_operon PCR_1	CGATGATATCGCCGGACTAC
5'_operon PCR_2	TATTCAGCTCTTCGCGCG
3'_operon PCR_2	TCCTGCCAGTGCTGTTAC
5'_operon PCR_3	ACATACCCCCTAGCGG
3'_operon PCR_3	GTTCCGGCCTGGGTAGTAGTT
5'_operon PCR_4	CCGATGAAAGATCGGAGTGT
3'_operon PCR_4	TTGGGCCATATTATGTATTAGCG
DNase 1 Footprinting Primers	
5'_sequencing template	GGTACTGCGCCGTTTAC
3'_sequencing template	GAACAATCGCCGTAAGGTATTC
5'_digestion/sequencing (5' 6-FAM label)	CGCAAATTTCCAGCGCCTGCG (same as 5'_*pagR*_EMSA)
3'_digestion/sequencing (5' HEX label)	TGTCGTTCTTTGCTTCGCATGAC

Plasmid construction for complementation of function and gene overexpression. All plasmids used here are listed in Table 3.

Table 3. Plasmids used in this study

Plasmid	Genotype	Description	Refere
pCV1	*araC*$^+$ *bla*$^+$	P$_{araBAD}$ expression vector	(VanD
pTEV19	*bla*$^+$	Overexpression vector for MBP-H$_6$- *N*-terminally tagged rTEV-cleavable protease	(VanD
pPagR7	*pagR*$^+$ *bla*$^+$	*pagR*$^+$ (*stm2345*$^+$) cloned into pCV1	
pTktC1	*tktD*$^+$ *tktE*$^+$ *bla*$^+$	*tktD*$^+$ and *tktE*$^+$ cloned into pCV1	
pTktA1	*tktA*$^+$ *bla*$^+$	*tktA*$^+$ cloned into pCV1	
pTktB1	*tktB*$^+$ *bla*$^+$	*tktB*$^+$ cloned into pCV1	
pPagR-8	*pagR*$^+$ *bla*$^+$	*pagR* $^+$ *(stm2345*$^+$*)* cloned into pTEV19	
pCP20	*flp*$^+$ *bla*$^+$ *cat*$^+$	FLP expression plasmid, temperature sensitive above 37 °C	(Chere
pKG136	*lacZY*$^+$ *kan*$^+$	For FLP integration of *lacZY*$^+$ *kan*$^+$ cassette at FLP-resolved deletion sites	J. M. S

The high-efficiency cloning method of BspQI digestion described elsewhere (Galloway et al., 2013, VanDrisse and Escalante-Semerena, 2016) was used to clone *pagR* into plasmids pCV1 and pTEV19, and*tktD-tktE* , *tktA* and *tktB* into pCV1. All genes cloned were PCR amplified from *S.* Typhimurium strain JE6583 genomic DNA using PFU Ultra II Fusion DNA polymerase and PCR products were visualized as described above. PCR products were cleaned using the Promega Wizard? SV gel and PCR clean up kit, and cloning using the method cited above. After transformation into *E. coli* DH5α cells and colony PCR screening of correctly ligated plasmids, plasmids were isolated using the Wizard? Plus SV miniprep kit (Promega). Sanger DNA sequencing was performed by Eton Bioscience to rule out mutations and confirm insertions.

Culture media, chemicals, and *in vivo* growth analyses.Growth behavior studies were conducted as follows: i) starter bacterial cultures were grown from a single colony in nutrient broth (NB, Difco) with overnight shaking at 180 rpm at 37 ºC; ii) after ˜16 h of growth, cells (˜10^8 cfu) were sub-cultured into no-carbon essential (NCE) minimal medium (Berkowitz et al., 1968) containing $MgSO_4$ (1 mM), Wolfe's trace minerals (Balch and Wolfe, 1976), ampicillin (100 µg/mL), L-(+)-arabinose (concentration stated in figure legends), with sodium succinate (30 mM) as the sole carbon and energy source. Inoculum used was routinely 1% (v/v) of the final volume of the culture. Growth studies were performed in 96-well polystyrene (Falcon) microtiter plates with each well containing 198 µL of fresh medium and a 2-µL inoculum. Microtiter plates were incubated at 37 ºC shaking continuously inside a PowerWave microtiter plate reader (Bio-Tek Instruments). Density of cells was monitored at 630 nm and data were analyzed using Prism 9 (GraphPad).

RNA isolation. Strains JE22070 (*pagR* $^+$/ vector), JE21566 (*pagR1* ::*kan*$^+$ / vector), JE21577 (*pagR1* ::*kan*$^+$ / pPagR) were grown overnight in triplicate in nutrient broth (2 mL; NB, Difco) with shaking at 37 ºC. After incubation, cultures were diluted 1:100 into 5 mL of fresh lysogeny broth rich medium supplemented with L-(+)-arabinose (100 µM). Cultures were grown shaking at 37 ºC to an optical density of 0.5 at 600 nm, then 5 mL of each sample were quickly centrifuged in 15-mL Falcon tubes at 4000 x *g* , supernatant was removed, and pellets were flash-frozen in liquid nitrogen and kept on dry ice. RNA was isolated following the RNAsnap^{TM}protocol (Stead et al., 2012). Pellets were re-suspended in 150 µL of boil solution (ethylenediaminetetraacetic acid (EDTA, 18 mM), SDS (0.025%, w/v) formamide (95%, v/v; RNA grade), 2-mercaptoethanol (1% v/v) in RNase-free water) and were vortexed vigorously to break up the cell pellet. Pellets were incubated at 95 ºC for 7 min and centrifuged at 16,000 x *g* for 5 min at room temperature; 100 µL of supernatant was transferred to a fresh tube. A sodium acetate/ethanol RNA precipitation was then conducted by the addition of 400 µL of RNase-free water, 50 µL of sodium acetate (3 M, pH 5.2: final concentration of 0.3 M), and finally 1.65 mL of ice-cold absolute ethanol (100%), with mixing briefly before the addition of the next reagent. The mixture was incubated at -80 ºC for one hour, centrifuged at 16,000 x *g* for 30 min at 4 ºC, and ethanol was decanted. Ethanol (300 µL of cold 70% v/v) was added, and pellets were centrifuged at 8,000 x *g* for 5 min at 4 ºC in an Eppendorf 5415D centrifuge. Ethanol was removed and pellets

were allowed to dry. RNA pellets were re-suspended in RNase-free water. Subsequent RNase-free DNase I treatment was conducted using the Ambion Turbo DNA-free kit according to manufacturer's instructions (ThermoFisher Scientific). After DNA cleavage, a final sodium acetate/ethanol precipitation was performed as described above, except using 360 µL of water, 50 µL of 3 M sodium acetate, and 1.5 mL of cold 100% ethanol. After overnight incubation at -80 ºC, RNA was centrifuged at 16,000 x *g* for 30 min, then washed with 300 µL of cold 70% ethanol (v/v). Ethanol was decanted, and RNA pellets were dried for 20 min at room temperature. RNA was resuspended in 100 µL of water. A small sample of each preparation was used for quantification with the RNA Broad Range (BR) Assay kit by Qubit on a Qubit 4 fluorometer. A small amount of each preparation was also tested for quality and integrity using the Qubit RNA IQ Assay. Primers for qPCR were designed using Primer3 (Untergasser et al., 2012, Koressaar and Remm, 2007, Koressaar et al., 2018)

cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA (972 ng) from each sample was used for the synthesis of cDNA using the iScript^{TM} cDNA synthesis Kit from Bio-Rad Laboratories according to manufacturer's protocol. Each cDNA reaction was then diluted to 7.5 ng/µL and used as template for PCR. For real-time PCR, 20 µL reactions were prepared with 10 µL of 2X FastSYBR Green master mix (Applied Biosystems), 500 nM of each gene-specific primer (1 µL of 10 µM primer stock), and 15 ng of cDNA (2 µL of 7.5 ng/µL cDNA). The real-time PCR reaction was performed using a 7500 Fast real-time PCR system (Applied Biosystems). The threshold cycle value of *gyrB* were checked first to ensure it was optimal for use as reference genes for these strains under the conditions chosen for RT-qPCR. Cycle threshold (C_T) data were normalized to the *gyrB* gene. These normalized values ([?]C_T) were transformed using the 2(e-[?]C_T)/10^{-6} method (Livak and Schmittgen, 2001), and were reported as the gene expression ratio (2ˆ[?][?]C_T) of the mutant strains/the parent strain (JE22070 *pagR* $^+$). Mean 2ˆ[?][?]$C_T$values were used to calculate the standard error of the mean (SEM) using Prism9 (GraphPad) from three biological replicates that were each tested in technical triplicate. Differences in 2ˆ[?][?]$C_T$between strains were compared using Welch's *t* -test with Prism9 software.

β-Γαλαχτοσιδασε ασσαψς. Plasmids pCV1 and pPagR7 were independently transformed into JE27072 (*pagR* ::*lacZY*$^+$ *kan*$^+$). Three independent colonies of each strain were grown overnight in 2 mL of NB plus ampicillin (100 µg/mL), then sub-cultured 1:100 into 5 mL of LB plus ampicillin (100 µg/mL) and arabinose (100 µM). Cells were grown shaking at 180 rpm at 37C until an OD_{600} nm of 0.4-0.6, and β-galactosidase units were measured as described (Miller and Hershberger, 1984).

Operon PCR. As described above, total RNA was isolated from strain JE21107 (*pagR1* ::*kan*$^+$) and was used to generate cDNA. cDNA and genomic DNA isolated from strain JE21107 were used in PCR reactions containing Green GoTaq (Promega) master mix to amplify overlapping genes within the *stm2340* -*stm2344* operon. Primer pairs are listed in Table S2.

Purification of PagR protein. PagR protein was purified to homogeneity from plasmid pPagR-8 encoding a PagR protein with a maltose binding protein-hexahistidine (MBP-H6) tag fused to its N terminus. The tag was removed after incubation with recombinant tobacco etch virus (rTEV) protease since the plasmid used to produce MBP-H_6-PagR (pTEV19) contained an rTEV protease cleavage site (VanDrisse and Escalante-Semerena, 2016). A sample (10 mL) of an overnight culture of *E. coli* C41 (λDE3) / pPagR-8 strain was used to inoculate one liter of lysogeny broth containing 100 µg/mL of ampicillin. Cells were grown shaking at 125 rpm at 37 °C until the culture reached an optical density at 600 nm of 0.7. Expression of genes of interest encoded by the plasmids was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.25 mM) followed by ˜12 h of overnight incubation at 37 °C. The next morning cells were harvested by centrifugation at 6,000 x *g* for 15 min using a refrigerated Beckman-Coulter Avanti J-20-XPI centrifuge equipped with a JLA 8.1 rotor. Cell pellets were resuspended in 20 mL of buffer A [(4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM, pH 7.5 at 4 ºC) containing NaCl (0.5 M), glycerol (20% v/v), and imidazole (20 mM)] and were sonicated thrice for 30-s intervals, and during each interval, sonication was on for 2 s and off for 2 s, at 60% amplitude. The resulting whole-cell lysates were centrifuged for 30 min at 40,000 x *g* and the supernatants were filtered with a 0.45-µm filter (VWR) to remove large

particulates. Each filtered lysate was applied onto a 1-mL nitrilotriacetic acid (NTA) affinity chromatography column pre-equilibrated with buffer A. Fractions were collected by gravity at 4 °C. The purification was performed as follows: After all the lysate was loaded onto the column, the column was washed with 10 column volumes (CV, *i.e.,* 10 mL) of buffer A, seven CV (*i.e.,* 7 mL) of buffer A containing 4% elution buffer B [HEPES buffer (50 mM, pH 7.5 at 4 ºC), NaCl (0.5 M), glycerol (20% v/v), and imidazole (0.5M)], and finally, MBP-H_6-PagR was eluted in two fractions, first with one CV (*i.e.,* 1 mL) of 100% elution buffer B, and the second fraction being four CV (*i.e.,* 4 mL) of 100% elution buffer B. Both elution fractions were pooled and MBP-H_6-PagR was cleaved with rTEV protease at a 1 mg 1:100 rTEV:PagR protein ratio while dialyzing at room temperature in HEPES buffer (50 mM, pH 7.5 at 4 ºC) containing NaCl (0.5 M), glycerol (10% v/v), and dithiothreitol (DTT, 1 mM). Cleaved protein was dialyzed twice more at 4 °C in buffer A. Cleaved PagR protein was loaded again onto a 2-mL NTA column to remove MBP-His and rTEV protease, both of which were fused to a hexahistidine tag. Tag-less PagR protein did not interact with the NTA resin and was collected in the flow-through fraction. To further remove MBP from PagR, flow-through fractions from the second NTA purification were run over a 1-mL amylose resin to remove contaminating MBP. Pure, tag-less PagR protein was dialyzed overnight against HEPES buffer (50 mM, pH 7.5 at 4ºC) containing NaCl (150 mM) and glycerol (20% v/v) at 4@C. Fifteen mL of dialyzed PagR solution was dispensed into Eppendorf microcentrifuge tubes, flash frozen in liquid nitrogen and stored at -80@C until used. Protein concentration was determined using the Qubit™ Protein Assay Kit (ThermoFisher) and the Qubit™ 4 fluorometer.

Electrophoretic mobility shift assays (EMSAs). EMSAs were performed to quantify PagR binding to DNA. EMSAs were performed as follows: purified PagR protein was incubated at 0, 0.5, 1.0, 2.5, 5.0 and 10.0 pmol of protein with 0.5 pmol of a 5(6)-carboxyfluorescein (5(6)-FAM) and hexachlorofluorescein (HEX)-labeled DNA probe (1: from 2,457,048 to 2,457,454 nt of the chromosome; 406 nt, 2: from 2,457,048 to 2,457,339 nt of the chromosome; 291 nt). EMSA buffer [HEPES buffer (50 mM, pH 7.5 at 4ºC) containing NaCl (150 mM), and glycerol (10% v/v)] was added to the reaction mixture (total volume = 25 µL) and DNA and protein were incubated at room temperature for 40 min. During incubation, a 7.5% Tris-Boric acid-EDTA (TBE) polyacrylamide gel was pre-developed at 100 V for 40 min in 0.5X TBE buffer at 4@C. After incubation, 5 µL of glycerol (50% v/v) was added to the reaction mixtures, and 20 µL of each reaction mixture was resolved by the polyacrylamide gel. A lane of xylene cyanol and bromophenol blue dye was added as a tracking indicator, and the gel was run until bromophenol blue reached the bottom of the gel. The gel was imaged using a Typhoon Trio Imager (GE Healthcare) at 525 nm with the 488 (Blue) filter.

DNase I footprinting. The promoter region of *stm2344* and*pagR* (from 2,457,048 to 2,457,339 nt of the chromosome; 291 nt) was PCR amplified using a 5(6)-FAM-labeled primer and a HEX-labeled primer from JE6583 genomic DNA. The product was purified with the Wizard? SV Gel and PCR Cleanup System (Promega). 7.5 pmol of 5(6)-FAM/HEX-labeled probe was incubated with either no PagR protein or 75 pmol PagR for 40 min at room temperature in 250 µL EMSA Buffer. Twenty-five ng of DNase I (Sigma) was added to each reaction and incubated for 5 min at room temperature. DNase was heat inactivated at 80°C for 10 min. Digested fragments were purified with the Wizard? SV Gel and PCR Cleanup System (Promega), eluted from the column with 30 µL diH_2O, and diluted 1:2 in diH_2O for analysis. Fragment analysis by capillary electrophoresis was performed at the University of Illinois DNA Core Sequencing facility using the Applied Biosystems 3730xl DNA Analyzer. The results were processed with GeneMapper6 and aligned to the sequencing results to determine the protected region(s).

Dideoxy Sanger sequencing. The promoter region of*stm2344 / pagR* (from 2,456,991 to 2,457,412 nt of the chromosome; 421 nt) was PCR amplified from JE6583 genomic DNA and purified with the Wizard? SV Gel and PCR Cleanup System (Promega) to create a template for dideoxy termination sequencing. This template was sequenced using the USB® ThermoSequenase Cycle Sequencing Kit (Affeymetrix) with the HEX and 5(6)-FAM labeled primers used to generate the digested fragment. Sequencing was performed following the manufacturer's instructions for dideoxy termination sequencing using 2pmol of primer, 200ng template, and 60 cycles for each reaction. Samples were diluted 1:2 in deionized water and analyzed at the University of Illinois DNA Core Sequencing facility using the Applied Biosystems 3730xl DNA Analyzer.

The resulting electropherograms were analyzed with GeneMapper Software 6 (Thermo Fisher Scientific).

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Conflict of interest statement. The authors have no conflict of interest to declare.

Data availability. All the data generated by these studies is reported in this paper and its Supplementary Material file.

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Supporting Information

File Name	Description
Figure S1, 44.2MB tif file	**PagR purified to >95% homogeneity.** PagR was purified as described in *Experimental p*
Figure S2, 53.6 MB tif file	**PagR generates the same banding pattern when assayed against two probes of th**
Figure S3, 113.8 MB tif file	**Identification of PagR binding sites in the promoter region of the *stm2344-stm23***