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YmoA Negatively Regulates Expression of Invasin from *Yersinia enterocolitica*

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***inv* encodes invasin, which is the primary invasion factor of *Yersinia enterocolitica*. *inv* expression in vitro is regulated in response to temperature, pH, and growth phase. In vitro, *inv* is maximally expressed at 26°C and repressed at 37°C at neutral pH but, when the pH of the media is adjusted to 5.5, levels of *inv* expression at 37°C are comparable to those at 26°C. A previous genetic screen for regulators of *inv* identified RovA, which was found to be required for activation of *inv* in vitro under all conditions tested as well as in vivo. Here we describe a screen that has identified a negative regulator of *inv* expression, *ymoA*. The *ymoBA* locus was identified by transposon mutagenesis as a repressor of *inv* expression in vitro at 37°C at neutral pH. This mutant shows increased *inv* expression at 37°C. The mutant can be fully complemented for *inv* expression by a plasmid expressing *ymoA*. These results indicate that YmoA plays a role in the negative regulation of *inv*.**

Yersinia enterocolitica is a gram-negative human pathogen capable of colonizing the gastrointestinal tract. Once the organism establishes itself within a host, it is able to cause a variety of syndromes including enterocolitis, mesenteric lymphadenitis, and terminal ileitis. *Y. enterocolitica* is normally acquired through ingestion of contaminated food or water, with swine serving as a major reservoir for strains pathogenic to humans (5).

Colonization of the intestinal epithelium first requires that the organism be able to survive the gastric barrier of the stomach. Once in the small intestine, the organism is able to adhere to specialized cells in the small intestine called M cells (14, 16). *Y. enterocolitica* is able to transverse these cells, ending up in the underlying lymphoid tissue (Peyer's patches), where it is able to replicate and spread to the mesenteric lymph nodes (7, 38).

Y. enterocolitica contains a variety of adherence and invasion factors to aid the bacterium in establishing an infection. The major adhesion and invasion molecules identified in *Y. enterocolitica* are invasin, which is the primary invasion factor (38) and which initiates cell penetration by binding to a subset of β_1-integrins (19) found on the surface of M cells (8), YadA, which is believed to be involved in adherence to intestinal brush border membranes as well as mucus (24, 35, 39), and Ail, which is involved in adherence and invasion as well as the promotion of serum resistance (4, 27, 28, 40, 45).

Invasin is an outer membrane protein found in *Y. enterocolitica* and *Yersinia pseudotuberculosis* (18, 19, 28, 37). When expressed in *Escherichia coli*, invasin is sufficient to promote invasion of tissue culture cells (18, 28). *inv* mutants do not colonize host tissues as well as wild-type bacteria at early time points. However, *inv* mutants eventually colonize to wild-type levels, presumably due to YadA and possibly Ail (38, 39). *inv* is maximally expressed in late exponential to early stationary phase in vitro and is regulated in response to changes in growth conditions. When cultures are grown at 26°C at neutral pH, *inv* expression is activated. In contrast, cultures grown at 37°C at neutral pH repress *inv* expression. However, when the pH of the media is adjusted to 5.5, levels of *inv* expression at 37°C are comparable to those observed at 26°C (36).

A previous genetic screen for regulators of *Y. enterocolitica* *inv* identified RovA (41), which was found to be required for activation of *inv* in vitro under all conditions tested. Subsequently, a homologue of RovA was identified as the regulator of *inv* in *Y. pseudotuberculosis* (44). Comparing *inv* expression in wild-type *Y. enterocolitica* to *inv* expression in a *rovA* mutant showed robust expression of *inv* in the wild type and little to no expression in the *rovA* mutant. Additionally, Western blot analysis of invasin in Peyer's patches from mice 2 days postinfection demonstrated that RovA is also required for in vivo expression of *inv* (41). However, preliminary analysis of the *inv* promoter suggested there may also be a negative regulator of *inv*. In this study we describe a screen that has identified YmoA as a negative regulator of *inv* expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The "v" designation refers to strains harboring the virulence plasmid; "c" refers to strains which have been cured of the virulence plasmid. Bacterial cultures were grown in Luria-Bertani (LB) broth at 26 or 37°C. When appropriate, antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 (for growth of *E. coli*) and 12.5 μg ml^{-1} (for growth of *Y. enterocolitica*); kanamycin, 50 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; streptomycin, 50 μg ml^{-1}.

Strain and plasmid construction. To construct the *inv-gfp* transcriptional fusions, *gfp* was amplified from pKENmut2 (9) with primer GFP#1, which has a *Sac*II linker (5′-TCC CCG CGG GGA AAG AAG GAG ATA TAC ATA TGA GT-3′), and primer GFP#2, which has a *Sac*I linker (5′-CGA GCT CGT ATT TGT ATA GTT CAT CCA TGC C-3′). The amplified fragment contained a ribosome binding site and the coding region of the *gfp* gene. The PCR product

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
Y. enterocolitica strains[a]		
JB580v	Serogroup O:8 Nal^r Δ*yenR*(r$^-$ m$^+$)	20
JB580c	Serogroup O:8 Nal^r Δ*yenR*(r$^-$ m$^+$)	20
JB41v	*inv′-′phoA inv*$^+$ Nal^r Cm^r	1
YVM567c	*inv′-′phoA inv*$^+$ *ymoB*::mTn*5*Kn2 Nal^r Cm^r	This work
YVM923c	Δ*ymoB* Nal^r	This work
YVM967c	Δ*ymoB inv′-′phoA inv*$^+$ Nal^r Cm^r	This work
YVM976c	*ymoB*::mTn*5*Kn2 Nal^r	This work
YVM1009c	*rovA-lacZ rovA*$^+$ Nal^r Cm^r	This work
YVM1010c	*ymoA-lacZ ymoB*$^+$ Nal^r Cm^r	This work
YVM1012c	*ymoB*::mTn*5*Kn2 *ymoA-lacZ* Nal^r Cm^r	This work
YVM1013c	*ymoB*::mTn*5*Kn2 *rovA-lacZ rovA*$^+$ Nal^r Cm^r	This work
Plasmid		
pBAD33	Expression vector controlled by *araBAD* promoter, Cm^r	15
pCR2.1	TA cloning vector, Kn^r Amp^r	Invitrogen
pHG329	pUC19 polylinker, pBR322 *ori*, Amp^r	43
pKENmut2	*gfp* vector, Amp^r	9
pSR47s	Suicide plasmid with *sacB* as a counter-selectable marker, Kn^r	25
pUTminiTn*5*Kn2	Transposon vector, Km^r	17
pWKS30:StrSpec	pWKS30, which has a Str^r-Spc^r cassette in the *Hind*III site of the polylinker, Amp^r Str^r Spc^r	Laboratory collection
pWKS30	cloning vector, Amp^r	46
pBY1	pCR2.1 with *gfp* mut2 from pKENmut2, Amp^r	This work
pBY8	pWKS30::Str^r-Spc^r with *gfp* mut2 from pBY1, Amp^r Str^r Spc^r	This work
pBY9	pGEM5 with 600-bp *inv* promoter (−415 to +170 of coding region), Amp^r	This work
pBY10	pBY8 with 600-bp *inv* promoter from pBY9, Amp^r Str^r Spc^r	This work
pBY11	pCR2.1 with 215-bp *inv* promoter (−69 to +170 of coding region), Amp^r	This work
pBY12	pBY8 with 215-bp *inv* promoter from pBY11, Amp^r Str^r Spc^r	This work
pBY34	pHG329 containing *ymoBA* from 11F3, Amp^r Kn^r	This work
pELL13	pBAD33 containing Δ*ymoB* and *ymoA* and upstream promoter region, Cm^r	This work
pELL16	pFuse carrying sequence just upstream of *rovA*, Cm^r	This work
pELL21	pWKS30::Str^r-Spc^r carrying Δ*ymoB* and *ymoA* and upstream promoter region, Cm^r	This work
pKN8	Suicide *lacZYA* reporter vector pFUSE with *Bgl*II linker cloned into *sma*I site, Cm^r	Laboratory collection
pKN38	pSR47S carrying upstream and downstream regions of *ymoB*, Kn^r	This work
pKN46	pKN8 carrying sequence just upstream of *ymoA*, Cm^r	This work
pKN47	pWKS30:Str^r-Spc^r carrying *ymoBA* and upstream promoter region, Cm^r	This work
pREV2	pCR2.1 carrying *inv* promoter region −415 to +170	Laboratory collection

[a] All strains listed are derivatives of strain JB580.

was initially subcloned into the TA cloning vector pCR2.1 (Invitrogen) to generate pBY1. Subsequently, the *Sac*II/*Sac*I *gfp* fragment was subcloned into the *Sac*II/*Sac*I sites of pWKS30:StrSpec (46) to generate pBY8. To generate the *inv-gfp* transcriptional fusion, the full-length *inv* promoter region −415 to +170 (36) was subcloned from pREV2 via the 5′ *Bam*HI site and the 3′ *Not*I site into pBY8 to generate pBY10. Primer inv#3 (5′-GCA TTT CAT TTG TCA TTG C-3′) and primer inv#1 (5′-CCG ATC GAT AAT ATT AGC C-3′) were used to amplify the 215-bp (−69 to +170) *inv* promoter fragment, which was cloned into pCR2.1 to generate pBY11. The 215-bp fragment was then subcloned into pBY8 via 5′ *Bam*HI and 3′ *Not*I sites within the polylinker of pCR2.1, generating pBY12. Plasmids were confirmed by sequencing.

To complement the mutation in YVM567c, plasmids pKN43 and pELL21 were constructed. To generate pKN43, *ymoB* was amplified from JB580v with primer ybaJ-5.2, which contains an *Eco*RI linker (5′-GGA ATT CCG GTG AGC GGA GAA ATG ATT TAT ATT AAT A-3′), and primer ybaJ-3.2, which contains an *Eco*RI linker (5′-GGA ATT CCT CAA TAT AAA AAT AAT ATA GGG AAA CTA TCC-3′). The resulting product was cloned into the *Eco*RI site of pWKS30:StrSpec. pELL21 was constructed by digesting pELL13 with *Eco*RI/*Xba*I; the resulting fragment contained a deletion of *ymoB*, leaving *ymoA* under the control of its native promoter. The fragment was subcloned into the *Eco*RI/*Xba*I sites of pWKS30:StrSpec. Plasmids were confirmed by sequencing.

lacZ transcriptional fusions were generated by cloning PCR fragments into pKN8 (3) and mating recipient (*Y. enterocolitica*) and donor (*E. coli*) strains of bacteria. To construct pKN46, primer ymoA-5L, which contains an *Xba*I linker (5′-GCT CTA GAC ACA TAT ACT CTG TTT AGT AGT TAC GGA ATC-3′), and primer ymoA-3L, which contains a *Bgl*II linker (5′-GAA GAT CTG TTG CCA TAC AGT AGG TGG AAT TTTA TCA T-3′), were used to amplify a 360-bp product that contained most of the *ymoA* gene. The resulting product was digested with *Xba*I/*Bgl*II and cloned into the same sites in pKN8. To generate pELL16, the promoter region (500 bp upstream of the start codon) of *rovA* was amplified from JB580v with primer rovAFusexba, which contains an *Xba*I linker (5′-CGT CTA GAT TCC ACA TCC ACC AAC-3′), and primer rovA-2, which has a *Bam*HI linker (5′-CGC GGA TCC TGC TAA ATC AGA TCC TAA TGT CGA TTC CAA-3′). After amplification the products were digested with *Xba*I/*Bam*HI and cloned into the *Xba*I/*Bgl*II sites in pKN8. Plasmids were confirmed by sequencing.

Strains JB580c (20) and YVM976c were mated with S17-λ*pir* (29) containing either pKN46 or pELL16 to generate chromosomal *lacZ* fusions. Briefly, 400 μl of overnight cultures was mixed and resuspended in 200 μl of LB broth and spotted on LB agar. The plate was incubated overnight at 26°C, and the bacteria were harvested and resuspended in 1 ml of LB broth. Dilutions of 10^{-1} and 10^{-2} were spread on LB agar containing nalidixic acid (to select against the donor *E. coli* strain), and chloramphenicol (to select for the recipient *Y. enterocolitica* strain). Integration was confirmed by PCR with a primer within *lacZ* and a primer 5′ of the homologous region used for recombination.

Green fluorescent protein assays. Cultures of JB580v containing *inv-gfp* transcriptional fusions were grown in 4 ml of LB broth overnight at 26°C with selection and then subcultured to an optical density at 600 nm (OD_{600}) of 0.2 in 3 ml of LB broth. Cultures were grown at 26 or 37°C for 24 h, and levels of fluorescence intensity were compared. Fluorescence intensity was determined with a Beacon 2000 variable-fluorescence polarization system (Panvera). The fluorescence was calculated by dividing the average fluorescence intensity by the

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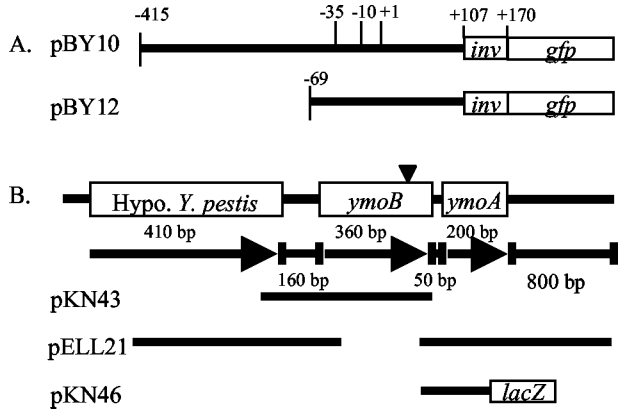

FIG. 1. Schematic of *inv-gfp* promoter fusions and *ymoBA* locus. (A) The predicted −35, −10, and +1 sites, along with the start codon (+107) and fusion junction (+170) are marked on pBY10. (B) The *ymoBA* locus contains a hypothetical *Y. pestis* gene just upstream of *ymoB* and no open reading frames in the first 800 bp downstream of *ymoA*. Arrows indicate the direction of transcription, and numbers of base pairs between the genes are also indicated. Inverted triangle, point of transposon insertion; solid black lines below the locus, regions of the locus cloned into plasmids pKN43 (*ymoB*), pELL21 (*ymoA*), and pKN46 (*ymoA-lacZ*).

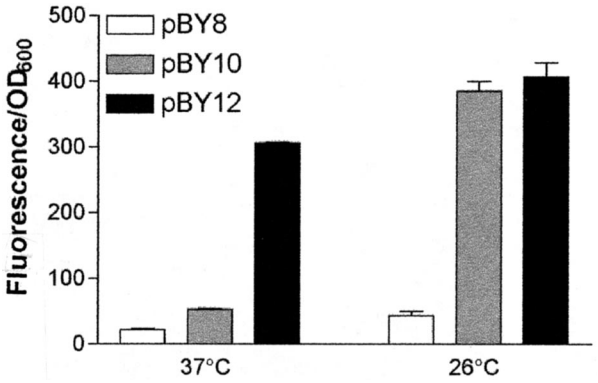

FIG. 2. Effect of promoter truncation on *inv-gfp* expression. Overnight cultures of *Y. enterocolitica* strain JB580v carrying the indicated plasmids were grown in triplicate at 26°C, subcultured to an OD_{600} of 0.2, and grown for 24 h. Fluorescence from the *inv-gfp* fusions was calculated by dividing average fluorescence by the OD_{600}. pBY10 (−415 to +170), which has the full-length *inv* promoter, and pBY12 (−69 to +170), which has a truncated *inv* promoter, were compared. pBY8 is the vector with promoterless *gfp* and was used to determine background levels of fluorescence.

culture OD_{600}. In all cases, JB580v with pBY8 was used to determine background fluorescence.

Screen for negative regulators of *inv*. Strain JB41v (1) was mutagenized with mTn*5*Kn2 by mating with S17-λ*pir* containing pUTmTn*5*Kn2 as previously described (17). To select for mutants that showed increased *inv* expression at 37°C and neutral pH, as shown by their dark blue appearance, plates containing nalidixic acid (to select against the donor *E. coli* strain), chloramphenicol (to select for the recipient *Y. enterocolitica* strain), kanamycin (to select for transposon insertions), and 5-bromo-4-chloro-3-indolylphosphate (XP; 40 μg ml^{-1}; to screen for alkaline phosphatase [AP] activity indicating alterations in *inv* promoter activity) were incubated at 37°C for 2 days. Colonies were then screened for *inv-phoA* expression. Of 300 mutants selected from plates 2, 6B4 and 11F3, showed increased expression of *inv-phoA* at 37°C when tested by a quantitative AP assay.

Southern blotting was performed to determine the sizes of the Kn^r-encoding fragments from mutants 6B4 and 11F3. The blots were probed with the *Eco*RI Kn^r fragment from the pUTminiTn*5*Kn2 plasmid. The 11F3 fragment was cloned into pHG329 (43) by making a subgenomic library and selecting for Kn^r to generate pBY34. However, the 6B4 mutation could not be cloned by this method. Since mutant 6B4 was not malleable to cloning via a subgenomic library, the location of the mutation was determined by Southern blotting using the Kn^r-encoding fragment and *ymoA* as probes and PCR with primers ymo1 (5′-GAA GAT CTT GCT ATT TCA CAT GTT GCC-3′), which anneals to the 3′ end of *ymoA* and reads into *ymoA*, P6 (5′-CCT AGG CGG CCA GAT CTG AT-3′), which anneals to the Tn*5* I repeat and reads away from Tn*5*, and P7 (5′-GCA CTT GTG TAT AAG AGT CAG-3′), which anneals to the Tn*5* O repeat to read away from Tn*5*.

β-Galactosidase assays. Cultures were grown in triplicate overnight in 2 ml of LB broth at 37 or 26°C. β-Galactosidase activity was measured according to previously described methods (26).

AP assays. Cultures were grown in triplicate overnight in 2 ml of LB broth at 37 or 26°C. AP activity was measured according to previously described methods (23).

Nucleotide sequence accession number. The GenBank accession number assigned to the *ymoBA* locus is AY387659.

RESULTS

Truncation of the *inv* promoter results in constitutive expression at 37 and 26°C. To determine if sequences upstream of the −35 region of the *inv* promoter were important for promoter function, a full-length promoter and a truncated promoter were fused to *gfp* (Fig. 1). The plasmids were transformed into JB580v, and levels of expression of the *inv-gfp* fusions at 37 (temperature where the *inv* promoter is repressed) and 26°C (temperature where the *inv* promoter is activated) were compared (Fig. 2). pBY10 (−415 to +170) shows normal regulation at 37 and 26°C, with *inv* expression elevated at 26°C compared to that at 37°C. pBY12 (−69 to +170) shows derepression of the *inv* promoter at 37°C, with levels of promoter activity comparable to those at 26°C. These results suggest the presence of a repressor binding site between positions −415 and −69 on the *inv* promoter.

Identification of a negative regulator of *inv*. Strain JB41v, which contains a translational fusion of *inv-phoA* integrated on the chromosome, was mutated with mTn*5*Kn2. To identify mutants that had altered regulation at 37°C, colonies were screened on indicator plates containing XP, which turns blue in the presence of AP. Colonies that showed normal repression of *inv* appeared light blue at 37°C. Colonies with a mutation in a potential repressor appeared dark blue on the indicator plates due to the increased expression of *inv-phoA*. Eleven independent matings, yielding approximately 28,000 mutants, were performed. Of the 28,000 mutants 300 appeared to have increased *inv-phoA* expression at 37°C. These were further characterized by performing AP assays at 26 and 37°C. Of the 300 mutants, 6B4 and 11F3 showed increased expression of *inv-phoA* at 37°C (data not shown). These two mutants were from independent matings.

To determine the sequences of the mutated genes in mutants 6B4 and 11F3, Southern blotting was performed to identify the size of the Kn^r-encoding fragments in both mutants (data not shown). The fragment from 11F3 was cloned into pHG329, generating pBY34. Sequence analysis of the transposon-chromosome junction in pBY34 indicated that the transposon had inserted just upstream of a previously identified gene, *ymoA*. Because mutant 6B4 was not malleable to cloning via a subgenomic library, the location of the mutation was determined by Southern blot and PCR analysis (data not

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shown). The results indicated that the transposon insertion was in the same general location as, but in the opposite orientation to, that in 11F3. The PCR product for 6B4 obtained with primers P6 and ymo1 was sequenced directly with primer ymo1, and this confirmed that the transposon was inserted 2 bp upstream of, but in the opposite orientation as, the insertion in 11F3.

It was determined by sequencing that both 6B4 and 11F3 contained a mutation in a gene we named *ymoB*. YmoB has homology to a hypothetical protein YbaJ from *E. coli*, which has no known function (64% identity and 82% similarity). Examination of the genetic locus revealed differences between *E. coli* and *Y. enterocolitica*. The sequence upstream of *ybaJ* in *E. coli* contains *acrB*, while the upstream sequence in *Y. enterocolitica* contains a hypothetical *Yersinia pestis* gene of unknown function. *hha* is located just downstream of *ybaJ* in *E. coli*, and further sequencing revealed that *ymoA*, a gene that has been previously shown to influence regulation of *Y. enterocolitica* virulence factors (10), is just downstream of *ymoB*. YmoA and Hha are members of a new class of proteins that regulate genes in response to different environmental conditions (21). This raised the possibility that the insertion in *ymoB* has a polar effect on the expression of *ymoA*, reducing the level of *ymoA* transcription, leading to increased *inv-phoA* expression at 37°C. 6B4 was chosen from the two mutants for further study and was designated strain YVM567c.

***ymoA* is able to restore wild-type *inv-phoA* expression in YVM567c.** To test the ability of *ymoB* and *ymoA* to restore wild-type *inv-phoA* expression in YVM567c, plasmids pKN43 (*ymoB*) and pELL21 (*ymoA*) were transformed into YVM567c, generating strains YVM567c(pKN43) and YVM567c (pELL21). Levels of *inv-phoA* expression from these strains were compared to those from wild-type strains JB41c(pKN43) and JB41c(pELL21). All cultures were grown in LB broth overnight at 26 or 37°C. Strain YVM567c(pKN43) showed levels of *inv-phoA* expression comparable to those of YVM567c, indicating that *ymoB* is not able to complement the mutation in YVM567c. Strain YVM567c(pELL21) showed levels of *inv-phoA* expression comparable to those seen in strain JB41c(pELL21), indicating that *ymoA* alone was able to complement the mutation in YVM567c (Fig. 3). JB41c and YVM567c containing the vector showed no difference in regulation compared to JB41c and YVM567c with no plasmids (data not shown). To further test whether or not *ymoB* was involved in the repression of *inv*, an in-frame *ymoB* deletion mutant was constructed and tested for *inv* expression. The Δ*ymoB* mutant, YVM967c, showed normal levels of *inv* expression compared to JB41c (data not shown). Together these data suggested that the phenotype of loss of *inv* repression observed with the transposon insertion mutation in *ymoB* was probably due to a polar effect on *ymoA* expression rather than loss of *ymoB*. We first tried to make an in-frame deletion of *ymoA* using two different suicide plasmids, but both strategies failed. Generation of an insertional *ymoA* mutant was also unsuccessful. All attempts to construct a mutation in *ymoA* were unsuccessful unless *ymoA* was supplied in *trans* on a plasmid, indicating that *ymoA* is probably an essential gene for this particular strain of *Y. enterocolitica*.

The data in Fig. 3 also suggested that YVM567c has increased *inv-phoA* expression at 26°C compared to JB41c.

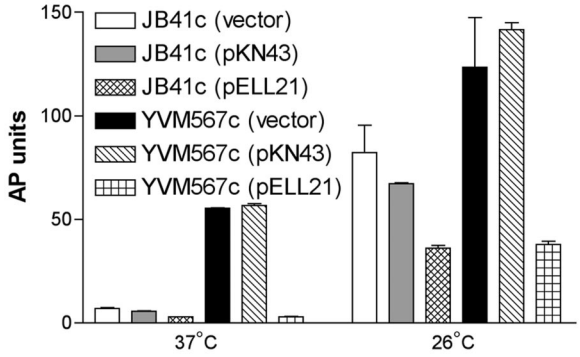

FIG. 3. Effect of complementing YVM567c with *ymoA* and *ymoB*. Cultures were grown in triplicate overnight at 26 or 37°C and assayed for AP activity. All strains have a chromosomal *inv-phoA* fusion to monitor promoter activity. JB41c is used as the wild-type control, YVM567c contains a *ymoB*::mTn*5*Kn2 mutation, YVM567c(pKN43) contains *ymoB*::mTn*5*Kn2 plus *ymoB* complementing clone, and YVM567c(pELL21) contains *ymoB*::Tn*5*Kn2 plus *ymoA* complementing clone.

These results are consistent with previously published reports that show a mutation in *ymoA* derepressed *virF* and the *yop* regulon of the pYV virulence plasmid of *Y. enterocolitica* strain W22711, which is a derivative of W22703, a wild-type O:9 strain, leading to higher levels of expression at both 26 and 37°C (10). Our data show the same type of derepression of *inv* in strain YVM567c, with higher expression of *inv-phoA* at both 37 and 26°C.

mTn*5*Kn2:*ymoB* has a polar effect on the expression of *ymoA*. To determine if the mutation in strain YVM567c has a polar effect on the expression of *ymoA*, chromosomal *lacZ-ymoA* fusions were constructed, generating a merodiploid. The fusion was constructed in strains JB580c and YVM976c, generating strains YVM1010c and YVM1012c, respectively. Cultures were grown in LB broth overnight at 26 or 37°C, and

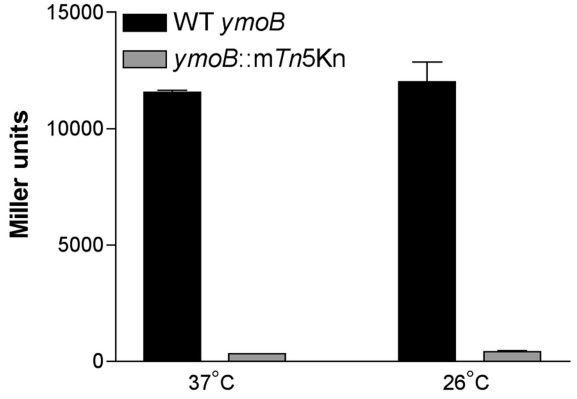

FIG. 4. Effect of *ymoB*::mTn*5*Kn2 on the expression of *ymoA*. Cultures were grown in triplicate overnight at 26 and 37°C and assayed for β-galactosidase activity. Both strains contain a chromosomal *ymoA-lacZ* fusion to monitor promoter activity. YVM1010c (black bars) is used as the wild-type (WT) control and is compared to YVM1012c (gray bars), which contains the *ymoB*::mTn*5*Kn2 mutation.

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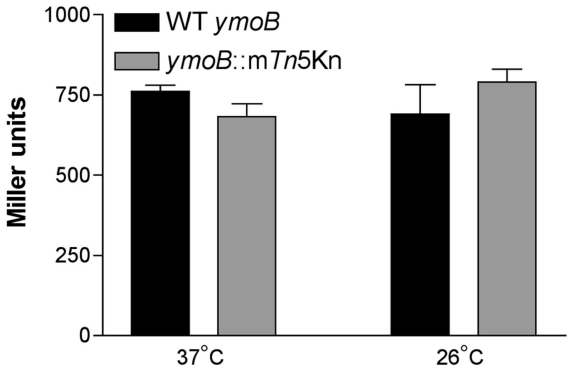

FIG. 5. Effect of *ymoB*::mTn*5*Kn2 on *rovA* expression. Cultures were grown in triplicate overnight at 26 and 37°C and assayed for β-galactosidase activity. Both strains contain a chromosomal *rovA-lacZ* fusion to monitor promoter activity. YVM1009c (black bars) is used as the wild-type (WT) control and is compared to YVM1013c (gray bars), which contains the *ymoB*::mTn*5*Kn2 mutation.

promoter activity was monitored by measuring β-galactosidase activity (Fig. 4). There was greatly reduced β-galactosidase activity in strain YVM1012c, which contains the *ymoB*:mTn*5*Kn2 mutation, compared to that in the wild-type strain YVM1010c. To rule out the possibility that YmoB is required for the expression of the *ymoBA* locus, we complemented YVM1012c with plasmid pKN43 and saw no difference in the expression of *lacZ* compared to that of YVM1012c (data not shown). The vector alone had no effect on expression of *lacZ* in YVM1012c (data not shown). These results reveal that the mutation in strain YVM567c exerts a polar effect on the transcription of *ymoA*. Taken together with previous data, YmoA appears responsible for the derepression of *inv* observed in strain YVM567c.

Increased levels of *rovA* are not responsible for the increase in *inv-phoA* expression seen in strain YVM567c. RovA has previously been shown to be required for the expression of *inv* in *Y. enterocolitica* and *Y. pseudotuberculosis* (30, 41). To rule out the possibility that increased *rovA* expression in strain YVM567c was responsible for the increased levels of *inv* expression, chromosomal *lacZ* fusions to the *rovA* promoter were constructed in strains JB580c and YVM976c, yielding strains YVM1009c and YVM1013c, respectively. Cultures were grown overnight in LB broth at 26 and 37°C, and levels of β-galactosidase activity for the two strains were compared (Fig. 5). The data show no difference in the level of *rovA-lacZ* expression between strains YVM1009c and YVM1013c. These results indicate that the increased *inv* expression observed in YVM567c was not due to increased levels of *rovA* transcription in strain YVM567c.

DISCUSSION

Invasin is an important virulence factor in *Y. enterocolitica*, allowing attachment and efficient translocation of the bacteria through M cells (38). Without *inv* the progress of *Y. enterocolitica* through the infection process is delayed (38). Previous work has shown that *inv* is positively regulated by RovA, a transcriptional regulator in the MarR/SlyA family (41). In this study we have found that YmoA plays a role in the negative regulation of *inv*. Promoter truncation experiments revealed the presence of a negative regulatory sequence in the *inv* promoter involved in temperature control of *inv* expression, and a subsequent genetic screen showed that the *ymoBA* locus was involved in the derepression of *inv* at 37°C. Complementing the mutation in the *ymoBA* locus with *ymoB* and *ymoA* revealed that *ymoA* and not *ymoB* was able to restore wild-type expression of *inv*. A *lacZ* fusion to *ymoA* showed that the mutation in the *ymoBA* locus exerted a polar effect on the transcription of *ymoA*. We have also shown that the mutation in the *ymoBA* locus does not affect the transcription of *rovA*. These results suggest that YmoA plays a negative regulatory role in the expression of *inv*.

YmoA is a member of a growing class of transcriptional regulators with homology to histone-like proteins (21). YmoA previously was shown to be involved in the regulation of Yop proteins and YadA in *Y. enterocolitica* (10). It is not clear why a *ymoA* mutant in the strain W22711 background was viable whereas a *ymoA* mutation in the 8081 strain background appears to be lethal. However, the W22711 mutant was isolated in the O:9 serotype background, and different serotypes of *Y. enterocolitica* are known to have genetic differences (6, 13). It is also possible that the *ymoA* mutant in the W22711 background simultaneously picked up a suppressor mutation. Another member of this family, Hha, has been implicated in the regulation of *hly*, a pore-forming toxin, in *E. coli* (31). It has also been reported that the Hha homologue from *Salmonella enterica* serovar Typhimurium is involved in repressing the invasion phenotype by altering expression of the regulatory gene *hilA* (12). It is interesting that other examples of H-NS-like proteins that regulate virulence factors have been shown to increase expression when the temperature is shifted from 26 to 37°C. This is not the case for *inv*, as expression is downregulated at 37°C and upregulated at 26°C, making regulation of *inv* by YmoA distinct.

Recent studies have shown that Hha from *S. enterica* serovar Typhimurium is able to bind specifically to the *hilA* promoter (12). Other studies on Hha from *E. coli* have shown that Hha is not able to bind the *hly* promoter specifically (33). However, recent work has described the ability of Hha and YmoA to interact with H-NS (32). Subsequent work done on the regulation of the *hly* promoter showed that Hha and H-NS act together to negatively regulate expression of hemolysin, with H-NS providing the binding specificity for the *hly* promoter (22). It has been suggested that H-NS and Hha form a multiprotein complex on the *hly* promoter and repress transcription. YmoA and Hha are functionally interchangeable, suggesting similar regulatory mechanisms (2). Further evidence to support a role for H-NS in the regulation of *inv* comes directly from the sequence of the *inv* promoter, which contains an AT-rich stretch of 30 bp centered on the −35 sequence, raising the possibility that the promoter may be intrinsically bent. It is widely accepted that H-NS binds to bent DNA (11, 42). Based on the data for the regulation of *hly* and the ability of YmoA to interact with H-NS, it is reasonable to speculate that YmoA and H-NS may form a repression complex at the *inv* promoter.

RovA is a member of the MarR family of transcriptional regulators, and has significant amino acid identity with SlyA

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from *S. enterica* serovar Typhimurium (41). When the *slyA* homologue from *E. coli* is overexpressed, it is able to activate *clyA* (cytotoxin) in laboratory strains of *E. coli* (34). It was recently found that H-NS plays a role in silencing expression of this gene in *E. coli* and that SlyA is able to overcome this silencing and activate transcription from the *clyA* promoter (47). Likewise, RovA is able to activate expression of the *inv* promoter, and we hypothesize that it may do so by overcoming the repression by YmoA. It remains to be seen what role if any is played by H-NS in the regulation of *inv*. RovA has been shown to bind the *inv* promoter (30), and it is possible that under some conditions (e.g., 37°C) YmoA or a YmoA–H-NS complex can interfere with RovA binding or alter the interaction of RovA with the transcriptional machinery. Further experiments with purified YmoA and RovA investigating their interaction with the *inv* promoter will provide further evidence for the mechanism by which the *inv* promoter is both repressed and activated.

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