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H-NS Represses *inv* Transcription in *Yersinia enterocolitica* through Competition with RovA and Interaction with YmoA

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Yersinia enterocolitica **is able to efficiently invade Peyer's patches with the aid of invasin, an outer member protein involved in the attachment and invasion of M cells. Invasin is encoded by** *inv***, which is positively regulated by RovA in both** *Y. enterocolitica* **and** *Yersinia pseudotuberculosis* **while negatively regulated by YmoA in** *Y. enterocolitica* **and H-NS in** *Y. pseudotuberculosis***. In this study we present data indicating H-NS and RovA bind directly and specifically to the** *inv* **promoter of** *Y. enterocolitica***. We also show that RovA and H-NS from** *Y. enterocolitica* **bind to a similar region of the** *inv* **promoter and suggest they compete for binding sites. This is similar to recently published data from** *Y. pseudotuberculosis***, revealing a potentially conserved mechanism of** *inv* **regulation between** *Y. enterocolitica* **and** *Y. pseudotuberculosis***. Furthermore, we present data suggesting H-NS and YmoA form a repression complex on the** *inv* **promoter, with H-NS providing the binding specificity and YmoA interacting with H-NS to form a repression complex. We also demonstrate that deletion of the predicted H-NS binding region relieves the requirement for RovA-dependent transcription of the** *inv* **promoter, consistent with RovA acting as a derepressor of H-NS-mediated repression. Levels of H-NS and YmoA are similar between 26°C and 37°C, suggesting that the H-NS/YmoA repression complex is present at both temperatures, while the levels of** *rovA* **transcript are low at 37°C and high at 26°C, leading to expression of** *inv* **at 26°C. Expression of RovA at 37°C results in transcription of** *inv* **and production of invasin. Data presented here support a model of** *inv* **regulation where the level of RovA within the cell governs** *inv* **expression. As RovA levels increase, RovA can successfully compete for binding to the** *inv* **promoter with the H-NS/YmoA complex, resulting in derepression of** *inv* **transcription.**

Yersinia enterocolitica is a gram-negative human pathogen capable of colonizing the gastrointestinal tract. *Y. enterocolitica* is normally acquired through ingestion of contaminated food or water, with swine serving as a major reservoir for human pathogenic strains (4). Invasin is an outer membrane protein found on the surface of *Y. enterocolitica* and *Yersinia pseudotuberculosis* (23, 24, 38, 47) that is responsible for binding to β1-integrins (24) on the apical surface of M cells and initiating uptake of the organism (7, 48). Migration through these cells leads to the accumulation of bacteria in the underlying lymphoid tissue (Peyer's patches) and spread to the mesenteric lymph nodes (6, 18, 20, 48). Once *Y. enterocolitica* establishes an infection, it is able to cause a variety of syndromes, including enterocolitis, mesenteric lymphadenitis, and terminal ileitis (4).

While a considerable amount of work has demonstrated a role for invasin during *Yersinia* infection, less is understood about the mechanisms controlling the expression of the gene encoding invasin (*inv*). Expression of *inv* in *Y. enterocolitica* responds to both temperature and pH (46). *inv* expression is higher at 26°C than 37°C during in vitro growth, with maximal expression occurring during late logarithmic to early stationary phase; however, expression of *inv* at 37°C can be restored to levels comparable to 26°C by adjusting the pH of the medium to 5.5 (46). Presence of invasin has also been shown in the Peyer's patches of infected animals during infection (46, 50).

Two regulators have been identified through genetic screens that affect the expression of *inv*: RovA (41, 50), which is required for *inv* expression, and YmoA (12), which is involved in the negative regulation of *inv*.

RovA is a member of the MarR/SlyA family of regulators and is present in all three pathogenic species of *Yersinia*. Members of this family are widely distributed in nature, with at least 336 putative members in 45 species of bacteria and 13 species of archaea present in the Clusters of Orthologous Groups database (http://www.ncbi.nlm.nih.gov/COG/). Members of this family regulate a wide variety of biological processes, including antibiotic resistance (MarR in *Escherichia coli*) (15), antimicrobial agents (Rap in *Serratia marcescens*), and virulence (SlyA in *Salmonella enterica* serovar Typhimurium) (5, 10, 26, 29). In many cases the mechanism by which these proteins repress or activate gene expression is unknown. However, SlyA appears to activate *hlyE* (also known as *clyA* or *sheA*) transcription in *E. coli* by competing for binding to the *hlyE* promoter with the negative regulator H-NS (60). Specifically, SlyA binds to two sites within the *hlyE* promoter that overlap with H-NS binding site I, suggesting that binding of SlyA may block H-NS binding and relieve H-NS-mediated repression (60). Recently, a similar mechanism was proposed for the regulation of *inv* in *Y. enterocolitica* (11, 12) and further explored in *Y. pseudotuberculosis* (21). In *Y. pseudotuberculosis*, RovA and H-NS bind to multiple overlapping sites within the *inv* promoter, indicating that like SlyA, RovA interactions with the *inv* promoter may inhibit H-NS binding and quell H-NS-mediated repression (21).

YmoA is a member of a growing family of proteins that have homology to the N-terminal dimerization domain of H-NS

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source
Y. enterocolitica strains[a]		
JB580c	Serogroup O:8 Nal^r Δ*yenR*(R$^-$ M$^+$)	27
JB41v	*inv'-'phoA inv$^+$* Nal^r Cm^r	2
MBL014	*inv'-'lacZ inv$^+$* Nal^r Cm^r	This work
MBL015	Δ*rovA inv'-'lacZ inv$^+$* Nal^r Cm^r	This work
YVM567c	*inv'-'phoA inv$^+$ ymoB*::mTn5Kn2 Nal^r Cm^r	This work
YVM927c	Δ*rovA* Cm^r	This work
E. coli strains		
BL21(DE3)	F$^-$ *ompT hsd*S$_B$(r$_B^-$ m$_B^-$) *gal dcm* (DE3), a λ prophage carrying the T7 RNA polymerase gene	55
MC4100	F$^-$ *araD139* Δ(*lacIOPZYA*)U169 *rpsL thiA*	14
RO949	MC1000 *hns205*::T*n10* Tet^r	R. Osuna
VM1303	MC4100 *hns205*::T*n10* Tet^r	This work
Plasmids		
pBAD33	Expression vector containing P$_{BAD}$ promoter, Cm^r	19
pBAD*rovA*:kn^r	pBAD33 containing Kn^r cassette in the SphI site and *rovA*, Cm^r	This work
pWKS30:StrSpec	pWKS30 with a Str/Spec cassette in the HindIII site of the polylinker, Amp^r Str^r Spec^r	Lab collection
pELL21	pWKS30::StrSpec carrying Δ*ymoBymoA* and upstream promoter region	12
pET-24+	Overexpression vector with T7 promoter and C-terminal His tag	Novagen
pET*rovA*-his	RBS plus *rovA* open reading frame cloned into pET	This work
pET*hns*-his	RBS plus *hns* open reading frame cloned into pET	This work
PGEX-6P-1	Overexpression vector with *tac* promoter and cleavable N-terminal GST tag	Pharmacia
pGST-*ymoA*	*ymoA* open reading frame cloned into pGex	This work
p*hns*	pWKS30::StrSpec carrying *hns* and upstream promoter region	This work
pROBE-*gfp*[LVA]	*gfp* with LVA tag to decrease its half-life, Kn^r	39
p*inv*16	pROBE-*gfp*[LVA], containing *inv* promoter fragment −305 to +146	This work
p*inv*29	pROBE-*gfp*[LVA], containing *inv* promoter fragment −276 to +146	This work
p*inv*30	pROBE-*gfp*[LVA], containing *inv* promoter fragment −239 to +146	This work
p*inv*31	pROBE-*gfp*[LVA], containing *inv* promoter fragment −190 to +146	This work
p*inv*17	pROBE-*gfp*[LVA], containing *inv* promoter fragment −158 to +146	This work
p*inv*12	pROBE-*gfp*[LVA], containing *inv* promoter fragment −69 to +146	This work

[a] All *Y. enterocolitica* strains listed are derivatives of JB580v.

(30). Studies with the YmoA homolog Hha in *E. coli* revealed that Hha interacts with H-NS to negatively regulate the expression of the *hly* operon of plasmid pHly152 (31, 43). The mechanism of repression is not completely understood, but it is believed that H-NS provides DNA binding specificity and Hha interacts with H-NS to form a higher-order complex to regulate *hly* expression (31, 43). YmoA has been shown to interact with H-NS (42), leading us to hypothesize that YmoA may act in a similar fashion to repress *inv* expression indirectly by forming a complex with H-NS on the *inv* promoter.

While RovA is required for expression of *inv* in both *Y. enterocolitica* and *Y. pseudotuberculosis* (41, 50), it is unclear if it acts on the *Y. enterocolitica inv* promoter in the same manner as reported for *Y. pseudotuberculosis*; two lines of evidence indicate that the regulatory mechanism of *inv* may differ between the two species. First, restoration of *inv* expression at 37°C by changes in medium pH has not been observed in *Y. pseudotuberculosis* (41). Secondly, comparison of the two promoters reveals significant sequence differences between *Y. enterocolitica* and *Y. pseudotuberculosis*. Based on the differences in regulation and sequence divergence in the promoter regions of the two species, our goals in this work were as follows: (i) to determine if H-NS was involved in the negative regulation of *inv* in *Y. enterocolitica*; (ii) to further investigate the mechanism by which RovA activates *inv* transcription in *Y. enterocolitica*;

and (iii) to better define the mechanism of YmoA-mediated repression of the *inv* promoter in *Y. enterocolitica*.

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 that have been cured of the virulence plasmid. Bacterial cultures were grown in Luria-Bertani (LB) broth at 26°C or 37°C as indicated. When appropriate, antibiotics were used at the following concentrations: chloramphenicol, 25 μg ml^{-1} (for growth of *E. coli*) and 12.5 μg ml^{-1} (for growth of *Y. enterocolitica*); ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; streptomycin, 50 μg ml^{-1}; tetracycline, 15 μg ml^{-1}.

Strain and plasmid construction. Primers used in this study are listed in Table 2. To construct an *hns* mutant in *E. coli* strain MC4100, the mutant allele *hns205*::T*n10* was introduced by P1 transduction from strain RO949.

To generate C-terminal histidine (His)-tagged recombinant RovA and H-NS, the genes were ligated into pET-24(+) (Novagen). The genes were amplified using primers RovAFRBSR1 and RovAXhoR3His for *rovA* and H-NSFnotHis and H-NSRR1His for *hns*. Both products contained the native ribosome binding site (RBS) and were digested with the appropriate restriction enzymes and ligated into pET-24(+). To generate N-terminal glutathione *S*-transferase (GST)-tagged YmoA, *ymoA* was amplified by PCR with primers ymoAgstFR1 and ymoAgstRXho and cloned into pGEX-6P-1 (Pharmacia). Plasmids p*rovA*-his, p*hns*-his, and pGST-*ymoA* were checked by DNA sequencing to ensure no errors had been introduced by PCR. Plasmid p*inv-gfp* was constructed by amplifying the *inv* promoter using primers invgfpH1F and invgfpR1R, generating a ~500-bp fragment that was digested and ligated into pROBE-*gfp*(LVA) (39). *inv* promoter truncations were subcloned from previously constructed plasmids

TABLE 2. Primers

Primer	Sequence (5′–3′)[a]
RovAFRBSR1	CGGAATTCGGTAGTTATGCTAGCACGC
RovAxhoR3His	GGTCTCGAGCTTACTTTGTAGTTGAATAATGTTTCTCTCAAGC
H-NSFnotHis	ATAAGAATGCGGCCGCCAGCAGGAAATCATCCAG
H-NSRR1His	CGGAATTCCACTCTATTATTATCCAGAC
YmoAgstFR1	GGAATTCATGACAAAAACTGACTACCT
YmoAgstRxho	CCGCTCGAGCTATTTCACATGTTGCCATA
InvgfpH1F	CGGATCCGATAAGTGAAGGAGCAAC
InvgfpR1R	GGAATTCCTAATTATTTTAGTCACAGTTAGC
H-NsccxbaF	TGCTCTAGATGATATGATCAAGGCTCGCC
H-NsccnotR	ATAAGAATGCGGCCGCGAACATTAACCAGGTTAGAGCG
Inv500F	GATAAGTGAAGGAGCAAC
Inv300F	AGTCTATTTTATGAGACGAATG
Inv150F	GTAAGTTCTGCTATTCTATTG
Inv300R	CATTCGTCTCATAAAATAGACT
Inv200R	GCAATGACAAATGAAATGC
Inv150R	CAATAGAATAGCAGAACTTAC
Inv3R	TATTAGCCTGCTAATTATTTTAGTCACAGTTAGCG
Inv194F	GCAGAGAGTCTATTTTATGAGACGAATGTAAAC
Inv34R	AAAATTAAAAATAACAGCAATGACAAATGAAATGC

[a] Restriction sites are indicated by underlining.

pAJD-16, pAJD-29, pAJD-30, pAJD-31, pAJD17, and pAJD-12 by digesting with XbaI/NruI. The digested fragments were then ligated into the XbaI/SmaI sites in pROBE-gfp(LVA) (39), generating plasmids p*inv-gfp*-16, p*inv-gfp*-29, p*inv-gfp*-30, p*inv-gfp*-31, p*inv-gfp*-17, and p*inv-gfp*-12, respectively. To construct an H-NS-complementing plasmid, *hns* was amplified using primers H-NSccXbaF and H-NSccNotR. The resulting fragment was digested and ligated into pWKS30:StrSpec, generating p*hns*.

Purification of RovA-His, H-NS-His, and YmoA. Overnight cultures of BL21(DE3) containing either p*rovA*-his, p*hns*-his, or p*GST-ymoA* were diluted 1/200 and allowed to grow at 37°C until an optical density at 600 nm (OD$_{600}$) of 0.5 was reached. To induce protein expression, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and growth was continued for 5 h. Cells were harvested by centrifugation at 7,000 rpm for 30 min; cell pellets were resuspended in 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole pH 8.0 for His tag purification or 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 140 mM NaCl, 2.7 mM KCl pH 7.3 for purification of GST-YmoA and frozen at −80°C. The cell suspension was removed from −80°C and allowed to thaw at room temperature. The samples were sonicated five times for 30 s with a 1-min incubation on ice between sonication steps. To isolate the soluble fraction, the samples were centrifuged at 10,000 × g for 30 min and transferred to a clean 50-ml conical tube. Five ml of cleared lysate was added to 0.5 ml of presettled Ni-nitrilotriacetic acid agarose (QIAGEN) or glutathione-Sepharose 4B (Amersham Biosciences) in 1-ml polypropylene columns (QIAGEN) and allowed to flow over the column at room temperature. For purification of His-tagged proteins, the column was then washed with 5 ml of 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 5% glycerol, 1% Triton X-100, 20 mM imidazole pH 8.0, followed by 5 ml of 50 mM NaH$_2$PO$_4$, 1 M NaCl, 20 mM imidazole pH 8.0. Excess salt was removed by washing the column with 5 ml of 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole pH 8.0. The wash procedure was the same for GST-YmoA purification except that the base buffer was 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 140 mM NaCl, 2.7 mM KCl pH 7.3. The His-tagged proteins were eluted from the column in 2 ml of 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole pH 8.0 or 50 mM Tris-HCl, 10 mM reduced glutathione pH 8.0 for GST-YmoA purification. To remove excess imidazole, protein samples were run over PD-10 desalting columns (Amersham) and exchanged into buffer containing 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 15% sucrose pH 8.0; 50-μl aliquots were stored at −80°C. To remove the GST tag from YmoA, purified protein was added to PD-10 desalting columns (Amersham) and exchanged into 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 140 mM NaCl, 2.7 mM KCl, pH 7.3. This was applied to a new 1-ml glutathione-Sepharose 4B column and allowed to bind at 4°C. The column was equilibrated with 10 ml of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0. Eighty units of PreScission protease (Amersham) was diluted with 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, and

mixed with glutathione-Sepharose 4B containing the bound GST-YmoA. This mixture was incubated at 4°C for 4 h, and then YmoA was collected from the column by washing with 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0. Purified YmoA was concentrated and exchanged into buffer containing 20 mM Tris-HCl, 5% glycerol, 20 mM NaCl, 5 mM MgCl$_2$. For all proteins, the concentration was determined by using the Bradford protein assay (Bio-Rad).

EMSA. Primers used to generate *inv* promoter fragments used in electrophoretic mobility gel shift assay (EMSA) experiments are listed in Table 2. An internal 500-bp fragment of the *lacZ* gene was used as a nonspecific control. EMSA experiments were performed at room temperature in binding buffer containing 20 mM Tris-HCl, 15% glycerol, 50 mM NaCl, 5 mM MgCl$_2$, and 1 μg of salmon sperm DNA. Random ^{32}P-labeled DNA was generated by PCR with [α-^{32}P]dATP and added to protein to a final activity of 3,000 cpm in 10-μl reaction mixtures. The reaction mixtures were incubated at room temperature for 15 min and then run on a 3.5%, 5%, or 8% polyacrylamide gel, depending on the size of the fragment. For the competition experiments, RovA was added and allowed to bind for 5 min before the addition of H-NS. The reaction mixtures were then incubated for 10 min before loading onto a 5% polyacrylamide gel. Competition experiments were preformed in binding buffer containing 20 mM Tris-HCl, 15% glycerol, 80 mM NaCl, 5 mM MgCl$_2$, 100 ng of salmon sperm DNA, and 1 μg of bovine serum albumin.

Northern blot assays. To examine the levels of *hns* mRNA in JB41c and YVM567c, the strains were grown overnight at 37°C, subcultured to an OD$_{600}$ of 0.2, and allowed to grow at 37°C to an OD$_{600}$ of 2.0; RNA was harvested using the RiboPure-Bacteria extraction kit (Ambion). To determine the levels of *inv*, *rovA*, *hns*, and *ymoA* mRNA, overnight cultures were grown at either 26°C or 37°C and subcultured to an OD$_{600}$ of 0.2, and samples were harvested at early log (0.5 to 1.0 OD$_{600}$), middle log (1.1 to 2.5 OD$_{600}$), and late log (2.6 to 3.5 OD$_{600}$); RNA was harvested using the RiboPure-Bacteria extraction kit (Ambion). Equal amounts of purified RNA were run on a 1% agarose gel containing 1× morpholineethanesulfonic acid and 6.7% formaldehyde and transferred overnight to a nitrocellulose membrane. The membranes were hybridized overnight at 42°C with either ^{32}P-labeled *inv*, *rovA*, *hns*, or *ymoA* probes consisting of the genes' corresponding open reading frames and exposed to an imaging screen. The image was scanned on an FLA-5000 phosphorimager and analyzed using Image Gauge (version 4.2; Fujifilm).

Green fluorescent protein assays. Strains were grown in triplicate overnight in 2 ml of LB at 26°C with aeration, and the levels of fluorescence intensity were determined as previously described (12).

AP assays. Strains were grown in triplicate overnight in 2 ml of LB at 37°C with aeration. Alkaline phosphatase (AP) activity was measured according to previously described methods (32).

β-Galactosidase assays. Cultures were grown in triplicate overnight in 2 ml of LB broth at 37°C or 26°C containing 0.002, 0.0002, 0.00002, 0.000002, or 0% arabinose. β-Galactosidase activity was measured according to previously described methods (37).

Western blot assays. One OD equivalent of cells was harvested and pelleted for 3 min at 13,000 rpm; the resulting pellets were resuspended in 50 μl Laemmli buffer containing 10% β-mercaptoethanol. A 0.1 OD equivalent was loading onto a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and run at 200 V for 1 h. Proteins were transferred to a nitrocellulose membrane, and RovA was detected using a polyclonal anti-RovA antibody (Covance) at a 1:1,000 dilution. H-NS was detected using a 1:2,000 dilution of anti-H-NS antibody kindly supplied by Yeong-Jae Seok, Seoul National University (53). YmoA was detected using a 1:1,000 dilution of anti-YmoA antibody kindly supplied by Gregory V. Plano (25).

RESULTS

***hns* silences expression of the *inv* promoter.** To determine if *hns* was involved in the negative regulation of *inv*, we initially attempted to inactivate *hns* in *Y. enterocolitica*. Several attempts to generate an *hns* mutant were unsuccessful, suggesting that *hns* is essential for viability of *Y. enterocolitica* strain JB580. Similar results have been reported for *Y. pseudotuberculosis* (21) and were also found for *ymoA*, a negative regulator of *inv*, in *Y. enterocolitica* strain JB580 (12). However, *hns* has been successfully inactivated in *E. coli*, and we tested the effect of *hns* on *inv* expression in this surrogate background. Plasmid
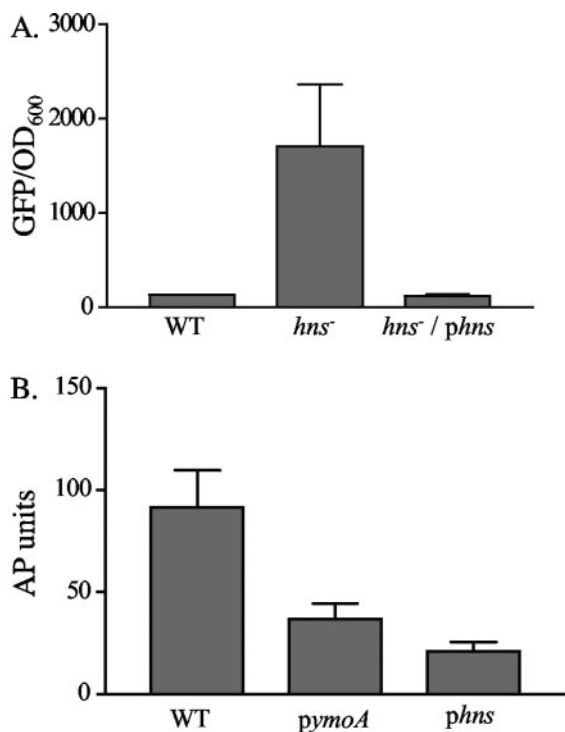
FIG. 1. Effect of *hns* on the expression of *inv*. (A) Cultures of MC4100 containing p*inv-gfp* and VM1303 (an *hns* mutant) containing p*inv-gfp* or p*inv-gfp* and p*hns* were grown overnight in triplicate at 26°C. Fluorescence was calculated by dividing average fluorescence by the OD_{600}. WT refers to MC4100 containing p*inv-gfp*, *hns*⁻ refers to VM1303 containing p*inv-gfp*, and *hns*⁻/p*hns* refers to VM1303 containing p*inv-gfp* and p*hns*. (B) Cultures of JB41v containing the plasmids p*hns*, p*ymoA* (pEll21), or pWKS30strspec were grown overnight in triplicate at 26°C and assayed for expression of *inv-phoA*. WT refers to JB41v containing pWKS30strspec as a vector control.

p*inv-gfp*, containing an *inv-gfp* transcriptional fusion, was transformed into wild-type (WT) *E. coli* MC4100 and an isogenic *hns* mutant of MC4100 (VM1303). These strains were grown overnight at 26°C and assayed for expression of *inv-gfp*. MC4100 and MC4100/p*inv-gfp* both demonstrated similar levels of fluorescence, indicating that *inv-gfp* is expressed at low levels in WT *E. coli*, while a mutation of *hns* resulted in a ~7-fold increase in *inv-gfp* expression (Fig. 1A). Complementation of the *hns* mutant with *hns* from *Y. enterocolitica* restored the level of *inv-gfp* expression to that of MC4100 harboring p*inv-gfp*. These data suggest that *Y. enterocolitica* H-NS is able to repress transcription of *inv*.

To determine if H-NS is able to repress *inv* expression in *Y. enterocolitica*, the *inv-phoA* reporter stain (JB41c) was transformed with either p*hns* or the vector control (pWKS30: Strspec) and assayed for expression of *inv-phoA* (Fig. 1B). The strain containing pWKS30:StrSpec exhibited high levels of AP activity as expected when cultured at 26°C. When p*hns* was present, a decrease in AP activity was observed. Supplying excess amounts of a second negative regulator of *inv*, YmoA, in *trans* also resulted in a reduction of *inv* expression in *Y. enterocolitica* (Fig. 1B). These data suggest that H-NS negatively regulates expression of *Y. enterocolitica inv* and strengthen
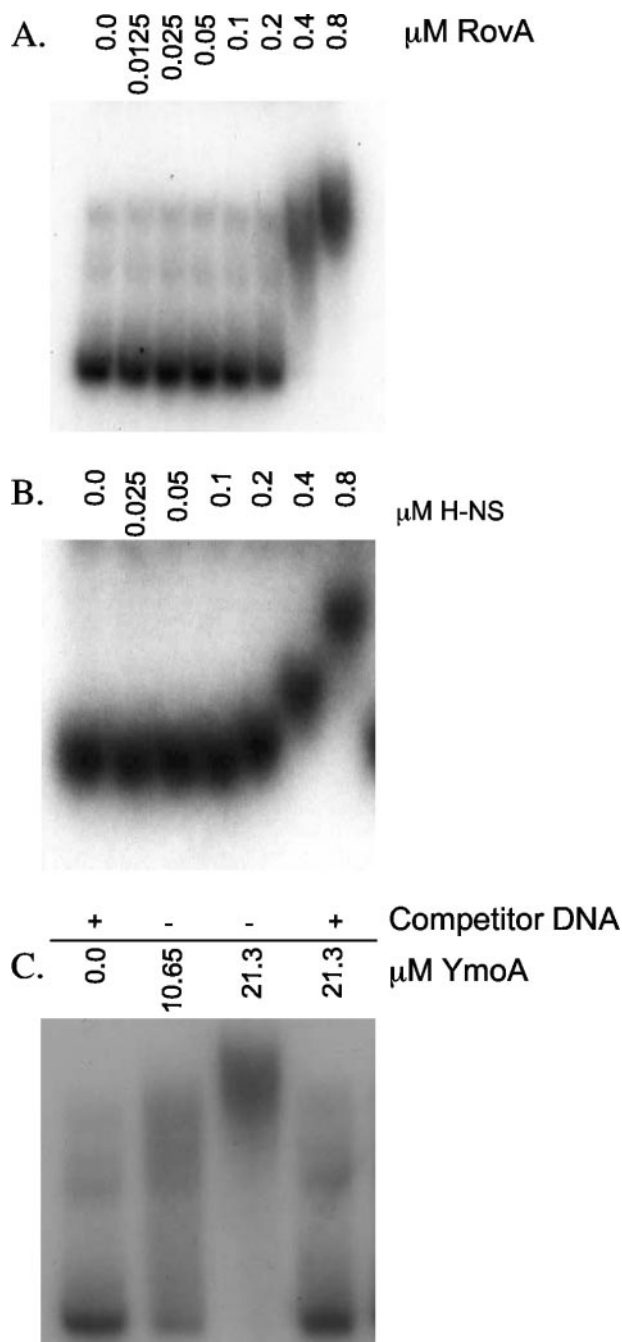

FIG. 2. Ability of RovA, H-NS, and YmoA to interact with *inv*. Randomly ^{32}P-labeled *inv* (−344 to +146) was incubated with purified RovA, H-NS, or YmoA at room temperature for 15 min and run on a 3.5% or 5% nondenaturing polyacrylamide gel. (A) RovA; (B) H-NS. In panels A and B, 1 μg of salmon sperm was included in all of the reaction mixtures. (C) YmoA. The + indicates the presence of 1 μg of salmon sperm; − indicates the absence of salmon sperm. The concentration of protein is shown across the top of the gel (in μM).

previous data showing that YmoA negatively regulates the expression of *inv*.

RovA and H-NS interact specifically with the *inv* promoter. To gain insight into the interaction of RovA, H-NS, and YmoA with the *inv* promoter, EMSA experiments were performed.
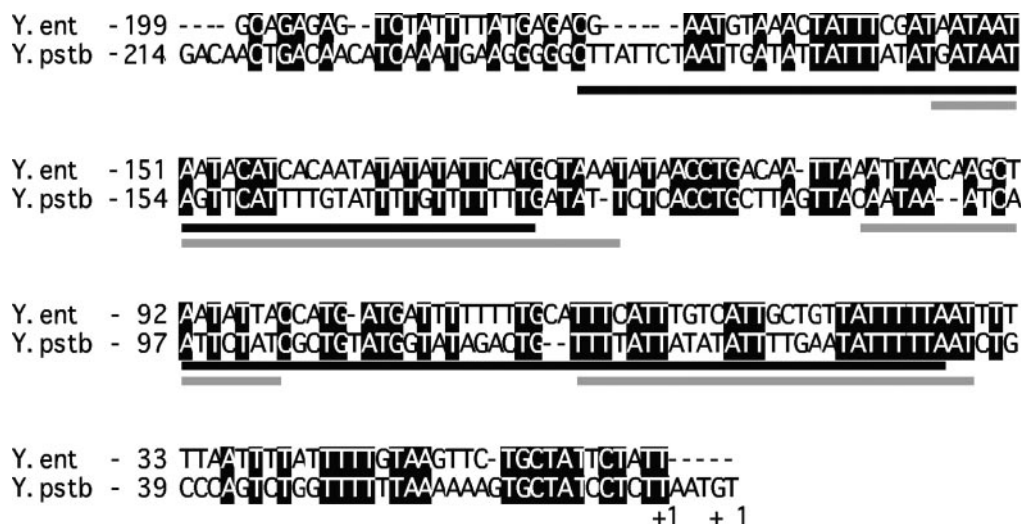
FIG. 3. Alignment of *inv* promoters. Shown is an alignment of the RovA and H-NS binding region of *inv* from *Y. enterocolitica* and *Y. pseudotuberculosis*. Conserved bases are shaded in black, RovA binding sites are underlined in black, and H-NS bindings sites are indicated by a gray underline. The RovA and H-NS binding sites are based on DNase I protection data from *Y. pseudotuberculosis* (21). The start of transcription, marked +1, was previously determined (41, 46).

Recombinant C-terminal His-tagged RovA and H-NS were generated and purified as described in Materials and Methods. Purified YmoA was obtained by purifying an N-terminal GST fusion protein that was later cleaved to yield a protein with a six-amino-acid N-terminal extension. We chose to use an N-terminal cleavable GST tag because previous work done with *E. coli* Hha showed that a C-terminal His tag rendered the protein unable to interact with H-NS (42). Purified proteins were incubated with a random ^{32}P-labeled *inv* fragment (-344 to $+146$) and 1 μg of salmon sperm DNA as nonspecific competitor. Both RovA and H-NS bound the *inv* promoter in the presence of excess competitor DNA (Fig. 2A and B). Binding specificity of RovA was demonstrated by an inability of the recombinant protein to bind the *ysaE* promoter from *Y. enterocolitica* under the same conditions tested for *inv* (data not shown). H-NS binding was unaffected by excess nonspecific competitor DNA, but H-NS did not bind ^{32}P-labeled *inv* probe in the presence of excess unlabeled *inv* promoter, indicating specific binding to the *inv* promoter (data not shown). Unlike RovA and H-NS, YmoA was unable to bind the *inv* promoter in the presence of nonspecific competitor DNA, even at elevated concentrations of purified protein (Fig. 2C). YmoA was able to bind DNA in the absence of nonspecific competitor DNA (Fig. 2C), consistent with previous reports for Hha, which revealed no binding specificity for Hha to the *hly* operon of plasmid pHly152 (31, 43). Hha and YmoA both have been shown to interact with H-NS, raising the possibility that our YmoA preparations are contaminated with H-NS (42). However, our data show that there is little contaminating H-NS protein in our YmoA preparations, because there is no binding to the *inv* promoter in the presence of nonspecific competitor DNA. These data indicate that RovA and H-NS bind specifically to the *inv* promoter while suggesting that YmoA does not.

RovA and H-NS bind similar sites on the *inv* promoter. RovA and H-NS bind to overlapping binding sites within the *inv* promoter of *Y. pseudotuberculosis* (21). These binding regions display considerable sequence differences in *Y. enterocolitica*, and we were unsure whether these differences affected RovA or H-NS binding in *Y. enterocolitica* (Fig. 3). To identify regions bound by RovA and H-NS, a set of fragments (Table 3) representing the *inv* promoter were generated by PCR and incubated with purified protein. EMSA experiments were performed as described above, and both RovA and H-NS demonstrated preferential binding to the same *inv* promoter fragments (Table 3). The fragments bound indicated that the binding sites are located between bp -194 and -10 relative to the $+1$ transcriptional start site. To confirm that RovA and H-NS bound to this region, a PCR product encompassing bp -194 to -34 was generated and the ability of the two proteins to bind this product was determined (Table 3). EMSA experiments revealed that RovA and H-NS independently bind this region of the *inv* promoter, raising the possibility that RovA and H-NS bind overlapping sites within the *inv* promoter of *Y. enterocolitica*.

H-NS and RovA compete for binding sites on the *inv* promoter. As described previously and recently shown by DNase I footprinting in *Y. pseudotuberculosis* (21), RovA and H-NS bind to overlapping sites within the *inv* promoter. These data suggest that RovA and H-NS compete for binding to the *inv* promoter. To test this directly, EMSA experiments were used

TABLE 3. Binding to *inv* fragments[a]

Fragment	5′ primer	3′ primer	Region	RovA	H-NS
A	Inv500F	Inv3R	-343 to $+146$	+	+
B	Inv300F	Inv3R	-193 to $+146$	+	+
C	Inv150F	Inv3R	-19 to $+146$	−	−
D	Inv500F	Inv150R	-343 to $+2$	+	+
E	Inv500F	Inv200R	-343 to -49	+	+
F	Inv500F	Inv300R	-343 to -171	−	−
G	Inv194F	Inv34R	-194 to -34	+	+

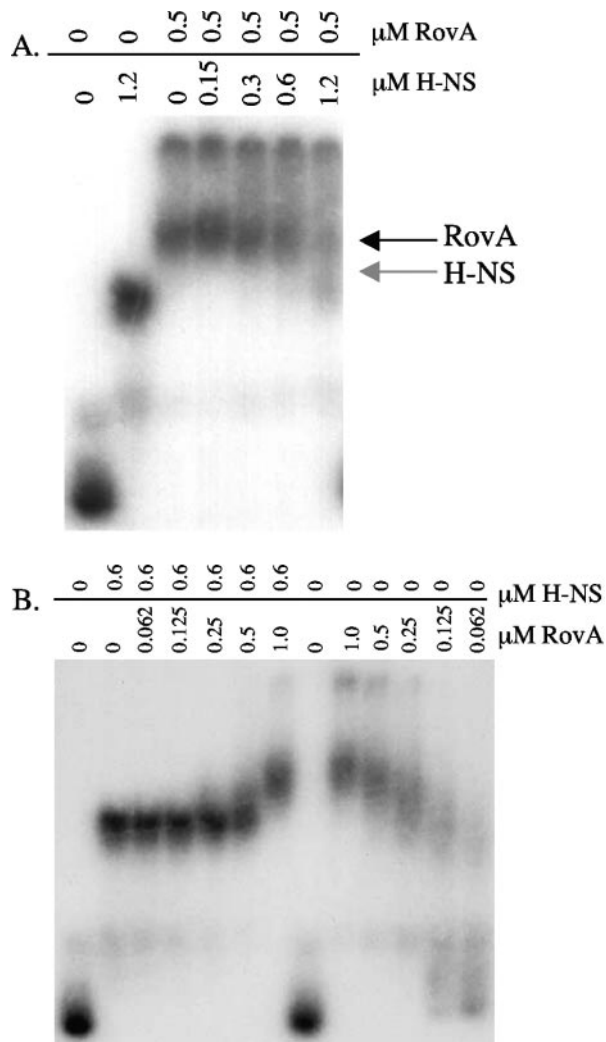[a] + indicates binding; − indicates no binding.

FIG. 4. Competition between RovA and H-NS for the *inv* promoter. (A) Randomly ^{32}P-labeled *inv* (−194 to −34) was incubated for 5 min with purified RovA (0.5 μM), shown across the top of the gel. To these reaction mixtures increasing concentrations of H-NS were added, as shown across the top of the gel (in μM), and incubated for 10 min. Samples were run on a 5% nondenaturing polyacrylamide gel. (B) Randomly ^{32}P-labeled *inv* (−194 to −34) was incubated for 5 min with purified H-NS (0.6 μM), shown across the top of the gel. To these reaction mixtures increasing concentrations of RovA were added, as shown across the top of the gel (in μM), and incubated for 10 min. Samples were run on a 5% nondenaturing polyacrylamide gel.

FIG. 5. Levels of *hns* in YVM567c. JB41c (WT) and YVM567c (*ymoB*::Tn*10*) were grown overnight at 37°C, and cultures were then subcultured to an OD_{600} of 0.2 and grown to an OD_{600} of 2.0. Transcriptional levels of *hns* were determined by Northern blotting. Levels of H-NS in the two strains are shown below the graph from samples grown in the same manner as for the Northern blot assay. Equal OD equivalents were loaded for each strain for the Western analysis, and data shown are from the same gel.

to determine if RovA and H-NS were able to replace one another once bound to *inv* (−194 to −34). RovA (0.5 μM) and H-NS (0.6 μM) were added in separate reaction mixtures at a concentration required to completely shift *inv*. To replace the prebound protein, increasing amounts of H-NS or RovA were added to the reaction mixtures (Fig. 4A and B). The data indicate that H-NS was able to displace RovA once bound to the *inv* promoter, as increasing concentrations of H-NS produced a mobility shift identical to the mobility shifts seen with H-NS incubated alone with *inv* (Fig. 4A). Similarly, increasing concentrations of RovA were able to displace prebound H-NS from the *inv* promoter, producing a shift identical to the mobility shifts seen with RovA incubated alone with *inv* (Fig. 4B).
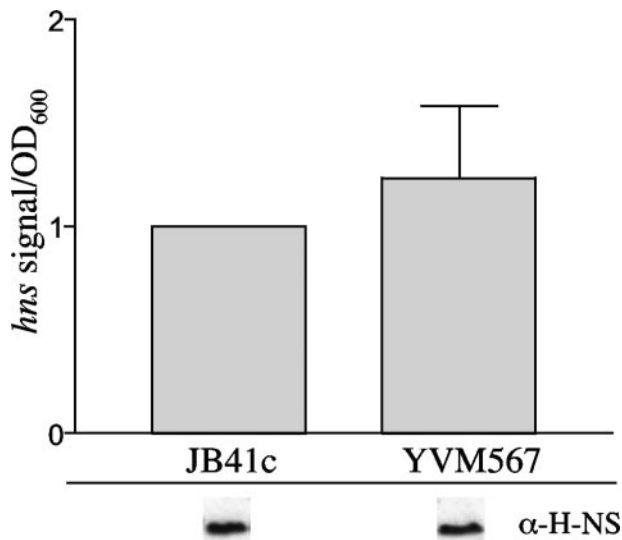
In both experiments, increasing amounts of RovA or H-NS were required to displace the prebound protein, suggesting that RovA and H-NS compete for binding sites on the *inv* promoter. We do not believe that the two proteins are able to bind simultaneously, as no cooperative binding (i.e., an additional shifted species) was observed at lower concentrations of protein known to shift the *inv* promoter (Fig. 4A and B). These data indicate that RovA and H-NS compete for binding sites within the *inv* promoter.

***hns* expression is unaffected in the *ymoB*::Tn*10* mutant.** Due to the essential nature of YmoA and H-NS for growth of *Y. enterocolitica*, we have been unable to successfully inactivate either gene. This has made it difficult to address the individual contribution of YmoA and H-NS in the regulation of *inv*. However, a previously characterized transposon insertion mutation of *ymoB* (YVM567c), the gene upstream of *ymoA*, exhibits a strong polar effect on *ymoA* expression (12). Strain YVM567c demonstrates a drastically reduced level of *ymoA* transcription (12), which leads to an increase in the expression of *inv* at 37°C. This increase in *inv* expression is not due to elevated levels of *rovA* transcription (12) or increased levels of RovA protein, as equivalent amounts of RovA are present in the parental strain (JB41c) and YVM567c (data not shown). Another possibility for the increase in *inv* expression at 37°C in YVM567c is a decrease in the levels of *hns* transcript, leading to a reduction in H-NS bound to the *inv* promoter and higher levels of *inv* expression at 37°C. This would correlate with recently published data suggesting that Hha positively regulates *hns* expression in *E. coli* (45). To determine if YmoA affects *hns* expression, RNA was harvested from JB41c and YVM567c cultures grown with aeration at 37°C to an OD_{600} of 2.0, and levels of *hns* transcript were compared (Fig. 5). North-
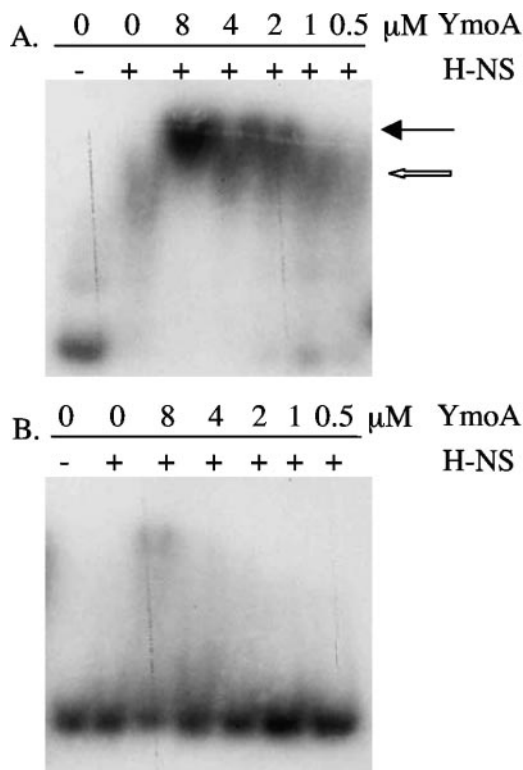
FIG. 6. Ability of H-NS and YmoA to form a repression complex on the *inv* promoter. (A) Randomly ^{32}P-labeled *inv* (−344 to +146) was incubated with purified H-NS and YmoA at room temperature for 15 min and run on a 5% nondenaturing polyacrylamide gel. (B) Randomly ^{32}P-labeled *lacZ* was incubated with purified H-NS and YmoA at room temperature for 15 min and run on a 5% nondenaturing polyacrylamide gel. The concentration of YmoA is shown across the top of the gel (in μM), and H-NS was added at a constant concentration of 0.1 μM. The open arrow indicates an H-NS–*inv* complex, with the closed arrow indicating an H-NS/YmoA–*inv* complex.

FIG. 7. Effect of promoter truncations on the expression of *inv-gfp* in WT and Δ*rovA* strains. (A) Schematic of the *inv* promoter with the predicted −35, −10, and +1 sites, along with the start codon (+107) and fusion junction (+170). The predicted RovA binding sites (black lines) from *Y. pseudotuberculosis* are shown above *inv*16, and the predicted H-NS binding sites (gray lines) are shown below (21). (B) JB580 (WT) and YVM927 (Δ*rovA*) strains were transformed with the *inv-gfp* promoter truncations and grown overnight at 26°C. Fluorescence was calculated by dividing the average fluorescence by OD$_{600}$. V indicates the pROBE-*gfp*[LVA], containing no promoter, and the numbers below the bars represent the different *inv-gfp* truncations shown in panel A. Shown below the numbers are the strains in which the truncations were tested.

ern blot analysis demonstrated no detectable difference in the expression of *hns* in the *ymoA* mutant. To determine if the expression levels of *hns* correlated with levels of H-NS protein, Western blot assays were performed. Similar levels of H-NS protein were detected in the two strains, suggesting that the levels of H-NS protein were unaffected in strain YVM567c (Fig. 5). Our result is different from the published work on the previously mentioned *hha* mutation in *E. coli* that showed a reduction in *hns* levels (45). This could be due to the fact that YVM567c still expresses low levels of *ymoA*, which could be enough to sustain wild-type expression of *hns*, could reflect differences between YmoA and Hha, or could reflect differences between *E. coli* and *Y. enterocolitica*. These data suggest that the increased expression of *inv* at 37°C in the *ymoA* mutant is not due to a reduction in both H-NS and YmoA but that the reduction in *ymoA* expression alone is responsible for the elevated levels of *inv* expression.

Interaction of YmoA and H-NS leads to the formation of a repression complex. While it has been demonstrated that YmoA influences the expression of *inv*, it appears that this is not by binding specifically to the *inv* promoter. It has been shown that the YmoA homolog Hha regulates the *hly* operon of plasmid pHly152 through specific interactions with H-NS
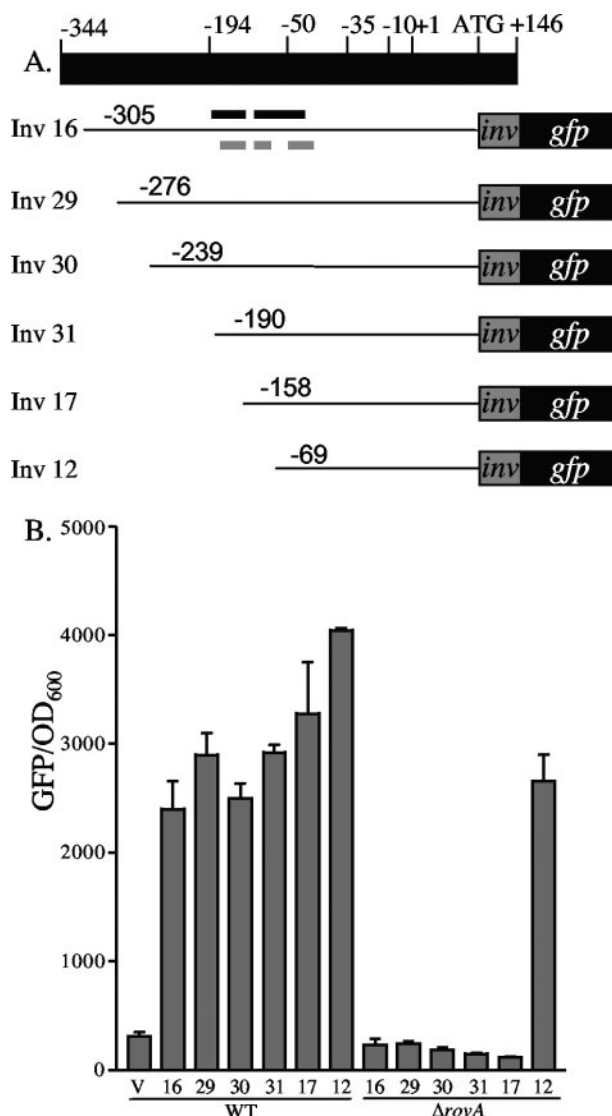
(43). In pull-down experiments, YmoA has also previously been shown to interact with H-NS (42). We therefore hypothesized that YmoA may regulate *inv* expression through a similar mechanism by interacting with H-NS at the *inv* promoter. To determine if YmoA and H-NS form a complex in the presence of *inv*, EMSA experiments were used to determine if migration of the H-NS-*inv* complex was altered by addition of YmoA to the reaction. Purified YmoA and H-NS were incubated with ^{32}P-labeled *inv* promoter (−344 to +146), and
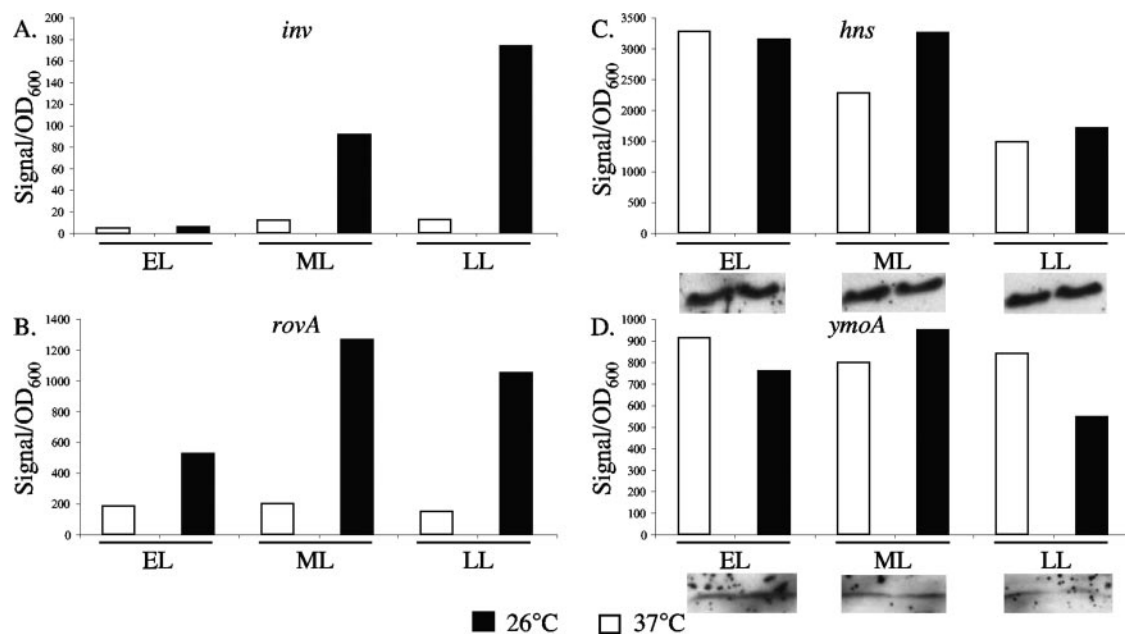
FIG. 8. Expression levels of *inv*, *rovA*, *ymoA*, and *hns* at early, middle, and late log phases of growth. (A) *inv*; (B) *rovA*; (C) *hns*; (D) *ymoA*. Cultures of WT bacteria were grown overnight and subcultured to an OD_{600} of 0.2. Approximately 4 OD_{600} of cells were taken at early log (0.5 to 1.0 OD_{600}), middle log (1.1 to 2.5), and late log (2.6 to 3.5) phases of growth, and the RNA was extracted. Three biological replicates were performed, but because of high signal variability between replicates, representative graphs are shown for *inv*, *rovA*, *ymoA*, and *hns*. The trends shown here were the same for all of the replicates, and the expression levels of *hns* and *ymoA* between 26°C and 37°C never differed by more than twofold. Corresponding levels of H-NS and YmoA, determined by Western analysis as described in Materials and Methods, are indicated below their graphs. For both H-NS and YmoA, the inserts were taken from the same gel and the same exposure time.

mobility of the labeled DNA was determined on a nondenaturing polyacrylamide gel (Fig. 6A). We observed that addition of YmoA to the reaction resulted in slightly slower migration of the H-NS-*inv* complex, suggesting an interaction of YmoA with H-NS. To show that the H-NS/YmoA complex was specific for the *inv* promoter, the same assay was performed using an internal ~500-bp fragment of *lacZ* (Fig. 6B). The data show that H-NS was unable to bind the *lacZ* fragment under the conditions tested and that very little H-NS/YmoA complex formed with the *lacZ* fragment under these conditions, indicating that the H-NS/YmoA complex is specific for *inv*. The ability to shift *lacZ* was only seen at the highest concentrations of YmoA and most likely represents nonspecific binding by YmoA to *lacZ*, as YmoA slightly shifts *inv* and *lacZ* when incubated alone at the highest concentration tested in this experiment (data not shown). Nevertheless, H-NS and YmoA still formed a complex with the *inv* promoter at lower concentrations of YmoA, at which there is an absence of nonspecific shifting. The H-NS/YmoA complex, like the H-NS/Hha complex, is also stable in the presence of excess competitor DNA (data not shown). These data indicate that YmoA and H-NS are able to form a complex in the presence of the *inv* promoter.

RovA acts as a derepressor of H-NS/YmoA-mediated repression of the *inv* promoter. While RovA is required for the expression of *inv*, it is unclear if regulation is mediated by RovA activating *inv* expression or through a mechanism of derepression by limiting the availability of H-NS/YmoA binding sites in the *inv* promoter. We hypothesized that if RovA is not required for activation then loss of H-NS/YmoA binding to the *inv* promoter would not alter expression of *inv* in the

absence of RovA. To test this hypothesis, a series of *inv* promoter truncations fused to *gfp* were generated (Fig. 7A) and transformed into WT *Y. enterocolitica* (JB580c) and Δ*rovA* (YVM927c). Cultures were inoculated and incubated with aeration overnight at 26°C and assayed for *inv-gfp* expression (Fig. 7B). All promoter truncations showed high levels of *inv-gfp* expression in WT *Y. enterocolitica*. In the Δ*rovA* mutant, five of the six truncations demonstrated no expression of *inv-gfp*, but the shortest truncation (p*inv*12), which lacks two of the three predicted H-NS binding sites (21), showed robust expression, indicating RovA is not required to activate *inv* expression. EMSA experiments revealed that H-NS was still able to bind this fragment; however, we hypothesize that without the other H-NS binding sites upstream, efficient repression does not occur (data not shown).

Expression levels of H-NS and YmoA are similar at 26°C and 37°C. *inv* expression has been shown to increase when cultures are grown at 26°C and repressed when grown at 37°C (41, 46); similar results have also been shown for the expression of *rovA* (41). However, the expression levels of *hns* and *ymoA* have yet to be investigated at 26°C and 37°C in *Y. enterocolitica*. To test if changes in *hns* and *ymoA* expression were involved in the regulation of *inv* between 26°C and 37°C, levels of *hns* and *ymoA* were determined using Northern blotting. Samples from early, middle, and late log phases of growth at 26°C and 37°C were harvested and assayed for *inv*, *rovA*, *ymoA*, and *hns* expression. As expected, expression of *inv* at 37°C was low and increased from early to late log phases of growth at 26°C (Fig. 8A). A similar pattern of expression was observed for *rovA*, with low expression at 37°C and increasing
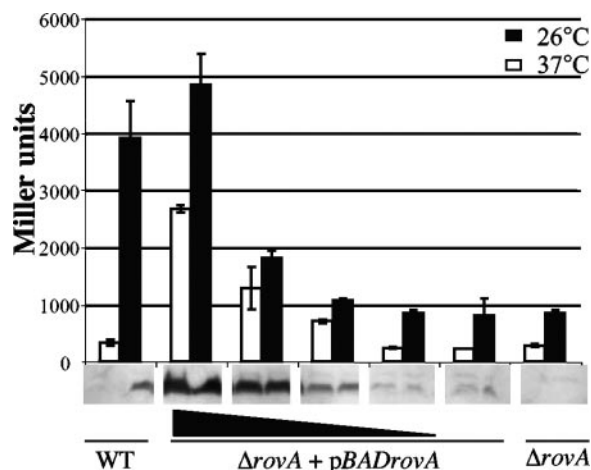
FIG. 9. Expression of *inv* at 37°C by induction of *rovA*. Cultures were grown overnight at 26°C or 37°C in triplicate and assayed for *inv-lacZ* expression and relative levels of RovA, which are shown below the graph. WT and Δ*rovA* strains are shown on the ends of the graph, and the Δ*rovA*+pBAD*rovA* mutant is shown in the middle; increasing concentrations of arabinose (0.000002, 0.00002, 0.0002, and 0.002%) are indicated by the black triangle.

expression as growth progressed at 26°C (Fig. 8B). The expression levels of *hns* and *ymoA* were similar between 26°C and 37°C at early, middle, and late log phases of growth (Fig. 8C and D). To determine if the expression data correlated with levels of protein, Western blot assays were done on H-NS and YmoA (Fig. 8C and D). These data suggest that the expression and the levels of H-NS and YmoA remain relatively constant between 26°C and 37°C and the increased expression of *inv* at 26°C is not associated with a decrease in H-NS or YmoA relative to 37°C.

Increasing the levels of RovA at 37°C leads to expression of *inv*. Based on our previous observations, we reasoned that the levels of RovA are a critical component for controlling the expression of *inv*, and increasing the expression of *rovA* under conditions where *rovA* is normally repressed (37°C at neutral pH) would activate *inv* transcription. To test this hypothesis, strain MBL015, a *rovA* mutant containing an *inv-lacZ* chromosomal fusion and an arabinose-inducible *rovA* construct (pBAD*rovA*), was grown overnight at 26°C and 37°C in the presence of increasing concentrations of arabinose and assayed for *inv-lacZ* expression and protein levels of RovA (Fig. 9). The expression of *inv-lacZ* showed a dose-dependent increase in *inv* expression as the levels of RovA increased at both 26°C and 37°C (Fig. 9). The presence of invasin was also detected by Western blotting of cells grown at 37°C and 26°C and showed similar results (data not shown). To control for effects of arabinose on the expression of *inv*, strain MBL014 (containing an *inv-lacZ* chromosomal fusion) was grown in the presence of the same concentrations of arabinose used in the previous experiments, and no effect on the expression of *inv* at 26°C or 37°C was observed (data not shown). The expression of *inv* was also unaffected in strain MBL015 harboring the vector control (data not shown). These data indicate that increasing the levels of RovA under conditions of *inv* repression activate expression of *inv*, supporting a model where the levels of RovA in the cell are a key determinant of expression levels of *inv*.

DISCUSSION

Invasin is expressed by both *Y. pseudotuberculosis* and *Y. enterocolitica* and is an important virulence factor involved in attachment and invasion of M cells (7, 18, 20, 23, 33, 38). Considerable work has been done to understand interactions between invasin and host ligands (7, 24) and the role of invasin in invasion of the host (6, 48); however, the mechanism of *inv* regulation has only recently begun to emerge.

The regulators RovA and H-NS previously have been reported to bind to the *inv* promoter in *Y. pseudotuberculosis* (21, 41), and here we demonstrate that these two regulators bind to a similar region within the *inv* promoter of *Y. enterocolitica*, suggesting a conserved mechanism of *inv* regulation between the two species. DNase I protection assays in *Y. pseudotuberculosis* revealed that the binding sites for RovA and H-NS overlap (21). We also showed that RovA and H-NS are unable to cooperatively bind to the *inv* promoter and that they compete with each other for binding to *inv*. Like H-NS, *Y. pseudotuberculosis* RovA also binds AT-rich regions of DNA (21), and we suspect that this will hold true in *Y. enterocolitica*, as the promoter fragments bound by RovA and H-NS also contain AT-rich segments. However, H-NS is believed to recognize DNA conformation (9, 61) rather than a consensus sequence. Recently, we identified additional genes regulated by RovA in *Y. enterocolitica* and *Yersinia pestis* and are currently analyzing the promoters to determine if a consensus sequence may be present that is not obvious from examination of the *inv* promoters alone (J. Cathelyn, D. W. Ellison, and V. L. Miller, unpublished data).

While RovA is required for expression from the native *inv* promoter, we demonstrated that truncation of the promoter and loss of putative H-NS binding sites results in *inv* expression in the absence of RovA. Our current interpretation of these results is that RovA mediates *inv* expression by competing for binding to the promoter with H-NS and counteracting H-NS-mediated repression. This is similar to the mechanism proposed for derepression of the *hlyE* promoter by SlyA (60) and is supported by data from Heroven et al. that show *inv* expression increases in an *hns* mutant *E. coli* but expression is not enhanced by addition of RovA to the mutant (21). While RovA is not required for the expression of *inv*, recent data from in vitro transcription assays indicate that RovA is able to enhance transcription of *inv* by interaction with RNA polymerase (57). This is similar to the regulation of *tcp* in *Vibrio cholerae*, where ToxT counteracts H-NS repression and enhances transcription through stimulation of RNA polymerase (44, 62). The identification of other genes regulated by RovA poses the question of whether or not RovA regulates expression of these targets in the same fashion as it does with *inv*.

We have also shown for the first time in *Y. enterocolitica* that an H-NS/YmoA complex is involved in the negative regulation of gene expression. Although YmoA has the ability to bind to the *inv* promoter, this interaction does not appear to be specific, as addition of nonspecific competitor DNA prevents binding. Our data suggest that YmoA interacts with H-NS at the *inv* promoter to form a negative regulatory complex. However, YmoA may still interact with DNA to stabilize the repression complex or provide further specificity in promoter binding within the H-NS/YmoA complex. Furthermore, inter-

actions between YmoA homologs and H-NS may be a conserved regulatory mechanism, as a similar regulatory scheme has been proposed in *E. coli* for *hly* regulation by Hha (43). In the future it will be interesting to determine if the H-NS/YmoA complex represses other genes regulated by RovA in a similar manner. The regulation of *rovA* in *Y. pseudotuberculosis* shows similar regulation to *inv*. It is positively autoregulated by RovA and negatively regulated by H-NS through competition for binding sites within the *rovA* promoter (21, 41). These data provide evidence that RovA and H-NS may regulate multiple genes in a similar manner.

The regulation of *hns* and *ymoA* in response to temperature had not previously been investigated in *Y. enterocolitica*. Previously, YmoA was shown to be present at both 26°C and 37°C in *Y. pestis*. However, YmoA was shown to be degraded more rapidly at 37°C than at 26°C in *Y. pestis* (25). Our data indicate little difference in the steady-state expression or protein levels of H-NS and YmoA at 26°C versus 37°C in *Y. enterocolitica*. This led us to hypothesize that the repression complex forms at both 26°C and 37°C and expression of RovA at 26°C would relieve repression of *inv*, presumably by competing for binding with H-NS. Therefore, the activation of *inv* expression is largely dependent on the concentration of RovA in the cell and its ability to compete for binding with the H-NS/YmoA complex. To test this hypothesis, we expressed RovA under conditions where *inv* is normally repressed (37°C) and we were able to activate the expression of *inv*, lending support for our model. These results are similar to data for VirB from *Shigella flexneri* that show that under H-NS-repressed conditions increasing the levels of VirB leads to activation of transcription of VirB-dependent promoters (3). While the concentration of RovA in the cell has been shown to be important for *inv* regulation, the factors that control the RovA concentration within the cell remain an open question in *Y. enterocolitica*.

Nucleoid proteins have been shown to repress transcription of genes through several different mechanisms (for reviews, see references 1 and 35). Here we have demonstrated that H-NS preferentially binds to a region of the *inv* promoter just upstream of the −35 to −10 region, similar to results observed in *Y. pseudotuberculosis* using DNase I footprinting (21). This led us to propose three possible models for H-NS-mediated repression of *inv*: (i) the upstream region of *inv* could provide a high-affinity H-NS binding site, allowing nucleation of H-NS and recruitment of YmoA to form a higher-order repression complex that blocks RNA polymerase from binding to the promoter (51). (ii) Formation of an H-NS/YmoA repression complex upstream of the −35 sequence could cause silencing of the promoter by locally constraining negative supercoils (16, 17, 40, 58). (iii) The H-NS/YmoA complex could trap RNA polymerase in an open complex that is incapable of proceeding into the elongation phase (52, 53). While none of these can be ruled out, the repression of *inv* could involve more than one of the mentioned mechanisms.

Temperature has been shown to be involved in the regulation of many virulence genes (for reviews, see references 22, 28, 34, and 36). H-NS is involved in silencing a number of virulence genes at ambient temperature, and this is released when organisms are shifted to 37°C (49, 56, 59). This is thought to be due to structural changes in the DNA that reduce the ability of H-NS to repress transcription (13). Thermoregula-tion of *inv* is unique in that *inv* is highly expressed at 26°C and repressed by an H-NS/YmoA complex at 37°C, whereas other YmoA-regulated genes are expressed primarily at 37°C. It is interesting that YmoA is involved in the repression of genes at both 26°C and 37°C in *Y. enterocolitica* (8, 12). The ability of H-NS to form tetramers is important for its function, and recent research has shown that as the growth temperature decreases so does the ability of H-NS to form tetramers (54). The reduced ability of H-NS to form tetramers at lower temperature could have an impact on the regulation of *inv*. However, further investigation is needed to determine what role a reduction in H-NS tetramer formation may have on *inv* transcription. Temperature has also been shown to affect the structure of DNA. Changes in DNA bending and/or supercoiling could have an effect on the regulation of *inv*, as these structural changes can affect binding by regulatory proteins such as H-NS. It will be interesting in the future to investigate the influence that temperature may have on the structure of the *inv* promoter and how these changes affect *inv* regulation.

This work has demonstrated that the regulation of *inv* in *Y. enterocolitica* and *Y. pseudotuberculosis* appears to be mechanistically conserved, with RovA acting primarily as a depressor of *inv* by competing with H-NS for binding sites within the *inv* promoter. We have also furthered the understanding of *inv* regulation by showing that *inv* is repressed by an H-NS/YmoA protein complex. We have determined that the concentration of RovA within the cell is critical for the activation of *inv* and presented a model where the H-NS/YmoA complex is able to repress the transcription of *inv* under conditions of low RovA concentration, but under conditions of increased RovA concentration RovA is able to successfully compete with the H-NS/YmoA complex and activate transcription of *inv*. While the levels of RovA within the cell are clearly important for activation of *inv*, there are other factors that may play a significant role in the regulation of *inv*. These include changes in DNA structure, changes in the oligomeric state of H-NS, and interaction with RNA polymerase and other proteins that have yet to be identified. Understanding the interactions between H-NS, YmoA, and RovA may provide insight into the global regulation of other potential virulence factors.

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