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Comparative Analysis of the Regulation of *rovA* from the Pathogenic Yersiniae[▽]

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RovA is a MarR/SlyA-type regulator that mediates the transcription of *inv* in *Yersinia enterocolitica* and *Y. pseudotuberculosis*. In *Y. pseudotuberculosis*, *rovA* transcription is controlled primarily by H-NS and RovA, which bind to similar regions within the *rovA* promoter. At 37°C, *rovA* transcription is repressed by H-NS. Transcription of *rovA* results when RovA relieves H-NS-mediated repression. The region of the *rovA* promoter that H-NS and RovA bind is not conserved in the *Y. enterocolitica* promoter. Using green fluorescent protein reporters, we determined that the *Y. enterocolitica rovA* ($rovA_{Yent}$) promoter is weaker than the *Y. pseudotuberculosis* promoter. However, despite the missing H-NS/RovA binding site in the $rovA_{Yent}$ promoter, H-NS and RovA are still involved in the regulation of $rovA_{Yent}$. DNA binding studies suggest that H-NS and RovA bind with a higher affinity to the *Y. pseudotuberculosis*/*Y. pestis rovA* ($rovA_{Ypstb/Ypestis}$) promoter than to the $rovA_{Yent}$ promoter. Furthermore, H-NS appears to bind to two regions in a cooperative fashion within the $rovA_{Yent}$ promoter that is not observed with the $rovA_{Ypstb/Ypestis}$ promoter. Finally, using a transposon mutagenesis approach, we identified a new positive regulator of *rovA* in *Y. enterocolitica*, LeuO. In *Escherichia coli*, LeuO regulates gene expression via changes in levels of RpoS and H-NS, but LeuO-mediated regulation of $rovA_{Yent}$ appears to be independent of either of these two proteins. Together, these data demonstrate that while the *rovA* regulatory factors are conserved in *Yersinia*, divergence of *Y. enterocolitica* and *Y. pseudotuberculosis*/*Y. pestis* during evolution has resulted in modifications in the mechanisms that are responsible for controlling *rovA* transcription.

There are three pathogenic species of *Yersinia* that cause disease in humans. *Y. pestis* is a vector-borne pathogen that causes plague and has been responsible for three major pandemics, including an ongoing pandemic that began in the 1860s (45). *Y. enterocolitica* and *Y. pseudotuberculosis* are gastrointestinal pathogens that cause milder manifestations of disease and enter the host through consumption of contaminated food or water (2, 41). Sharing the same route of infection, *Y. enterocolitica* and *Y. pseudotuberculosis* utilize similar virulence factors that appear to be inactivated in *Y. pestis*, including the colonization factors invasin (Inv) and YadA. These adhesins are important for colonization by the oral pathogens but are no longer expressed in *Y. pestis* due to naturally occurring mutations (51, 52).

Invasin is the major adherence factor encoded in both *Y. enterocolitica* and *Y. pseudotuberculosis* (20, 36). Inv is believed to promote efficient entry into the Peyer's patches of the small intestine through interactions with β1 integrins on the surface of the M cells overlying these lymphoid tissues (4, 21, 31, 49). During in vitro culture, *inv* transcription is regulated by temperature and growth phase. The highest levels of Inv expression are observed in early-stationary-phase cultures incubated at 23 to 26°C. The pH of the culture medium has also been shown to alter the expression of *inv* in *Y. enterocolitica* cultures grown at 37°C (22, 43). Work to understand the regulation of *inv* has identified three proteins involved in modulating the levels of Inv (10, 12, 39, 40). H-NS and YmoA have been implicated in repression of *inv* transcription. H-NS is a histone-like protein that is important for the proper nucleoid packaging of the bacterial chromosome (1) and is involved in the regulation of multiple genes, including virulence factor genes, in response to temperature (1, 16, 38). YmoA is a member of the Hha/YmoA family of regulatory proteins (5, 29). Studies in *Escherichia coli* with the YmoA homolog Hha were the first studies to demonstrate that Hha/YmoA members interact with H-NS to enhance repression mediated by H-NS (42). Ellison and Miller extended these observations to *Yersinia* and demonstrated that YmoA interacts with H-NS during the regulation of *inv* transcription (10). The current model for *inv* repression proposes that H-NS recognizes and binds to the *inv* promoter, where YmoA interacts with H-NS to propagate the formation of a regulatory complex, blocking recognition of the promoter by RNA polymerase.

The third protein involved in *inv* regulation is RovA, which is necessary for the expression of Inv (40, 46). RovA is a member of the SlyA/MarR regulatory family, which contains homologs in several species of bacteria and archaea, including all three pathogenic species of *Yersinia* (11). SlyA/MarR family members have been shown to regulate a variety of functions, including resistance to antibiotics (15, 54), production of antimicrobial agents (55), and expression of virulence factors (19, 27, 46, 47). In *Yersinia*, microarray data suggest that RovA regulates the expression of multiple genes in addition to *inv* (3; J. S. Cathelyn and V. L. Miller, unpublished data). During *inv* regulation in *Y. enterocolitica*, RovA appears to act as a derepressor to relieve the negative regulation of the H-NS/YmoA complex. In vitro analysis of RovA and H-NS binding to the *inv* promoter indicates that RovA and H-NS bind to similar overlapping regions in both *Y. pseudotuberculosis* and *Y. enteroco-*

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litica. Furthermore, RovA can displace H-NS bound to the *inv* promoter (10). Therefore, in vivo it is predicted that RovA binding either displaces H-NS/YmoA or relieves topological restraints imposed by H-NS/YmoA on the promoter to allow access to RNA polymerase, resulting in transcription of *inv*. This model is supported by work demonstrating that the RovA level relative to the H-NS/YmoA level within the cell is a key determinant of *inv* expression and that RovA is not required for *inv* expression in *Y. enterocolitica* if the *inv* promoter is truncated to remove the H-NS binding site (10). Thus, an understanding of how *rovA* is regulated is necessary to understand the expression of *inv* and other RovA-regulated genes.

The regulation of *rovA* in *Y. pseudotuberculosis* appears to use a mechanism similar to that observed for *inv* regulation in *Y. enterocolitica*. Heroven et al. have demonstrated that H-NS and RovA regulate the levels of *rovA* transcription in an *E. coli* surrogate system, suggesting that these proteins regulate the expression of *rovA* in *Y. pseudotuberculosis* as well (18). These authors reported that *rovA* transcription levels are low in wild-type *E. coli* but increase with either the addition of RovA or the inactivation of the *E. coli hns* gene. In *Y. pseudotuberculosis*, addition of a plasmid encoding a trans copy of *hns* also decreased the levels of RovA. In vitro, H-NS and RovA bind to similar regions within the *Y. pseudotuberculosis rovA* promoter, suggesting that RovA may use a conserved mechanism of H-NS derepression to regulate the expression of genes. Data suggest that RovA may have an additional negative role in autoregulation. As levels of RovA were increased in an *E. coli* strain carrying a *rovA*::*lacZ* reporter, a moderate decrease in *rovA* transcription (~2-fold) was observed (18). In vitro data also suggest that RovA binds to a second region within the *Y. pseudotuberculosis rovA* promoter, although at a lower affinity than to the previously described binding site. Heroven et al. have suggested that as levels of RovA increase, RovA may bind to this second binding site, resulting in decreased transcription of the gene (18).

Recently, a third protein has been implicated in *rovA* regulation in *Y. pseudotuberculosis*. RovM (modulator of *rovA* expression) is a LysR-like protein that represses the expression of *rovA* in response to growth in minimal medium (17). Further, Heroven et al. demonstrated in vitro binding of recombinant RovM to the *rovA* promoter and suggested that RovM directly represses *rovA* expression through this interaction. RovM also autoregulates its own expression and affects the motility of *Y. pseudotuberculosis* independent of *rovA*. Interestingly, a *rovM* mutant is hypervirulent during oral infection of the mouse model, consistent with previous studies suggesting that there is a requirement for RovA in virulence (9, 46).

Comparisons of the DNA sequences upstream of *rovA* from *Y. pseudotuberculosis*, *Y. pestis*, and *Y. enterocolitica* reveal considerable divergence in the *Y. enterocolitica rovA* promoter. Notably, while the *Y. pseudotuberculosis* and *Y. pestis* promoter sequences are identical to each other (the *rovA* coding regions are also identical in these two species), there are two predicted open reading frames (ORFs) upstream of *rovA* in *Y. enterocolitica* that are not found in *Y. pseudotuberculosis* and *Y. pestis*. Furthermore, these ORFs appear to have replaced the predicted H-NS/RovA binding region reported in *Y. pseudotuberculosis* and *Y. pestis*. These significant differences in the *Y. enterocolitica rovA* ($rovA_{Yent}$) promoter suggest that the regulatory mechanism of *rovA* could differ in *Y. enterocolitica*. To understand how these differences in the *rovA* promoter affect the transcription of *rovA* in *Y. enterocolitica*, we defined the promoter of *rovA* in *Y. enterocolitica*, compared the expression of this promoter to the expression of the *Y. pseudotuberculosis*/*Y. pestis* promoter in different *Yersinia* backgrounds, and elucidated the effects of RovA, H-NS, and RovM on *rovA* transcription in *Y. enterocolitica*. Furthermore, we used transposon mutagenesis to identify a fourth regulator of *rovA* in *Y. enterocolitica*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Y. enterocolitica* and *E. coli* strains were grown in Luria-Bertani (LB) broth at 26°C or 37°C as indicated below. *Y. pestis* strains were grown in brain heart infusion (BHI) broth at 26°C. When appropriate, antibiotics were used at the following concentrations: nalidixic acid, 20 μg ml^{-1}; tetracycline, 15 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; and spectinomycin, 100 μg ml^{-1}. Primers used in plasmid construction in this study are listed in Table 2. To generate in-frame *lacZ*, *rovA*, *leuO*, and *rpoS* deletion strains, the regions flanking the genes were amplified by PCR, digested with SalI, NotI, and BamHI, and ligated into the suicide plasmid pSR47s (34). The suicide plasmids were introduced into *Y. enterocolitica* through conjugation, and mutants were selected as described previously (59). The YVM1251 *rovA*::*lacZ* reporter was generated using the system described by Maxson and Darwin (32). To construct *rovA*::green fluorescent protein (GFP) reporters, regions of the promoter were amplified by PCR, digested, and ligated into the low-copy-number vector pPROBE-gfp[LVA] (37). pLEUO was generated by amplifying *leuO* and 500 bp upstream of *leuO* by PCR and introducing the product into pCR2.1 by TOPO cloning (Invitrogen, Carlsbad, CA).

Primer extension. RNA was extracted from *Y. enterocolitica* using a RiboPure-Bacteria isolation kit (Ambion, Austin, TX) and treated with DNase I (Ambion) as described by the manufacturer. Primers PE1 and PE2 (Table 2) were labeled with ^{32}P using T4 DNA polynucleotide kinase (New England Biolabs, Beverley, MA). One picomole of labeled primer was hybridized with 10 μg of total RNA and incubated with 20 U of Superscript III reverse transcriptase (Invitrogen). The reaction was terminated by addition of stop solution, and primer-extended products were separated on 8% polyacylamide-8 M urea gels (58).

GFP assays. For *Y. enterocolitica* and *E. coli*, strains were grown in triplicate overnight in 2 ml of LB broth at 26°C with aeration and diluted to an optical density at 600 nm (OD_{600}) of 0.1 in media containing the appropriate antibiotics. Then 0.7 ml of each diluted culture was inoculated into individual wells in a 48-well plate and grown with shaking for 10 h at 26°C in a Synergy HT kinetic plate reader (BioTek, Winooski, VT). The OD_{600} and fluorescence (measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm) of the cultures were determined at numerous intervals. For *Y. pestis*, bacteria were grown overnight in 2 ml of BHI broth at 26°C with aeration and diluted to an OD_{600} of 0.2 in 20 ml of BHI broth containing the appropriate antibiotics. The cultures were grown for 6 h with aeration, and the OD_{600} and fluorescence of the cultures were determined. Data for *Y. pestis* studies were normalized and expressed as fluorescence (in relative light units)/OD_{600}.

EMSA. Primers used to generate *rovA* promoter fragments used in an electrophoretic mobility gel shift assay (EMSA) are listed in Table 2. An approximately 500-bp fragment of the *ysaE* promoter, which is not regulated by RovA or H-NS, was used as a control for nonspecific binding. Protein purification and binding reactions were performed as described previously (10).

Transposon mutagenesis. YVM1251 was mutagenized with the Tn*5* transposon Tn*Mod*-RKm′ (6). Tn*Mod*-RKm′ contains an R6K origin of replication and a kanamycin resistance cassette which are integrated into the target DNA during successful transposition. Transposon mutants were selected on MacConkey plates with kanamycin containing 1% lactose incubated at 26°C and initially screened for effects on *rovA*::*lacZ* expression levels by determining colorimetric differences in colonies. Colonies with altered intensities of the red color compared to the majority of the colonies were purified, inoculated into 2-ml broth cultures, and grown overnight at 26°C, and β-galactosidase activities were determined (35). Mutants that demonstrated at least a 20% change in activity were stored at −80°C for future analysis.

To rule out false-negative mutants that affected the reporter and not the native *rovA* gene, anti-RovA Western blotting was performed as a secondary screen.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Y. enterocolitica strains		
YVM150	JB580 Δ*yenR* (R^- M^+)	23
YVM927	JB580 Δ*yenR* (R^- M^+) Δ*rovA*	10
YVM1251	JB580 Δ*yenR* (R^- M^+) pYV^- Δ*lacZ* Δ*araGFB*::[ϕ($rovA_{Yent}$-*lacZY*)]	This study
YVM1252	JB580 Δ*yenR* (R^- M^+) pYV^- Δ*lacZ* Δ*araGFB*::[ϕ($rovA_{Yent}$-*lacZY*)] Δ*leuO*	This study
YVM1253	JB580 Δ*yenR* (R^- M^+) Δ*rpoS*	This study
YVM1254	JB580 Δ*yenR* (R^- M^+) Δ*rovM*	This study
TM102	JB580 Δ*yenR* (R^- M^+) pYV^- Δ*lacZ* Δ*araGFB*::[ϕ($rovA_{Yent}$-*lacZY*)] *leuO*::miniTn*5* Km^r	This study
Y. pestis strains		
YP6	CO92 $pCD1^-$	7
YP12	CO92 $pCD1^-$ Δ*rovA*	3
E. coli strains		
MC4100	F^- *araD139* Δ(*lacIOPZYA*)*U169 rpsL thiA*	14
VM1303	MC4100 *hns205*::Tn*10* Tet^r	10
Plasmids		
pPROBE-gfp[LVA]	Promoterless *gfp* with LVA tag to decrease GFP half-life, Km^r	37
pYEL	pPROBE-gfp[LVA] containing $rovA_{Yent}$ promoter fragment from nt −614 to 170	This study
pYES	pPROBE-gfp[LVA] containing $rovA_{Yent}$ promoter fragment from nt −445 to 170	This study
pYPL	pPROBE-gfp[LVA] containing $rovA_{Ypstb/Ypestis}$ promoter fragment from nt −622 to 170	This study
pYPS	pPROBE-gfp[LVA] containing $rovA_{Ypstb/Ypestis}$ promoter fragment from nt −443 to 170	This study
pWKS30::StrSpec	pWKS30 with a Str/Spec cassette in the HindIII site of the polylinker, Amp^r Str^r $Spec^r$	10
pHNS	pWKS30::Str/Spec::*hns* with native promoter region	10
pLEUO	pCR2.1::*leuO* with native promoter region	This study

Bacteria were grown in LB broth at 26°C to mid-logarithmic phase, harvested, and resuspended in Laemmli buffer containing 10% β-mercaptoethanol. A volume of each sample equivalent to an OD_{600} of 0.1 was separated on 15% polyacrylamide-sodium dodecyl sulfate gels and transferred to a polyvinylidene difluoride membrane. RovA was detected using rabbit polyclonal anti-RovA serum (1:5,000) as described previously (10). Levels of RovA from the transposon mutants were compared to levels of the protein from YVM1251. To verify that samples having an OD_{600} of 0.1 were equivalent, protein samples were also analyzed by Coomassie blue staining.

The R6K origin of replication within the transposon was used to reisolate the transposon along with flanking chromosomal DNA from the mutants as described below. Genomic DNA was isolated as previously described (33), and 50 μl was digested with EcoRV (New England Biolabs) overnight at 37°C. This restriction enzyme does not digest the DNA within the transposon, and therefore, the digested fragment containing the transposon had flanking ends that could be sequenced to identify the location of the transposon insertion within the *Y. enterocolitica* genome. The digested DNA was diluted 10-fold and ligated with T4 DNA ligase (New England Biolabs) overnight at 16°C. The ligated DNA was desalted and concentrated by ethanol precipitation to 30 μl. The entire ligation preparation was transformed into *E. coli* S17 λ *pir* by electroporation. Bacteria containing the religated transposon were selected on LB agar containing kanamycin. Three clones from each ligation were selected for sequencing using primer TNF. Sequence data were compared to the *Y. enterocolitica* genome from the *Y. enterocolitica* Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/Y_enterocolitica/) using BLAST.

Western blot analysis. *Y. enterocolitica* cultures were grown overnight in 2 ml of LB broth at 26°C with aeration, and volumes equivalent to 1.0 OD_{600} unit were harvested by centrifugation and resuspended in Laemmli buffer containing 10% β-mercaptoethanol. Whole-cell extracts equivalent to an OD_{600} of 0.1 were separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. To confirm that protein samples were equivalent, total proteins were also analyzed by Coomassie blue staining prior to Western blot analysis. H-NS was detected with mouse anti-H-NS antibody kindly supplied by Yeong-Jae Seok, Seoul National University (50).

RESULTS

***rovA* promoter in *Y. enterocolitica*.** The DNA upstream of *rovA* in *Y. enterocolitica* differs greatly from the *Y. pseudotuberculosis* and *Y. pestis* DNA, with increasing divergence further 5′ of the gene (Fig. 1). In contrast, the *Y. pseudotuberculosis* and *Y. pestis* sequences are identical. The differences include dramatic changes in the region of the *Y. enterocolitica* promoter that corresponds to a predicted H-NS/RovA binding site of the *Y. pseudotuberculosis rovA* promoter. Furthermore, two putative ORFs upstream of *rovA* in *Y. enterocolitica* are absent in *Y. pseudotuberculosis*/*Y. pestis*. The ORFs overlap or replace regions that have been shown to be important for *rovA* regulation in *Y. pseudotuberculosis* (18). These variations in important regulatory domains of the *Y. pseudotuberculosis* promoter suggested that *rovA* regulation might differ in *Y. enterocolitica*.

To begin our comparison of the *rovA* promoters (the $rovA_{Yent}$ and *Y. pseudotuberculosis*/*Y. pestis rovA* [$rovA_{Ypstb/Ypestis}$] promoters), we first determined the transcriptional initiation sites of $rovA_{Yent}$ using primer extension. In addition to the two initiation sites previously reported in *Y. pseudotuberculosis* (18), a third *rovA* putative transcript initiates downstream of P1 in *Y. enterocolitica* (Fig. 2). P2 begins at the same nucleotide in *Y. enterocolitica* that has been reported for *Y. pseudotuberculosis* and shares a very similar predicted −10/−35 region (one nucleotide difference in *Y. enterocolitica*) that is a strong σ^{70} consensus sequence. P1 of $rovA_{Yent}$ appears to initiate approximately 3 nucleotides (nt) upstream of P1 in $rovA_{Ypstb/Ypestis}$ but shares a conserved predicted −10/−35 region. P3 is predicted to begin ~35 bp downstream of P1 and has a −10/−35 region with weaker similarity to the σ^{70} consensus sequence.

A subset of environmental conditions that may affect the expression of *rovA* were tested, and only temperature influenced *rovA* transcription. As reported for *Y. pseudotuberculosis*, we observed an increase in the *rovA* transcript by Northern analysis in

TABLE 2. Primers used in this study

Primer	Sequence[a]
Deletion constructs	
lacZ	
lacZ5′F	ACGCGTCGACGCCAGCAATACCCCATTTAGC
lacZ5′R	CGGGATCCAATTTCAGCCTTATCTTTTACGAAAGTTAGC
lacZ3′F	CGGGATCCGCAACAATATCAACACAGAATTTCTAATACGC
lacZ3′R	ATAAGAATGCGGCCGCTGCTGGGCTATAATCTGGTGC
leuO	
leuO5′F	ACGCGTCGACGGATTGGTTCATGCTTCTTATATTTTATGGCT
leuO5′R	CGGGATCCTACTAAGTTGTGTTCAAACATGCTTAACTCCAC
leuO3′F	CGGGATCCTAGTTATTATCATTAAGTCCTGCTGCTGAAGTTG
leuO3′R	ATAAGAATGCGGCCGCACGCTCTTGGATGGCAGC
rpoS	
rpoS5′F	ACGCGTCGACTGTCGCTACAACCGCACC
rpoS5′R	GGAAGATCTCATATGCTGCTCCTACCCGTG
rpoS3′F	GGAAGATCTTAGCGATACTCTCGCAAACAGTCTG
rpoS3′R	ATAAGAATGCGGCCGCCCGTATCAAAGCCATGACGCTA
Reporters	
rovA::*lacZ*	
rlacZF	TGCTCTAGAGGGTCAATGACAAATAATAAGCCTCCAGT
rlacZR	CGGGATCCACCAATCGCTTTCGCCAGTTG
rovA::*gfp*	
pYELF	CGGGATCCTGGTTATCATGAACTAATATTTTAACCAATCGGC
pYESF	CGGGATCCAGGGGGATTGCATATAATAATTCCACA
pYEL-2R	GGAATTCACCAATCGCTTTCGCCAGTTG
pYPLF	CGGGATCCTGCCGCCTTCCTGCAA
pYPSF	CGGGATCCTTTGAAATATTGATGATTCATATCAATTTACCCAAGTC
pYPL-2R	GGAATTCACCAATCGCTTTCGCCAGTTG
Complementation clone: pLEUO	
pLEUOF	ACGCGTCGACGGATTGGTTCATGCTTCTTATATTTTATGGCT
pLEUOR	GGGGTACCTTAAGAAGGAATATTAAGCTGGCTGAGTAATTC
Primer extension	
PE1	CTAATCGTGCTAAATCAGATCC
PE2	AAAATTATGTATTTACTAAAATTACCTCTTAAGGA
EMSA	
YP1-2F	TGC CGC CTT CCT GCA A
YP3-4F	TTTGAAATATTGATGATTCATATCAATTTACCCAAGTC
YP1-4R	ACCAATCGCTTTCGCCAGTTG
YP2-3R	GGACAATAGCAATAAATACGGGGAA
YE1-2F	TGGTTATCATGAACTAATATTTTAACCAATCGGC
YE3F	AGGGGGATTGCATATAATAATTCCACA
YE4F	ATTTAAGAGACTGATGATTCATATCAATTTACCAAC
YE5F	TTGGATGCCAGATATCACCC
YE6F	ACCTAGCATAACCGCCTTAAAAATT
YE1-4-5-6R	ACCAATCGCTTTCGCCAGTTG
YE2-3R	GGACAATAGCAATAAATACGGGGAA
Transposon sequencing	
TNF	CCCATGTCAGCCGTTAAGTGT

[a] Underlining indicates restriction sites.

cultures grown at 26°C compared to cultures grown at 37°C (approximately fourfold higher at 26°C). This transcription profile correlated directly with protein levels in the cultures (10; data not shown). We did not observe a change in transcription in response to nutrient limitation, changes in the concentrations of iron or magnesium, increases in $NaCl_2$ concentrations, or alterations in the pH of the medium (data not shown).

Transcription of *rovA* in *Y. enterocolitica* and the influence of expression by RovA. To further characterize the $rovA_{Yent}$ promoter and compare the expression to that of the $rovA_{Ypstb/Ypestis}$

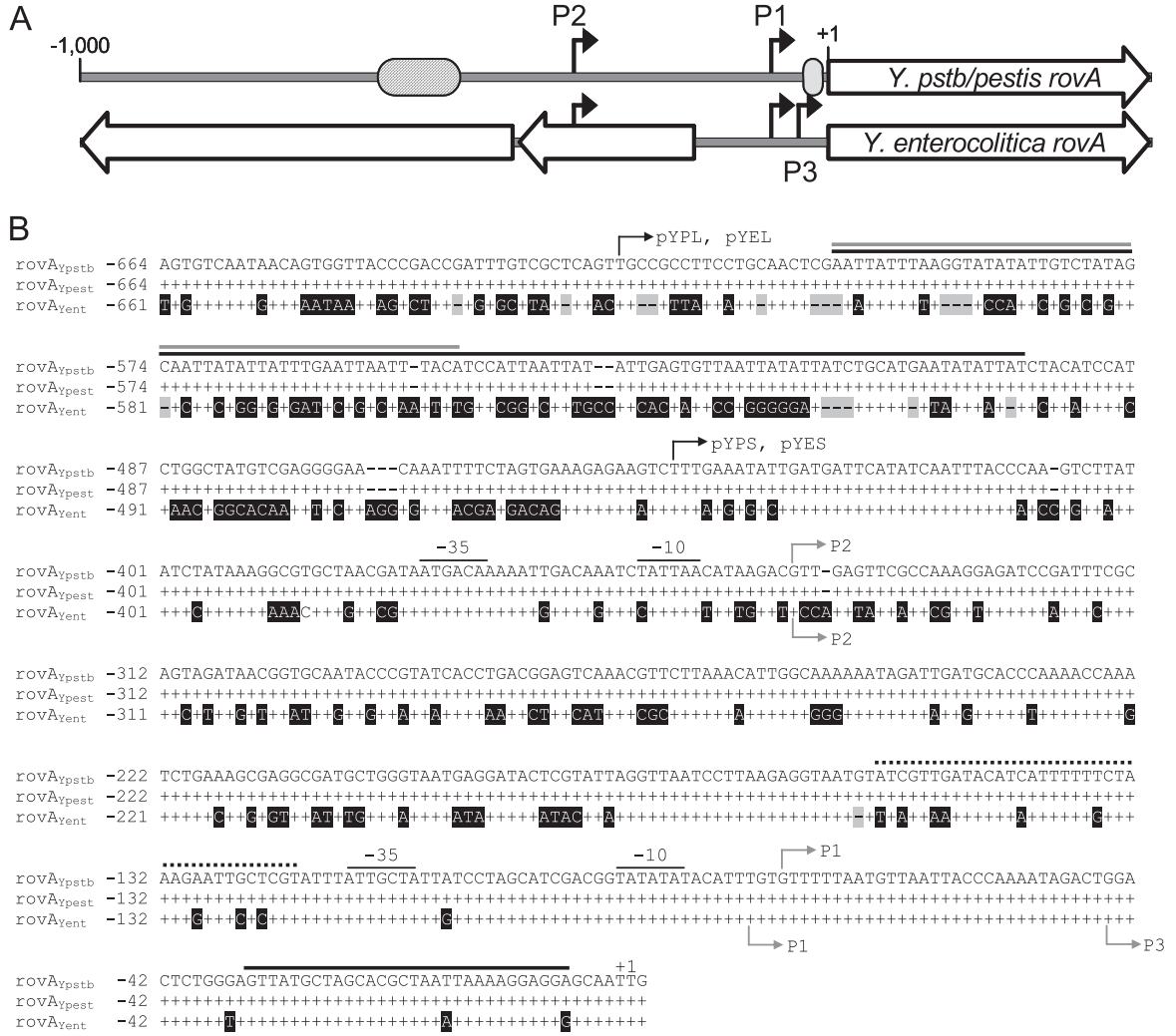

FIG. 1. *rovA* promoters of *Yersinia*. (A) Linear representation of comparisons of the *rovA* regions from *Y. pseudotuberculosis*/*Y. pestis* (*Y.pstb*/*pestis rovA*) and *Y. enterocolitica* (*Y. enterocolitica rovA*). The black arrows indicate transcriptional initiation sites. The cross-hatched oval represents the predicted high-affinity RovA/H-NS binding site. The gray oval represents the predicted low-affinity RovA binding site. The large open arrows represent ORFs. (B) Sequence alignment of DNA upstream of *rovA* in *Y. pseudotuberculosis* ($rovA_{Ypstb}$) with similar regions in *Y. pestis* ($rovA_{Ypest}$) and *Y. enterocolitica* ($rovA_{Yent}$). Conserved nucleotides are represented by plus signs, and divergent nucleotides are indicated by a black background. Gaps are indicated by shaded dashes. The initiation codon for *rovA* is designated +1. The gray arrows indicate mRNA initiation sites for *Y. pseudotuberculosis* (above the sequences) or *Y. enterocolitica* (below the sequences). Predicted −35 and −10 regions for P1 and P2 in *Y. pseudotuberculosis* are also shown. Solid black, gray, and dotted lines above the sequences indicate predicted RovA, H-NS, and RovM binding sites, respectively, in *Y. pseudotuberculosis* (17, 18). The black arrows indicate the locations of the most 5′ nucleotides of reporter constructs.

promoter, we generated a series of GFP reporters fused to promoter regions from either $rovA_{Yent}$ or $rovA_{Ypstb/Ypestis}$ (Table 1 and Fig. 1). The first pair of reporters (pYEL from *Y. enterocolitica* and pYPL from *Y. pseudotuberculosis*/*Y. pestis*) includes the first 170 bp of *rovA* and approximately 620 bp of upstream DNA. pYPL includes the predicted H-NS and RovA binding sites of *Y. pseudotuberculosis* (Fig. 1) (18). The second pair of reporters (pYES and pYPS) also includes 170 bp of the *rovA* coding region but only approximately 445 bp of the upstream region, resulting in loss of the predicted H-NS/RovA binding site of *Y. pseudotuberculosis*. These reporters were transformed into wild-type and Δ*rovA* strains of *Y. enterocolitica* to compare the activities of the two promoters.

The pYPL reporter demonstrated approximately fivefold-higher activity than the equivalent *Y. enterocolitica* reporter, pYEL, suggesting that the $rovA_{Ypstb/Ypestis}$ promoter is a stronger promoter than the $rovA_{Yent}$ promoter in *Y. enterocolitica* (Fig. 3). pYPL also demonstrated a greater requirement for RovA than pYEL. The activity of pYPL decreased almost 20-fold when it was analyzed in a Δ*rovA* background. While a decrease in pYEL activity was also observed in the Δ*rovA* strain, it decreased by only 2.5-fold and was still expressed at levels above background levels. As expected, when we removed the H-NS/RovA binding site from the *Y. pseudotuberculosis*/*Y. pestis* promoter (pYPS), the requirement for RovA for expression was lost. Interestingly, when a similar amount of DNA was removed from the 5′ end of the *Y. enterocolitica* promoter (pYES), we observed a partial dependence on RovA. Similar

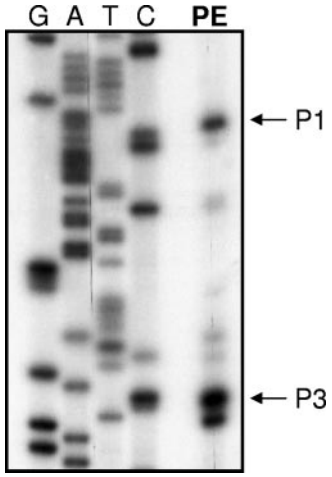

FIG. 2. Mapping of *rovA* transcriptional initiation sites in *Y. enterocolitica*. Primer extension (lane PE) was performed to determine if transcription in *Y. enterocolitica* initiated from the same promoters that have been reported for *Y. pseudotuberculosis*. P3 indicates that there is a potential third transcript in *Y. enterocolitica* that initiates downstream of the conserved promoter P1. Sequencing ladders shown on left side of the gel.

differences in promoter strengths were observed when the reporters were transformed into *Y. pestis* (Fig. 3C). pYPL expression was more than threefold higher than pYEL expression during late stationary phase, and both reporters demonstrated negligible expression in the *rovA* mutant. These results indicate that the $rovA_{Yent}$ promoter is less active than the $rovA_{Ypstb/Ypestis}$ promoter, but regardless of the promoter activities, both promoters require RovA for maximal expression.

H-NS regulation of *rovA*. Heroven et al. previously demonstrated that H-NS is a negative regulator of *rovA* in *Y. pseudotuberculosis*, with repression achieved through direct interactions with a region of the promoter missing in the $rovA_{Yent}$ promoter (Fig. 1) (18). Since $rovA_{Yent}$ is temperature regulated and H-NS is a major mediator of temperature regulation in prokaryotes, we suspected that H-NS mediates *rovA* transcription even in the absence of the $rovA_{Ypstb/Ypestis}$ H-NS binding site. *hns* is apparently required for growth of *Yersinia* as we and others have been unable to inactivate the gene in *Y. enterocolitica* or *Y. pseudotuberculosis* (17; M. B. Lawrenz, D. W. Ellison, C. Affolter, and V. L. Miller, unpublished data). To overcome this obstacle, two independent systems were utilized to provide insight into the role of H-NS regulation of $rovA_{Yent}$. Similar systems have been used previously to determine the impact of H-NS on *Yersinia* gene regulation (10, 18). We determined the expression of the *rovA* reporters in an *E. coli hns* mutant and compared the pattern to expression in wild-type *E. coli* or in the *hns* mutant complemented with *hns* from *Y. enterocolitica* expressed ectopically on a low-copy-number plasmid (pHNS). Activity of the promoters was determined as a function of fluorescence. For clarity, data from cultures at an OD_{600} of approximately 0.5 are displayed, but the trends were conserved throughout the growth curve. As reported previously, $rovA_{Ypstb/Ypestis}$ transcription was dependent on the presence of H-NS (Fig. 4A). In the wild-type MC4100 background no fluorescence in the culture above the fluorescence of the

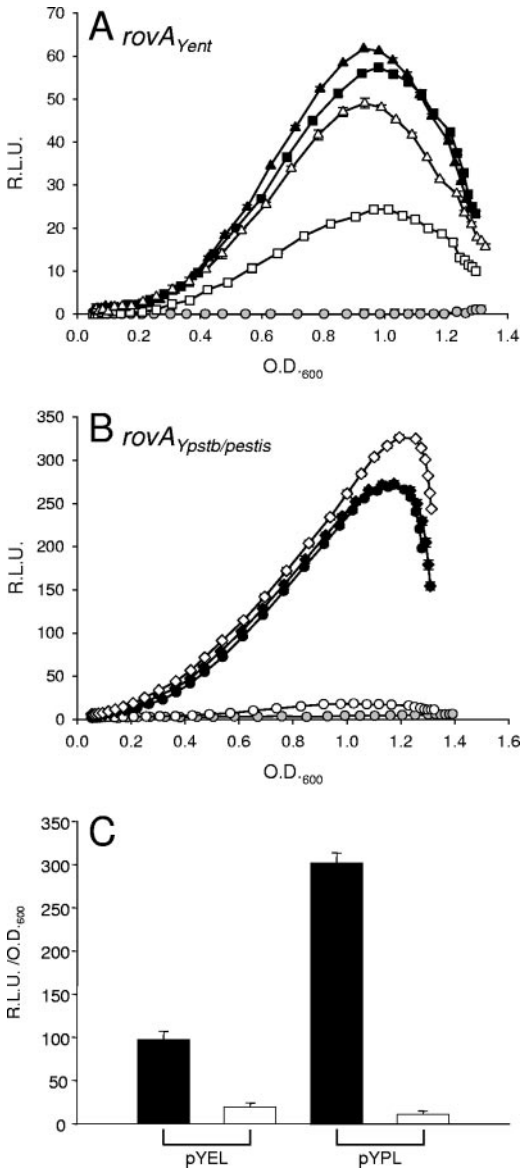

FIG. 3. Influence of RovA on the expression of *rovA*. To determine the effect of RovA on the $rovA_{Yent}$ (A) and $rovA_{Ypstb/Ypestis}$ (B) promoters, expression patterns were determined in wild-type *Y. enterocolitica* (filled symbols) and a Δ*rovA* mutant (open symbols). Four GFP reporters were utilized: *Y. enterocolitica rovA* reporter fusions (squares, pYEL; triangles, pYES) and *Y. pseudotuberculosis*/*Y. pestis rovA* reporter fusions (circles, pYPL; diamonds, pYPS). Gray circles show the results for vector-only controls. The data are expressed in relative light units (R.L.U.) as a function of the OD_{600}. (C) Expression of pYPL and pYEL in wild-type *Y. pestis* (filled bars) and a Δ*rovA* mutant (open bars). The data are expressed in relative light units (R.L.U.)/OD_{600}. Experiments were performed in triplicate, and error bars were plotted but are typically too small to see clearly.

control strain (vector only) was observed. Inactivation of *hns* resulted in dramatic increases in pYPL expression, which were more than 11-fold at this time point and 30-fold at the peak of the GFP expression (data not shown). $rovA_{Yent}$ transcription followed a similar pattern (Fig. 4B). While *hns* inactivation also resulted in increased transcription of the $rovA_{Yent}$ promoter, a more moderate increase was observed (the greatest

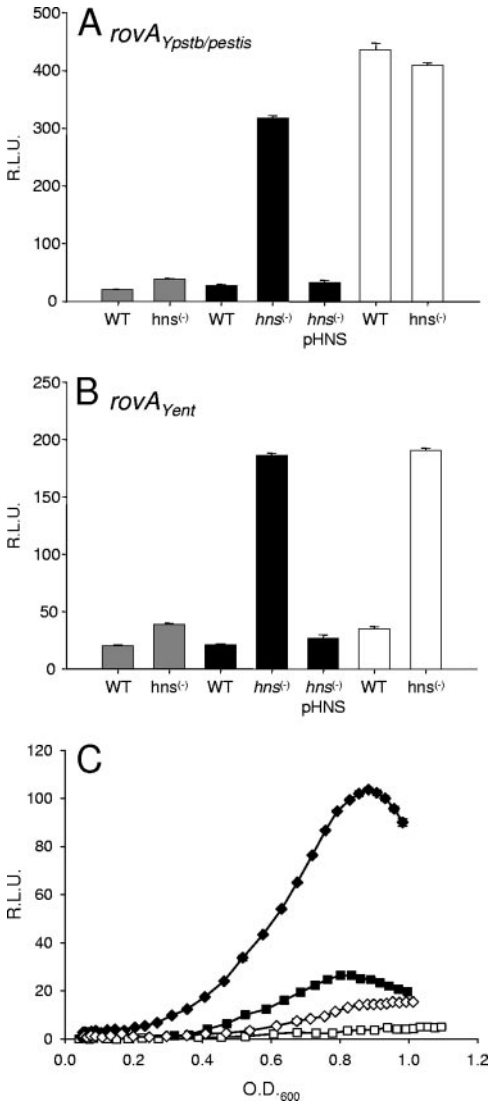

FIG. 4. Influence of H-NS on the expression of *rovA*. To determine the effect of H-NS on the $rovA_{Ypstb/Ypestis}$ (A) and $rovA_{Yent}$ (B) promoters, expression patterns were determined in *E. coli* MC4100 (WT), an *hns* mutant [$hns^{(-)}$], or an *hns* mutant complemented with *Y. enterocolitica hns* [$hns^{(-)}$ pHNS]. Filled bars, pYPL (A) or pYEL (B); open bars, pYPS (A) or pYES (B); gray bars, vector control. (C) Expression of pYPL (diamonds) and pYEL (squares) in wild-type *Y. enterocolitica* (filled symbols) and pHNS-complemented *Y. enterocolitica* (open symbols). The data are expressed in relative light units (R.L.U.) as a function of the OD_{600}.

increase observed was ninefold), and the levels of fluorescence from the pYEL reporter never reached those from the *Y. pseudotuberculosis*/*Y. pestis* reporter. Complementation with pHNS resulted in an expression pattern similar to that of wild-type MC4100 and expression of pYPL and pYEL at background levels. These results strongly indicate that H-NS negatively regulates $rovA_{Yent}$, at least in a surrogate *E. coli* background.

To support our *E. coli* data, we next investigated H-NS regulation of $rovA_{Yent}$ in native *Y. enterocolitica* to determine if the regulation was conserved in the wild-type background. We transformed the reporters into a strain of *Y. enterocolitica* that also contained pHNS. The levels of H-NS in this strain are artificially high at 26°C due to the increased copy number of the gene (10; data not shown). The expression of both pYPL and pYEL was decreased in this strain by approximately 7.5-fold compared to the expression in wild-type *Y. enterocolitica* (Fig. 4C). Taken together with the data from *E. coli*, these results suggest that $rovA_{Yent}$ is modulated by H-NS.

Deletion of 175 bp from the 5′ end of pYPL resulted in *rovA* transcription in wild-type *E. coli* (Fig. 4A), supporting the presence of an H-NS binding site within this region as predicted by in vitro DNA binding experiments of Heroven et al. (18). Deleting a similar region (based on distance from the *rovA* initiation codon) in *Y. enterocolitica* did not relieve H-NS-mediated repression (Fig. 4B). These results demonstrate that while H-NS represses *rovA* transcription in *Y. enterocolitica*, H-NS binds to a different region in the $rovA_{Yent}$ promoter than in *Y. pseudotuberculosis*.

Binding of RovA and H-NS to the *rovA* promoter. In vitro analysis of *Y. pseudotuberculosis* revealed that RovA and H-NS bind to a similar region in the *rovA* promoter (18). Despite the absence of this binding site in the *Y. enterocolitica* promoter, our in vivo data demonstrate that both proteins influence the expression of $rovA_{Yent}$. To determine whether these proteins directly regulate $rovA_{Yent}$ through interactions with the *Y. enterocolitica* promoter, we compared binding of recombinant RovA and H-NS from *Y. enterocolitica* to fragments of the $rovA_{Yent}$ and $rovA_{Ypstb/Ypestis}$ promoters using an EMSA (Fig. 5). As predicted by Heroven et al. (18), RovA bound to fragments of the $rovA_{Ypstb/Ypestis}$ promoter that contained the 5′ RovA binding site (YP1 and YP2) and bound more weakly (requiring at least twofold more protein to bind) to a fragment that contained only the 3′ RovA binding site (YP4) (Fig. 5B). The absence of both sites resulted in loss of RovA binding (YP3). Approximately fourfold-higher concentrations of RovA were required to initiate changes in the mobility of the *Y. enterocolitica* promoter (YE1), and these concentrations approached concentrations leading to nonspecific binding of the negative control (*ysaE*) (Fig. 5B).

H-NS bound to the *Y. pseudotuberculosis*/*Y. pestis* promoter at a concentration approximately threefold lower than the concentration at which it bound to the *Y. enterocolitica* promoter (YP1 and YE1), indicating that H-NS had a higher affinity for $rovA_{Ypstb/Ypestis}$ (Fig. 5C). This interaction with the $rovA_{Yent}$ promoter appears to be specific, as concentrations required for binding YE1 did not bind the *ysaE* control promoter. These results correlate directly with the degree of regulation by RovA and H-NS for these promoters. Both proteins bind to the *Y. pseudotuberculosis*/*Y. pestis* promoter with a greater affinity than they bind to the *Y. enterocolitica* promoter and have greater influence on the expression of $rovA_{Ypstb/Ypestis}$.

In vivo data demonstrated that truncation of the $rovA_{Yent}$ promoter by 175 nt did not alter the effects of H-NS on transcription (Fig. 4A), indicating that the H-NS binding site is not present within this region. To further narrow the region within the *Y. enterocolitica* promoter that H-NS binds, we generated PCR fragments representing truncated regions of the promoter and analyzed the ability of H-NS to bind to these fragments. Deletion of 265 nt from the 5′ end of the promoter resulted in a dramatic decrease in H-NS binding (YE6), which

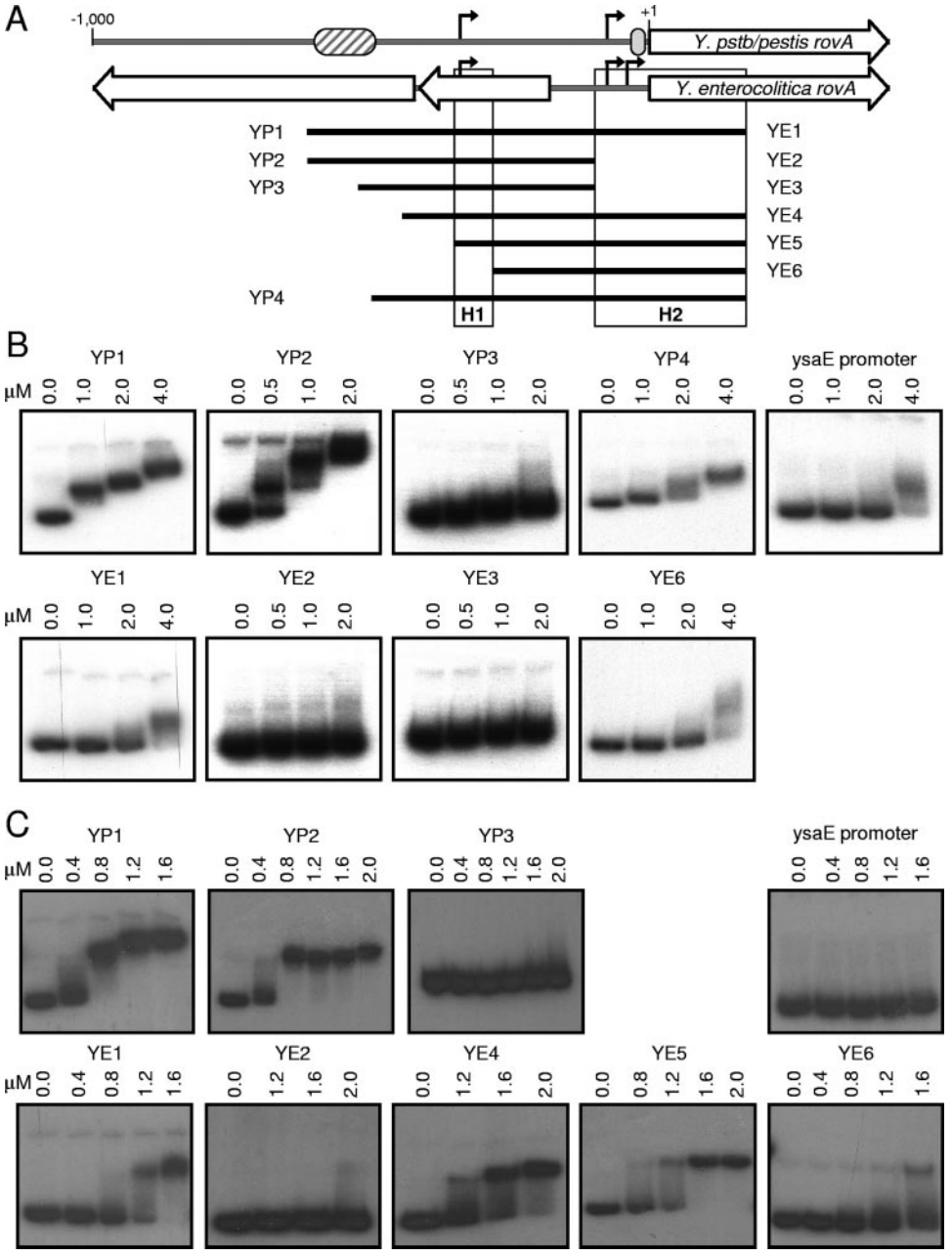

FIG. 5. Ability of RovA and H-NS to bind to the *rovA* promoters. (A) Schematic representation of the *rovA* promoters from *Y. pseudotuberculosis*/*Y. pestis* (*Y.pstb*/*pestis rovA*) and *Y. enterocolitica* and the PCR products used in EMSA analysis. The striped and gray ovals represent the predicted high-affinity RovA/H-NS and predicted low-affinity RovA binding sites, respectively, from *Y. pseudotuberculosis*. The black arrows represent transcriptional initiation sites. The black bars represent PCR products used for the EMSA from *Y. pseudotuberculosis*/*Y. pestis* (YP) and *Y. enterocolitica* (YE). H1 and H2 represent regions of H-NS binding in the *Y. enterocolitica* promoter. (B) EMSA performed with recombinant RovA-His. (C) EMSA performed with recombinant H-NS–His. The fragment used in each panel and the concentrations of the protein added to each reaction mixture are shown. The *ysaE* promoter is not regulated by either RovA or H-NS and was included as a negative control for binding.

was not restored until the fragment included the region 349 to 279 nt upstream of the *rovA* initiation codon (YE5). These results suggest the presence of an H-NS binding site in this region. Interestingly, H-NS did not bind to YE2, which contains this region but lacks sequence in the 3′ region of the promoter. This finding may indicate that a second H-NS binding site is present within the 3′ region of the promoter. A second low-affinity H-NS binding site within this region could also explain the weak binding observed at higher H-NS concentrations in YE6.

RovM regulation of *rovA* in *Y. enterocolitica*. Recently, it was shown that RovM modulates the expression of *rovA* in *Y. pseudotuberculosis* (17). RovM is also present in *Y. enterocolitica* (ORF YE1343), and the region within the *rovA* promoter where RovM is predicted to bind is considerably more conserved between the species (76% identity) than the RovA/

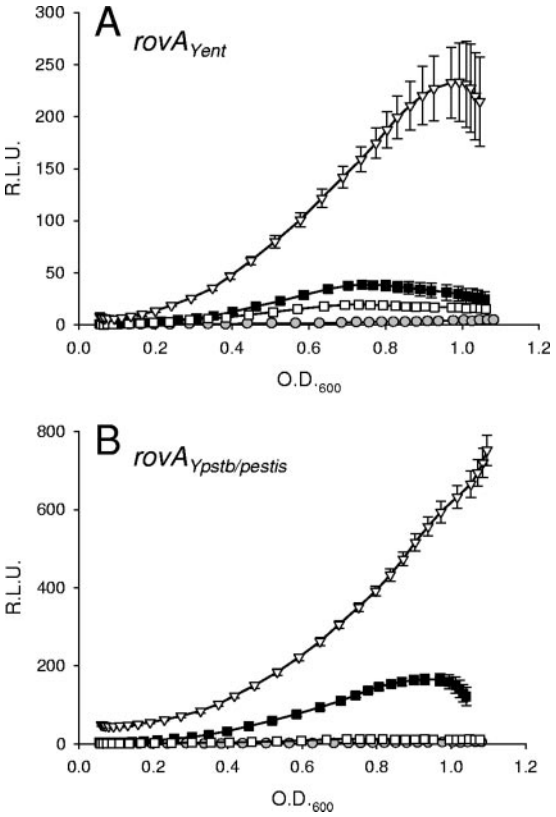

FIG. 6. Influence of RovM on the expression of *rovA*. To determine the effect of RovM on the $rovA_{Yent}$ pYEL (A) and $rovA_{Ypstb/Ypestis}$ pYPL (B) promoters, expression patterns were determined in wild-type (■), Δ*rovA* mutant (□), and Δ*rovM* mutant (▽) *Y. enterocolitica*. The results for the vector-only control are indicated by gray circles. The data are expressed in relative light units (R.L.U.) as a function of the OD_{600}.

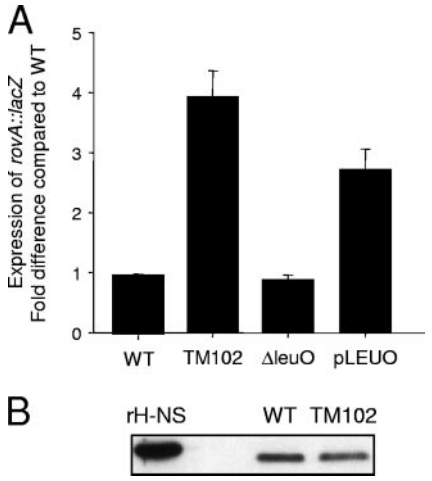

FIG. 7. LeuO regulation of *rovA* in *Y. enterocolitica*. (A) To determine the effect of LeuO on *rovA* transcription in *Y. enterocolitica*, the levels of β-galactosidase activity of the *rovA*::*lacZ* reporter were compared in the wild type (WT), a transposon mutant with a mutation in the promoter of *leuO* (TM102), a mutant with an in-frame deletion of *leuO* (ΔleuO), and a strain containing *leuO* on a multicopy plasmid (pLEUO). The expression is shown as the fold change compared with wild-type expression. (B) Whole-cell proteins (equivalent to 0.1 OD_{600} unit) from wild-type (WT) and TM102 cultures were harvested and analyzed by Western blotting with anti-H-NS antibody. Purified His-tagged H-NS (rH-NS) served as a positive control.

H-NS binding site (Fig. 1B). Therefore, we hypothesized that RovM is involved in the regulation of *rovA* in *Y. enterocolitica*. To determine the role of RovM in the regulation of $rovA_{Yent}$, we generated an in-frame deletion of *rovM* in *Y. enterocolitica* and transformed the pYPL and pYEL reporters into the strain. Cultures were grown as described above, and levels of *rovA* transcription in the *rovM* mutant were determined as a function of fluorescence and compared to levels in wild-type bacteria. As observed in *Y. pseudotuberculosis*, deletion of *rovM* in *Y. enterocolitica* resulted in an increase in *rovA* transcription (Fig. 6). Near peak $rovA_{Ypstb/Ypestis}$ expression in wild-type *Y. enterocolitica* (OD_{600}, ~0.9), the levels of transcription in the *rovM* mutant increased approximately 3.2-fold. A moderately higher increase was observed for the $rovA_{Yent}$ promoter (approximately 4.8-fold). These results support the hypothesis that RovM has a conserved role in the modulation of *rovA* expression in *Yersinia*.

LeuO is a positive regulator of *rovA*. In order to identify novel factors involved in the regulation of *rovA* expression in *Y. enterocolitica*, we initiated a transposon mutagenesis screen in a strain of *Y. enterocolitica* with a second copy of the *rovA* promoter fused to a *lacZ* reporter integrated in the arabinose operon (YVM1251). Approximately 42,000 colonies from 21 independent conjugations were screened. There were 150 mutants that displayed at least a 20% variation in β-galactosidase activity compared to YVM1251 in an independent analysis in liquid culture. Eight of these mutants showed a reproducible >2-fold effect on *rovA*::*lacZ* expression. Upon Western blot analysis of native RovA levels, only four of the eight mutants demonstrated a difference in native RovA protein levels (data not shown). TM102 demonstrated the greatest activation of *rovA* of these four mutants (approximately threefold increase in *rovA*::*lacZ* expression) (Fig. 7) and was selected for further characterization.

TM102 contains an insertion upstream of ORF YE0655 (56). YE0655 encodes a homolog of the LeuO regulator of *E. coli* (75% similarity and 62% identity; $P = 9.9e^{-91}$). LeuO is a member of the LysR family of transcriptional regulators and was originally identified as a regulator of the cryptic *bgl* operon of *E. coli* (28). Subsequently, it was shown that *leuO* expression is ppGpp dependent and involved in the stringent response of bacteria (13, 30). It was unclear whether the location of the transposon insertion within the promoter of *leuO* resulted in inactivation of the gene or induction of *leuO* transcription. To determine which event occurred, we generated two *Yersinia* strains in the YVM1251 background: a strain with an in-frame deletion of *leuO* (YVM1252) and a strain containing an additional copy of *leuO* controlled by its native promoter on a multicopy plasmid (pLEUO). Deletion of the gene resulted in no changes in *rovA*::*lacZ* expression, while increased β-galactosidase levels were observed in the pLEUO-complemented strain (Fig. 7). These results indicate that LeuO is a positive regulator of *rovA* in *Y. enterocolitica*.

In addition to the *bgl* operon, LeuO has been shown to positively regulate *osmY* in *E. coli* (24). *osmY* expression is mediated by σ^S and induced during the transition from logarithmic to stationary phase and in response to osmotic stress

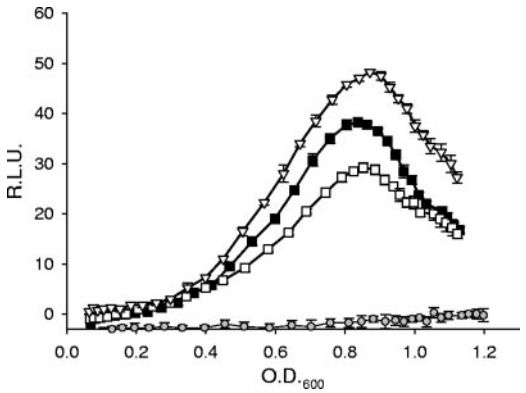

FIG. 8. Influence of σ^s on the expression of *rovA*. To determine the effect of σ^s on the expression of $rovA_{Yent}$ (pYEL), expression patterns were determined in wild-type (■), Δ*rovA* mutant (□), and Δ*rpoS* mutant (▽) *Y. enterocolitica*. The results for the vector-only control are indicated by gray circles. The data are expressed in relative light units (R.L.U.) as a function of the OD_{600}.

(25, 60). Klauck et al. (24) demonstrated that LeuO indirectly regulated *osmY* by inducing the expression of a small regulatory RNA, *dsrA*, which altered the levels of σ^S in the bacterium. The changes in σ^S levels in the bacterium in turn influenced the expression of *osmY*. The similarities in *osmY* and *rovA* expression patterns (peak expression during the transition to stationary phase and regulation by LeuO) suggested that σ^S may also be a regulator of *rovA* in *Y. enterocolitica*. To address this possibility, we generated an in-frame deletion of *rpoS* in *Y. enterocolitica* and determined the effects on *rovA* using the pYEL reporter. Inactivation of *rpoS* did not result in a significant change in *rovA*::GFP expression compared to wild-type *Y. enterocolitica* (Fig. 8), demonstrating that σ^S is not a regulator of *rovA*. Furthermore, these results suggest that LeuO-mediated regulation of *rovA* is independent of σ^S.

LeuO has also been implicated in the regulation of H-NS translation in *E. coli* (24, 26). Since H-NS is a negative regulator of *rovA*, it is possible that the changes in *rovA* transcription in TM102 are a result of alterations in the steady-state levels of H-NS in the mutant. To determine if H-NS levels are affected by increased LeuO expression, total protein was harvested from wild-type and TM102 cultures and the levels of H-NS were compared by Western blot analysis (Fig. 7B). No significant differences in H-NS levels were observed, indicating that the increased expression of LeuO did not significantly alter the translation of H-NS in *Y. enterocolitica*. These data indicate that LeuO is unlikely to regulate $rovA_{Yent}$ expression by altering the levels of H-NS in the cell.

DISCUSSION

Y. enterocolitica and *Y. pseudotuberculosis* share conserved virulence mechanisms to cause gastrointestinal infection. Invasin, the major adhesin for both species, interacts with β_1-integrins on the surface of human cells (21). This interaction mediates invasion of host cells and may promote efficient translocation across the epithelial barrier during initial stages of colonization (20, 36, 44). Interestingly, *inv* has been inactivated in *Y. pestis*, which relies primarily on an arthropod vector to colonize a new host (51). The regulation of *inv* transcription is also conserved between *Y. enterocolitica* and *Y. pseudotuberculosis*. Interactions between H-NS and RovA have been shown to mediate the expression of *inv* in both species (10, 40). These proteins have also been implicated in the regulation of the *rovA* gene in *Y. pseudotuberculosis* (18). While *rovA* is highly conserved in all three species, inspection of the region upstream of *rovA* revealed that the promoter in *Y. enterocolitica* has greatly diverged from that in the other two pathogens. The differences in the promoters result in significantly lower levels of transcription in *Y. enterocolitica* from the *rovA* promoter. This decreased $rovA_{Yent}$ activity may be due to transcription of the divergent ORFs upstream of $rovA_{Yent}$ occurring at 26°C (Lawrenz and Miller, unpublished). However, we were interested in determining whether mechanisms reported for *rovA* regulation in *Y. pseudotuberculosis* are active in *Y. enterocolitica*.

Despite the divergence of the *Y. enterocolitica rovA* promoter from the promoter of the other two species and the absence of the $rovA_{Ypstb/Ypestis}$ predicted H-NS/RovA binding site, we observed that *rovA* regulation in *Y. enterocolitica* remains responsive to H-NS and RovA. As reported for *Y. pseudotuberculosis*, H-NS represses $rovA_{Yent}$ transcription; however, regulation is mediated through interactions with different regions within the promoter. *rovA* is also autoregulated in *Y. enterocolitica*, and RovA is required for maximal transcription. It should be noted that both regulators affect the level of transcription from the $rovA_{Ypstb/Ypestis}$ promoter to a greater degree than that seen for $rovA_{Yent}$. These results support the hypothesis that H-NS and RovA mediate transcription in *Y. enterocolitica*. Interestingly, transcription of pYPL and pYEL is detectable in a *rovA* mutant, and the levels of activity from the reporters are similar. These data indicate that the basal levels of RovA in the repressed state are similar for both promoters. This low level of expression may be important to maintain a pool of RovA that it is available to quickly initiate *rovA* transcription in response to stimuli. However, the mechanism(s) that leads to derepression of *rovA* is not yet fully understood. Transcription may result from a combination of factors, including changes in affinity for the promoter by RovA or H-NS, alterations of the DNA structure within the promoter, stability of the regulators, and/or activity of other regulators.

The differences in transcriptional modulation by RovA and H-NS correlate directly with the ability of the proteins to bind to the promoters. Both proteins bind to the *Y. pseudotuberculosis*/*Y. pestis* promoter at lower concentrations and affect levels of transcription to higher degrees. The ability of the $rovA_{Ypstb/Ypestis}$ promoter to bind the proteins at lower concentrations can be attributed mostly to the region identified by Heroven et al. that is absent in the $rovA_{Yent}$ promoter (18). The current model for RovA/H-NS-mediated regulation in *Y. pseudotuberculosis* suggests that the two proteins compete for binding to this site, so that successful binding by RovA derepresses H-NS repression. In corroboration of this hypothesis, in vivo data presented here demonstrate that deletion of the RovA/H-NS binding site from the *Y. pseudotuberculosis*/*Y. pestis* promoter eliminated the requirement of RovA for expression of the pYPS reporter. We were also able to observe weak RovA binding to

YP4, which includes a second proposed RovA binding site near the $rovA_{Ypstb/Ypestis}$ coding region (Fig. 1) (18). Herevon et al. have suggested that this low-affinity site may be involved in negative auto-feedback regulation to repress *rovA* transcription when levels of RovA in the cell reach a certain value. This low-affinity site is conserved within the *Y. enterocolitica* promoter, with only three nucleotide changes.

The interactions between RovA and H-NS that mediate the transcription of $rovA_{Yent}$ are less obvious due to the lack of observable RovA binding to the *Y. enterocolitica* promoter. While our in vivo data suggest that autoregulation occurs in *Y. enterocolitica*, our in vitro data indicate that RovA does not bind specifically to the $rovA_{Yent}$ promoter. These in vitro results suggest that RovA regulation may occur through an indirect mechanism. However, we cannot eliminate the possibility that RovA derepresses H-NS directly in *Y. enterocolitica* and the level of specific binding of RovA to $rovA_{Yent}$ is below the sensitivity of our assay. The possible limitation of our EMSA conditions to detect low-affinity RovA binding to $rovA_{Yent}$, and therefore direct regulation by RovA, is supported by the lack of specific RovA binding to the conserved RovA low-affinity binding site in the *Y. enterocolitica* promoter. Modifications in EMSA conditions have yet to demonstrate specific RovA binding, but future variations of in vitro binding conditions and/or in vivo binding assays, such as chromatin immunoprecipitation, may result in more sensitive assays that could aid in determining if RovA interacts with the *Y. enterocolitica* promoter.

Tran et al. have reported that RovA can directly activate transcription of $rovA_{Ypstb/Ypestis}$ through interactions with RNA polymerase in vitro (57). Deletion of the high-affinity RovA/H-NS binding site did not alter expression of $rovA_{Ypstb/Ypestis}$ in wild-type *Y. enterocolitica*. These results support the hypothesis that binding of RovA to the RovA/H-NS binding site in the *Y. pseudotuberculosis*/*Y. pestis* promoter primarily relieves negative regulation by H-NS. Furthermore, since expression of pYPS did not decrease in wild-type *Y. enterocolitica*, RovA binding to the high-affinity binding site in pYPL does not appear to significantly activate *rovA* transcription in vivo. If RovA actively induces transcription of the *Y. pseudotuberculosis*/*Y. pestis* promoter, these data suggest that it occurs through interactions with other regions of the promoter.

In vitro DNA binding assays indicate the presence of two H-NS binding sites in *Y. enterocolitica*: between nt −349 and −279 and between nt −128 and 171 (in relation to the initiation codon). H-NS demonstrates much lower affinity for either of these sites than for the reported *Y. pseudotuberculosis* site. H-NS also appears to cooperatively bind to these sites, as loss of the H2 site results in loss of binding to the H1 site (compare binding to YE2 and binding to YE4). The presence of multiple binding sites within a promoter is a common theme for H-NS-mediated repression and has been well described for the *rrnB* P1 and *proU* promoters (48, 53). Dorman and Deighan proposed that binding to two regions, in conjunction with protein oligomerization, leads to formation of a loop within these promoters that traps RNA polymerase and blocks initiation of transcription (8). A similar mechanism could occur within the $rovA_{Yent}$ promoter. Binding to the H1 and H2 regions may result in loop formation that occludes all three promoters from interactions with RNA polymerase, leading to repression of transcription.

Based on sequence similarity alone, one would suspect that H-NS should also bind the same H2 region in *Y. pseudotuberculosis*/*Y. pestis*; however, binding to this region has not been observed by us or reported by others. The lack of data indicating a second binding site in *Y. pseudotuberculosis* may be due to weak binding of H-NS to the H2 site that is below the sensitivity of the footprinting analysis used to map interactions of H-NS with the $rovA_{Ypstb/Ypestis}$ promoter (18). Alternatively, binding to the H2 region may have been missed because the probes used for footprinting the 5′ region of the promoter did not include both binding sites on the same fragment. In *Y. enterocolitica*, binding of H-NS to either the H1 or H2 site required the presence of the other site, indicating cooperative binding. The presence of two binding sites may also be necessary for H2 binding in *Y. pseudotuberculosis*. Finally, the strong binding of H-NS to the high-affinity site may mask binding to a second site. In *Y. pseudotuberculosis*/*Y. pestis*, the high-affinity site does not appear to require a second binding site. Therefore, promoter probes for EMSA that are generated without the H2 site would still interact with H-NS, masking the presence of this binding site in the promoter.

Heroven and Dersh previously demonstrated that RovM regulates the transcription of *rovA* in *Y. pseudotuberculosis* (17). Unlike the predicted H-NS/RovA binding site, the RovM predicted binding site is conserved in *Y. enterocolitica*. We demonstrated here that *rovA* transcription in *Y. enterocolitica* is also mediated by RovM. It seems likely that RovM binds to the same region in the *Y. enterocolitica* promoter and directly represses *rovA* transcription, as reported for *Y. pseudotuberculosis*.

In addition to H-NS, RovA, and RovM, we identified a fourth regulator involved in *rovA* regulation in *Y. enterocolitica*. *leuO* encodes a LysR-like regulator and is induced in the stringent response that occurs during amino acid starvation. Also, it is required to resume growth after starvation (30). The conditions for LeuO expression suggest that *rovA* responds to nutrient limitation or another stress signal. It has been shown that *rovA* transcription in *Y. pseudotuberculosis* is altered in cultures grown in minimal medium compared to cultures grown in rich medium (17, 40); however, we did not observe a similar pattern in *Y. enterocolitica*. The higher levels of *rovA* transcription from the *Y. pseudotuberculosis* promoter may allow detection of subtle changes in *rovA* expression that are not as easily observed for the weaker *Y. enterocolitica* promoter. The *rovA* response to starvation or decreased availability of nutrients could also explain why peak expression of *rovA* and the *rovA*-regulated gene *inv* occurs upon entry into late-logarithmic/early-stationary growth, when nutrient levels in the medium are starting to decline.

LeuO has been implicated in the regulation of several genes in *E. coli*, including the *bgl* operon and *osmY* (24, 28). In the case of *osmY* regulation, increased expression of LeuO represses the transcription of the small regulatory RNA *dsrA*, resulting in destabilization of the *rpoS* message and down regulation of *osmY*. Due to the difficulty in predicting small regulatory RNAs, it has yet to be determined if a *dsrA*-like homolog is present in *Y. enterocolitica*. However, we did not observe a requirement for *rpoS* for *rovA* regulation, indicating that LeuO affects the transcription of *rovA* by a mechanism that differs from that of *osmY*. It is unclear at this time whether regulation occurs through direct interaction between LeuO

and the *rovA* promoter or if LeuO modulates an additional regulator that in turn acts on the *rovA* promoter. However, LeuO does not appear to affect *rovA* transcription by modulating H-NS steady-state levels in the bacterium.

In conclusion, we have demonstrated that conserved factors are active in the regulation of *rovA* in *Y. enterocolitica* and *Y. pseudotuberculosis*/*Y. pestis* despite considerable differences in the relative strengths and sequences of the *rovA* promoters. However, divergence between *Yersinia* species during evolution has resulted in modifications in the regulatory mechanisms. The identification of the stringent response protein LeuO as a *rovA* regulator strengthens the hypothesis that starvation or nutrient limitation may be an additional signal to *Yersinia* to induce *rovA* transcription, leading to induction of *inv* and other genes that prepare the bacterium to colonize its host. Future analysis of the mechanism by which LeuO regulates *rovA* may provide insight into whether a small regulatory RNA such as DsrA is also involved in *rovA* regulation.

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