The Leucine-Responsive Regulatory Protein, Lrp, Activates Transcription of the *fim* Operon in *Salmonella enterica* Serovar Typhimurium via the *fimZ* Regulatory Gene[▽]

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The *fim* operon of *Salmonella enterica* serovar Typhimurium encodes type 1 fimbriae. The expression of *fim* is controlled in response to environmental signals through a complex regulatory cascade involving the proteins FimW, FimY, and FimZ and a genetic locus, *fimU*, that encodes a rare arginine tRNA. We discovered that a knockout mutation in *lrp*, the gene that codes for the leucine-responsive regulatory protein (Lrp), inhibited *fim* transcription. The loss of *fim* gene expression was accompanied by a corresponding loss of the mannose-sensitive hemagglutination that is a characteristic of type 1 fimbriae. Normal type 1 fimbrial expression was restored following the introduction into the knockout mutant of a plasmid carrying a functional copy of the *lrp* gene. Electrophoretic mobility shift analysis revealed no interactions between purified Lrp protein and the regulatory region of the *fimA*, *fimU*, or *fimW* gene. Instead, Lrp produced protein-DNA complexes with the regulatory region of the *fimZ* gene, and the nature of these complexes was leucine sensitive. DNase I footprinting showed that Lrp binds within a region between −65 and −170 with respect to the *fimZ* transcription start site, consistent with the binding and wrapping of the DNA in this upstream region. Ectopic expression of the *fimZ* gene from an inducible promoter caused Lrp-independent type 1 fimbriation in serovar Typhimurium. These data show that Lrp makes a positive contribution to *fim* gene expression through direct interaction with the *fimZ* promoter region, possibly by antagonizing the binding of the H-NS global repressor protein.

Type 1 fimbriae were the first bacterial fimbriae to be described (13, 30, 31), and most members of the *Enterobacteriaceae* express them (2, 3, 22, 35, 44, 47). These proteinaceous appendages are arranged peritrichously on the cell surface, where they facilitate bacterial adhesion to a variety of eukaryotic cells through interactions with mannosylated glycoproteins (50). *Salmonella enterica* serovar Typhimurium possesses 13 putative fimbrial operons (10, 33, 42, 48, 56). One of these is *fim*, the operon encoding type 1 fimbriae. The *fim* genes are expressed in vitro in serovar Typhimurium cultures grown statically in Luria-Bertani (LB) broth and in bacteria growing in vivo in ligated bovine ileal loops (42). However, they are not expressed by bacteria growing on Luria agar plates under standard laboratory growth conditions (49). Thus, type 1 fimbriae are subject to environmental regulation, and they have been described as having an on-off phase-variable expression pattern (73). They are also contributors to bacterial virulence in a number of hosts (4, 5, 8, 32, 53, 71).

The mechanism of phase-variable *fim* gene expression in serovar Typhimurium differs substantially from that in *Escherichia coli*, which has by far the best-characterized type 1 fimbrial gene regulatory system. In *E. coli*, the inversion of a DNA element known as *fimS* is mediated by a site-specific recombination mechanism that alternatively connects and disconnects the *fimA* structural gene to and from its promoter (1, 34). In serovar Typhimurium, *fim* gene expression is not regulated through the reversible inversion of a *cis*-acting regulatory DNA motif. Instead, control is exerted through a cluster of four regulatory genes, *fimU*, *fimW*, *fimY*, and *fimZ*, located downstream of the main *fim* structural operon (Fig. 1). Transcriptional control of the structural genes is achieved primarily through the regulation of the *fimA* gene promoter. The FimZ transcription factor activates this promoter directly, and it also positively regulates the transcription of its own gene, *fimZ* (Fig. 1) (82). The FimZ protein is an "orphan" member of the response regulator family of transcription factors, that is, one for whom no cognate histidine protein kinase partner has been identified (83, 84). The FimY protein is an essential coregulator that cooperates with FimZ in activating the *fimA* promoter through a mechanism that does not involve a direct interaction between FimY and *fimA* (72). A third regulatory protein, FimW, exerts a negative effect on *fimA* expression through a FimW-FimZ protein-protein interaction that is inhibitory toward the positive influence of the FimZ protein (74).

Posttranscriptional control is a feature of both the serovar Typhimurium and *E. coli fim* operons. In serovar Typhimurium, the *fimU* gene specifies a rare arginine tRNA that modulates the translation of the mRNA expressed by the *fimY* regulatory gene (73). In *E. coli*, a detachable Rho-dependent transcription terminator modulates the rate of turnover of an mRNA expressed by one key regulatory gene (40, 45) while a rare leucine tRNA encoded by the *leuX* gene influences the rate of translation of another (65). Mutations in the serovar
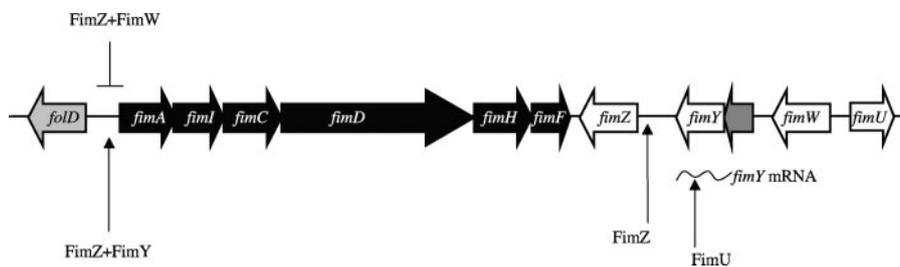
FIG. 1. Regulation of *fim* gene expression in serovar Typhimurium. The diagram summarizes the structure of the *fim* gene cluster. Filled black arrows represent structural *fim* genes, while unfilled arrows represent regulatory *fim* genes. Other genes are shown in gray. In each case, the direction of transcription is shown by the orientation of the arrow. Positive regulatory inputs are shown using upward-pointing arrows, while a negative input exerted by a FimZ-FimW complex is represented by an inverted "T" symbol. FimZ is an activator of the *fimA* and *fimZ* promoters. The *fimU* gene acts at the level of *fimY* mRNA translation, and a wavy line represents the *fimY* transcript.

Typhimurium *fimU* locus inhibit *fimY* translation and lead to an afimbriate phenotype (73).

The leucine-responsive regulatory protein (Lrp) is an 18.8-kDa DNA binding protein that acts globally to influence transcription and other DNA transactions (12, 14, 16, 20, 26, 43, 55, 59, 69, 81). Its activity can be enhanced, attenuated, or unaffected by L-leucine in different Lrp-dependent systems. Leucine also influences the oligomeric structure of the protein, with the formation of octamers being favored when it is present (19, 21, 62). Lrp is known to regulate many fimbrial genes, and its contributions have been studied in considerable detail in the cases of the *E. coli pap* and *fim* systems. In the *E. coli fim* system, it serves to modulate the efficiency of the site-specific recombination reaction that inverts the *cis*-acting *fimS* regulatory element (6, 46, 66); in the *pap* system, it impedes the methylation of key regulatory sequences by the DNA adenine methylase (Dam) protein (11, 39, 76). In both cases, Lrp directly influences the phase-variable expression of these *E. coli* fimbrial structural genes. The *pef* genes on the pSLT virulence plasmid in serovar Typhimurium encode Pef pili and are regulated by a mechanism that is strikingly similar to that found in the *E. coli pap* system (60). Pef fimbriae are expressed in vitro only in static cultures growing in rich media at acid pHs (5). The on-off *pef* switch is controlled by the differential methylation of 5′-GATC-3′ sites in *pef* regulatory DNA by Dam. Dam competes with Lrp for access to these sites (60), although the distribution of high- and lower-affinity sites within the *pef* regulatory region is thought to differ from that in *pap* (39). Dam-Lrp competition has also been described in the cases of pili encoded by *daa* (F1845 pili), *fae* (K88 pili), and *sfa* (S pili) from *E. coli* (39).

We aimed to discover whether Lrp could influence type 1 fimbrial gene expression in serovar Typhimurium; here, we report a role for Lrp as a positive regulator of the serovar Typhimurium *fim* genes and present details of the regulatory mechanism.

MATERIALS AND METHODS

Growth media and conditions. Strains were routinely grown in LB broth at 37°C with shaking at 250 rpm or on LB agar. The expression of fimbriae in the cultures was induced by aerobic passage in static broth at 37°C for 48 to 72 h. Motility was examined using swarm plates as described previously (46). To analyze pBAD*fimZ* functionality, 0.2% glucose or 0.2% arabinose was incorporated into the swarm agar.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The *lrp* knockout mutant was complemented using plasmid pKMC102. This plasmid was made by amplifying the *lrp* gene from strain SL1344 by PCR using primer pair fwd1.3 and rev1.3 (Table 2). The resulting amplicon was digested with AatII. The single-copy-number plasmid pZC320 was digested with PmlI and AatII and ligated with the *lrp* gene-containing fragment. The *fimZ* gene was placed under the control of the arabinose-inducible P$_{BAD}$ promoter. First, the open reading frame of the *fimZ* gene was amplified by PCR using the primer pair *fimZ*-BAD_F and *fimZ*-BAD_R-H and the resulting amplicon was digested with NcoI. Both the linearized vector and the *fimZ* DNA fragment were treated with the Klenow fragment of DNA polymerase to create blunt ends. Digestion with HindIII was used to facilitate the insertion and ligation of the *fimZ* gene into pBAD24 in the appropriate orientation for transcription from the P$_{BAD}$ promoter. The plasmid pBSK*fimZ* that was used for DNase I footprinting and DNA sequencing was generated by PCR with primer pair *fimZ*300_F.BamHI and *fimZ*300_R.EcoRI, followed by cloning into the multiple cloning site of pBluescript II SK(−).

Construction of the *lrp* mutant of SL1344. CJD3130, the *lrp* mutant derivative of SL1344 (Table 1), was constructed by cloning the *lrp* gene plus flanking regions into the low-copy-number plasmid vector pCL1921 using primer pair fwd1.3_EcoRI and rev1.3_PstI (Table 2). The BamHI fragment of pHP45-ΩKm, containing an Ω-flanked kanamycin cassette, was ligated into a BglII site located 29 bp into the coding region of the cloned *lrp* gene. The disrupted gene was PCR amplified using primer pair fwd1.3 and rev1.3, digested with DpnI, and using pKOBEGA as described previously (17), transduced into the strain LT2. The disrupted gene was retransduced into SL1344 using P22. The structure of the insertion mutation was confirmed by PCR and by Southern blotting (67). SL1344 and the isogenic *lrp* mutant of SL1344 were analyzed for growth in LB broth at 37°C with shaking (250 rpm). A typical growth curve showed a slight growth defect of the *lrp* mutant: SL1344 showed a mean doubling time of ~28 min, while the SL1344 *lrp* mutant displayed a mean doubling time of ~30 min.

RT-PCR. RNA was isolated from cultures using the SV total RNA isolation kit (Promega). The RNA concentration was determined by spectrophotometry at A_{260}. Reverse transcription-PCR (RT-PCR) was carried out with the OneStep RT-PCR kit (QIAGEN) using sample RNA at 0.6 µg µl^{-1} and the oligonucleotides listed in Table 2. The primer melting temperature was calculated to be 52°C, and the number of cycles was set to 24. Samples were separated by gel electrophoresis in 1% agarose–Tris-acetate-EDTA. Densitometry was performed using the Quantity One program (Bio-Rad).

Mannose-sensitive-hemagglutination assay. Agglutination tests were performed by incubating 50-µl aliquots of bacterial cultures with equal optical densities at 600 nm with 50-µl samples of 6% guinea pig erythrocytes. Mannose sensitivity was shown by a failure to agglutinate following the incorporation of D-mannose at a final concentration of 3% (wt/vol).

Lrp protein purification. The *lrp* gene was amplified from SL1344 genomic DNA using primer pair forNdeI-*lrp* and revXhoI-*lrp* (Table 2), incorporating NdeI and XhoI restriction sites. The amplicon was cloned into pET22b, the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible protein expression plasmid, to produce pKMC301, which expresses Lrp with a C-terminal His tag. Lrp expression was induced and the protein was purified as described previously (46).

Electrophoretic mobility shift assay (EMSA). DNA probes were amplified using biotinylated primers for the region of interest (Table 2) and were subse-

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source or reference
Strains		
Salmonella		
CJD3130	*lrp* mutant of SL1344	This study
CJD3131	CJD3130(pKMC102)	This study
CJD3134	SL1344(pBAD24)	This study
CJD3135	SL1344(pBAD*fimZ*)	This study
CJD3136	CJD3130(pBAD*fimZ*)	This study
SL1344	Virulent wild-type strain; *rpsL hisG*	41
E. coli XL1-Blue	Routine cloning strain	Stratagene
Plasmids		
pBAD24	Arabinose-inducible expression vector; Amp^r	38
pBAD*fimZ*	Arabinose-inducible *fimZ* gene vector; Amp^r	This study
pBluescriptII SK(−)	Cloning vector	Stratagene
pBSK*fimZ*	Template for DNase I footprinting	This study
pCL1921	Low-copy-number vector; Spc^r	51
pET22b	IPTG-inducible protein expression vector; Amp^r	Novagen
pHP45ΩKm	Contains Ω-flanked Km^r element	7
pKMC102	Construct with *lrp* gene cloned into pZC320	This study
pKMC301	IPTG-inducible Lrp expression vector; Amp^r	This study
pKOBEGA	Arabinose-inducible λ Red system vector; Amp^r; carries temperature-sensitive origin of replication	17
pZC320	Carries F replicon; Amp^r	68

quently purified by gel electrophoresis. Aliquots of approximately 50 pg of biotinylated DNA probes were incubated with binding buffer for 5 min at room temperature as described previously (15). Increasing concentrations of Lrp protein were added, and the mixtures were incubated for 20 min at room temperature. Protein-DNA complexes were formed in a reaction volume of 20 μl, including L-leucine where applicable. Ten-microliter samples of the protein-DNA mixtures, plus loading dye, were subjected to electrophoresis at 100 V on Novex 6% DNA retardation gels (Invitrogen) at room temperature. Gels were transferred onto membrane using the Novex XCell II blot module (Invitrogen), UV cross-linked, and developed using the chemiluminescent nucleic acid detection module (Pierce).

DNase I footprinting. DNase I footprinting was carried out as previously described (75). The probes used for DNase I footprinting were generated by PCR using primer pair *fimZ*300_F.BamHI and T7_for or *fimZ*300_R.EcoRI and T7_rev (Table 2). The sequences complementary to the T7 oligonucleotides are located outside the multiple cloning site of pBluescript. T4 polynucleotide kinase was used to label the DNA probes at both ends with [γ-^{32}P]ATP, and the probes were then digested to remove the label from one end, depending on the strand to be analyzed. Lrp protein-DNA binding reactions used for DNase I footprinting were identical to those used for EMSA analysis, except that these reactions were performed in 50-μl volumes. DNA sequencing reactions using dideoxy chain terminators were performed as previously described (75) by using the T4 DNA sequencing kit (USB) and appropriate sequencing primers. The products of the DNase I footprinting and the DNA sequencing reactions were resolved by electrophoresis through a 6% urea-polyacrylamide gel.

RESULTS

Expression of *fim* genes is strongly reduced in an SL1344 *lrp* mutant. Lrp is known to regulate the fimbrial genes of a number of bacterial species (11, 60, 76), including the *fim* genes encoding the type 1 fimbriae of *E. coli* (6, 46, 66). In this study, we examined the effect of an *lrp* knockout mutation on type 1 fimbriation in serovar Typhimurium. A characteristic of type 1 fimbriae is their ability to bind to a mannosylated glycoprotein, laminin, on the surfaces of erythrocytes. The serovar Typhimurium strain used in this study, SL1344, strongly agglutinates erythrocytes (Fig. 2). We constructed an SL1344 *lrp* knockout mutant and investigated whether it displayed altered levels of

type 1 fimbriation. Hemagglutination assays were performed in the presence and absence of D-mannose. SL1344, the SL1344 *lrp* mutant, and the SL1344 *lrp* mutant complemented with pKMC102 (*lrp*$^+$) were incubated with guinea pig erythrocytes in the presence or absence of D-mannose, as described in Materials and Methods. The *lrp* mutant displayed no visible agglutination activity (Fig. 2). As expected, the wild-type strain and the complemented *lrp* mutant showed obvious agglutination in the absence of mannose and this hemagglutination was inhibited in the presence of mannose (Fig. 2).

Lrp regulates type 1 fimbrial genes in *E. coli* by participating in a DNA inversion event that is mediated by site-specific recombination. However, the serovar Typhimurium *fim* gene cluster does not possess an invertible DNA switch. Since Lrp operates as a transcription factor at promoters throughout the genome (43), its ability to influence the transcription of genes within the serovar Typhimurium *fim* gene cluster was assessed by RT-PCR. The genes tested were the structural genes *fimA*, *fimF*, and *fimH* and the regulatory genes *fimW*, *fimY*, and *fimZ*. RNA for RT-PCR analysis was extracted from SL1344, the SL1344 *lrp* mutant, and the SL1344 *lrp* mutant complemented with pKMC102 (*lrp*$^+$) at the stationary phase of growth in LB broth cultures. The results obtained showed that the transcript levels of the *fimA* and *fimZ* genes were reduced dramatically in the *lrp* mutant, by approximately 5- and 10-fold, respectively, compared to those in the wild type and the complemented mutant (Fig. 3A). The levels of transcripts of *fimH* (encoding the fimbrial adhesin), *fimF* (encoding a putative fimbrial protein), and *fimY* (a positive regulator) were down-regulated by 2-, 1.4-, and 2-fold, respectively, in the *lrp* mutant. Importantly, *fimW*, a negative regulator of fimbrial expression, did not show a significant difference in transcript level in the *lrp* mutant compared to the wild type or the complemented mutant (Fig. 3A). This result showed that the negative effect associated with

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)
fimA-RT_F	GTT GCG GCT GAT CCT AC
fimA-RT_R	GTC CGC AGA GGA GAC AG
fimF-RT_F	TTC ATC GCT ATC GGT TGT
fimF-RT_R	CAG CAA GCG CCA GTA AT
fimH-RT_F	CGC GCT CTT TTT CAC C
fimH-RT_R	CGC CCA GAA GGT AGT CA
fimW-RT_F	AAC AGT CAC TTT GAG CAT GG
fimW-RT_R	ATT TTC GGG GTA ATT TCT TC
fimY-RT_F	TGA CAA CTA CCT CGG CTA TTC
fimY-RT_R	GCC ATA CGG ATA AAC TGT G
fimZ-RT_F	ATA ACA GAT GCA GGT TTC ATT G
fimZ-RT_R	ACT ACT CAA TGT CAA CTC TAA AG
*fimZ*300_F.BamHI	ATA GGA TCC GAT TAT ACC TGG TCT GAT TTC
*fimZ*300_R.EcoRI	ATA GAA TTC TAT TTA CAA CTC GTC CTG GTA AG
T7_for	GCG CGC GTA ATA CGA CTC AC
T7_rev	AGC GCG CAA TTA ACC CTC AC
fimZ-BAD_F	AAA CCT GCA TCT GTT ATC ATT ATG GAC G
fimZ-BAD_R-H	ATA AAG CTT AAC GCG GAT GCG ACC TTC
fimA-EMSA_F	CCT GAA CTT TTT GAG CAA CCT C
fimA-EMSA_R	GGA GTA GGA TCA GCC GCA AC
fimW-EMSA_F	TGA CTG TTT TGT TCC TTA ATA GC
fimW-EMSA_R	GCG CCT TGT GAA GTG AAG AC
*fimZ*_F.3	AAT AAC AGA AAG ATG TTG ACG C
*fimZ*_F.4	GTG GAG CTA TTT TCT TTA GAG TTG
*fimZ*_F.5	GAT TAT ACC TGG TCT GAT TTC TC
*fimZ*_F-BIO	ATA ACA GAT GCA GGT TTC ATT G
*fimZ*_R	ACT ACT CAA TGT CAA CTC TAA AG
*fimZ*_R.2	TAT TTA CAA CTC GTC CTG GTA AG
rev1.3	GGG CAA TAA GTA TCA ACA ACG CTT CCA AAA G
fwd1.3	GTT GTT GGC AGA CAA TGA GCA GAA TTG TAG G
forNdeI_*lrp*	TAC CAT ATG GTA GAT AGC AAG AAG CGC
revXhoI_*lrp*	GGC TGC TCG AGG CGT GTC TTA ATA ACC AG
fwd1.3_EcoRI	ATA GAA TTC GTT GTT GGC AGA CAA TGA GCA G
rev1.3_PstI	ATA CTG CAG GGG CAA TAA GTA TCA ACA ACG C

the loss of *lrp* gene expression was not shared among all the genes in the *fim* cluster.

The RNA used in this experiment was isolated from bacteria grown under incubation conditions that were suboptimal for fimbriation. We therefore used RNA isolated from cultures that had been grown under conditions that were ideal for the expression of type 1 fimbriae (see Materials and Methods) and analyzed the transcript levels expressed by the major fimbrial subunit gene (*fimA*) and the three essential regulatory genes (*fimW*, *fimY*, and *fimZ*) by RT-PCR. This analysis showed that both the *fimA* and *fimZ* genes had decreased transcript levels in the *lrp* mutant (approximately threefold lower than those in the wild type) whereas their expression in the complemented mutant showed no difference compared to that in the wild type (Fig. 3B). In addition, the presence of the *lrp* mutation had no
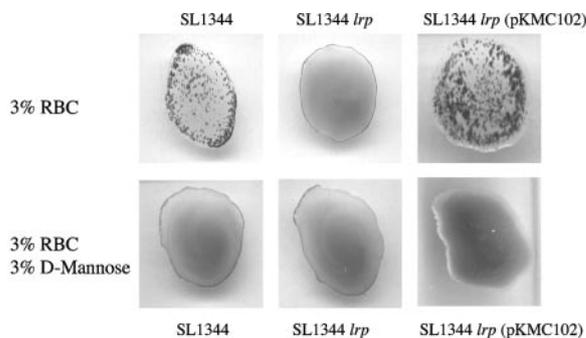

FIG. 2. Effect of an *lrp* knockout mutation on mannose-sensitive hemagglutination activity in serovar Typhimurium. The six panels show 3% red blood cell (RBC) suspensions to which SL1344, the SL1344 *lrp* knockout mutant (SL1344 *lrp*), or the complemented SL1344 *lrp* knockout mutant [SL1344 *lrp* (pKMC102), which harbors a plasmid, pKMC102, that carries a functional copy of the *lrp* gene] has been added. Incubations were carried out in the absence (top row) or the presence (bottom row) of 3% mannose, an inhibitor of hemagglutination mediated by type 1 fimbriae.

effect on either the *fimW* or the *fimY* transcripts. Taken together, these RT-PCR data suggested that Lrp had a positive role in controlling the transcription of the *fimA* and *fimZ* genes.
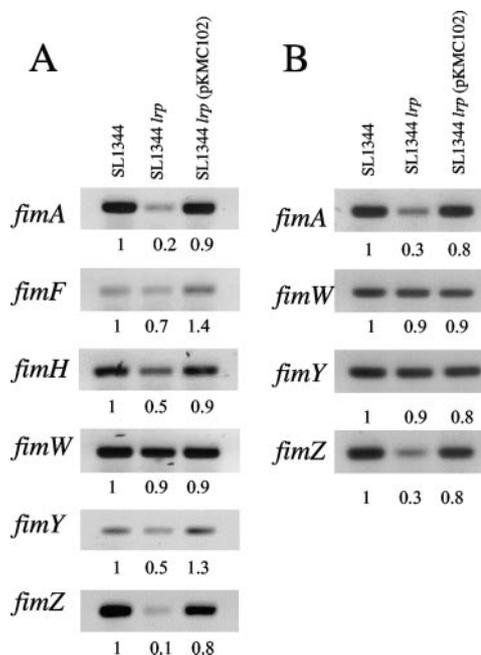

FIG. 3. Effect of an *lrp* knockout mutation on transcription within the *fim* gene cluster. Reverse transcription-PCR assays were used to monitor *fim* gene transcription in wild-type SL1344, the SL1344 *lrp* knockout mutant (SL1344 *lrp*), and the complemented SL1344 *lrp* knockout mutant that harbors pKMC102, a plasmid expressing functional Lrp protein [SL1344 *lrp* (pKMC102)]. Results are presented for bacteria grown to stationary phase (A) or grown under conditions that stimulate fimbriation (B). The intensities of the bands were determined by densitometry and expressed relative to the value for SL1344. The experiment was performed on two separate occasions, with similar results.
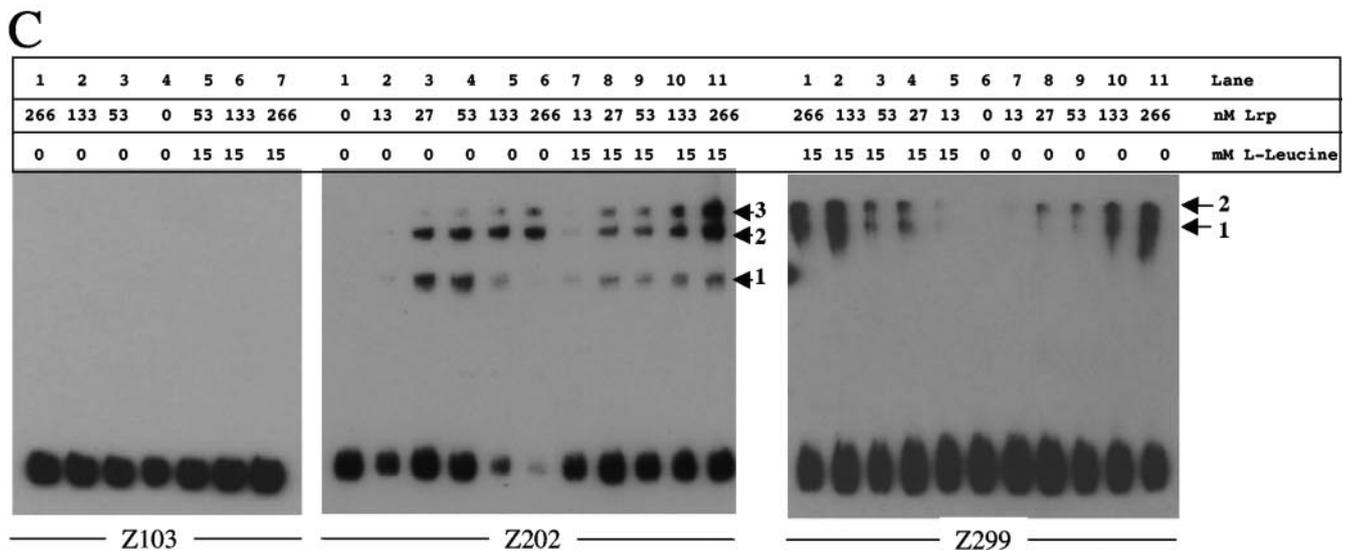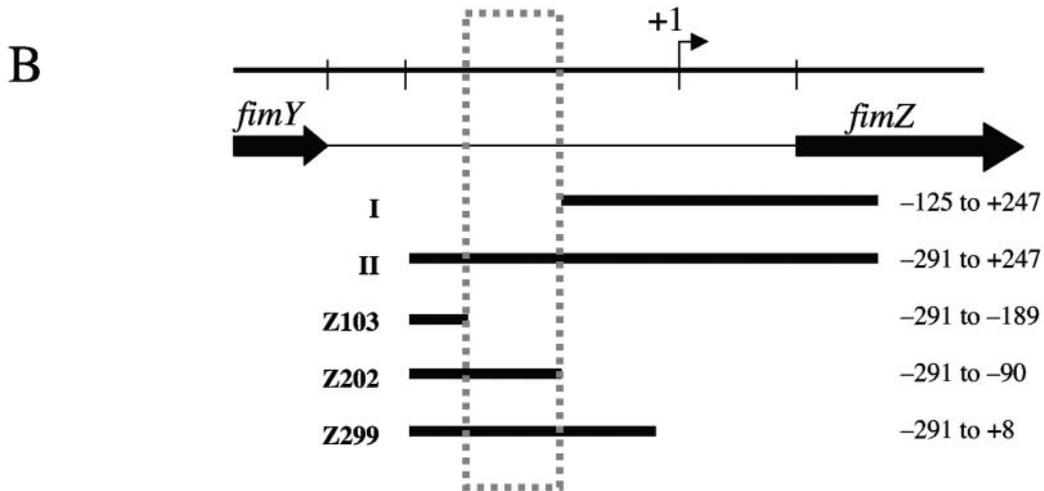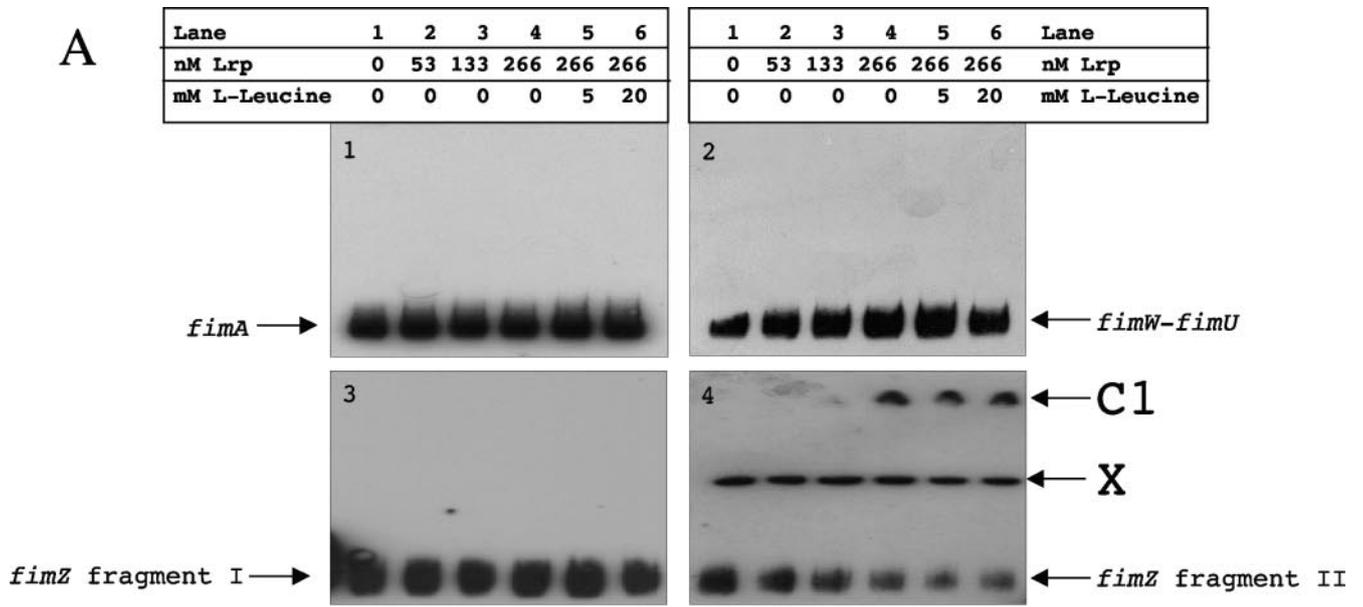
FIG. 4. Detection of Lrp-DNA interactions within the *fim* gene cluster by EMSA. (A) DNA fragments from the regulatory regions of the *fimA*, *fimW-fimU*, and *fimZ* genes were incubated with increasing concentrations of purified Lrp protein in the presence or absence of L-leucine. Gel

Lrp binds to the *fimZ* promoter region in vitro. To determine whether the effect of Lrp was direct or indirect, the interactions of this protein with the promoter regions of *fimA* and *fimZ* and with the *fimW-fimU* intergenic region were analyzed by EMSA. The *fimA* fragment was 699 bp in length (extending from −425 to +274 with respect to the transcription start site [84]). The *fimW-fimU* intergenic region is 294 bp in length and extends from position 35 in the *fimW* open reading frame to a location that is 5 bp upstream of the start point of the *fimU* tRNA. These EMSA analyses showed no direct interaction of Lrp either with the promoter region of *fimA* or with *fimW-fimU*, either in the presence or in the absence of leucine (Fig. 4A, panel 1 and 2).

The promoter region of *fimZ* was also examined for Lrp binding. The *fimZ* transcriptional start site is located 227 bp upstream of the *fimZ* start codon (84). When a 372-bp region encompassing the *fimZ* transcriptional start site (+1) plus 125 bp upstream (fragment I) was incubated with increasing concentrations of Lrp, no interaction was seen (Fig. 4A, panel 3). However, when this region was extended to 291 bp upstream of the *fimZ* transcriptional start site (fragment II), an interaction with Lrp was demonstrated (Fig. 4A, panel 4). This interaction did not appear to be modulated by leucine.

The region of Lrp binding to *fimZ* was characterized in more detail by EMSA in combination with a deletion analysis using nested probes, as shown in Fig. 4B. The far-upstream probe Z103 did not show an interaction at 53, 133, or 266 nM Lrp, in either the presence (lanes 1 to 3) or the absence (lanes 5 to 7) of 15 mM leucine (Fig. 4C). Probe Z202 (−291 to −90) formed three complexes, and extending the length of the probe to encompass the DNA from −291 to +8 did not result in the formation of additional complexes (Fig. 4C). This finding showed that the region important for the interaction of Lrp with the *fimZ* promoter is located between −90 and −190 with respect to the *fimZ* transcription start site (84), a region encompassed by probe Z202 (Fig. 4B). Three complexes with Z202 were observed, suggesting that Lrp binds to up to three sites within this region (Fig. 4C). In the absence of leucine (Fig. 4C, middle panel, lanes 2 to 6), complexes 1 and 2 predominated at Lrp concentrations in the range from 27 to 53 nM (lanes 3 and 4), with increasing formation of complex 3 at Lrp concentrations in the range from 27 to 266 nM (lanes 3 to 6). The addition of leucine altered the relative concentrations of the three complexes, but no new complexes were seen (lanes 7 to 11). This result suggested that leucine influenced the distribution of Lrp across the sites.

DNase I footprinting analysis of Lrp binding at the *fimZ* promoter. A DNase I protection assay was used to analyze in more detail the nature of the interaction of Lrp with the *fimZ*

regulatory region and the effect of leucine on this interaction. Both the coding (Fig. 5A) and the noncoding (Fig. 5B) strands of the *fimZ* regulatory region were examined, both in the absence and in the presence of leucine. This analysis was done with a DNA fragment extending from positions −300 to +17 of *fimZ*, which was approximately equivalent to probe Z299 (Fig. 4B). The regions of protection and hypersensitivity are summarized in Fig. 5C. Lrp protected a 90-bp region on the coding strand, between approximately −85 and −170 with respect to the transcriptional start site (+1), from DNase I digestion (Fig. 5A). In addition, periodic hypersensitive regions, which are indicative of the bending of DNA and characteristic of Lrp, were detected at positions −71, −84, −112, −113, −144, −145, and −157. This finding suggested that Lrp had bent and/or wrapped the DNA to form a nucleoprotein complex. The hypersensitive reaction at position −71 was seen only in the presence of leucine, as was a small region of DNase I protection extending from −70 to −65.

DNase I footprinting of the top, noncoding strand of the *fimZ* regulatory region revealed a pattern of protection by Lrp that was strikingly similar to that seen on the coding strand (Fig. 5B and C). These data correlated with those from the EMSA analysis (Fig. 4). Hypersensitive bases were also visible on the noncoding strand, at positions equivalent to those on the coding strand.

Ectopic expression of FimZ restores fimbriation in an SL1344 *lrp* mutant. To examine whether the positive regulatory effect of Lrp on *fimZ* is required for the expression of fimbriae, an arabinose-inducible, glucose-repressible *fimZ* expression plasmid, pBAD*fimZ*, was constructed (see Materials and Methods). The wild-type strain SL1344 was transformed with plasmids pBAD*fimZ* and pBAD24, the vector control. The pBAD*fimZ* construct was tested for its functionality by inoculation onto swarm plates including 0.2% arabinose or 0.2% glucose. The overexpression of FimZ has been shown previously to inhibit motility by the down-regulation of the *flhDC* master regulatory genes, which activate flagellar expression (23). As expected, only the SL1344(pBAD*fimZ*) construct displayed inhibition of motility in the presence of arabinose, which was consistent with the expression of functional FimZ protein from pBAD*fimZ*.

The expression of fimbriae by SL1344 wild-type and SL1344 *lrp* mutant strains containing pBAD*fimZ* was induced as described in Materials and Methods. SL1344(pBAD*fimZ*) showed similar levels of hemagglutination in the presence of arabinose and glucose (Fig. 6). The pBAD*fimZ*-containing *lrp* mutant showed no visible agglutination of red blood cells in the presence of glucose, which inhibited the expression of FimZ from this construct. In the presence of 0.2% arabinose,

electrophoresis was used to detect changes in the mobility of the labeled DNA fragments caused by the formation of Lrp-DNA complexes. A complex (C1) was detected only in the case of the 538-bp *fimZ* DNA fragment II (panel A4). A nonspecific band (X) was detected in all lanes of panel A4, whether Lrp protein was present or not. (B) The origin of the 538-bp *fimZ* DNA fragment is depicted in panel B, together with that of the 372-bp *fimZ* fragment I used for panel A3 and the shorter *fimZ* regulatory region fragments Z103, Z202, and Z299 used in the experiments with results shown in panel C. (C) The 103-bp Z103 *fimZ* fragment did not form a complex with Lrp, while both the Z202 and Z299 fragments did. Probe Z202 (−291 to −90) formed three complexes (arrows), and increasing the length of the probe to include the DNA from −291 to +8 (Z299) did not result in the formation of additional complexes: at least two (arrows) were detectable with the Z299 probe. Taken together, the data showed that the Lrp interaction with *fimZ* DNA occurred within the region bounded by the dashed box in panel B that was common to Z202 and Z299.
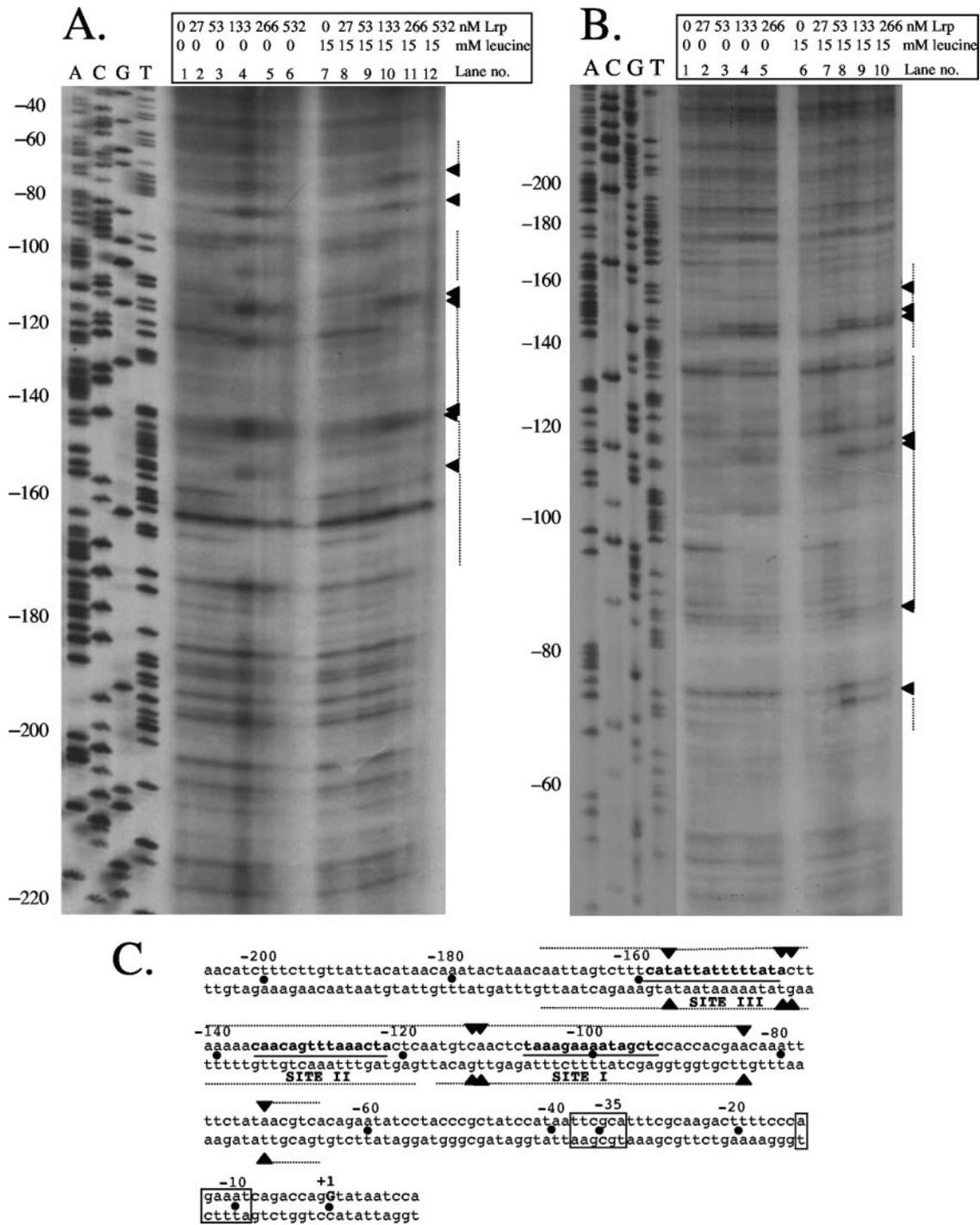
FIG. 5. Detection of Lrp interactions with the *fimZ* regulatory region by DNase I protection assay. (A and B) Lrp protein binding to the coding (A) and noncoding (B) strands of the regulatory region of the *fimZ* gene was detected by DNase I footprinting. Purified Lrp protein was added to *fimZ* regulatory region DNA at the indicated concentrations in the presence or absence of L-leucine, as indicated above each lane. DNA sequencing reactions were run in the lanes labeled A, C, G, and T for the coding (A) and noncoding (B) DNA strands. Vertical dashed lines show approximately those regions where Lrp protein has protected the DNA from DNase I digestion, while arrowheads indicate bases that show hypersensitivity to the enzyme. (C) A summary of the data from panels A and B is shown in panel C. Bases in the *fimZ* regulatory-sequence DNA are numbered with reference to the transcription start site (+1), and the numbers correspond to those shown next to the DNA sequencing reaction results in panels A and B. Dashed horizontal lines indicate approximately the regions that are protected by Lrp, and arrowheads show bases displaying hypersensitivity to DNase I in the presence of Lrp. Filled circles between the DNA strands show the positions of the numbered coordinates. The sequences showing homology to the consensus sequence for Lrp binding sites are in boldface type and are underlined and labeled sites I, II, and III. The boxed motifs are possible binding sites for RNA polymerase located around positions −10 and −35.
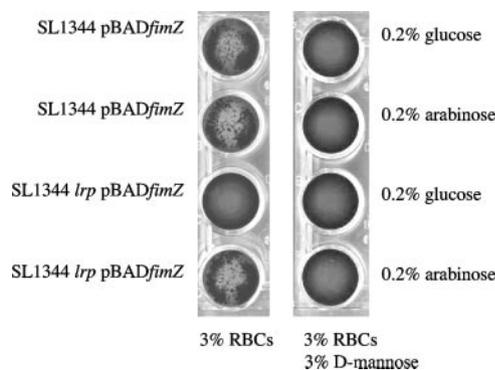
FIG. 6. Ectopic expression of the FimZ regulatory protein reverses the effect of the *lrp* mutation on mannose-sensitive hemagglutination. Hemagglutination assays were carried out with variants of strain SL1344 and the SL1344 *lrp* mutant containing the *fimZ* gene under the control of the arabinose-inducible and glucose-repressible P_BAD promoter in recombinant plasmid pBAD*fimZ* (SL1344 pBAD*fimZ* and SL1344 *lrp* pBAD*fimZ*, respectively). Equivalent numbers of bacterial cells were mixed with red blood cells (RBCs) in the absence or presence of 3% (wt/vol) D-mannose.

which induced the P_BAD promoter, the SL1344 *lrp* mutant showed agglutination of red blood cells at levels that were comparable to those seen with the wild-type strain SL1344. In all cases, this agglutination was demonstrated to be mannose sensitive (Fig. 6).

DISCUSSION

This study establishes that the leucine-responsive regulatory protein, Lrp, plays a positive role in controlling the expression of type 1 fimbriae in serovar Typhimurium. The same protein is a regulator of type 1 fimbrial gene expression in *E. coli*, but it acts there through a completely different mechanism. The role of Lrp in regulating the *E. coli fim* genes is intimately linked to the site-specific recombination mechanism that inverts the 314-bp *fim* DNA switch that harbors the promoter for the transcription of the *fim* structural genes (6, 66). Lrp binds within this invertible DNA element but does not influence transcription from the *fimA* promoter (29). Instead, it acts as an architectural element within the *fim* invertasome and contributes to the distinguishing of the on and off forms of the switch at the level of the nucleoprotein complex (46, 66).

The serovar Typhimurium *fim* genes are not controlled by a DNA inversion mechanism but rely instead on a complicated regulatory cascade involving at least four regulatory genes (Fig. 1). Our data have identified a role for the Lrp protein acting directly at the *fimZ* regulatory gene. The inactivation of the *lrp* gene by mutation results in a loss of mannose-sensitive hemagglutination that is consistent with a failure to express type 1 fimbriae (Fig. 2), which is accompanied by a reduction of at least threefold in *fimA* and *fimZ* transcription (Fig. 3). Both hemagglutination and *fimA* and *fimZ* transcription are restored in the *lrp* knockout mutant following the introduction of a recombinant plasmid that contains a functional copy of the *lrp* gene (Fig. 2 and 3). The expression of the *fimH* gene was not as strongly affected as that of the other *fim* genes by the *lrp* mutation (Fig. 3A). This finding raises the possibility that the

FimH adhesin was still present, albeit in highly truncated fimbriae. If this was so, the adhesin had little ability to agglutinate red blood cells.

We used a combination of electrophoretic mobility shift and DNase I protection assays to locate the Lrp binding site(s) at the *fimZ* gene (Fig. 4 and 5). Although a search was made using bioinformatic methods and EMSA, we found no evidence for Lrp binding in the promoter region of the *fimA*, *fimU*, or *fimW* gene (Fig. 4). All our results indicate that Lrp exerts its positive effect on type 1 fimbriation in serovar Typhimurium through the modulation of *fimZ* regulatory gene transcription.

The EMSA data suggest that Lrp forms three complexes upstream of the *fimZ* promoter and that this interaction is modulated by L-leucine (Fig. 4 and 5). Although Lrp can form high-affinity nonspecific complexes with DNA, these complexes are not usually influenced by leucine (62). The DNase I protection studies permitted the identification of regions upstream of *fimZ* that became hypersensitive to DNase I digestion in the presence of the Lrp protein. Lrp likely binds among those hypersensitive regions, bending the DNA between its binding sites and opening the DNA duplex there to enhanced DNase I digestion, as has been described in previous Lrp studies (79, 80). These findings, along with data from the EMSA analysis, suggest that there are three binding sites for Lrp amid the hypersensitive bases in the protected region. These regions were examined for sequences with similarity to the Lrp consensus sequence YAGHAWATTWTDCTR (24, 25, 63). Three sites (Fig. 5C) show mismatches of three, four, and five bases compared to the consensus sequence. Site I, centered at −100 and having a mismatch of five bases, is located in the region covered by *fimZ* probe I (Fig. 4B), which did not show any interaction with Lrp in the EMSA. It is tempting to hypothesize that Lrp binding at site I may require cooperative binding to sites II and III, centered at −129 and −152, respectively, and that complex 3, seen in the EMSA analysis with probe Z202, indicates the occupation of this site. Certainly, the cooperative binding of DNA is a recurring theme in studies of Lrp (20, 36, 37, 62, 63). Purified Lrp protein protects bases in the regions extending from approximately −85 to −119, −121 to −140, and −146 to −170 on both strands from DNase I digestion in vitro (Fig. 5). Each of these regions of protection contains a match to the consensus sequence for Lrp binding sites (Fig. 5C). The presence of the Lrp protein results in hypersensitivity to DNase I digestion in the case of certain bases on both the coding and noncoding strands. This elevated sensitivity is known to be consistent with the wrapping of the DNA around the protein, which results in the exposure of specific bases to enhanced DNase I cleavage (61, 77). The EMSA data suggest that leucine influences the cooperativeness of Lrp binding to *fimZ* DNA (Fig. 4). However, the pattern of DNase I hypersensitivity seen in the *fimZ* regulatory region was independent of leucine except in the region from −65 to −71 (Fig. 5). This result suggests that once purified Lrp protein has bound to its target sequences, leucine has only a modest influence on the protein-DNA interaction. However, the EMSA data indicate that leucine can also influence Lrp-DNA interaction further upstream, within the sequences encompassed by probe Z202 (Fig. 4C). Thus, leucine

does have an influence on Lrp-*fimZ* interaction, but it is a subtle one that requires further study.

The ectopic expression of the *fimZ* regulatory gene from plasmid pBAD*fimZ* made the expression of mannose-sensitive hemagglutination independent of the *lrp* gene. This result provides strong evidence that *fimZ* is the primary point at which the Lrp protein interacts with the *fim* gene cluster. It is also completely consistent with data from EMSA and DNase I footprinting experiments that show physical interaction of the Lrp protein with the *fimZ* regulatory region.

The Lrp binding sites identified in the *fimZ* regulatory region are located far upstream from the transcription start site. The closest is centered at position −100, making it unlikely that the positive effect of Lrp on *fimZ* transcription involves direct protein-protein interaction with RNA polymerase unless the intervening DNA is looped. FimZ is known to regulate the expression of its own gene positively, raising the possibility that Lrp can potentiate the positive effect of FimZ at the P$_{fimZ}$ promoter. A 7-bp sequence (5′-AATAAGA-3′) that is known to be required for FimZ binding at *fimA* is centered at position −352 upstream of *fimZ* (84). Binding at this far-upstream location may mean that FimZ interaction with RNA polymerase requires the intervening DNA to be bent by the Lrp protein. Certainly, the relative locations of the putative FimZ binding site at −352 and the Lrp sites at −152 and −100 are consistent with such a model. Another attractive mechanism involves the Lrp-mediated (or Lrp- and FimZ-mediated) remodeling of the *fimZ* promoter region to displace a transcriptional repressor.

The H-NS protein is a global repressor of transcription in gram-negative bacteria, and it has high affinity for A+T-rich DNA sequences (9, 27, 28, 54, 58, 64, 70). The *fimZ* gene is unusually A+T rich, and two independent chromatin immunoprecipitation studies have shown that the H-NS protein binds to it (54, 57). Moreover, the inactivation of the *hns* gene resulted in the up-regulation of *fimZ* transcription by ~17-fold in a previous transcriptomic experiment (57). Our finding (Fig. 2) that Lrp can activate *fimZ* transcription by at least 10-fold under growth conditions comparable to those used in the H-NS transcriptomic experiments is consistent with the hypothesis that Lrp derepresses *fimZ* by the displacement of H-NS. In light of the positive regulation of *fimZ* by the FimZ protein, H-NS displacement could be achieved by Lrp alone or by Lrp acting in combination with FimZ. This type of antirepression mechanism is a common theme in studies of H-NS-mediated negative regulation of transcription, and it is becoming clear that a wide variety of DNA binding proteins are capable of dislodging the repressor (18, 52, 75, 78). The involvement of the global regulators Lrp and H-NS in addition to the *fim*-specific proteins FimW, FimY, and FimZ is likely to make the *fim* gene cluster sensitive to environmental stimuli and to the physiological state of the cell. In the future, it will be important to discover what role, if any, these factors play in the phase variation of serovar Typhimurium type 1 fimbriae.

ACKNOWLEDGMENTS

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