The Leucine-Responsive Regulatory Protein, Lrp, Activates Transcription of the fim Operon in Salmo nella 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 II-NS global repressor.

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 mann 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

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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 1-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 E. coli adenine methylase (Dam) protein (11, 39, 76). In both cases, 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).

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 fimbriate 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 pBADfimZ 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 Pbad 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 Pbad promoter. The plasmid pBSfimZ that was used for DNase I footprinting and DNA sequencing was generated by PCR with primer pair fimZ300_F,BamHI and fimZ300_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-Okm, containing an 11-flanked kanamycin cassette, was ligated into a BgII 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 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 260 nm. Reverse transcription-PCR (RT-PCR) was carried out with the OneStep RT-PCR kit (QUIAGEN) using sample RNA at 0.6 coated with 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. The lrp protein 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-
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 (lp′) 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 (encoding a putative fimbrial protein), 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 (lp′) 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
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<tbody>
<tr>
<td>fimA-RT_F</td>
<td>GTT GCG GCT GAT CCT AC</td>
</tr>
<tr>
<td>fimA-RT_R</td>
<td>GTC CGC AGA GGA GAC AG</td>
</tr>
<tr>
<td>fimF-RT_F</td>
<td>TTG ATC GCT ATC GGT TGT</td>
</tr>
<tr>
<td>fimF-RT_R</td>
<td>CAG CAA GGG CCA GTA AT</td>
</tr>
<tr>
<td>fimH-RT_F</td>
<td>CGC GGT CTT TTT CAC C</td>
</tr>
<tr>
<td>fimH-RT_R</td>
<td>GGC CCA GAA GGT AGT CA</td>
</tr>
<tr>
<td>fimW-RT_F</td>
<td>AAC AGT CAT CTT TTA GAG CAT GG</td>
</tr>
<tr>
<td>fimW-RT_R</td>
<td>ATT TTC CGG GTA ATT TTC TT</td>
</tr>
<tr>
<td>fimY-RT_F</td>
<td>TGA CAA CTA CCT CGG GTA TTT</td>
</tr>
<tr>
<td>fimY-RT_R</td>
<td>GCC ATA CGG ATA AAC TGT G</td>
</tr>
<tr>
<td>fimZ-RT_F</td>
<td>ATA ACA GGA GGT TTC ATT G</td>
</tr>
<tr>
<td>fimZ-RT_R</td>
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</tr>
<tr>
<td>fimZ-BAD_F</td>
<td>AAA CCT GCA TCT GTC ATT ATG GAC G</td>
</tr>
<tr>
<td>fimZ-BAD_R-H</td>
<td>ATA AAG CTC ACC CTA GCG GAC ACC TTC</td>
</tr>
<tr>
<td>fimA-EMSA_F</td>
<td>CTT GAA CCT TTT GAG CAA CCT C</td>
</tr>
<tr>
<td>fimA-EMSA_R</td>
<td>GGA GTA GGA TCA GCC GCA AC</td>
</tr>
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<td>TGA CGT TGG TCC TTA GTA AGC</td>
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<td>fimW-EMSA_R</td>
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<td>fimZ_F.4</td>
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<tr>
<td>forNdeI_lrp</td>
<td>TAC CAT ATG GTA GAT AGC AAG AAG CGC</td>
</tr>
<tr>
<td>revXhol_lrp</td>
<td>GCC TGC TGC AGG CGT GTC TTA GTA ACC AG</td>
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<tr>
<td>fwa1.3_EcoRI</td>
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</tr>
<tr>
<td>rev1.3_PstI</td>
<td>ATA CTC CAG GGG CAA TAA GTA TCA ACA AGC G</td>
</tr>
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</table>

The RNA used in this experiment was isolated from bacteria grown under infection 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 fimbral 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 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.

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

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.

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, pBADfimZ, was constructed (see Materials and Methods). The wild-type strain SL1344 was transformed with plasmids pBADfimZ and pBAD24, the vector control. The pBADfimZ 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(pBADfimZ) construct displayed inhibition of motility in the presence of arabinose, which was consistent with the expression of functional FimZ protein from pBADfimZ.

The expression of fimbriae by SL1344 wild-type and SL1344 lrp mutant strains containing pBADfimZ was induced as described in Materials and Methods. SL1344(pBADfimZ) showed similar levels of hemagglutination in the presence of arabinose and glucose (Fig. 6). The pBADfimZ-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,
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.
which induced the pBAD 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 fimbrae. 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 YAGHAVATTWTDCRT (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 pBADfimZ 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 PfimZ 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, 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 transcriptional 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 Typhi-

memurium type 1 fimbriae.

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REFERENCES


Lrp REGULATION of fim OPERON in SEROVAR TYPHIMURIUM


