Lrp Acts as Both a Positive and Negative Regulator for Type 1 Fimbriae Production in *Salmonella enterica* Serovar Typhimurium

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Abstract

Leucine-responsive regulatory protein (Lrp) is known to be an indirect activator of type 1 fimbriae synthesis in *Salmonella enterica* serovar Typhimurium via direct regulation of FimZ, a direct positive regulator for type 1 fimbriae production. Using RT-PCR, we have shown previously that *fimA* transcription is dramatically impaired in both *lrp*-deletion (*Δlrp*) and constitutive-*lrp* expression (*lrp*) mutant strains. In this work, we used chromosomal *PfimA-lacz* fusions and yeast agglutination assays to confirm and extend our previous results. Direct binding of Lrp to *PfimA* was shown by an electrophoretic mobility shift assay (EMSA) and DNA footprinting assay. Site-directed mutagenesis revealed that the Lrp-binding motifs in *PfimA* play a role in both activation and repression of type 1 fimbriae production. Overproduction of Lrp also abrogates *fimZ* expression. EMSA data showed that Lrp and FimZ proteins independently bind to *PfimA* without competitive exclusion. In addition, both Lrp and FimZ binding to *PfimA* caused a hyper retardation (supershift) of the DNA-protein complex compared to the shift when each protein was present alone. Nutrition-dependent cellular Lrp levels closely correlated with the amount of type 1 fimbriae production. These observations suggest that Lrp plays important roles in type 1 fimbriation by acting as both a positive and negative regulator and its effect depends, at least in part, on the cellular concentration of Lrp in response to the nutritional environment.

Introduction

Type 1 fimbriae are mannose-sensitive agglutination factors that mediate bacterial adhesion to a broad range of eukaryotic cells by interactions with mannolysed glycoproteins [1–3]. Most members of the family Enterobacteriaceae, including *Salmonella enterica* serovar Typhimurium, produce type 1 fimbriae that are believed to contribute to pathogenesis by facilitating the initial interaction with host cells [3–5]. The *fim* gene cluster, responsible for type 1 fimbriae production, is composed of six structural genes, *fimAICDHF* transcribed as an operon from the *fimA* promoter, three regulatory genes, *fimZ*, *fimI*, and *fimW*, and an arginine tRNA gene, *fimU* [6]. The structural gene products are the major type 1 fimbrial subunit FimA [7,8], fimbilin-like protein FimI [9], periplasmic chaperone FimC [10], outer membrane usher protein FimD [6], minor fimbrial subunit FimH (adhesin) [11], and fimbrial-like protein FimF [6,11]. The regulatory *fimZ*, *fimI*, and *fimW* genes are expressed from independent promoters [12–14]. The FimZ regulator activates expression of the *fimAICDHF* operon by binding to the *fimA* promoter [15]. In serovar Typhimurium, FimY and FimW act as a transcriptional coactivator and repressor, respectively, through protein-protein interactions with FimZ [12,14]. However, Saini et al. reported that FimY independently activates the *fimA* promoter, and FimW acts as a negative regulator by repressing FimY transcription [16]. The *fimU* gene product arginine tRNA acts as a posttranscriptional regulator by affecting FimY translation [17,18].

Bacteria are efficient at switching between type 1 fimbriate and non-fimbriate status in response to environmental conditions [3,19]. The mechanism of phase-variable type 1 fimbriation has been well characterized in *Escherichia coli* [20]. FimB and FimE recombinases mediate site-specific recombination of the *fimA* promoter region, resulting in alteration of orientations allowing or blocking transcription [20,21]. Nucleoid-binding global regulators that modulate DNA topology, such as Lrp, integration host factor (IHF), and H-NS affect phase variation and synthesis of type 1 fimbriae in *E. coli* [22–27]. In addition, McClain et al. suggested that there is an inversion-independent phase variation mechanism [28]. Despite significant homology between the *fim* structural genes, the mechanism by which type 1 fimbriae synthesis is regulated in *S. enterica* serovar Typhimurium differs substantially from that in *E. coli*. The serovar Typhimurium *fimA* promoter does not possess a cis-acting regulatory DNA element for reversible inversion-dependent regulation of type 1 fimbriate expression [29]. Moreover, homologs of the *E. coli* FimB and FimE recombinase are not present in serovar Typhimurium [30,31]. Conversely, no homologs for serovar Typhimurium FimZ, FimY and FimW regulators have been found within the *E. coli* fim gene cluster [32]. In serovar Typhimurium, Lrp is required for type 1 fimbriation production by activating FimZ synthesis [33], whereas in *E. coli*, Lrp is involved in inversion-dependent phase variation [26]. Lrp activates *fimZ* expression by binding directly to the *PfimA* promoter [33]. FimZ is an essential positive regulator for type 1 fimbriate...
production in serovar Typhimurium [13]. Thus an hph-deletion mutant cannot produce FimZ, and is blocked for type 1 fimbriation [33].

Although, the mechanism for type 1 fimbriae production in bacteria has been extensively studied, no clear mechanism for on-off switching in response to environmental cues has been demonstrated. We have proposed that dynamic change in cellular Lrp levels in response to nutritional state (feast or famine) is important for coordinating virulence traits in Salmonella [34]. In this study, we address the effect of Lrp on type 1 fimbriation in Salmonella.

**Results**

**Lrp acts as both positive and negative regulator for fimA expression**

In our previous study using RT-PCR, we observed that an lrp expression mutation abrogated fimA transcription [34]. The lack of fimA expression in the hph strain was unexpected, since Lrp is known to be an indirect positive regulator for type 1 fimbriae production by enhancing expression of the positive regulator FimZ [33]. To further define the role of Lrp in fimA expression, we determined the activity of PfimA using PfimA-lacZ fusions in wild-type strain 3761, and isogenic Δhp and hph mutant strains (Table 1) by measuring the β-galactosidase activity in each of these strains after static, 24 h growth in LB medium at 37°C (Fig. 1A). The lack of β-galactosidase synthesis in the Δhp and hph mutant backgrounds indicates that transcription from P fimA is not active in the absence of Lrp or when Lrp is overproduced.

Next, we measured fimbriation production by determining the ability of static cultures of strains 3761, 9411 (Δhp) and 9448 (hph) to agglutinate yeast cells. Wild-type cells displayed mannose-sensitive agglutination, while both mutant strains were deficient in this phenotype (Fig. 1B), consistent with our observations that no fimA transcript was detected in the strains. We further confirmed

| Table 1. Bacterial strains and plasmids used in this study. |
|-------------------------------------------------------------|
| **Strains** | **Description[^p]** | **Source** |
|--------------------------|----------------------|-----------|
| S. Typhimurium | | |
| 3761 | Wild-type strain UK-1, highly virulent for chicks and mice | [52,53] |
| 9411 | Δlp-13 (hp-deletion mutation) [3761] | [34] |
| 9448 | hp-1281 (ΔPmr-Pm, hp, chromosomal deletion-insertion mutation to drive constitutive expression of Lrp (hp^C)) [3761] | [34] |
| 9449 | ΔrelA198::carrC P^rhuA21 lacI TT ΔaraBAD23 hp-1281 [9509] | [34] |
| 9455 | P^rac::pYA4311 (P fimA-lacZ), Amp’ Gm’ [3761] | This study |
| 9467 | Δlp-13 P^maa::pYA4311 (P fimA-lacZ), Amp’ Gm’ [9411] | This study |
| 11107 | P fimA413 (Δ mutation) [3761] | This study |
| 11111 | P fimA529 (Δ mutation) [3761] | This study |
| 11115 | P fimA1325 (Δ2 mutation) [3761] | This study |
| 11153 | hp-1281 P fimA1225 [9448] | This study |
| 11263 | hp-1281 P fimA::pYA4311 (P fimA-lacZ), Amp’ Gm’ [9448] | This study |
| 11264 | P fimA413::pYA4311 (P fimA1225-lacZ), Amp’ Gm’ [11107] | This study |
| 11265 | P fimA2925::pYA4311 (P fimA-lacZ), Amp’ Gm’ [11111] | This study |
| 11266 | P fimA1325::pYA4311 (P fimA1225-lacZ), Amp’ Gm’ [11115] | This study |
| 11267 | hp-1281 P fimA1225::pYA4311 (P fimA1225-lacZ), Amp’ Gm’ [11153] | This study |
| 11377 | P fimA1325 (Δ2 double mutation) [11115] | This study |
| 11378 | P fimA1225 (Δ2 double mutation) [11110] | This study |
| 11379 | P fimA2925 (Δ2 double mutation) [11115] | This study |
| 11380 | P fimA529 (Δ2 triple mutation) [1379] | This study |
| E. coli | | |
| MGN-617 (7213) | thr-1 leuB6 fhuA21 lacY1 glnV44 recA1 ΔasdA4 thy-1 RP4-2-Tc::Mu [l-pir], Km’ | [54] |
| **Plasmids** | | |
| pRE112 | Positive selection suicide vector (R6K ori) for gene replacement, Cm’ | [55] |
| pSG3 | a suicide vector (R6K ori) for construction of promoter-lacZ fusion into chromosome | [49] |
| pWSK29 | a low-copy-number cloning vector (pSC101 ori), Amp’ | [47] |
| pYA4124 | Derivative of pET containing His Tag from pET-14b, Km’ | [34] |
| pYA4311 | Derivative of pSG3 for insertion of the P fimA-lacZ fusion into the chromosome, Amp’ Gm’ | This study |
| pYA4758 | Derivative of pRE112 for replacement of the P fimA with P fimA413, Cm’ | This study |
| pYA4759 | Derivative of pRE112 for replacement of the P fimA with P fimA2925, Cm’ | This study |
| pYA4801 | Derivative of pRE112 for replacement of the P fimA with P fimA1225, Cm’ | This study |
| pYA4865 | Derivative of pWSK29 harboring a recombinant fimZ gene, Amp’ | This study |

[^p]: Amp’, ampicillin resistance; Gm’, gentamicin resistance; Cm’, chloramphenicol resistance; Km’, kanamycin resistance.

doi:10.1371/journal.pone.0026896.t001
these results by transmission electron microscopy (TEM). Typical type 1 fimbriae appendages were detected on the cell surface of wild-type strain \( \chi3761 \), while no type 1 fimbriae were detected on the cell surface of the \( Dlrp \) or the \( lrpC \) strains (data not shown). These results demonstrate that deletion of \( lrp \) or overproduction of Lrp has a strong negative effect on type 1 fimbriae synthesis by directly influencing \( fimA \) transcription.

**Lrp directly interacts with \( P_{fimA} \)**

Based on our previously described consensus sequences [34], we detected four putative Lrp-binding motifs in the \( PfimA \) region. All four motifs are located upstream of the \( fimA \) transcription start site (\(+1\)) [15]. The DNA motifs 1 \([-308 \text{ to } -301]\), 2 \([-112 \text{ to } -105]\), and 3 \([-35 \text{ to } -28]\) belong to Lrp-binding consensus IV, \( 5'-\text{GNN(N)TTTT} -3' \) [34,35] and DNA motif 4 \([-31 \text{ to } -20]\) belongs to Lrp-binding consensus III, \( 5'-\text{HNDWTTATTHND} -3' \) [where \( H \neq \text{ not G}; W = \text{ A or T}; D = \text{ not C}; N = \text{ all bases}; \) and \( (N) = \text{ all bases or none} \) ] [34]. DNA motif 2 lies just upstream and motifs 3 and 4 lie just downstream of the FimZ binding site \([-98 \text{ to } -47]\) [33]. These observations led us to postulate that Lrp acts as both an activator and a repressor for type 1 fimbriae expression mediated by differential interactions with Lrp-\( P_{fimA} \), depending on cellular concentration of Lrp and on environmental conditions. To address this hypothesis, we tested the direct interaction between Lrp and \( P_{fimA} \) using the electrophoretic mobility shift assay (EMSA). Lrp directly interacted with \( P_{fimA} \) in a concentration-dependent manner (Fig. 2). These results are in contrast to the study by McFarland et al who did not detect an Lrp-\( P_{fimA} \) interaction in gel shift assays [33]. We noted differences in the binding buffer used in their study compared to ours. Of particular interest was the fact that their binding buffer included MgCl\(_2\), while ours did not include divalent cations and, instead included EDTA to chelate any divalent cations present. We performed the EMSA assay using McFarland’s binding buffer and, like McFarland et al, did not detect binding (data not shown), suggesting that the magnesium concentration may play a role in regulating Lrp binding to \( P_{fimA} \).

A DNase I footprinting analysis was performed to elucidate in more detail the molecular nature of the Lrp-\( P_{fimA} \) interaction with both coding (Fig. 3A) and non-coding (Fig. 3B) strands. A 388-bp DNA probe extending from -334 to +54 with respect to transcriptional start site (+1) was used, which includes the entire \( P_{fimA} \) region. The footprint was estimated by densitometry comparing two lanes for 150 nM and 0 nM Lrp. Sites protected from or hypersensitive to DNase I are summarized in Fig. 3C. All four putative Lrp-binding motifs were protected by Lrp (Fig. 3A and B). We observed strong protection of the DNA region \([-326 \text{ to } -257]\) containing the Lrp-binding motif 1, while protection of the DNA regions \([-123 \text{ to } -102]; -39 \text{ to } -33; \) and \([-26 \text{ to } -6]\) of the Lrp-binding motifs 2, 3, and 4, respectively, was weaker. The DNA region \([-5 \text{ to } +4]\) in immediate downstream of the Lrp-binding motif 4 also showed weak protection. The AT-rich overlapping region \([-34 \text{ to } -28]\) within Lrp-binding motif 3 and 4 was highly resistant to DNase I digestion. The FimZ-binding region partly overlapped with the Lrp-binding region \([-59 \text{ to } -47]\) (Fig. 3C). This result suggests that both Lrp and FimZ competitively interact with the overlapped motif in \( P_{fimA} \). Two

![Figure 1. Expression of type 1 fimbrial operon and the associated phenotype in the wild-type (WT, \( \chi3761 \)), \( \Delta lrp \) (\( \chi9411 \)), and \( lrpC \) (\( \chi9448 \)) strains. (A) \( \beta \)-galactosidase assay for the \( P_{fimA} \)-\( lacZ \) fusions in each strain is shown. *, \( P<0.05 \) (B) Mannose-sensitive yeast agglutination assay to assess type 1 fimbriae synthesis. Representative images from several experiments are shown. Bacterial cells were statically grown in LB broth for 24 h at 37°C. doi:10.1371/journal.pone.0026896.g001](https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0026896)

![Figure 2. Binding of the purified Lrp to the wild-type \( P_{fimA} \). Binding reactions were carried out in various Lrp concentrations: 0, 50, 100, 150, and 200 nM. The 178-bp DNA fragment from pBluescript multi-cloning sites was used as the negative control. doi:10.1371/journal.pone.0026896.g002](https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0026896)
super-hypersensitive positions (red arrowheads in Fig. 3A and B), −193 T on coding strand and −130 G on non-coding strand, were detected. None of tested DNA fragments showed non-specific degradation in the absence of DNase I (data not shown).

Each of the Lrp-binding motifs in P \(_{fimA}\) plays a distinct role in regulating type 1 fimbriae production

To dissect the role of Lrp interactions with the promoter region of \(fimA\), we constructed \(P_{fimA}\) mutations, \(P_{fimA\_A1225}\), and \(P_{fimA\_A1225}\), designated \(\alpha\), \(\beta\), and \(\gamma\), respectively, in the Lrp-binding motifs by site-directed mutagenesis (Table 1 and Fig. 3C). Our strategy for these mutations was to change multiple bases in each of the Lrp-binding motifs to increase the likelihood of disrupting the Lrp-DNA interaction and to reduce the likelihood of reversion. In addition, the GC content (25% or less) of the Lrp-binding motifs was increased by the changes to achieve a GC content closer to the average for \(Salmonella\), approximately 52% [9]. The GC content was raised to 50% by the \(\alpha\) and \(\beta\) mutations and to 44% by the \(\gamma\) mutation. None of the changes affected bases known to be part of the RNA polymerase binding site for \(P_{fimA}\).

To determine the influence of the \(P_{fimA}\) mutations on expression, we estimated the levels of mannose-sensitive fimbriae by a yeast agglutination assay, the synthesis of FimA by western expression, we estimated the levels of mannose-sensitive fimbriae degradation in the absence of DNase I (data not shown).

Each of the Lrp-binding motifs in \(P_{fimA}\) contributes to Lrp-\(P_{fimA}\) interaction

To address the mechanism behind the Lrp-mediated dual (activation/repression) regulation, interactions between Lrp and each of the mutant \(fimA\) promoters were evaluated using EMSA. Lrp binding to the the \(\alpha\), \(\beta\), \(\gamma\), \(\alpha \gamma\), \(\alpha \beta\), and \(\beta \gamma\) (single and double) mutant promoters was indistinguishable from binding to \(P_{fimA\_A1225\_WT}\) regardless of Lrp concentrations. In contrast, Lrp binding to the \(\alpha \beta \gamma\) triple mutant promoter was impaired when the Lrp concentration was reduced to 100 nM or 50 nM (Fig. 5). This indicates that each of the Lrp-binding motifs in \(P_{fimA}\) contributes to Lrp-\(P_{fimA}\) interactions. Leucine had a minor effect on the band pattern, but we observed no significant effect on the binding affinity of Lrp to \(P_{fimA\_A1225}\) (data not shown). In addition, there was no significant effect of leucine on the Lrp-\(P_{fimA}\) footprint (data not shown).

Lrp acts as both positive and negative regulator of \(fimZ\) expression

The increase in \(fimA\) expression observed in the \(\beta\) mutant (Fig. 4B) raised the possibility that the binding of Lrp to motif 2, adjacent to the \(fimZ\) binding site (Fig. 3C), may affect \(fimZ\) binding. If the \(\beta\) mutation precluded Lrp binding, this could allow for greater accessibility of \(fimZ\) to its binding site in \(P_{fimA}\), thereby accounting for the observed hyper-fimbriation phenotype of the \(\beta\) mutant. To address this possibility, we investigated whether the \(\beta\) mutation could relieve the observed repression of \(fimA\) in the \(\beta\) mutant. Therefore, we introduced the \(\beta\) mutation into the \(\beta\) mutant and evaluated the resulting strain. We found that introduction of the \(\beta\) mutation did not alleviate the \(fim\) phenotype in \(\beta\) mutant (Fig. 4). The new strain was essentially identical to the \(\beta\) mutant carrying the \(\beta\) mutant (WT). It did not produce any detectable FimA (Fig. 4A), did not agglutinate yeast cells, and no \(fimA\) transcription was detected from the \(P_{fimA\_A1225}\) promoter (Fig. 6A). Because FimZ is a positive activator of \(fimA\) expression, we assessed \(fimZ\) expression in the \(\beta\) mutant by RT-PCR analysis. We found that \(fimZ\) expression was undetectable in both the \(\Delta \beta p\) and \(\beta\) mutants (Fig. 6A). The results within the \(\Delta \beta p\) mutant are consistent with previous observations that Lrp is a positive activator of \(fimZ\) [33]. The lack of \(fimZ\) expression in the \(\beta\) mutant indicates that Lrp can also act as a negative regulator of \(fimZ\). In addition, complementation with plasmid-borne (Lrp-
independent) fimZ can overcome the loss of type 1 fimbriae production in the lrpC mutant in both wild-type PfimA and PfimA529 backgrounds (Fig. 6B). Therefore, it appears that even if Lrp binding is reduced at the Lrp-binding motif 2 when the mutation is present, no fimA is expressed due to repression of fimZ. In addition, positive regulation of fimA expression by FimZ is dominant over the negative regulation by Lrp when fimZ is overexpressed.

Both Lrp and FimZ independently bind to PfimA

To gain greater insight into the regulation of fimA expression by Lrp and FimZ, we evaluated the binding of Lrp and FimZ to PfimA at several different molar ratios. First, we confirmed that purified FimZ directly interacted with PfimA (Fig. 7A). When the concentration of FimZ was held constant at 50 nM, the intensity of shifted DNA-protein complex was increased by adding Lrp in a concentration dependent manner (Fig. 7B). Similarly, when the Lrp concentration was held constant at 50 nM, the intensity of shifted DNA-protein complex was increased by adding FimZ in a concentration dependent manner (Fig. 7B). In contrast to these conditions, in presence of Lrp (50 nM) or FimZ (12.5 nM) alone, the PfimA-Lrp or PfimA-FimZ complexes ran as a smear in the gel (Fig. 7A and B). These results indicate that both Lrp and FimZ independently bind to PfimA. Although, DNase I footprinting analysis showed that the FimZ-binding motif partly overlapped with the Lrp-binding region (Fig. 3B and C), we could not obtain any evidence for competitive binding of Lrp and FimZ to PfimA using EMSA. In addition, in the presence of both Lrp and FimZ,
we observed an increase in the apparent size of the $P_{\text{fimA}}(\text{WT})$ complex (supershift) compared to the shift when each protein was present alone (Fig. 7C). To identify the Lrp-binding motif(s) responsible for the supershift, we estimated the binding of Lrp and FimZ to each of the mutant promoters, $P_{\text{fimA}413}$ ($\alpha$), $P_{\text{fimA}529}$ ($\beta$), and $P_{\text{fimA}1225}$ ($\gamma$). The $\alpha$ mutation in motif 1 abrogated the supershift of the DNA-protein complex, while the $\alpha$ and $\beta$ mutations maintained the supershift (Fig. 7C). Remarkably, the $\alpha$ mutation led to a very strong supershift (Fig. 7C). These results indicate that the Lrp-binding motif 1 and the FimZ-binding motif in $P_{\text{fimA}}$ allow the supershifting of the PfimA complex by binding of both Lrp and FimZ to PfimA.

The cellular level of Lrp is a key factor for on/off switching of type 1 fimbriae production in serovar Typhimurium

To examine Lrp-dependent on/off switching of type 1 fimbriae production, we employed $S$. Typhimurium strain $\chi 9449$ harboring an arabinose-dependent Lrp expression system ($\text{araC, PBAD, lacI}$ and $\text{Ptrc, lrp}$) [34]. In the presence of arabinose, lacI expression is induced and $\text{lrp}$ expression, transcribed from the lacI-regulated $\text{Ptrc}$ promoter, is repressed. Conversely, in the absence of arabinose, no LacI is produced and Lrp is synthesized (Fig. 8A). In strain $\chi 9449$, Lrp synthesis and the ability to agglutinate yeast cells were dependent on the arabinose concentration in LB medium (Fig. 8A).

To evaluate whether the nutrition-dependent cellular Lrp levels are related to on-off switching of type 1 fimbriation, wild-type strain $\chi 3761$ was statically grown in MOPS minimal broth, LB broth, and MOPS plus LB (MOPS-LB) broth mixed in several different ratios. Cells from these cultures were harvested and tested for mannose-sensitive yeast agglutination. Cell lysates were analyzed by western blot using anti-Lrp mouse serum. As shown in Fig. 8B, Lrp synthesis was proportional to the nutritional content of the growth medium: with more Lrp produced under poor nutritional conditions and less Lrp produced under rich nutritional conditions. Mannose-sensitive yeast agglutination was dramatically reduced in the $S$. Typhimurium cells grown in MOPS minimal medium, which is the condition generating the highest cellular Lrp level (Fig. 8B). Mannose-sensitive agglutination was partially recovered in the bacterial cells grown in MOPS minimal broth supplemented with 1% (v/v) LB broth (Fig. 8B). The wild-type $S$. Typhimurium completely recovered mannose-sensitive type 1 fimbriation in the MOPS-LB media containing 10% (v/v) LB broth or more (Fig. 8B). These results imply that the nutrition-
dependent intracellular Lrp concentration is important for regulating type 1 fimbriation.

To get a better sense of how much Lrp is present in cells, we estimated the number of Lrp molecules per cell when cells were grown in MOPS minimal broth, LB broth, and MOPS plus LB (MOPS-LB) broth mixed in several different ratios by comparing the western blot shown in Fig. 8B with a western blot loaded with known amounts of purified Lrp (Fig. 8C). Based on our densitometry calculations using the standard curve in Fig. 8C, the wild-type *Salmonella* strain *x*3761 produced about 6,000 Lrp molecules (3,000 dimers) per cell when grown in MOPS minimal medium. This is the same number of Lrp molecules calculated for *E. coli* cells grown in a glucose-based minimal medium [36]. The wild-type *Salmonella* strain *x*3761 produced about 6,000 Lrp molecules (3,000 dimers) per cell when grown in MOPS minimal medium. This is the same number of Lrp molecules calculated for *E. coli* cells grown in a glucose-based minimal medium [36]. The wild-type *Salmonella* strain *x*3761 produced about 6,000 Lrp molecules (3,000 dimers) per cell when grown in MOPS minimal medium. This is the same number of Lrp molecules calculated for *E. coli* cells grown in a glucose-based minimal medium [36].

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**Figure 7. Binding of the purified Lrp and FimZ proteins to P<sub>fimA</sub>.** (A) Binding of FimZ to P<sub>fimA</sub>(WT). (B) Binding of Lrp and FimZ to P<sub>fimA</sub>(WT). (C) Binding of Lrp and FimZ to P<sub>fimA</sub>(WT), P<sub>fimA413</sub> (α), P<sub>fimA529</sub> (β), or P<sub>fimA1225</sub> (γ). The white arrowheads indicate super-shifted Lrp-P<sub>fimA-FimZ</sub> complexes. To enhance resolution of the super-shifted DNA-protein complexes, the running time of the polyacrylamide gel in panel C was extended from 1 h (used in panels A and B) to 2 h. Schematic diagrams of the wild-type and mutant fimA promoters are shown under the gel.

doi:10.1371/journal.pone.0026896.g007

Lrp is required for synthesis of type 1 fimbriae [33]. Previous studies have shown that FimZ is required for fimA expression and Lrp is required for fimZ expression [33]. Lrp binds to the fimZ promoter region and can thereby enhance fimZ expression [33]. FimZ binds to P<sub>fimA</sub> and activates fimA expression. Our previous report showed that while Δlp mutants did not express fimA as expected, neither did PfimA mutants [34]. To address the basis for these apparently contradictory phenomena, we investigated the role of Lrp in regulating type 1 fimbriation in more detail. Using both genetic and molecular approaches, we found that high cellular levels of Lrp repressed fimA expression, with a concomitant loss of the type 1 fimbriae-associated mannose-sensitive agglutination phenotype (Fig. 4). Under these conditions, production of FimZ is also abrogated (Fig. 6). Site-directed mutagenesis of putative Lrp-binding sites in the fimA promoter indicated that binding of Lrp to the fimA promoter is necessary for both activation and repression of type 1 fimbriae expression (Fig. 4).
The γ mutation in the Lrp-binding motif 1 (Fig. 3B) abolished type 1 fimbiae synthesis, as judged by fimA expression and the yeast agglutination phenotype (Fig. 4). These results suggest that Lrp-binding motif 1 in PfimA may play a crucial role in Lrp-mediated fimA transcription, as judged by fimA expression and elimination of any detectable yeast agglutination (Fig. 6B). This result is most easily explained by the lack of fim expression in these cells (Fig. 6A), as the synthesis of type 1 fimbiae is restored by overexpression of fim (Fig. 6B).

The δ mutation in Lrp-binding motif 2 (Fig. 3B) enhanced fimA transcription and FimA synthesis (Fig. 4), indicating that the Lrp-binding motif 2 in PfimA is important for repression of type 1 fimbiae production. Based on our results showing that Lrp binds to motif 2 (Fig. 3B), we infer that repression via motif 2 is Lrp-mediated. Introduction of the lipC mutation into the β mutant (PfimA::lac) repressed fimA expression and eliminated any detectable yeast agglutination (Fig. 6B). This result is most easily explained by the lack of fim expression in these cells (Fig. 6A), as the synthesis of type 1 fimbiae is restored by overexpression of fim (Fig. 6B).

The PfimA region of S. Typhimurium includes four Lrp-binding motifs, 1, 2, 3, and 4 (Fig. 3C). Interestingly, three motifs are located immediately upstream (motif 2) and downstream (3 and 4) of the FimZ-binding cis element in PfimA [33]. Motif 1 is located far upstream (−308 to −301) from the fimA transcription start site [15]. Moreover, two potential high-affinity H-NS binding sites [41], 5′- AAAATAAGA -3′ (−100 to −92) and 5′- AT- TAAAAGA -3′ (−51 to −43), are located immediate downstream of Lrp-binding motif 2 and upstream of Lrp-binding motif 3, respectively, and overlap with the FimZ-binding motif (Fig. 3). This result suggests that Lrp and FimZ compete for binding to PfimA at the overlapping sites. However, the EMSA results indicated that Lrp and FimZ independently bind to PfimA without competitive exclusion.

Many Lrp-regulated genes include multiple Lrp-binding motifs in their promoter region. Cooperative binding of Lrp to these motifs is an important factor for Lrp-mediated gene regulation [38]. Cooperative interactions between Lrp and other nucleoid-binding proteins such as H-NS are thought to repress transcription of some genes [39]. While Lrp has been shown to act as a positive or negative regulator for each of the genes in the Lrp regulon, no systematic study of the mechanism has been undertaken. Although it is unusual for Lrp to be both a positive and a negative regulator in the same operon, this type of dual regulation has been reported for the papBA operon [40]. In that case, Lrp interacts with H-NS for repression and PapI for activation.

The PfimA region of S. Typhimurium includes four Lrp-binding motifs, 1, 2, 3, and 4 (Fig. 3C). Interestingly, three motifs are located immediately upstream (motif 2) and downstream (3 and 4) of the FimZ-binding cis element in PfimA [33]. Motif 1 is located far upstream (−308 to −301) from the fimA transcription start site [15]. Moreover, two potential high-affinity H-NS binding sites [41], 5′- AAAATAAGA -3′ (−100 to −92) and 5′- AT- TAAAAGA -3′ (−51 to −43), are located immediate downstream of Lrp-binding motif 2 and upstream of Lrp-binding motif 3, respectively, and overlap with the FimZ-binding motif site. This observation suggests that Lrp binding to Lrp-binding motif 2 and 3 may facilitate binding of the silencing protein H-NS. Site-directed mutagenesis revealed that motifs 1 (distal locus for γ mutation) and 2 (proximal locus for β mutation) are important determinants for activation and repression, respectively, of type 1 fimbiae production (Fig. 4). This result is consistent with previous reports that Lrp acts as a repressor when bound to motifs closer to or within the promoter and as an activator when bound to motifs further upstream [35,42]. Similarly, we also found seven Lrp-binding motifs in the PfimA region (603-bp, between fimI stop codon and fimZ start codon) (data not shown). Two motifs belong to Lrp-binding consensus III (5′- HNDWTTATTHND -3′) and five motifs belong to Lrp-binding consensus IV (5′-
GNN[NS][TTT T-3'] [34,35]. Furthermore, three of the seven motifs in P\textsubscript{JunA} are strong Lrp-binding DNA sequences as identified by DNA footprint analysis in a previous study [33]. One Lrp-binding motif in P\textsubscript{JunA} is also located far upstream (--353 to --546) from the fim\textsubscript{Z} transcription start site [33] similar to motif 1 in P\textsubscript{JunA} (Fig. 3C). A specific feature of the P\textsubscript{JunA} region is that two Lrp-binding motifs are located between the transcription start site and the start codon of fim\textsubscript{Z} gene. Although the double and triple mutations, P\textsubscript{JunA1329} (\gamma\beta\gamma), P\textsubscript{JunA1329} (\gamma\beta\gamma), P\textsubscript{JunA2925} (\gamma\beta\gamma), and P\textsubscript{JunA935} (\gamma\beta\gamma) still can interact with Lrp, all of the multiple mutants produced wild-type levels of yeast agglutination on both day 1 and day 3 (data not shown). These results suggest that for Lrp to exert its regulatory effect, it must bind to at least two Lrp-binding motifs in P\textsubscript{JunA}. In the absence of cooperative binding, as is the case in the double or triple mutants, P\textsubscript{JunA} expression would not be under direct Lrp control. However, transcription from these mutant promoters is still sensitive to regulation by FimZ. Thus these promoters can be activated by FimZ and produce wild-type levels of type 1 fimbrae when grown in LB broth. In addition, we believe that the DNA motif 3 and 4 (\alpha mutation position) can facilitate cooperative binding of Lrp to the DNA motif 1 and motif 2, even if \alpha mutation itself does not have any effect on type 1 fimbrae production. Therefore, the effects of \beta and \gamma mutations can be suppressed by adding \alpha mutation. These observations indicate that the cooperative binding of Lrp to multiple Lrp-binding motifs in P\textsubscript{JunA} is important for Lrp-mediated regulation of type 1 fimbrae production. Based on results from site-directed mutagenesis of the multiple Lrp-binding motifs in P\textsubscript{JunA}, we assume that the organization (proximity, number, and orientation) of Lrp-binding motifs and their cooperative interaction with Lrp play a crucial role for on/off switching of the fim\textsubscript{Z} gene expression.

Saini et al. suggested that inhibition of fim gene expression occurs through the direct repression of P\textsubscript{JunA} by FimW, resulting in prevention of FimY-mediated fim\textsubscript{Z} activation [16]. FimY is also a transcriptional activator for fim\textsubscript{A}, fim\textsubscript{W}, and itself [14,16]. However, the positive and negative feedback loops are not sufficient to explain the regulation of type 1 fimbrae synthesis, since type 1 fimbrae synthesis under inducing conditions is continuous or rheostat-like rather than an autocatalytic or switch-like response [16]. These observations suggest that expression of type 1 fimbrae in Salmonella cells is a collective and continuous event in response to environmental milieu. These phenomena could be well explained if we assume that Lrp can act as both positive and negative regulators for type 1 fimbrae production depending on intracellular levels of Lrp, which are closely related with the nutritional environment [34,43]. In addition, mannose-sensitive yeast agglutination correlated with nutrition-dependent cellular Lrp levels (Figs. 8A and B). Based on the Lrp titration results from the western blot analyses in Fig. 7B, we conclude that mannose-sensitive type 1 fimbraion in Salmonella is inhibited by Lrp at a concentration of 3,000 or more Lrp dimers per cell (Fig. 8B) under nutrient-poor conditions. However, mannose-sensitive yeast agglutination was observed in Salmonella producing about 2,400 or fewer Lrp dimers per cell in nutrient-rich environments (Fig. 8B). We estimated that the lrp\textsuperscript{78} mutant \chi\textsuperscript{944} produces at least 6,000 Lrp dimers per cell grown in LB medium. This cellular Lrp concentration is enough to inhibit type 1 fimbrae production, even when the Salmonella cells are grown in LB broth, a rich medium. Therefore, our results indicate that a narrow range of Lrp concentrations governs activation of fim\textsubscript{A} transcription and production of type 1 fimbrae. Too much or too little Lrp results in no type 1 fimbrae production, allowing the cell to tightly regulate production of these complex extracellular structures in response to the nutritional environment.

Based on these data, we propose here a revised model for the fim gene regulatory circuit in S. Typhimurium (Fig. 9A). In our model, Lrp modulates expression of fim\textsubscript{A} and fim\textsubscript{Z} either positively or negatively, depending on growth conditions and the amount of Lrp present. When Lrp is present in excess (more than 3,000 dimers per cell), as is the case in the lrp\textsuperscript{78} mutant or during growth in nutrition-poor media, no fim\textsubscript{Z} is expressed, and Lrp binds to all four motifs 1, 2, 3, and 4 in P\textsubscript{JunA} resulting in complete repression of fim\textsubscript{A} (Fig. 9B). We assume that the binding of Lrp to motifs 2 and 3 may allow binding of the silencing factor H-NS to the high affinity H-NS-fim\textsubscript{Z} binding motifs in the FimZ-binding region, and competitively exclude FimZ binding to the P\textsubscript{JunA}. This feature is similar to the collaborative competition mechanism in eukaryotic gene regulatory regions typically encompassing multiple DNA target sites for two or more regulatory proteins within a space of a few hundred base pairs or less [44]. At a lower range of Lrp concentrations (about 1,000~2,400 dimers per cell), the levels of FimZ are high, such as occurs when cells are grown in nutrient-rich conditions (e.g. LB broth). FimZ is better able to occupy its activation site in P\textsubscript{JunA} presumably due to the fact the affinity of Lrp to motifs 2, 3, and 4 is relatively weak (Fig. 3B) and there is an abundance of FimZ. Due to the requirement of motif 1 for Fim\textsubscript{A} and type 1 fimbrae synthesis (Fig. 4), we infer an interaction between Lrp and FimZ under these conditions that enhances FimZ-mediated activation of fim\textsubscript{A} expression by changing the regional DNA secondary structure. Finally, in the lrp\textsuperscript{78} strain, neither fim\textsubscript{Z} nor fim\textsubscript{A} are expressed. We conclude that Lrp is a key regulator to direct on-off switching of type 1 fimbrae production by the concentration-dependent dual regulation in S. Typhimurium in contrast to the recombination-mediated phase-variable type 1 fimbraion in E. coli.

**Materials and Methods**

**Bacterial strains, plasmids, culture conditions, and reagents**

Bacterial strains and plasmids used in this study are listed in Table 1. S. Typhimurium and E. coli strains were routinely grown in LB broth [45]. For analysis of type 1 fimbrae production, S. Typhimurium strains were grown statically in MOPS minimal broth [46] or LB broth at 37°C for 24 h or 3 days. Diaminopimelic acid (DAP, 50 \mu g/ml) was added to LB medium for growing \Delta\textsuperscript{aod} mutant strains. Antibiotics were used as needed at the following concentrations: ampicillin, 100 \mu g/ml; chloramphenicol, 20 \mu g/ml; gentamicin, 20 \mu g/ml; kanamycin, 50 \mu g/ml; and tetracycline, 10 \mu g/ml. All antibiotics and chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Inc (Pittsburgh, PA).

**DNA manipulations**

The primers used in this study are listed in Table 2. Plasmid DNA was isolated by using QiAprep Spin Miniprep Kit (QiAGEN, Valencia, CA). Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturers (Promega, Madison, WI or New England Biolabs, Ipswich, MA).

To produce a His-tagged FimZ protein, a DNA fragment containing the entire fim\textsubscript{Z} ORF was amplified from strain \chi\textsuperscript{944} by PCR using primers, RCB-52 and RCB-53 (Table 2). This PCR product was digested with NcoI/BamHI and ligated into expression vector pYA14124 digested with the same enzymes [34]. The recombinant fim\textsubscript{Z} gene was excised from the resulting plasmid using XbaI/BamHI and cloned into a low-copy number plasmid pWSK29 [47] using the same enzyme sites, to create pYA4065.

![PLoS ONE | www.plosone.org 10 October 2011 | Volume 6 | Issue 10 | e26896](Image 58x24 to 76x41)
Purification of FimZ

χ9448 (pYA4065) (Table 1) was used for synthesis of the His-tagged FimZ fusion protein. Cells were grown to an early stationary phase (optical density at 600 nm [OD600] of 1.2) in LB medium at 37°C and harvested by centrifugation at 3,300 × g for 15 min at 4°C. The His-tagged FimZ fusion protein was purified by using a nickel affinity gel system, Ni Sepharose 6 Fast Flow (Amersham Bioscience).

Construction of the PfimA413, PfimA529, and PfimA1225 mutations

The fimA promoter (PfimA) region was amplified from χ3761 by PCR using primers RCB-56 and RCB-57 (Table 2). The resulting PCR product was digested with ApaI/SacI and ligated with ApaI/SacI-digested pBluescript SK-. The resulting plasmid was used as a template DNA for site directed mutagenesis of the Lrp-binding motifs in P_fimA by inverse PCR using primers RCB-46 and RCB-47 for PfimA413, RCB-48 and RCB-49 for PfimA529 and RCB-50 and RCB-51 for PfimA1225 (Table 2). The inverse PCR products were digested with XhoI, BglII, or XbaI, and self-ligated, to create plasmids carrying PfimA413, PfimA529, and PfimA1225 mutations, respectively. The PfimA413, PfimA529, and PfimA1225 DNA fragments were excised using KpnI/SacI restriction enzymes, and cloned into the same restriction enzyme sites of suicide vector pRE112, to create pYA4758, pYA4759, and pYA4801, respectively. These suicide plasmids were introduced into serovar Typhimurium strains by conjugation to construct the PfimA mutants, PfimA413, PfimA529, and PfimA1225, by allelic exchange as previously described [48]. Each of the PfimA413 and PfimA529 mutations were added to the PfimA1225 mutant to generate double mutants PfimA1325 and PfimA2925, respectively. The PfimA529 mutation was added to the PfimA413 mutant to generate a double mutant PfimA1329.

Figure 9. Model for Lrp and FimZ-mediated regulation of type 1 fimbriae production in Salmonella. (A) Summary of the regulatory circuit for type 1 fimbriae production. Arrowed and blunted lines indicate activation and repression, respectively. (B) Molecular model for Lrp and FimZ-mediated regulation of type 1 fimbriae production. Dotted arrows, access to binding sites; ×, competitive exclusion of competitor binding; and arrowed flag indicates the transcription start site [15]. [High], at high concentration of Lrp or FimZ; and [Low], at low concentration of Lrp or FimZ. doi:10.1371/journal.pone.0026896.g009

Wild-type Salmonella in a nutrient-rich environment (10 - 100% LB)

Wild-type Salmonella under nutrient-poor condition s (minimal media) or the trp” mutant

In the trp-deletion mutant

Regulation of Type 1 Fimbriae Production by Lrp
Table 2. Primers were used in this study.

| Name      | Sequence (5’ to 3’)                  | Related product |
|-----------|--------------------------------------|-----------------|
| RCB-24    | GACCTCTACTATTTGGGAG                 | fimA            |
| RCB-25    | TCACACGCGGCTGCTTC                   | fimA            |
| RCB-28    | CGGCGCTTTGCCCGGCTGAGAATGTTC         | murA            |
| RCB-29    | CGCAAGCTTTTTGCTACGGGTCAATTTTC       | murA            |
| RCB-42    | ACTAAAGGGGAACAAAAAGC                |                 |
| RCB-43    | GTAACCGAGCGCCGATG                   |                 |
| RCB-44    | CTTGAGCCCTTTGCTGTAATAG              |                 |
| RCB-45    | TTAGATCCATTTGACTTTCCCTGAG           |                 |
| RCB-46    | CTATTCTGGGATTGGAATTGTTATTATAC       |                 |
| RCB-47    | TAACCTGGAATATCCGGAACACTTTTAATG      |                 |
| RCB-48    | TGCAGATCCTCATACAATGATAGAGACCCTTTC   |                 |
| RCB-49    | TATGGAGATGTCTTATCCGCCGCGATG         |                 |
| RCB-50    | GACCTCTAGCATTGAACAGCGGCGCTCCGTAC   |                 |
| RCB-51    | GACGGCTAGATGACTCCCTTCGTACG          |                 |
| RCB-52    | ATACCACTGGGCGAGAGCGATCAGCACTACATCA | fimZ (RT)       |
| RCB-53    | CGCGGACGGCTTTGCTGTTACATTAC          | fimZ (RT)       |
| RCB-54    | TGTTGGATCATGCCAAGGGAGGATGAC         |                 |
| RCB-55    | CCTGAGATCCAGGCGGAGG                 |                 |
| RCB-56    | ATCCGGCCGATGACCGTCAGAC             |                 |
| RCB-57    | TTTGAGCTGCGGCTTACAGGTTGAAGA        |                 |

PfimA1329 mutation was added to the PfimA2925 double mutant to generate a triple mutant PfimA395.

**Construction of lacZ fusions**

A 357-bp DNA fragment containing the fimA promoter region was amplified from χ3761 by PCR using primers RCB-44 and RCB-45 (Table 2). The PCR product was digested with ApaI and BamHI, and was cloned into the unique ApaI/BamHI sites of RCB-46 (Table 2). The PCR product was digested with ApaI and used as non-specific control DNA (MCS-pBS, 178 bp) was amplified by PCR using primers, RCB-54 and RCB-55 (Table 2). The multi-cloning site of pBluescript SK- (MCS-pBS, 178 bp) was amplified by PCR using primers RCB-24 and RCB-25 for fimZ (757 bp); RCB-28 and RCB-29 for murA (725 bp) [34] (Table 2). PCR products were separated in a 1.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

**Yeast agglutination assay**

Bacteria were grown statically in various media at 37°C for 24 h and/or 3 days. Bacterial cells were harvested by centrifugation at 5,000×g for 5 min at room temperature. The cell pellet was gently resuspended in phosphate-buffered saline (PBS) and serially diluted in the same buffer to adjust the optical density at 600 nm [OD600] to 4.0, 0.8, or 0.16. Yeast cells were washed twice with PBS and diluted with the same volume of yeast cells in PBS at room temperature for 10 min with gentle orbital shaking. Mannose sensitivity was demonstrated by the absence of agglutination when the assay was performed in the presence of 2% (wt/vol) mannose.

**β-Galactosidase assay**

Bacterial cells were statically grown at 37°C for 24 h. β-Galactosidase activity was measured as previously described [50]. Means ± standard errors were calculated from four independent assays.

**Reverse transcription (RT)-PCR analysis**

A 0.5 ml aliquot of bacterial culture was mixed with 1 ml of RNAProtect Bacteria Reagent (QIAGEN, Valencia, CA) and incubated for 5 min at room temperature. Total RNA from the cell pellet was isolated using RNAsnap Mini Kit (QIAGEN). The RNA samples were treated with extra DNase I to avoid any DNA contamination, and repurified using the column in the kit. The DNA-free RNA samples were confirmed by PCR. A 200 ng sample of total RNA was used for semi-quantitative RT-PCR with the OneStep RT-PCR kit (QIAGEN). RT was performed for 30 min at 50°C, followed by heat inactivation of the reverse transcriptase for 15 min at 95°C. PCR amplification was performed in the same tube with the following cycling conditions: 25 cycles with 30 s at 95°C for primer annealing, and 1 min at 72°C for primer extension. The primers for RT-PCR (expected sizes of PCR products) were as follows: RCB-24 and RCB-25 for fimZ (427 bp) [34]; RCB-52 and RCB-53 for fimZ (757 bp); RCB-28 and RCB-29 for murA (725 bp) [34] (Table 2). PCR products were separated in a 1.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

**Electrophoretic Mobility Shift Assay (EMSA)**

The wild-type PfimA, PfimA1313, PfimA2925, PfimA1225, PfimA1329, PfimA395, and PfimA395 DNA fragments were amplified from wild-type and mutant strains by PCR using primers RCB-54 and RCB-55 (Table 2). The multi-cloning site of pBluescript SK-(MCS-pBS, 178 bp) was amplified by PCR using primers, RCB-42 and RCB-43 (Table 2) and used as non-specific control DNA [34]. These DNA fragments were tested for interaction with Lrp protein by EMSA as previously described [34]. The reactions
subjected to electrophoretic separation in a 5% polyacrylamide gel in 1 × Tris-borate-EDTA (TBE) (100 V for 1 h) or 1 × Tris-taurine-EDTA buffer (TTE) (75 V for 1 h). The gel was stained with 1 × SYBR Gold (Invitrogen), and DNA bands were visualized on a UV transilluminator.

For the competitive binding assay, the P<sub>flmA</sub> DNA fragment was mixed with different molar ratios of Lrp and FimZ in the DNA binding buffer [15] with minor modification: 5 mM Tris-HCl pH 7.5, 25 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, BSA (5 µg/ml), and 20% glycerol. The reaction mixture was incubated at room temperature for 15 min, and was subjected to electrophoresis in 1 × TBE as described above.

**DNase I footprinting**

RCB-54 and RCB-55 primers were labeled with [γ-<sup>32</sup>P]ATP (PerkinElmer) using DNA 5′ end-labeling system (Promega) for non-coding and coding strands, respectively. The DNA probes were amplified by PCR using primer sets, [γ-<sup>32</sup>P]ATP-labeled RCB-54 and unlabeled RCB-55 primers or vice versa. One pmol of the labeled probe was used for DNase I footprinting analysis. Lrp-DNA probe binding reactions were identical to the conditions for EMSA excepting addition of poly(dI-dC) at 10 µg ml<sup>-1</sup> (Sigma) instead of MCS-pBS control DNA. One µl volume of DNase I (0.1 unit, Ambion) in 10 mM Tris-HCl pH 7.5, 25 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol. The reaction mixture was incubated at 37°C for 10 min. The [γ-<sup>32</sup>P]ATP-labeled RCB-54 and RCB-55 primers were also used for DNA sequencing using Sequi_Therm EXCEL II DNA sequencing Kit (EPICENTRE Biotechnologies). The products of the DNase I footprinting and the DNA sequencing reactions were resolved by electrophoresis through a denaturing 7% polyacrylamide-7M urea gel in 1 × Tris-taurine-EDTA buffer. The gel was subjected to autoradiography.

**Western blot analysis**

Protein bands from a 12% SDS polyacrylamide gel were transferred to a nitrocellulose membrane. Western blot analysis was performed as previously described [51]. Blots were probed with rabbit anti-FimA, anti-Lrp, or anti-His<sub>6</sub> monoclonal mouse immunoglobulin G (IgG, Invitrogen). Alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Sigma) was the secondary antibody, as appropriate.

**Estimation of the number of Lrp molecules per cell**

The number of Salmonella cells per ml was estimated by turbidity and CFU. The Lrp concentration in the crude bacterial cell extract was determined by western blotting next to dilutions of purified Lrp using an anti-Lrp mouse antiseraum [34]. We calculated the number of Lrp molecules per ml based on the western blots and then divided the estimated number of Lrp molecules per ml by the number of cells per ml to obtain the number of Lrp molecules per cell.

**Densitometry**

The relative band intensities were obtained by using a computational densitometry program Quantity One (Bio-Rad).

**Statistical Analysis**

Statistical analysis was performed using t test (GraphPad). Results are presented as the mean and SEM. A P value<0.03 was considered statistically significant.

**Acknowledgments**

We thank Dr. Praveen Alamuri for editorial suggestions for this article.

**Author Contributions**

Conceived and designed the experiments: C-HB. Performed the experiments: C-HB. Analyzed the data: C-HB KLR RC. Contributed reagents/materials/analysis tools: C-HB H-YK. Wrote the paper: C-HB KLR RC.

References

1. Kukkonen M, Raunio T, Virkola R, Lahteenmaki K, Makela PH, et al. (1993) Basement membrane carbohydrates as a target for bacterial adhesion: binding of type 1 fimbriae of Salmonella enterica and Escherichia coli to laminin. Mol Microbiol 7: 229–237.
2. Baumler AJ, Tsolis RM, Heffron F (1997) Fimbrial adhesins of Salmonella enterica serovar Typhimurium. J Bacteriol 169: 5831–5834.
3. Kukkonen M, Raunio T, Virkola R, Lahteenmaki K, Makela PH, et al. (1993) Basement membrane carbohydrates as a target for bacterial adhesion: binding of type 1 fimbriae of Salmonella enterica and Escherichia coli to laminin. Mol Microbiol 7: 229–237.
4. Baumler AJ, Tsolis RM, Heffron F (1997) Fimbrial adhesins of Salmonella enterica serovar Typhimurium. J Bacteriol 169: 5831–5834.
5. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
6. Klemm P (1984) The fimU gene affects expression of type 1 fimbriae and is related to the Escherichia coli fimA promoter region and may regulate its own expression with FimY. Microbiol Immunol 48: 1–10.
7. Klemm P (1984) The fimU gene affects expression of type 1 fimbriae and is related to the Escherichia coli fimA promoter region and may regulate its own expression with FimY. Microbiol Immunol 48: 1–10.
8. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
9. Klemm P (1984) The fimU gene affects expression of type 1 fimbriae and is related to the Escherichia coli fimA promoter region and may regulate its own expression with FimY. Microbiol Immunol 48: 1–10.
10. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
11. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
12. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
13. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
14. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
15. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
16. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
17. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
18. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
19. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
20. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
21. Tinker JK, Clegg S (2000) Characterization of FimY as a coactivator of type 1 fimbrial expression in Salmonella enterica serovar Typhimurium. Infect Immun 68: 3035–3033.
22. Blomfield IC, Calie TJ, Eberhardt KJ, McClain MS, Eisenstein BI (1995) Lrp stimulates phase variation of type 1 fimbriae in Escherichia coli K-12. J Bacteriol 175: 27–36.

23. Blomfield IC, Kalaskarca DH, Eisenstein BI (1997) Integration host factor stimulates both FimB and FimE-mediated site-specific DNA inversion that controls phase variation of type 1 fimbriae expression in Escherichia coli. Mol Microbiol 23: 705–717.

24. Dorman CJ, Higgins CF (1987) Fimbrial phase variation in Escherichia coli: dependence on integration host factor and homologues with other site-specific recombinases. J Bacteriol 169: 3840–3843.

25. Eisenstein BI, Sweet DS, Friedman DI (1987) Integration host factor is required for the DNA inversion that controls phase variation in Escherichia coli. Proc Natl Acad Sci USA 84: 6506–6510.

26. Gally DL, Rucker TJ, Blomfield IC (1994) The leucine-responsive regulatory protein binds to the fim switch to control phase variation of type 1 fimbrial expression in Escherichia coli K-12. J Bacteriol 176: 5665–5672.

27. Kawula TH, Orndorff PE (1991) Rapid site-specific DNA inversion in Escherichia coli mutants lacking the histonelike protein H-NS. J Bacteriol 173: 4116–4123.

28. McClain MS, Blomfield IC, Eberhardt KJ, Eisenstein BI (1993) Inversion-independent phase variation of type 1 fimbriae in Escherichia coli. J Bacteriol 175: 4335–4344.

29. Clegg S, Hancox LS, Yeh KS (1996) Salmonella typhimurium fimbrial phase variation and FimA expression. J Bacteriol 178: 342–345.

30. Gally DL, Leathart J, Blomfield IC (1996) Interaction of FimB and FimE with the fim switch that controls the phase variation of type 1 fimbriae in Escherichia coli K-12. Mol Microbiol 21: 725–738.

31. Klemm P (1986) Two regulatory fim genes, fimR and fimE, control the phase variation of type 1 fimbriae in Escherichia coli. EMBO J 5: 1389–1393.

32. Svensson DL, Clegg S (1994) Salmonella fimbriae. p. 105-113. In: Klemm P, ed. Fimbriae: adhesion, genes, biochemistry and vaccines. Boca Raton, Fla.: CRC Press.

33. McFarland KA, Lucchini S, Hinton JC, Dorman CJ (2008) The leucine-responsive regulatory protein, Lrp, activates transcription of the fim operon in Salmonella enterica serovar Typhimurium via the fimR regulatory gene. J Bacteriol 190: 602–612.

34. Back CH, Wang S, Roland KL, Curtiss III R (2009) Leucine-responsive regulatory protein (Lrp) acts as a virulence repressor in Salmonella enterica serovar Typhimurium. J Bacteriol 191: 1275–1282.

35. Nou X, Braaten E, Kaltenbach L, Low DA (1995) Differential binding of Lrp to two sets of pap DNA binding sites mediated by Pap I regulates Pap phase variation in Escherichia coli. EMBO J 14: 5785–5797.

36. Williams DA, Ryan CW, Plauto JY, Calvo JM (1991) Characterization of Lrp, and Escherichia coli regulatory protein that mediates a global response to leucine. J Biol Chem 266: 10768–10774.

37. Beloin C, Jeuset J, Revert B, Mirambeau G, Le Hegarat F, et al. (2003) Contribution of DNA conformation and topology in right-handed DNA wrapping by the Bacillus subtilis LrpC protein. J Biol Chem 278: 5333–5342.

38. Chen S, Iannolo M, Calvo JM (2005) Cooperative binding of the leucine-responsive regulatory protein (Lrp) to DNA. J Mol Biol 345: 251–264.

39. Pal U, Warum R, Lux B, Meltzer M, Menzel A, et al. (2005) LRP and H-NS cooperative partners for transcription regulation at Escherichia coli rRNA promoters. Mol Microbiol 50: 864–876.

40. van der Woude MW, Kaltenbach LS, Low DA (1995) Leucine-responsive regulatory protein plays dual roles as both an activator and a repressor of the Escherichia coli pap fimbrial operon. Mol Microbiol 17: 303–312.

41. Beaufort E, Baille M, Badault C, Travers A, Rumsy S (2007) H-NS cooperative binding to high-affinity sites in a regulatory element results in transcriptional silencing. Nat Struct Mol Biol 14: 441–448.

42. Zhi J, Mathew E, Freundlich M (1999) Lrp binds to two regions in the dusLY promoter region of Escherichia coli to repress and activate transcription directly. Mol Microbiol 32: 29–40.

43. Landgraf JR, Wu J, Calvo JM (1996) Effects of nutrition and growth rate on Lrp levels in Escherichia coli. J Bacteriol 178: 6930–6936.

44. Miller JA, Widom J (2005) Collaborative competition mechanism for gene activation in vivo. Mol Cell Biol 23: 1623–1632.

45. Berti G (1951) Studies on lyogensis. I. The mode of phase liberation by lyogenic Escherichia coli. J Bacteriol 62: 293–300.

46. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. J Bacteriol 119: 736–747.

47. Wang RF, Kushner SR (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. Gene 100: 189–199.

48. Kaniga K, Delor I, Cornélis GR (1991) A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blad gene of Yersinia enterocolitica. Gene 109: 137–141.

49. Back CH, Kim KS (2005) lcrZ and aph-based reporter vectors for in vivo expression technology. J Microbiol Biotechnol 15: 872–880.

50. Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

51. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

52. Curtiss III R, S. B. Porter, M. Munson, S. A. Tinage, J. O. Hassan, C. Gentry-Weeks, M. Kelly S (1991) Nonrecombinant and recombinant avirulent Salmonella enterica serovar Typhimurium SL1344 and UK-1: O3p and A3I deletion mutants. Infect Immun 63: 3531–3537.

53. Zhang X, Kelly SM, Bollen WS, Curtiss III R (1997) Characterization and immunogenicity of Salmonella typhimurium SL1344 and UK-1: O3p and A3p deletion mutants. Infect Immun 65: 5301–5307.

54. Roland K, Curtiss III R, Sizemore D (1999) Construction and evaluation of a delta cya delta cya Salmonella typhimurium strain expressing avian pathogenic Escherichia coli O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis 43: 429–441.

55. Edwards RA, Keller LH, Schifferle DM (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 207: 149–157.