Leucine-Responsive Regulatory Protein Lrp and PapI Homologues Influence Phase Variation of CS31A Fimbriae

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CS31A, a K88-related surface antigen specified by the *clp* operon, is a member of the type P family of adhesive factors and plays a key role in the establishment of disease caused by septicemic and enterotoxigenic *Escherichia coli* strains. Its expression is under the control of methylation-dependent transcriptional regulation, for which the leucine-responsive regulatory protein (Lrp) is essential. CS31A is preferentially in the OFF state and exhibits distinct regulatory features compared to the regulation of other P family members. In the present study, surface plasmon resonance and DNase I protection assays showed that Lrp binds to the distal moiety of the *clp* regulatory region with low micromolar affinity compared to its binding to the proximal moiety, which exhibits stronger, nanomolar affinity. The complex formation was also influenced by the addition of PapI or FooI, which increased the affinity of Lrp for the *clp* distal and proximal regions and was required to induce phase variation. The influence of PapI or FooI, however, was predominantly associated with a more complete shutdown of *clp* expression, in contrast to what has previously been observed with AfaF (a PapI ortholog). Taken together, these results suggest that the preferential OFF state observed in CS31A cells is mainly due to the weak interaction of the leucine-responsive regulatory protein with the *clp* distal region and that the PapI homolog favors the OFF phase. Within the large repertoire of fimbrial variants in the P family, our study illustrates that having a fimbrial operon that lacks its own PapI ortholog allows it to be more flexibly regulated by other orthologs in the cell.

Bacterial infection includes a dynamic balance between the requirement of virulence factors that are produced by the microorganism and the production of an antigen recognized by the host immune system (1, 2). Phase variation is an example of the complex regulatory systems that pathogenic strains have developed to change their phenotype in response to environmental signals. This stochastic and reversible switch between an all or none (ON/OFF) phenotype is a key mechanism to generate phenotypic diversity in genetically identical cells, especially to survive in a hostile environment (3, 4). After division, most daughter cells retain the expression phase of the parent, whereas a minority of cells switch to the other phenotype. Despite its energetic cost and the risk of being deleterious to individual cells, phase variation confers a net advantage to the entire population by allowing it to anticipate host environmental changes without having to sense them. Thus, many virulence factors, such as fimbrial adhesins, are subject to phase variation, and pathogenic bacteria coordinately express more than one adhesin by phase variation (5, 6).

The *clp*-encoded CS31A surface antigen, found in septicemic and enterotoxigenic *Escherichia coli* strains of bovine and human origins, is required for full bacterial virulence (7, 8). It was first described in *E. coli* strains recovered from calves with septicemia or diarrhea. It is a capsule-like surface protein, immunologically related to F4 (K88) and F41 fimbriae (8). Experimental infections (9) and epidemiological data (10) suggest a close association of CS31A-producing strains with cases of septicemia (11). Pap-related fimbriae are the most frequent *E. coli* adhesins associated with human urinary tract infection, but they are also found in strains producing CS31A (12). There is a worldwide distribution of CS31A antigen in bovine *E. coli* strains (11, 13). CS31A is required for full bacterial virulence in the rat model (J.-P. Girardeau, personal communication), mediates adhesion to Caco-2 and Int-

407 cells (14), and protects bacteria from phagocytosis by bovine polymorphonuclear neutrophils (PMNs) (15).

While CS31A is genetically and immunologically related to K88, its expression is controlled by phase variation, like the well-characterized Pap-regulatory family of adhesive factors. It has also been proposed that CS31A depends on the expression of an additional fimbrial gene cluster that is also controlled by phase variation (15–17).

For Pap, expression is inherited in an epigenetic manner (6) because of methylation-controlled modifications within the regulatory region of the *pap* operon and the presence of the leucine-responsive regulatory protein (Lrp) (6). Lrp controls a large number of operons in *E. coli*, including those involved in amino acid biosynthesis, amino acid degradation, metabolite transport, and carbon metabolism (18–20). The regulatory region of *pap* consists of a DNA sequence of approximately 400 bp that is flanked by two divergently transcribed genes, *papI* and *papB* (Fig. 1). The intergenic region also includes 6 Lrp binding sites [all containing the consensus sequence GN(2/3)TTT, where N is any nucleotide], as well as 2 GATC sites spaced 102 bp apart (the proximal [GATC^{prox}] and distal [GATC^{dist}] GATC sites). Differences in the methylation status of these sequences determine the binding of Lrp (21, 22). Thus,

A

clp operon

B

FIG 1 Overview of the *clp* operon. (A) Schematic representation of the *clp* operon. The two GATC sites subject to methylation by Dam are indicated by GATC^{dist} and GATC^{prox}. Note the absence of a *papI* homologue in the *clp* operon. (B) Comparison of the nucleotide sequences of the regulatory regions of the *clp* and *pap* operons. Identical nucleotides in *clp* and *pap* are shown as boxed regions. Lrp-binding site positions 1 to 6 in the *pap* sequence are indicated with underlines, and letters a to g with overlines indicate the positions of Lrp-binding sites in the *clp* sequence, as identified by DNase I footprint. The regions corresponding to the PapB binding sites and the CAP binding site are represented with dashed underlines, and the putative promoter regions (-10 and -35 sites) with underlines. Red letters indicate GATC sites. Blue and red arrows represent the boundaries of the distal and proximal fragments, respectively, used in Biacore experiments.

switching from one phenotype to the other is the result of competition between the binding of Lrp at sites 1 to 3 or sites 4 to 6 and methylation by Dam methyltransferase at the opposite GATC site, which prevents the binding of Lrp (Fig. 1) (for reviews, see references 23, 24, and 25). When GATC^{dist} is fully methylated, Lrp cooperatively binds to sites 1 to 3, maintaining the cells in the OFF state, and when GATC^{prox} is fully methylated, Lrp binds to sites 4 to 6, maintaining the ON state (Fig. 1). Control of *pap* phase variation also requires the action of PapI, a positive regulator that increases the affinity of Lrp for sites 4 to 6 *in vivo* (25, 26), as well as PapB, a second specific regulator of the *pap* operon that coordinates the expression of the *pBA* and *pI* promoters (25). Taken together, the combined actions of Lrp, Dam methyltransferase, PapI, and PapB result in a finite probability that each cell will express Pap fimbriae (ON) or not (OFF) just after DNA replication.

Distinct regulatory features can be observed between members of the P-regulatory family. In particular, Lrp activates the expression of some operons, including *pap* and *foo*, and represses the expression of others, including *clp* (15, 16). In addition, for

CS31A, striking features include the absence of a PapI homologue encoded by the *clp* operon and a moderate level of *clp* transcription. However, the addition of the PapI homologue AfaF in *trans* promotes *clp* phase variation (15, 17), confirming that it is required during *clp* phase variation. Similar to the results for Pap, we have previously shown that CS31A phase variation in the presence of *afaF* is mediated by the global regulator Lrp and methylation protection of the two *clp* GATC sites (15, 17). However, CS31A is characterized by a higher level of OFF cells than Pap in the presence of *afaF* (15).

To date, direct evidence for the interplay between PapI homologues and phase variation has only been described with AfaF, the *papI* homologue found in *afa-3* (15, 17). This mechanism might not mimic what is found in natural isolates, since strains may possess adhesin gene clusters other than *afa-3*. For instance, Bertin et al. showed that the reference strain 31A encodes not only CS31A but also P fimbriae (12). Whether PapI homologues encoded by other *pap*-related operons in CS31A strains cross-regulate *clp* transcription remains to be investigated.

To provide direct evidence as to why *clp* expression (CS31A)

gives rise to a high level of OFF cells compared to *pap* expression (Pap) and to establish whether PapI homologues influence CS31A phase variation in an identical manner, we analyzed the interactions between Lrp, PapI, and/or FooI and the intergenic regions of the *clp* operon in our present work; notably, FooI is a PapI homologue whose sequence differs from that of PapI by only a single amino acid modification (D17N).

By combining *in vitro* binding experiments with genetic phase variation studies, we show that the preferential OFF state observed in CS31A-positive cells is mainly due to a weak interaction of Lrp with the distal region of *clp*. Moreover, the addition of PapI or FooI (a PapI homolog encoded by the *foo* operon) promoted CS31A phase variation to similar extents and, due to increased Lrp affinity for both GATC^{dist} and GATC^{prox}, the OFF state surpassed the levels observed previously in the presence of *afaF*. Furthermore, the overall affinity of the ternary complex (DNA together with Lrp and PapI or FooI) for the *clp* proximal region is so strong that only a small fraction of cells can switch to the ON state.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* strain MC4100.λ6 contains a single chromosomal copy of a *clp-lacZYA* fusion. ptrf5 and ptrf6, multicopy recombinant plasmids derived from ptrc99A (Pharmacia Biotech) express *fooI* and *papI*, respectively, under the control of the *trc* promoter. Luria-Bertani (LB) broth and M9 minimal broth were prepared as described previously (27, 28). When necessary, these media were supplemented with 100 μg · ml^{-1} ampicillin and 40 μg · ml^{-1} kanamycin (unless otherwise noted), 1mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg · ml^{-1} 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal). Cultures of strains harboring ptrf5 or ptrf6 were grown in the absence of IPTG to avoid too-high expression of *fooI* or *papI*.

Plasmid construction. For overexpression and purification of the FooI protein, the pET32a-fooI plasmid was constructed by first amplifying the *fooI* sequence from the chromosome of strain 4787 using the NcoI-fooIrg1F and XhoI-fooIrg1R primers, containing the NcoI and XhoI restrictions sites, respectively. After enzymatic digestion, the fragment was ligated into the NcoI/XhoI restriction sites of the expression plasmid pET32a. The inserts were sequenced for verification after being transformed into *E. coli* BL21(DE3)pLysS by electroporation. Plasmid pET32a-papI was constructed using the QuikChange site-directed mutagenesis system (Stratagene). Briefly, the expression plasmid pET32a-fooI was extracted from strain *E. coli* BL21(DE3)pLysS, purified, and used as the template for amplification using the fooIN17D-F and fooIN17D-R primers. PCR-amplified plasmids were purified and digested with the restriction enzyme DpnI to remove any trace of the pET32a-fooI template. The PCR-amplified plasmids were dialyzed and transformed into strain *E. coli* BL21(DE3)pLysS by electroporation.

Purification of recombinant proteins. *E. coli* strain CV1494 was used to purify His$_6$-tagged Lrp as previously described (29). A 1-liter culture of CV1494 was grown in LB broth at 37°C with shaking to an optical density at 600 nm (OD$_{600}$) of 0.7 to 0.8. IPTG was added to the culture at a final concentration of 0.5 mM for 6 h. Bacteria were harvested, resuspended in 30 ml of Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 0.5 M NaCl), and lysed with a French press and an ultrasonic processor. The soluble fraction was clarified by centrifugation at 15,000 × *g* for 20 min at 4°C. Proteins were purified using an ÄKTA purifier system with a 1-ml HisTrap HP column (Amersham Biosciences) according to the instructions of the manufacturer. Purified proteins were subsequently dialyzed against TG$_{50}$ED buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.1 mM dithiothreitol [DTT], 0.1 M NaCl), and the purity of the proteins was confirmed by SDS-PAGE and staining with Coomassie blue.

For purification of FooI and PapI, a single *E. coli* BL21(DE3)pLysS colony carrying plasmid pET32a-fooI or pET32a-PapI was incubated in LB broth with shaking at 37°C to an OD$_{600}$ of 0.4 to 0.6. The culture was

incubated for 3 h more in the presence of IPTG at a final concentration of 1 mM. Bacteria were harvested, resuspended in 30 ml of Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 0.5 M NaCl), and lysed with a French press and an ultrasonic processor. The soluble fraction was clarified by centrifugation at 15,000 × *g* for 20 min at 4°C. The His$_6$-Trx-FooI and His$_6$-Trx-PapI fusion proteins were purified using an ÄKTA purifier system using the same procedure as for His$_6$-tagged Lrp. FooI and PapI proteins were removed from their His$_6$-Trx tag after enzymatic digestion using enterokinase (New England BioLabs) in digestion buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 2 mM CaCl$_2$). Proteins were dialyzed against 20 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6.0, and purified using an ÄKTA purifier system with a mono S 5/50 GL column (Amersham Biosciences) according to the instructions of the manufacturer. The purity of FooI and PapI proteins was confirmed by SDS-PAGE and staining with Coomassie blue.

Surface plasmon resonance (SPR). The interactions between 5′ biotinylated, double-stranded oligonucleotides (*clp* distal region, 120 kDa; *clp* proximal region, 77 kDa; and *clp* distal and proximal region, 185 kDa) and three DNA-binding proteins (Lrp, 20 kDa monomer; FooI, 9 kDa; and PapI, 9 kDa) were examined using a Biacore 3000 SPR spectrometer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Experiments were performed on research-grade streptavidin (SA)-coated sensor chips (XanTec Bioanalytics GmbH, Muenster, Germany) at 25°C using filtered (0.2 μm) and degassed CALVO-P running buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.4 mM EDTA, 1 mM MgCl$_2$, 0.1 mM DTT, 0.005% [vol/vol] Tween 20). As recommended by the manufacturer, SA-coated sensor chips were preconditioned with three 1-min pulses of 1 M NaCl in 50 mM NaOH. The protein-grade detergents (Tween 20 and Empigen) were from Anatrace (Maumee, OH, USA), and fatty acid-free bovine serum albumin (BSA) was from Millipore (Billerica, MA, USA); all other chemicals were reagent-grade quality. Purified protein concentrations were determined using the bicinchoninic acid (BCA) assay, and protein purity (>95%) was assessed by SDS-PAGE (12.5% polyacrylamide) under reducing (5% [vol/vol] 2-mercaptoethanol) and nonreducing conditions (data not shown).

To examine binding with Lrp, the appropriate oligonucleotides were annealed and then captured (20 μl/min with 10 nM DNA oligonucleotides in running buffer containing 0.5 M NaCl) to generate low-density surfaces (<100 response units [RU] immobilized). Lrp (0 to 100 nM) or BSA (negative control) was injected over reference (i.e., SA only) and active (i.e., *clp* DNA fragments) surfaces in tandem using the Kinject mode (25 μl/min for 10 min association and 10 min dissociation). Between sample injections, surfaces were regenerated at 50 μl/min using two 30-s pulses of solution I (1 M NaCl, 0.02% [vol/vol] Empigen in CALVO-P), followed by Extraclean and Rinse procedures.

To examine binding with FooI and PapI, medium-density oligonucleotide surfaces (300 to 400 RU immobilized) were prepared as noted above. FooI or PapI (0 to 15 μM) was injected over reference and active surfaces in tandem using the Kinject mode (5 μl/min × 5 min association plus 10 min dissociation). Between sample injections, surfaces were regenerated as noted above. In additional experiments, Lrp (50 nM) was also injected in the presence of FooI and PapI (0.0 to 5.6 and 0.0 to 8.1 μM, respectively) using the Kinject mode (10 μl/min × 5 min association plus 10 min dissociation).

The mass transport-independent data were doubled referenced (30) and are representative of duplicate injections acquired from three independent trials. For each replicate series, a buffer blank was injected first, the highest titrant concentration second, and serial dilutions thereafter (from the lowest to the highest concentration); comparing responses between the two highest titrant injections verified consistent DNA surface activity throughout each assay. To estimate apparent equilibrium dissociation constants (K_D), steady-state binding responses (Req; average RU near the end of the association phase) were plotted as a function of protein concentration (C) and then subjected to nonlinear regression (1-site-specific binding with Hill slope or steady-state affinity model using Prism5

version 5.0c for Mac OS X; GraphPad Software, San Diego, CA, USA). To estimate individual dissociation rate constants (K_d), sensorgram profiles were analyzed using the Fit Kinetics Separate ka/kd tool (BIAevaluation version 4.1). Theoretical binding maxima were predicted using the equation $R_{max} = (MWA/MWL) \times RL \times n$, where R_{max} is the maximal binding response (RU) at saturating protein concentration, MWA is the molecular weight (kDa) of the protein injected in solution, MWL is the molecular weight (kDa) of the DNA fragment immobilized, RL is the amount (RU) of DNA immobilized, and n is the predicted binding stoichiometry (e.g., 1:1).

Footprint analysis. DNase I footprinting of free DNA and DNA-protein complexes was performed as described previously (31). The DNA fragment corresponding to the *clp* regulatory region (317 bp) was amplified using primers clp-F (5′-GCGCTACCGTTTTTTGACTCTCCC-3′) and clp-R (5′-GCAGCGAAGATTATCACGATGTTTTATAGCG-3′). A *clp* DNA fragment end labeled with ³²P (125,000 cpm, 0.3 nM) was subsequently incubated in a total volume of 20 μl with 2 μg of salmon sperm DNA and 2 μg of acetylated bovine serum albumin (New England Biolabs) in binding buffer (60 mM Tris-HCl [pH 7.5], 40 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT). After incubation for 10 min at room temperature, 2 μl of DNase I (200 U · ml⁻¹; New England BioLabs) containing 22 mM CaCl₂ and 22 mM MgCl₂ was added for 4 min (top fragment) and 6 min (bottom fragment). The reaction was stopped by the addition of 100 μl of stop buffer (10% glycerol, 2.5 mM EDTA, 0.6 M ammonium acetate, 2 mg yeast tRNA per ml) to each sample. DNA fragments were precipitated in ethanol, and amounts with equivalent cpm (5×10^4) from each reaction were loaded onto 6% polyacrylamide–7 M urea gels. Maxam-Gilbert A+G reactions (32) were carried out on the appropriate ³²P-labeled DNA fragments, and the products loaded alongside the DNase I footprinting reaction mixtures. The gels were dried and analyzed by autoradiography.

Phase variation and measurement of switching frequencies. The switch frequency was calculated as described previously (15, 33). Strains were inoculated onto LB-kanamycin agar. Colonies were excised from the agar and resuspended in M9 salts containing no carbon source. Appropriate dilutions were subsequently spread onto M9 X-Gal agar containing 0.2% glycerol and, after 36 h of growth at 37°C, plates were photographed using a digital camera. Three colonies showing a uniform Lac⁺ or Lac⁻ phenotype were excised from the agar, resuspended in M9 salts, and spread onto M9 X-Gal agar as described above. The passage from M9 X-Gal agar to fresh M9 X-Gal agar containing the same source of carbon was repeated twice more to follow the switch frequency through three generations. At least 2,000 colonies were scored for a Lac phenotype as described previously (33), and the switch frequencies were calculated according to the formula (M/N)/g, where M is the number of cells that underwent phase variation, N is the total number of cells evaluated, and g is the total number of generations (estimated to be 20 generations for all the strains tested in our experiments).

β-Galactosidase activity assay. A single bacterial colony was used to inoculate an overnight culture in M9 glycerol medium. This culture was diluted 50-fold and grown to an OD_{600} of 0.5 to 0.8 in M9 glycerol medium. The culture was then assayed for β-galactosidase activity as described previously (27). For strains showing phase variation, a blue or white colony was picked from M9 X-Gal glycerol agar plates as starting material for assays of the β-galactosidase activities of ON and OFF cultures, respectively. Each experiment was performed in duplicate at least three times.

RESULTS

Differences in complex formation between Lrp and *clp* proximal or distal region. Similar to the intensively studied *pap* operon, *clp* phase variation requires Lrp. The Lrp target regulatory region contains two GATC sites separated by 102 or 103 bp, and protection of these sites from methylation is Lrp dependent (15, 17). We hypothesized that the lower affinity of Lrp for the *clp* distal
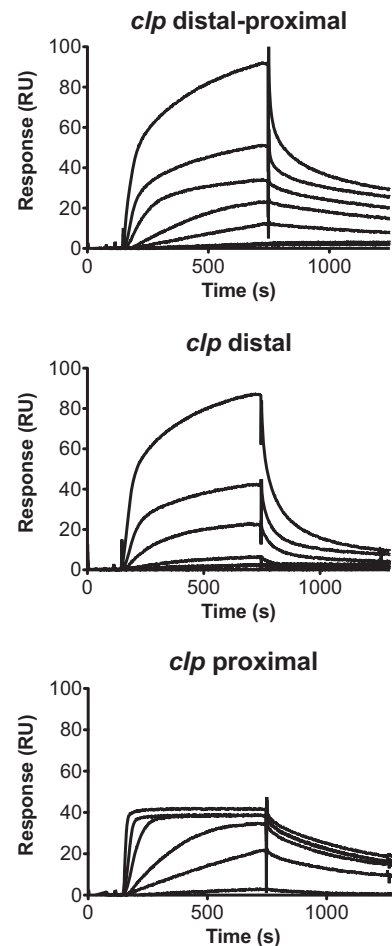
FIG 2 Association and dissociation kinetics of His₆-Lrp binding to *clp* regulatory regions. Representative SPR analyses of Lrp (0 to 100 nM; 2-fold dilution series) injected over *clp* distal, proximal, and distal-proximal fragments (95, 40, and 100 RU, respectively) at 25 μl/min (10 min association plus 10 min dissociation).

region than for the *clp* proximal region is responsible for the CS31A bias toward the OFF phase. To test for these interactions, label-free surface plasmon resonance (SPR) was used to monitor real-time binding of Lrp to unmethylated DNA fragments corresponding to the *clp* regulatory regions. Biotinylated oligonucleotide duplexes containing the *clp* distal, *clp* proximal, or *clp* distal-proximal region were immobilized on streptavidin-coated sensor chips, and His₆-Lrp was injected over the reference and DNA surfaces simultaneously. As shown by the results in Fig. 2, dose-dependent and/or saturable binding of Lrp to the *clp* regions was detected. In contrast to what has already been described for *pap* (22, 25), Lrp binding to the *clp* distal region was noticeably weaker (i.e., slow association and fast dissociation kinetics) than its binding to the *clp* proximal region (i.e., fast association and slow dissociation kinetics). A hybrid of the distal and proximal kinetics is reflected in the binding of Lrp (i.e., biphasic association and dissociation phases) to the full-length *clp* fragment.

Analysis of the data sets was performed according to previous reports in the literature indicating that Lrp binding is cooperative (i.e., sites 1 to 3 and 4 to 6) (34). Similar to SPR analyses of other cooperative protein-DNA kinetics (35), the *clp* proximal region

TABLE 1 Apparent equilibrium dissociation rate constants for Lrp, FooI, and PapI binding to *clp* regulatory regions[a]

clp fragment	Avg K_D ± SEM		
	Lrp (nM)	PapI (μM)	FooI (μM)
Distal	11,000 ± 1000	13 ± 1.0	14 ± 1.4
Proximal	10 ± 1	17 ± 1.1	14 ± 0.7
Distal-proximal	180 ± 15	ND	ND

[a] SPR binding responses (RU, average 710 to 730 s) were plotted as a function of concentration using Prism5 version 5.0c for Mac OS X (GraphPad Software, San Diego, CA, USA). Lrp constants were determined by fitting the experimental data to a 1-site-specific binding with Hill slope model. PapI and FooI constants were determined by fitting the experimental data to a steady-state affinity model. Each titration series was analyzed globally using the specified model, and estimates represent the average results of three independent trials. *clp* distal-proximal refers to the DNA fragment encompassing the *clp* distal and the *clp* proximal regions. ND, not done.

titration series fit well to a 1-site model with Hill slope, which indicated positive cooperativity (i.e., Hill coefficients of >1) (see Fig. S1 in the supplemental material). While Lrp binding to the proximal region exhibited low nanomolar affinity (K_D, ~10 nM) (Table 1), binding to the distal region exhibited significantly different micromolar affinity (K_D, ~11 μM). Analogous to other biomolecules where variable binding sites are critical for differential function (36, 37), we propose the following physiological duality with *clp*: the preferential OFF state in CS31A-positive cells is mainly due to Lrp interacting with the weak-affinity GATC[dist] site; in the presence of PapI or FooI, however, the increased Lrp affinity involves both the low-affinity GATC[dist] and high-affinity GATC[prox] sites. Notably, ternary complex formation between the DNA, Lrp, and the PapI homologue is so strong for the *clp* proximal region that only a small fraction of cells can switch to the ON state. Since Lrp can exhibit higher affinity for the *clp* proximal region than for the distal region, this differential property could block the entry of the RNA polymerase at the *clpBA* promoter, thereby yielding a predominant OFF phase.

Given that SPR showed that Lrp has a higher affinity for the *clp* proximal region than for the distal region, we hypothesized that such a difference could be due to differences in the proximal and distal Lrp-binding sites and/or to divergent nucleotide sequences surrounding the GATC sites. In contrast to *pap*, for which six Lrp-binding sites have been identified, the *clp* intergenic region contains more GN(2/3)TTT motifs of Lrp-binding sites (Fig. 1). Thus, we used DNase I footprint analysis to identify the Lrp-binding sites within the full-length *clp* regulatory region. The results in Fig. 3 show that the addition of His$_6$-Lrp was associated with the presence of multiple gel-protected regions, with four corresponding to the proximal region and three to the distal region. GATC[prox] and GATC[dist] sites were included within Lrp-binding sites of *clp*. In addition, DNase protection was observed at a lower concentration of His$_6$-Lrp for the *clp* proximal region than for the distal region, in agreement with the higher affinity of Lrp for the *clp* proximal region observed by SPR. In contrast, the *pap* proximal region was protected at an Lrp concentration 10 times lower than that at which the *clp* proximal region was protected (38). Thus, this unique pattern of Lrp-binding sites may result in a weak affinity for the *clp* distal region. In addition, DNase I footprinting shows periodic regions of DNase I protection and hypersensitivity, characteristic of bent DNA (39). This was also previously observed in *pap* and *foo* (38).

PapI and FooI direct CS31A phase variation toward the OFF state. In *E. coli* K-12, phase variation of the *clp* operon requires the presence in *trans* of a PapI homologue (15–17). So far, this has only been demonstrated with AfaF, a PapI homologue that controls the genetically distant operon *afa-3* and which is absent in the
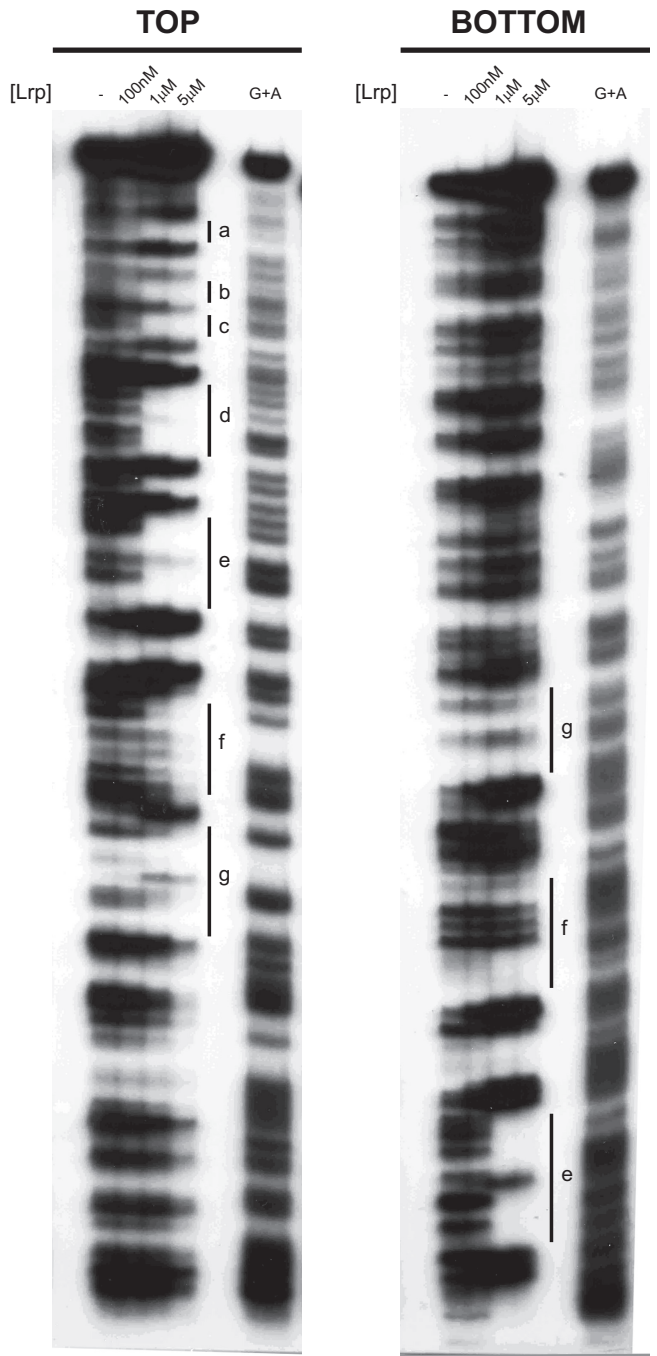

FIG 3 Lrp localization on the *clp* regulatory region. DNase I footprinting assays were performed with increasing amounts of Lrp. First to 4th lanes, DNase I footprinting assays with increasing amounts of Lrp on the *clp* regulatory region (317 bp) end labeled with ^{32}P on the top (left) and bottom (right) strands (1st lane, DNA alone; 2nd lane, Lrp at 100 nM; 3rd lane, Lrp at 1 μM; 4th lane, Lrp at 5 μM); 5th lane, Maxam-Gilbert A+G reactions. Letters and bars indicate the positions of Lrp-binding sites in the *clp* sequence.
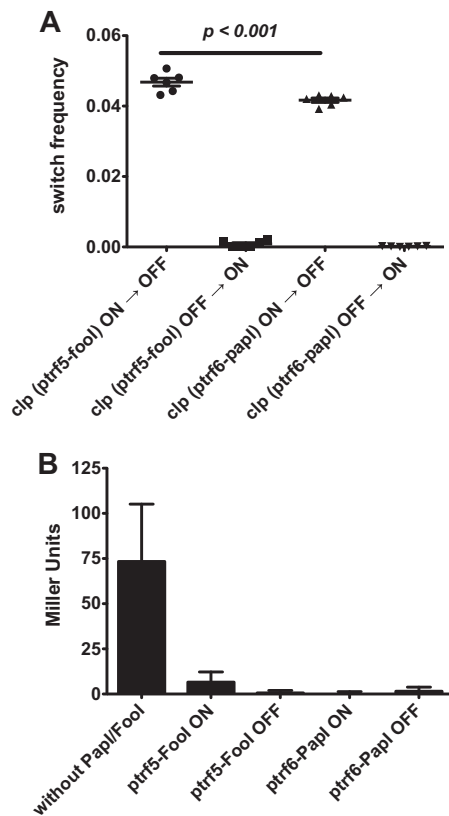
FIG 4 Effects of PapI homologues on *clp* basal expression. Lac$^+$ and Lac$^-$ phenotypes of *clp* strains expressing *papI* or *fooI*. (A) Switch frequencies were calculated using the formula $(M/N)/g$, where M is the number of cells that underwent phase transition, N the total number of cells evaluated, and g the number of generations, estimated to be 20 generations, that gave rise to the colony. Cells were grown on M9 X-Gal agar containing 0.2% glycerol for 36 h at 37°C. Horizontal bars represent the mean results. (B) Influence of PapI or FooI on the transcription levels of the *clp* operon as measured by the *lacZYA* reporter system. Bacteria were grown to an OD$_{600}$ of 0.5 to 0.8 in M9 glycerol medium. Cultures were then assayed for β-galactosidase activity as described previously (27). For strains showing phase variation, a blue or white colony was picked from M9 X-Gal glycerol agar plates as starting material for assays of β-galactosidase activities of ON and OFF cultures, respectively. β-Galactosidase activities are indicated in Miller units. Bars represent the mean results of at least three independent experiments. Error bars show standard deviations.

pathogenic reference strain *E. coli* 31A (17). However, strain 31A carries a *pap* fimbrial operon in its genome, so *clp* phase variation could result from the cross-regulation between *clp* and *pap* fimbriae through PapI supplied in *trans*. To assess whether PapI homologues can influence *clp* phase variation (as previously demonstrated with AfaF [17]), *clp* phase variation was measured *in vivo* by providing FooI or PapI in *trans*. Strain MC4100.λ6, carrying a chromosomal fusion between the *clp* regulatory region and the *lacZYA* reporter system, was transformed with plasmid ptrf5 or ptrf6 (expressing *fooI* and *papI*, respectively). The two transformed strains were then plated onto X-Gal M9 agar without IPTG (to avoid a too-high level of PapI or FooI protein in the cell), and the [Lac] phenotype was followed for up to two platings. This medium uses glycerol as the carbon source to avoid catabolite repression, which affects phase variation (28), and has no amino acids (40). While strain MC4100.λ6 led to a uniform phenotype characterized by light-blue colonies (data not shown), transformation with either ptrf5 or ptrf6 led to a majority of white colonies with few dark blue colonies (Fig. 4A and Table 2). Thus, PapI or FooI produced in *trans* induced phase variation of the *clp* operon. However, phase variation of *clp* was biased toward the OFF state by more than an order of magnitude, even when the starting phenotype was the ON state (Table 2). This was also observed when bacteria were grown in M9 glycerol liquid medium, with the β-galactosidase activity being lower in the presence than in the absence of any PapI homologue tested, regardless of the starting phenotype (Fig. 4B). This result is in agreement with the observation that Lrp shows a higher affinity for the *clp* proximal region than for the *clp* distal region whether a PapI homologue is present or absent (15, 17). As a result, the low affinity of Lrp for the *clp* distal region contributes greatly to the low level of ON cells. Interestingly, differences in the ability of PapI and FooI to promote switching toward the ON state were observed. Notably, the rate of switching to ON cells was higher in the presence of FooI (average of 0.8×10^{-3}) than in the presence of PapI (average of 0.1×10^{-3}) (Table 2). In contrast, no significant difference was seen in ON-to-OFF switching between these two orthologs. Thus, it seems that even if PapI homologues are all able to promote phase variation, they can still differentially influence the level of the ON population.

PapI and FooI preferentially increase Lrp association with and stability of its binding to the *clp* proximal region. To understand why FooI and PapI had such strong influences on the ON-

TABLE 2 Effects of PapI homologues on phase variation of *E. coli clp-lacZYA* fusion lysogen

Strain and phenotype	Colony	Total no. of colonies counted	No. of [Lac$^+$] colonies/no. of [Lac$^-$] colonies	% of [Lac$^+$] colonies	α or β switch frequency[a] ($\times 10^{-3}$)
MC4100.λ6 (ptrf5-*fooI*)					
[Lac$^-$]	1	5,889	239/5,650	4.05	α Þ 1.0
	2	4,838	57/4,781	1.18	α Þ 0.6
[Lac$^+$]	1	4,864	155/4,709	3.18	β Þ 49
	2	6,664	575/6,089	8.62	β Þ 44
MC4100.λ6 (ptrf6-*papI*)					
[Lac$^-$]	1	7,774	19/7,755	0.20	α Þ 0.82
	2	6,141	29/6,112	0.40	α Þ 0.2
[Lac$^+$]	1	8,701	72/8,629	0.80	β Þ 40
	2	7,099	149/6,950	2.10	β Þ 42

[a] The switch frequencies α (OFF to ON) and β (ON to OFF) were calculated using the formula $(M/N)/g$, where M is the number of cells that underwent phase transition, N is the total number of cells evaluated, and g is the total number of generations (estimated to be 20) that gave rise to the colony. Colony 1 corresponds to the initial plate analyzed; colony 2 corresponds to the second plate analyzed and was obtained directly from colony 1.
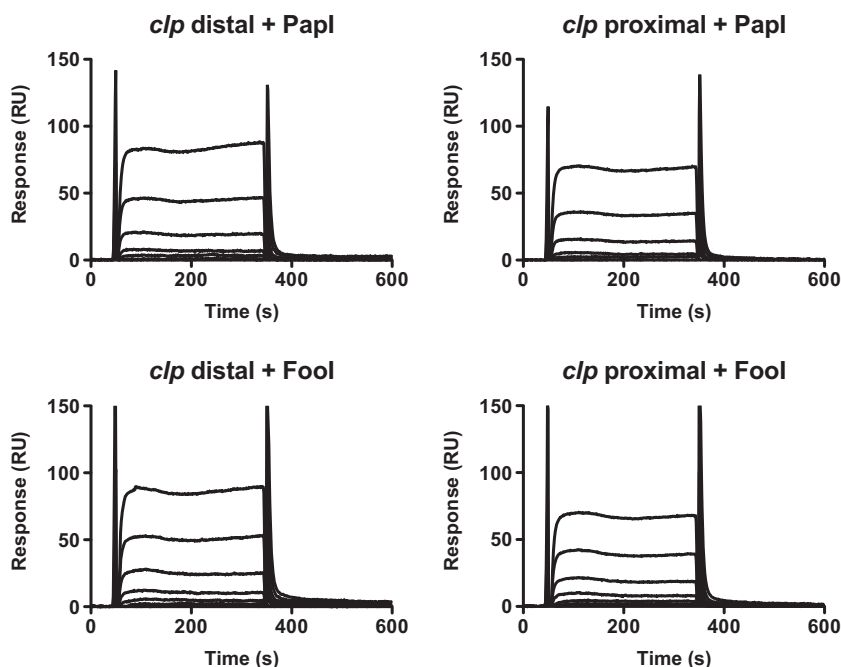
FIG 5 Association and dissociation kinetics of PapI and FooI when bound to the distal or proximal region of the *clp* operon. Representative SPR analyses of PapI or FooI (0 to 15 μM; 2-fold dilution series) binding to immobilized *clp* distal (400 RU) and proximal (300 RU) fragments at 5 μl/min (5 min association and 5 min dissociation).

to-OFF switching, we used SPR to investigate the direct binding of FooI or PapI to the *clp* distal and proximal regions. Rapid-ON, rapid-OFF binding of FooI and PapI to all *clp* fragments correlated with low micromolar overall binding affinities (Fig. 5 and Table 1), consistent with previous work by Kawamura et al., who showed direct binding of PapI to *pap* sites 2 and 5 with similarly weak affinities (41). However, no noticeable differences in the binding kinetics were observed between FooI and PapI with all *clp* fragments (Fig. 5; see also Fig. S2 in the supplemental material). In additional SPR experiments, a fixed concentration of His$_6$-Lrp was injected over the *clp* distal and proximal regions in the absence or presence of increasing PapI or FooI concentrations. While low micromolar injections of FooI or PapI (i.e., <8 μM) yielded small binding responses to the *clp* fragments on their own (Fig. 5), Lrp binding was significantly altered in the presence of FooI or PapI (Fig. 6). In a dose-dependent manner, increasing concentrations of FooI or PapI enhanced the overall amounts of Lrp bound in all cases (i.e., increased association). The stability of the resultant *clp* distal and proximal complexes was also increased (i.e., dissociation rates were approximately 50% lower with 6 μM FooI and 8 μM PapI).

Since the transcription of *papI* homologues on a ptrc99A vector is independent of the *clp* operon, a PapI homologue may contribute to enhancing the binding of Lrp at the *clp* proximal region, in combination with the action of the Dam methylase. Thus, these results suggest that PapI and FooI have two opposite functions. First, they contribute to switching ON the *clp* operon by increasing the association kinetics of Lrp for the *clp* distal region, and second, they strongly maintain cells in the OFF state by forming a particularly stable complex between Lrp and the *clp* proximal region.

DISCUSSION

The objective of the present study was to determine why *clp* expression (CS31A) gives rise to a higher level of OFF cells than does *pap* expression (Pap). Using *in vitro* techniques, we have demonstrated that the weak micromolar affinity of Lrp for the *clp* distal region likely contributes to the low level of ON cells observed. Moreover, we have also shown that PapI homologues, namely, FooI and PapI, favor the OFF state during CS31A phase variation, mainly through their capacity to increase the association and stability of Lrp binding to the proximal region of the *clp* operon.

During phase variation of P fimbriae, the competitive Lrp and Dam assembly on the regulatory region is important for the heritability of the OFF state. In phase OFF cells, Lrp binds the proximal region, which blocks the entry of the RNA polymerase and protects GATC^{prox} from Dam methylation. In phase ON cells, Lrp binds to the distal region and protects GATC^{dist} from methylation. The methylation of the GATC^{prox} site by Dam is required for transition to the phase ON state by specifically blocking PapI-dependent binding of Lrp to promoter proximal sites (25).

The regulation of the *clp* operon, encoding CS31A, shares similar mechanisms with the regulation of the expression of other P-related fimbrial operons (17). Notably, CS31A production is under phase variation control in strain 31A. The *clp* gene cluster does not carry any *papI* homologue, however, even though one is required for phase variation to occur. Only AfaF, a PapI homologue controlling the genetically distant operon *afa-3*, has been used to observe CS31A phase variation to date. Based on these observations, it was suggested that the presence of an additional *pap* operon on the chromosome of CS31A-positive strains may supply the PapI required in *trans*, resulting in *clp* phase variation (15). We have now used several techniques to demonstrate that
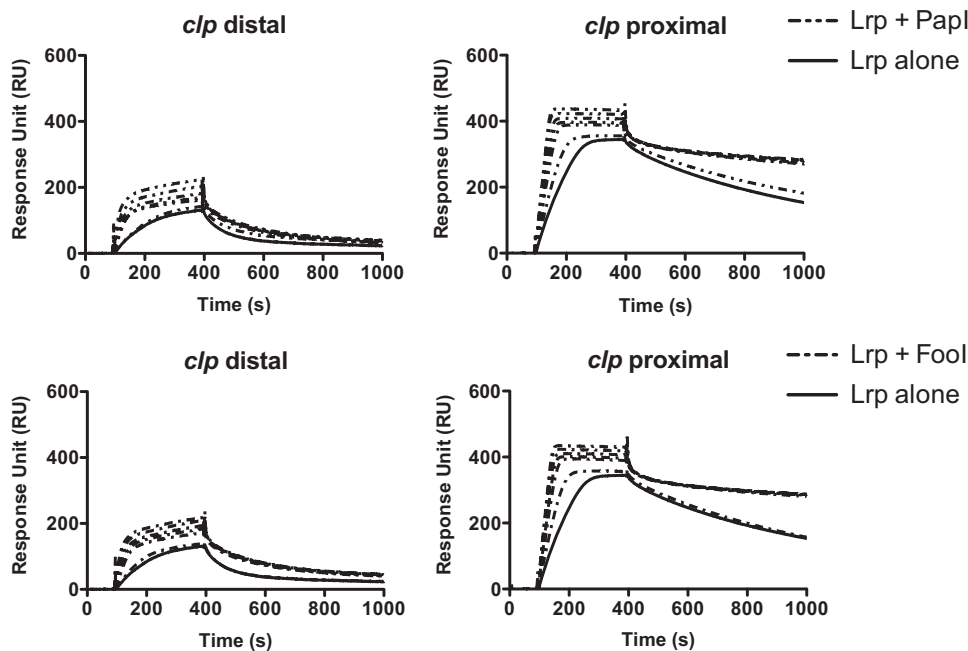
FIG 6 PapI and FooI increase both association and stability of Lrp at the *clp* proximal and distal regions. Representative SPR analyses of 50 nM Lrp binding to immobilized *clp* (95 RU distal, 40 RU proximal) fragments in the absence (solid lines) and presence (dashed lines) of FooI (0 to 6 µM; 2-fold dilution series) or PapI (0 to 8 µM; 2-fold dilution series).

PapI homologues (PapI or FooI) can induce phase variation of the *clp* operon, but overall *clp* expression is repressed due to their capacity to enhance Lrp binding at the proximal region of the *clp* operon. Moreover, we show here that the affinity of Lrp for the distal region is significantly lower (10^3-fold) than its affinity for the proximal region—this distinction contributes greatly to the overall repression of *clp*. Martin showed that, in the absence of a PapI homologue supplied in *trans* (i.e., in the absence of phase variation), Lrp protects the GATC sites from methylation and cannot bind methylated GATC sites ([17]). Here, we provide further information showing that Lrp possesses a lower affinity for $GATC^{dist}$ binding sites than for $GATC^{prox}$ binding sites in the *clp* regulatory region.

In the Pap model, the low nanomolar Lrp affinity is only 2-fold higher for the proximal sites 1 to 3 than for the distal sites 4 to 6 ([25]). Moreover, the concentration of Lrp must be relatively low for the system to work properly ([42]). Binding of Lrp at the proximal sites 1 to 3 reduces by 10-fold the affinity of Lrp for *pap* at the distal sites 4 to 6. This phenomenon, apparent when Lrp binding on plasmid DNA is analyzed, has been called "mutual exclusion" ([25]). However, no mutual exclusion *sensu stricto* could be observed in CS31A, since the Lrp affinity is 10^3-fold higher for the proximal region than for the distal region. Moreover, our findings are consistent with previous reports ([43]) that have proposed two distinct interaction states for Lrp, binding to the *clp* proximal region with low nanomolar affinity (in a range similar to what is shown when Lrp is considered a specific regulator) versus binding to the *clp* distal region with low micromolar affinity (in the same range as is shown when Lrp is considered a nucleoprotein). As a result, Lrp protects $GATC^{prox}$ from methylation and negatively controls basal *clp* expression.

By analyzing the phase variation control of $F165_1$, another member of the P family that includes Pap, we showed that a higher level of ON cells for $F165_1$ is due to an altered Lrp binding stability at the DNA repressor sites 1 to 3 ([38]). Furthermore, we identified nucleotides surrounding the Lrp-binding site 1 that are critical for maintaining a high OFF-to-ON switch rate during $F165_1$ phase variation, as well as for switching Pap fimbriae toward the OFF state by reducing Lrp dissociation from the proximal site. In the case of CS31A, the predominant OFF state is mainly due to the low Lrp affinity for the *clp* distal region. The presence of an additional Lrp binding site, if not directly involved in Lrp binding, probably influences the interaction of Lrp with the *clp* regulatory region. This suggests that the interaction of Lrp with its binding sites dictates their accessibility to methylation and, thus, influences the epigenetic process of phase variation. Given that phase variation of P fimbriae involves the action of multiple regulators, both local (e.g., PapB and PapI) and global (e.g., CpxAR and cyclic AMP-catabolite activator protein [cAMP-CAP]) regulators may contribute to phase variation differences between strains ([44–47]).

In *E. coli* reference strain 31A, CS31A is encoded by the high-molecular-weight plasmid p31A, which does not harbor a *clp* that is a specific homologue to *papI*. It was suggested that the phase variation control observed in wild-type strains could result from cross talk between *clp* and a chromosomal *pap*-related operon, as strain 31A encodes P fimbriae ([15], [16]). In this study, PapI and the homologue FooI restored the bistable state of phase variation when supplied in *trans*. Similar to *pap*, the affinity of Lrp for both the distal and proximal regions increases in the presence of PapI or FooI. Moreover, the significantly stronger affinity of the PapI-/FooI-Lrp complex for the proximal region means that only a minority of *clp* cells is in the ON phase. Furthermore, decreased dissociation of the PapI-/FooI-Lrp complex to the DNA target could also explain why the level of *clp* operon expression in the OFF phase is even lower than the moderate level observed in the absence of a PapI homologue (i.e., the stable PapI-Lrp complex

clp phase variation
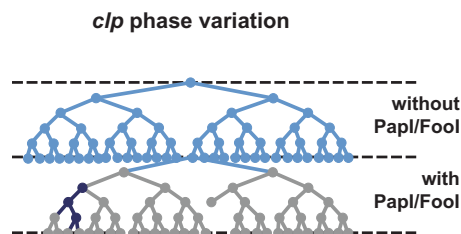
FIG 7 Schematic model representing *clp* phase variation, depending on the presence of PapI in *trans*. In this model, the control of *clp* expression by phase variation depends on the presence in *trans* of PapI expressed by a *pap*-related operon in the genome of the CS31A strain. Since the expression of *pap* fimbriae is also controlled by phase variation, the expression of *papI* reflects Pap ON bacteria. Thus, the presence/absence of PapI results in cross talk between *pap* and *clp* in *pap*-positive and *clp*-positive bacteria: for cells in the ON state for *pap*, PapI is produced, and then *clp* is mostly repressed, with a few cells that show a high level of expression for *clp*. Reciprocally, for cells in the OFF state for *pap*, PapI is not produced, and then *clp* is expressed at a low level.

blocks *clp* transcription more efficiently than Lrp alone or in the presence of AfaF [15]). Taken together, our results show that, in the case of *clp*, PapI homologues play a dual role, (i) promoting the ON phase when *papI* is not expressed (Fig. 7) and (ii) promoting the OFF phase when expressed, thus inducing phase variation in a manner different than what was observed with AfaF. The precise mechanism by which PapI homologues promote the switch from OFF to ON remains unresolved.

It is also noteworthy that the levels of ON colonies can vary greatly depending on which PapI homologue is present in the cell. Indeed, previous studies using AfaF (17) exhibited higher levels of ON cells than we observed in the present study, and our head-to-head comparison between FooI and PapI also demonstrated differences in the levels of ON cells. Considering that at least 16 *papI* alleles have been identified so far (45) and that CS31A is often associated with the presence of other type P-related fimbriae, such as Pap or F165$_1$ (7, 12, 48, 49), we suggest that PapI homologues can be seen as specific regulators that cocoordinately regulate the expression of more than one fimbrial operon (Fig. 7) (45). Similarly, Totsika et al., studying the cross-activation of *pap* variants by FooI and PapI, suggested that sequence variation among PapI homologues could affect their ability to activate *pap* transcription and that they may have evolved in order to prevent cross-activation of related proteins (45).

Strains producing CS31A are often associated with septicemia and have features of extraintestinal *E. coli*. While virulent extraintestinal pathogenic *E. coli* strains mainly belong to the phylogenetic groups B2 and D, reference strain CS31A belongs to the less virulent group A and is thus considered to be opportunistic, although it was isolated from a clinical case (9, 50). In certain environments, the expression of *pap* would reduce that of *clp* in CS31A. This may enhance its adaptation to fluctuating environments and contribute to its fitness.

Overall, the present study provides novel insights about why *clp* expression is repressed by Lrp alone and, in the presence of a PapI homolog, has a low switch rate, like *pap*. This is likely due to the very low affinity of Lrp to the *clp* distal site. Our data emphasize that the fine molecular interaction of regulatory proteins with their DNA-binding sites influences phase variation and the expression of fimbriae. Within the large repertoire of fimbrial operon variants in the P family, we show that *clp*, found in septi-

cemic and enterotoxigenic *E. coli* strains, is an exquisite example of finely tuned regulatory expression that arms the bacterium with strategies for adapting to more than one particular environment. In conclusion, our study exemplifies how bacteria may orchestrate a finely tuned regulatory expression with diverse adhesive factors, thus arming themselves for quick adaptation to changing environments.

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