

## The *lrp* Gene and Its Role in Type I Fimbriation in *Citrobacter rodentium*

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*Citrobacter rodentium* is a murine pathogen that is now widely used as an in vivo model for gastrointestinal infections due to its similarities with human enteropathogens, such as the possession of a locus for enterocyte effacement (the LEE island). We studied the *lrp* gene of *C. rodentium* and found that it encodes a product highly similar to members of the Lrp (leucine-responsive regulatory protein) family of transcriptional regulators, able to recognize leucine as an effector and to repress the expression of its own structural gene. In enterobacteria, Lrp is a global regulator of gene expression, as it controls a large variety of genes, including those coding for cell appendages and other potential virulence factors. Based on the well-established role of Lrp on the expression of pilus genes in *Escherichia coli*, we also studied the role of Lrp in controlling the formation of the type I pilus in *C. rodentium*. Type I pili, produced by the *fim* system, are virulence factors of uropathogens, involved in mediating bacterial adhesion to bladder epithelial cells. Yeast agglutination assays showed that Lrp is needed for type I pilus formation and real-time PCR experiments indicated that Lrp has a strong leucine-mediated effect on the expression of the *fimAICDFGH* operon. Mutant studies indicated that this positive action is exerted mainly through a positive control of Lrp on the phase variation mechanism that regulates *fimAICDFGH* expression. A quantitative analysis of its expression suggested that this operon may also be negatively regulated at the level of transcription.

Members of the Lrp (leucine-responsive regulatory protein) family of transcriptional regulators are small DNA-binding proteins, widely distributed among eubacteria and archaea and active as dimers, tetramers, octamers, or hexadecamers in such diverse organisms as *Escherichia coli*, *Agrobacterium tumefaciens*, *Pseudomonas putida*, *Sulfolobus solfataricus*, and *Pyrococcus furiosus* (1, 11, 13, 16, 27). Several Lrp homologues have been studied in detail and have been shown to be involved in the regulation of amino acid metabolism (1).

In enteric bacteria, Lrp appears to play more general role as a global regulator of metabolism (2, 6, 10, 17). The 3,000 dimers of Lrp estimated in *E. coli* cells were shown by two-dimensional electrophoresis comparison of wild-type and *lrp* mutant cells to affect the expression of at least 30 genes (5). More recently, a microarray analysis has expanded this view, suggesting that at least 10% of all *E. coli* genes are under Lrp control (24). In addition to genes involved in amino acid metabolism and transport, most of the genes expressed upon entry into the stationary growth phase belong to this expanded group (24). For some of these genes the interaction with leucine is responsible for the modulation of Lrp action, with cases in which leucine determines or potentiates and others in which it inhibits or reduces the Lrp effect. For a third class of genes, which includes the Lrp structural gene, *lrp*, leucine has no effect on Lrp action (25).

In pathogenic enterobacteria, Lrp controls some virulence-associated genes. Examples are genes required for conjugal transfer of the virulence plasmid of *Salmonella enterica* (3), the plasmid-encoded *spv* gene of *S. enterica* serovar Typhimurium (15), virulence-associated genes of *Proteus mirabilis* (8), and various fimbrial genes of *E. coli*, including the *fim*, *sfa*, *daa*, *pap*, and *fan* operons (14, 19).

The *fim* system is composed of a seven-cistron operon (*fimAICDFGH*), encoding the structural and regulatory components of the type I pilus, and by two independent genes, *fimB* and *fimE*, each encoding a specific recombinase needed to control by phase variation the expression of the *fimAICDFGH* operon (19). Depending on the orientation of FimS, a 314-bp element containing the promoter of the *fimAICDFGH* operon, pili are formed (phase ON) or not formed (phase OFF), with the FimB recombinase catalyzing both the ON to OFF and OFF to ON switches and FimE only catalyzing the ON to OFF switch. To allow phase variation, the two recombinases, bound to the two ends of FimS, have to physically interact with each other, which is only possible if the DNA separating them is bent (19). In addition to the *fimAICDFGH* promoter, the 314-bp element also contains multiple binding sites for the integration host factor (IHF) and for Lrp, two regulatory proteins known to promote DNA bending (19). Mutations in each one of three Lrp binding sites present within FimS, whose effect is to prevent Lrp binding, reduce both FimB- and FimE-dependent switching (7).

We studied the *lrp* gene and the regulatory role of its product on the expression of *fim* genes in *Citrobacter rodentium*, a mouse pathogen that belongs to a family of human and animal

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TABLE 1. Synthetic oligonucleotides

Gene and Name	Sequence (5'-3') <sup>a</sup>	Positions of annealing <sup>b</sup>
<i>lrp</i>		
A	GGGCCGGTCAGGTC	-732/-719
B	CGACACACGGACCTACG	+427/+443
C	TTAGCGCGTCTTAATAAACC	+477/+495
D	AGCTGGGATCCGAGG	+773/+787
E	ACCAGAAAGTGACGCATCC	+210/+227
F	GTAGGGAATTTACCGGC	-505/-489
G	GGTGAGAAAATGACGATTTG	-373/-354
H	GTGTTATCTGTGTGTCGC	-252/-234
I	GTCAGGCAGGAATAGGG	-41/-25
L	ATGGTAGATAGCAAGAAGCG	+1/+20
N	<u>GCATGCCTTCTTGCTATCTACCAT</u>	+1/+18
O	TGGATTGTAGGGAATTTACCGGC	-512/-489
P	CCGTTGCCTGACGGC	-148/-133
Q	GTGAGTAAACCTCGTTATCTTACC	+1/+24
X	GAAAATGACGATTTGACGCTGTTGGCAATGAATAACTGGgtgtagctggagctgcttc	+368/-329
Y	TTGCTCTGTTTGACTTCTCCATAACGACGTAGGTCCgcatatgaatatcctcctta	+432/+469
Z	CCGGTAAAGAAGTACAGGCTATGATGCTGGCCGCCCGCATGGCgtgtagctggagctgcttc	+23/+66
W	CGAATATTACGGTGGCCGAGATAATCCTGGATAAGCCGCGTcatatgaatatcctcctta	+457/+497
c1	TTATACGCAAGGCGACAAGG (4)	
<i>fim</i>		
fimA-rt1	CGCTGACGCCACCTTCA	+516/+532
fimA-rt2	GCCCTGAGTCACTCCCTGTCT	+556/+576
fimB-rt1	CGGGTACCGCTGGAGAT	+396/+413
fimB-rt2	GTTTGCCAAAGCGAAACCA	+441/+459
fimE-rt1	CGCCGCTGAAAATGG	+187/+203
fimE-rt2	AACCGCTTCCCCTCATC	+232/+249
oNs	AATAAAGAGGAAATATAAATCTGAACAAGTCA	-419/-400
oNa	AACCCCTCACAGGAAGCCAT	-480/-449
sig70s	TCCAGCGTAGAGTCCGAAATC	+259/+279
sig70a	TGCCCATTTTCGCGCATAT	+308/+325
A5	GCTGACGCCACCTTCAAAGT	+517/+536
Cs	CAGGAAATGACGGTG	-12/+3
C4	GGTGATTGCCCGTGTTTTTTT	+25/+45
Ds	GCCGCCGGGAACCTATCGCG	+219/+238
D1	CATTGAGGTAGATATCCACGCG	+245/+267
D6	GGTGAGCTCAGCGTCAACC	+1583/+1602
Da	GGTGCCGCTCAGATAGA	+1626/+1634
G5	TTGTCCTGAAGTTCGAGTGG	-1/+21
G4	GATGAAATGGTTTCAATCCGG	+318/+338
H3	CGACGTTCACTTGCGGTGTA	+151/+170

<sup>a</sup> Capital and lowercase letters indicate bases of *lrp* or *fim* DNA, respectively, and of an unpaired tail carrying a restriction site (underlined).

<sup>b</sup> Position of annealing refers to the *lrp* or *fim* sequence, with the first base of the translational initiation codon as +1.

pathogens, such as the clinically significant enteropathogenic and enterohemorrhagic *E. coli*. *C. rodentium* causes transmissible colonic hyperplasia in mice by means of attaching and effacing lesions through which it colonizes the host gastrointestinal tract (12). Since enteropathogenic and enterohemorrhagic *E. coli* and other human enteropathogens are not able to colonize mice, *C. rodentium* has been extensively used as a model of human gastrointestinal pathogens in in vivo experiments and has proven useful in revealing phenotypes for proteins, including fimbrial proteins, not revealed by in vitro infection models (26).

#### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *C. rodentium* ATCC 51459 was the parent of all recombinant strains described below. *E. coli* DH5 $\alpha$  (*supE44*  $\Delta$ *lacU169* [ $\phi$ 80 $\Delta$ *lacZM15*] *hsdR17 recA1*) (20) was used for all cloning experiments, while the *E. coli* strains CV975 (*ihvIH::lacZ*) and CV1008 (*ihvIH::lacZ* *lrp-35::Tn10*) (18) were used for the complementation experiments. Bacteria were grown at 37°C in rich or minimal medium supplemented with thiamine (5

$\mu$ g/ml), glucose (0.4%), and, when indicated, leucine (100  $\mu$ g/ml). Antibiotics were 100  $\mu$ g/ml ampicillin and 35  $\mu$ g/ml chloramphenicol. For  $\beta$ -galactosidase or  $\beta$ -glucuronidase activity assays, overnight cultures were diluted to an optical density at 600 nm of 0.1 in the appropriate medium, grown in shaking conditions at 37°C up to the log phase (optical density at 600 nm of approximately 0.8), collected by centrifugation, and stored at -80°C until the time of assay.

**DNA manipulations.** Plasmid and chromosomal DNA preparations, restriction digestions, ligation, bacterial transformation, and agarose gel electrophoresis were performed as previously described (20). DNA sequencing reactions were performed by using the T7 sequencing kit (USB corporation) with [ $\alpha$ -<sup>35</sup>S]thio-dATP (>1,000 Ci mmol<sup>-1</sup>; MP Biomedicals). Southern blot experiments were performed according to standard procedures (20) and using as probes DNA fragments labeled by means of Ready-ToGo DNA labeling beads kit (without dCTP) (Amersham) and with [ $\gamma$ -<sup>32</sup>P]dCTP (MP Biomedicals).

Plasmid pAC12 was obtained by cloning a 1,228-bp amplification product resulting from a PCR performed with *C. rodentium* chromosomal DNA as the template and synthetic oligonucleotides A and C as the primers (Table 1) into the commercial vector pGEMT-Easy (Promega). Plasmid pAC12 was used to transform competent cells of the *E. coli* strain CV1008. Ampicillin-resistant clones were screened on LB agar containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) and one clone, AC13, checked by nucleotide sequencing reaction, was selected for further study.

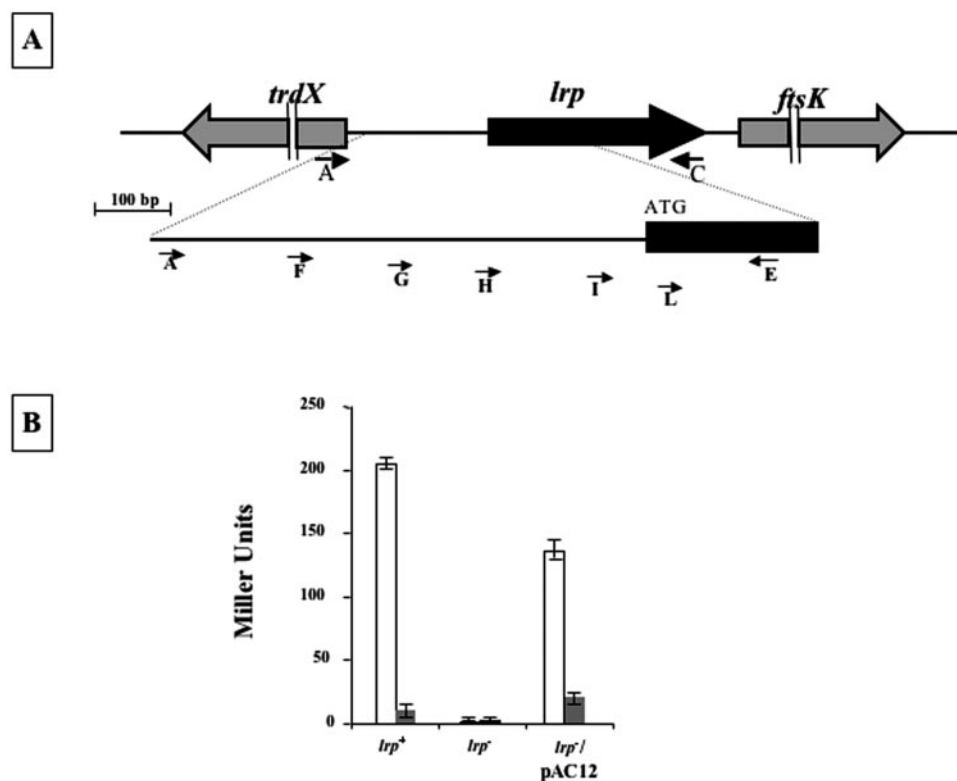


FIG. 1. (A) Schematic representation of the *trdX-lrp-fisK* region on the *C. rodentium* chromosome. Short arrows indicate the position of annealing of synthetic oligonucleotides. (B)  $\beta$ -Galactosidase assay performed with the *E. coli* strains CV975 (*ilvIH::lacZ*), CV1008 (*ilvIH::lacZ* *lrp::Tn10*), and AC13 (CV1008 carrying plasmid pAC12), indicated as *lrp*<sup>+</sup>, *lrp*<sup>-</sup>, and *lrp*<sup>l</sup>/pAC12, respectively. Cells were grown in minimal medium (white bars) and leucine-supplemented minimal medium (gray bars).

An *lrp:gusA* translational fusion was constructed by amplifying a 734-bp DNA fragment containing the *lrp* promoter and 18 nucleotides of the coding region using *C. rodentium* chromosomal DNA as the template primed with oligonucleotides A and N (Table 1). The PCR product was initially cloned into a pGEMT-Easy vector (Promega) and, upon digestion with SphI, transferred into pGusA, a pGEMT-Easy derivative containing the coding region of the *E. coli gusA* gene, in frame with the reporter gene. The obtained plasmid, pAC47, was checked by nucleotide sequencing reaction and used to transform wild-type and *lrp* null mutant *C. rodentium* (see below), yielding strains AC49 and AC52, respectively.

An entire copy of the *lrp* gene, released by NotI digestion from plasmid pAC12, was cloned into pAC47 previously linearized by SalI digestion. The noncohesive ends of the DNA fragments were filled by treatment with Klenow fragment (Biolabs), according to the manufacturer's instructions. The resulting plasmid, pAC61, was then used to transform the wild-type *C. rodentium*, generating strain AC62.

**Construction of *lrp* and *fimE* null mutations.** Null *C. rodentium* mutations in the *fimE* and *lrp* genes were constructed by using the Datsenko and Wanner (4) method. In brief, the chloramphenicol resistance cassette (*cat*) of plasmid pKD3 was PCR amplified by using oligonucleotides X and Y for *lrp* or Z and W for *fimE* (Table 1). These oligonucleotides were designed with 18 and 17 bases, respectively, complementary to their 3' ends, next to 40 bases complementary to regions adjacent to the *lrp* or *fimE* gene (-368 to -329 for X, +432 to +469 for Y, +23 to +66 for Z, and +457 to +497 for W, considering the first translated nucleotide as +1). The 1,091-bp (*lrp*) and 1,099-bp (*fimE*) amplification products were used to transform *C. rodentium* strain EM1, generated by transformation of the wild-type strain ATCC 51459 with the low-copy-number plasmid pKD46, expressing the lambda red recombinase and carrying an ampicillin resistance gene, by electroporation. Chloramphenicol-resistant clones were then cured of the pKD46 plasmid by repeated growth cycles at 37°C in the absence of ampicillin. The presence of the *cat* cassette within the *lrp* or *fimE* gene was verified by PCR with oligonucleotides c1 and O (*lrp*) or Q (*fimE*) (Table 1) and Southern blot. One positive clone for each transformation, EM2 (*lrp*) and EM3 (*fimE*), was selected for further studies.

**$\beta$ -Galactosidase and  $\beta$ -glucuronidase assays.**  $\beta$ -Galactosidase assays were performed as previously described (18). For each strain analyzed, units of activity (nanomoles of *o*-nitrophenyl galactoside hydrolyzed per minute) were calculated from  $A_{420}/V_e t$ , where  $V_e$  is the volume of permeabilized cells in ml and  $t$  is the time in minutes. Specific activity equals units per  $A_{590}$ .  $\beta$ -Glucuronidase assays were performed as previously described (28). For each sample a graph of  $A_{405}$  (y axis) versus time in minutes (x axis) was designed; the slope  $S$  of the graph in  $A_{405}$  units per minute was estimated and units of activity (nanomoles of *p*-nitrophenyl glucuronide hydrolyzed per minute) were calculated from  $S/V_e \times 0.02$ , where  $V_e$  is the volume of permeabilized cells in ml and 0.02 represents the  $A_{405}$  given relative to 1 nmol of product produced. Specific activity equals units per  $A_{590}$ . Values reported here were the average of at least three independent experiments. Statistical significance was determined by Student's *t* test and the significance level was set at  $P < 0.05$ .

**Isolation of total RNA and RT-PCR.** Total RNA was extracted using the QIAGEN mini kit (QIAGEN, Milan, Italy) using the manufacturer's instructions. Total RNAs were dissolved in 50  $\mu$ l of RNase-free water containing recombinant porcine RNasin (1U/ $\mu$ g of total RNA; Amersham Pharmacia Biotech) and stored at -80°C until used for the reverse transcription (RT)-PCR analysis. The final concentration and quality of the RNA samples were estimated using either a spectrophotometer or by agarose gel electrophoresis with ethidium bromide staining.

Prior to RT-PCR total RNAs were treated with RNase-free DNase (1 U/ $\mu$ g of total RNA; Turbo DNA-free, Ambion) for 30 min at 37°C and the reaction was stopped with DNase inactivation reagent; 2  $\mu$ M of oligonucleotide E was used to prime 4  $\mu$ g of total RNAs in a final volume of 15.5  $\mu$ l. The template and primer were incubated at 70°C for 10 min and then added to a reverse transcriptase reaction mixture containing 1 $\times$  reaction buffer, 3 mM MgCl<sub>2</sub>, 0.5 mM each of the deoxynucleoside triphosphates, 1 U/ $\mu$ l recombinant porcine RNasin (1 U/ $\mu$ g of total RNA; Amersham Pharmacia Biotech), and 1  $\mu$ l Improm II reverse transcriptase (Promega). The mixture was incubated at 25°C for 5 min, at 42°C for 60 min, and at 70°C for 10 min to inactivate the enzyme.

After reverse transcription the cDNA obtained was primed with oligonucleo-

tide pairs as indicated in Fig. 1A and 3A. For each oligonucleotide pair, a positive (chromosomal DNA as template) and two negative (no RT and no cDNA) control reactions were performed. PCR conditions were 5 min at 94°C, followed by 30 cycles of 95°C for 30 s, 52°C for 40 s, and 72°C for 1 min, concluding with extension at 72°C for 5 min. The products were separated on 1.0% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

**Real-time PCR.** Real-time PCRs were performed by using chromosomal DNA or bulk cDNA samples synthesized from total RNA extracted from *C. rodentium* cells grown in different growth phases and media, by using SuperScript III reverse transcriptase (Invitrogen) and random hexamers as primers. Templates were always diluted 200-fold. All primers, including those for the normalizing gene, *rpoD* (Table 1), were designed with ABI PRISM Primer Express software (PE Applied Biosystems). Real-time PCR was performed with each specific primer pair and with cDNA or genomic DNA as the template by using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Reactions were performed with an ABI PRISM 7500 sequence detection system (PE Applied Biosystems) and during the reactions, the fluorescence signal due to SYBER Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. The  $\Delta\Delta C_t$  method was used to calculate the relative amount of specific RNA present in a sample, from which the fold induction of transcription of the gene was estimated by comparison to values relative to the wild-type strain grown in minimal medium at early stationary phase.

Data were expressed as the mean  $\pm$  standard error of the mean. Statistical significance was determined by Student's unpaired *t* test and the significance levels are reported in the text.

Primer pair efficiency was tested by looking at how  $\Delta C_t$  (the difference between the two  $C_t$  values of two PCRs for the same template amount) varies with template dilution, as suggested by the manufacturer's instruction guide (PE Applied Biosystems). According to the instruction guide, the efficiency of amplicons was considered 100% when plots of log template amount versus  $C_t$  originated parallel lines with slope values between  $-3.1$  and  $-3.7$ .

**Yeast agglutination experiment.** The capacity of bacteria to express an  $\alpha$ -D-mannose binding phenotype was assayed by their ability to agglutinate *Saccharomyces cerevisiae* cells on glass slides, as previously reported (23); 10  $\mu$ l of liquid bacterial cultures at an optical density of 595 nm of 3 and 10  $\mu$ l of 3% (wt/vol) yeast cells, dissolved in phosphate-buffered saline, were gently mixed and the resulting suspension was incubated at room temperature for 2 min.

## RESULTS

***lrp* gene of *C. rodentium*.** In order to study the *lrp* gene of *C. rodentium* we compared the nucleotide sequence of the *Escherichia coli* K-12 chromosomal region containing the *lrp* coding part and 500 bp upstream and downstream of it (accession number EG10547), with sequences present in the data bank of the Sanger Institute, where the *C. rodentium* genome sequence has recently been completed but not annotated yet ([http://www.sanger.ac.uk/Projects/C\\_rodentium/](http://www.sanger.ac.uk/Projects/C_rodentium/)). This search identified a *C. rodentium* genome region highly homologous to the *lrp* region of *E. coli*, in which *lrp* is located between the *ftsK* and *trdX* genes, with the latter divergently oriented with respect to the other two (Fig. 1A). A computer-assisted search indicated that the putative product of the *C. rodentium lrp* gene was homologous to Lrp of various enterobacteria. In particular, it was identical to Lrp of *Salmonella enterica* serovar Typhimurium LT2 (accession number CB025049) and very similar to Lrp of *E. coli* K-12 (CB035580), *Shigella flexneri* (CB015933), *Proteus mirabilis* (GL086888), and *Yersinia enterocolitica* (GL082961) with 99.4%, 99.4%, 97.6%, and 93.9% amino acid identity, respectively.

A 1,228-bp PCR product, obtained using synthetic oligonucleotides A and C (Table 1 and Fig. 1A) to prime for the amplification of the *lrp* gene from the *C. rodentium* chromosome, was cloned in a pGEM-T-easy (Promega) vector. The recombinant plasmid obtained, pAC12, was used to transform the *E. coli* strain CV1008 (*ilvIH::lacZ lrp::Tn10*) (18). The

resulting recombinant strain, AC13, contained the *lrp*-controlled operon *ilvIH* (18) translationally fused to the *lacZ* reporter gene on the chromosome and the *C. rodentium lrp* gene on a plasmid as the only entire copy of this gene, since a Tn10 chromosomal insertion disrupted *lrp* of *E. coli* (18). The *lrp* gene of *C. rodentium* was able to complement the *lrp* null mutation of strain CV1008, restoring *ilvIH* expression to levels similar to those due to endogenous *lrp* (strain CV975) (Fig. 1B). In addition, like the *E. coli* protein (5), *C. rodentium* Lrp responded to the presence of leucine with a strong reduction of the transcriptional activation of the *ilvIH* operon (Fig. 1B).

To characterize the *lrp* promoter region we performed a series of RT-PCR experiments. Oligonucleotide E (Table 1, Fig. 1A) was used to prime total RNA with reverse transcriptase. The cDNA obtained was then primed with oligonucleotide pairs as indicated in Fig. 1A. Primer-pairs L-E, I-E, and H-E but not G-E, F-E, and A-E originated an amplification product of the expected size (data not shown), indicating that a DNA region up to 250 bp upstream of the translational start site is transcribed and that the transcriptional start point is in the region between oligonucleotides G and H. A similar situation has been observed for *E. coli*, where the *lrp* transcriptional start site has been mapped 267 bp upstream of the translational start site (25).

**Lrp negatively controls the expression of its own structural gene.** To study the expression of the *lrp* gene of *C. rodentium* we constructed an *lrp* null mutant by replacing the *lrp* gene with a chloramphenicol resistance cassette (*cat*) on the *C. rodentium* chromosome. The low-copy-number plasmid pKD46, encoding the  $\lambda$  Red recombinase (4), was used to transform wild-type *C. rodentium*. The resulting strain, EM1, was then transformed with a 1,091-bp PCR product, containing the *cat* cassette flanked by 40 bp of homologous to DNA adjacent to *lrp*. Chloramphenicol-resistant clones were the result of a double-crossover event. Several clones were checked by PCR and Southern blot, and one clone, EM2, was selected for further analysis.

An *lrp::gusA* translational fusion was then obtained as follows: a 734-bp DNA fragment containing the *lrp* promoter region and six N-terminal codons of the *lrp* open reading frame was amplified from *C. rodentium* chromosomal DNA by using oligonucleotides E and N as primers (Table 1). The PCR product was then fused in frame to the *gusA* gene of *E. coli* carried by plasmid pGusA, yielding plasmid pAC47. Plasmid pAC47 was then introduced into the wild-type *C. rodentium* strain ATCC 51459 and into its isogenic *lrp* null mutant EM2, yielding strains AC49 and AC52, respectively. AC49 showed a  $\beta$ -glucuronidase activity significantly higher than that observed with ATCC 51459 cells transformed with the vector plasmid pGusA (data not shown), excluding a potential interference of endogenous enzymes in our assays. As shown in Fig. 2, *lrp*-directed  $\beta$ -glucuronidase activity was slightly higher ( $P < 0.05$ ) in the *lrp* null mutant (AC52, white bar) than in the wild type (AC49, white bar), suggesting that Lrp autogenously repressed the expression of its own structural gene. Addition of 100  $\mu$ g/ml of exogenous leucine to the growth medium did not significantly ( $P > 0.05$ ) affect  $\beta$ -glucuronidase activity in either strain (Fig. 2, AC49 and AC52, gray bars), suggesting that the Lrp control on *lrp* was leucine independent.

To check whether the modest regulatory effect of Lrp on its



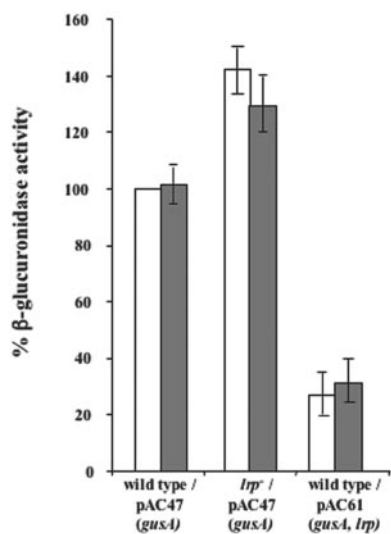


FIG. 2.  $\beta$ -Glucuronidase assay performed on *C. rodentium* strains AC49 (ATCC 51459, wild type carrying plasmid pAC47), AC52 (EM2, *lrp* null carrying plasmid pAC47), AC62 (ATCC 51459, wild type carrying plasmid pAC61), indicated as wild type/pAC47(*gusA*), *lrp*<sup>-</sup>/pAC47 (*gusA*), and wild type/pAC61 (*gusA lrp*), respectively. Cells were grown in minimal medium (white bars) and leucine-supplemented minimal medium (gray bars). The activity value obtained for strain AC49 grown in the absence of leucine was considered 100% activity. All values are the average of at least three independent experiments.

structural gene was limited by a titration of Lrp caused by the presence of Lrp target sites on the multicopy plasmid pAC47, we cloned the same DNA fragment used for the complementation experiment of Fig. 1B that contains the entire *lrp* gene, in plasmid pAC47 also carrying the *lrp::gusA* fusion, yielding plasmid pAC61. The introduction of pAC61 into a wild-type strain of *C. rodentium* yielded strain AC62, which showed an *lrp*-directed  $\beta$ -glucuronidase activity threefold ( $P < 0.05$ ) lower than that observed with strain AC49 carrying pAC47 (Fig. 2). Since pAC47 and pAC61 showed a similar copy number in *C. rodentium* (not shown), the result in Fig. 2 (AC62) confirmed the negative role of Lrp in the expression of its own structural gene, indicating that the presence of pAC47 caused an Lrp titration effect in wild-type cells of *C. rodentium* and, as a consequence, that at least one Lrp binding site is present in pAC47.

TABLE 2. RT-PCR experiments<sup>a</sup>

Oligonucleotide pair <sup>b</sup>	Specific amplification <sup>c</sup>
B1-B2 .....	+
E1-E2 .....	+
B1-E2 .....	-
E1-C4 .....	-
A5-C4 .....	+
C5-D1 .....	+
D5-Da .....	+
D6-G5 .....	+
G4-H3 .....	+

<sup>a</sup> *C. rodentium* total RNA was used to produce cDNA as described in Materials and Methods. cDNA was then amplified with the oligonucleotide pairs here indicated.

<sup>b</sup> Oligonucleotides are described in Fig. 3 and Table 1.

<sup>c</sup> For each oligonucleotide pair, a positive (chromosomal DNA as template) and two negative (RT and cDNA not added) control reactions were performed.

**Lrp positively controls the expression of *fim* genes.** In order to verify whether the *C. rodentium* chromosome contains genes encoding the type 1 pilus, we compared the nucleotide sequence of the *fim* chromosomal region of *Escherichia coli* K-12 (accession number U14003) with sequences present in the data bank of the Sanger Institute. This search identified a *C. rodentium* genome region highly homologous to the *fimAICDFGH* operon and to the two adjacent *fimB* and *fimE* genes of *E. coli*. In addition, a 314-bp region bounded by left and right inverted repeats (IRL and IRR) and identified as *fimS* in Fig. 3, located between the *fimAICDFGH* and *fimE* genes and containing the *fimAICDFGH* promoter, was also found. By homology with the same region of *E. coli*, we assumed that in *C. rodentium* *fimS* is involved in a phase variation mechanism controlling *fimAICDFGHI* expression.

To verify whether the *C. rodentium* *fim* genes are transcriptionally active, we performed a series of RT-PCR experiments priming the total RNA of *C. rodentium* with the synthetic oligonucleotides listed in Table 1 and graphically indicated in Fig. 3. These experiments, summarized in Table 2, indicated that *fimB* and *fimE* are transcribed but not cotranscribed (see Table 2, oligonucleotide pairs B1-B2, E1-E2, and B1-E2) and that *fimAICDFGH* is not cotranscribed with *fimE* (see Table 2, oligonucleotide pair E1-C4). Indication that *fimAICDFGH* is transcribed and forms a single transcriptional unit came from DNA sequence data revealing the presence of the open reading frames indicated in Fig. 3 and from RT-PCR data (see Table 2, oligonucleotide pairs A5-C4, C5-D1, D5-Da, D6-G5,

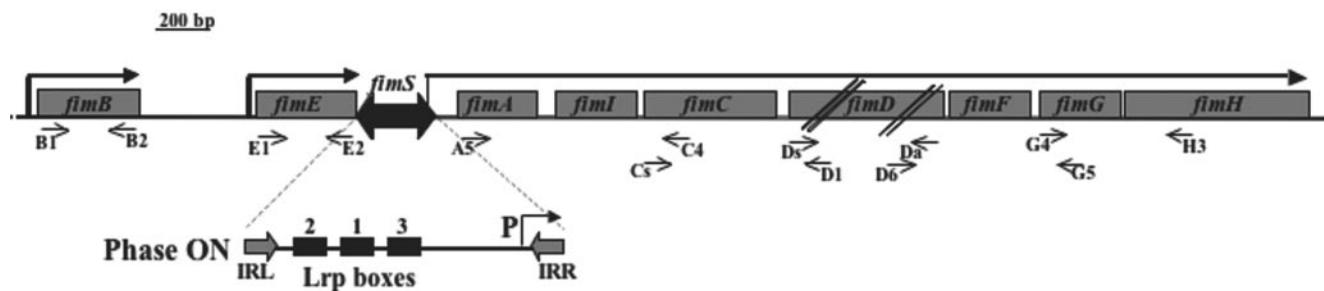


FIG. 3. Schematic representation of the *fim* region on the *C. rodentium* chromosome. Arrows indicate the transcription orientation; short arrows indicate the position of annealing of synthetic oligonucleotides. Enlarged is the *fimS* element in the ON orientation. Three Lrp boxes and two inverted repeats (IRL and IRR) are also indicated. The three Lrp boxes are indicated in the order 2-1-3 in homology to the *E. coli* model (19).

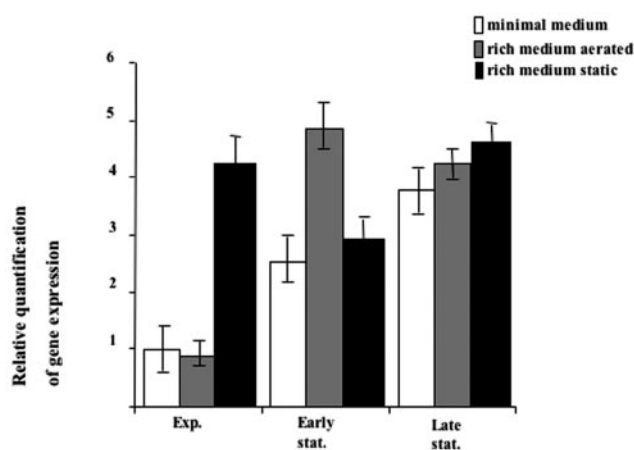


FIG. 4. Real-time PCR experiment performed to monitor *fimAICDFGH* expression in various growth conditions. Cells of the wild-type ATCC 51459 strain were grown in minimal medium (white bars), rich medium in aerated conditions (gray bars), and rich medium in static conditions (black bars) and collected during the exponential growth phase, at entry into stationary growth phase, or after 3 h of stationary growth phase. Total RNA was extracted, and cDNA was synthesized and used in the reactions with an ABI PRISM 7500 sequence detection system (PE Applied Biosystems). The fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. The  $\Delta\Delta C_t$  method was used to calculate the relative amount of specific RNA present in each sample, and the transcriptional induction was estimated by comparison to values relative to the wild-type strain grown in minimal medium at exponential phase.

and G4-H3). These results allowed us to conclude that the *fim* genes are transcribed and organized into three units of 6,596 bp (*fimAICDFGH*), 606 bp (*fimB*), and 597 bp (*fimE*).

To characterize the expression of the *fimAICDFGH* transcriptional unit, encoding the major pilin subunit, the tip constituents, and related regulatory proteins, we performed a real-time PCR analysis (see Materials and Methods) using cDNA synthesized from total RNA extracted from *C. rodentium* cells at different growth phases in minimal or rich medium and in static or aerated conditions. In agreement with observations obtained with other enterobacteria (21), our real-time PCR analysis showed that in cells grown in minimal or rich medium the expression of the *fimAICDFGH* operon is induced upon entry into the stationary growth phase, with an induction rate (relative expression in early stationary phase/relative expression in exponential phase) of 2.6 ( $P < 0.05$ ) in minimal medium and 5.4 ( $P < 0.05$ ) in rich medium (Fig. 4). In addition, *fimAICDFGH*-specific RNA levels of exponential cells growing in rich medium were significantly higher ( $P < 0.05$ ) in static conditions than in shaken conditions (Fig. 4). These levels were temporarily reduced in early stationary phase ( $P < 0.05$ ) and increased again in late stationary phase ( $P < 0.05$ ) (Fig. 4). The temporary decrease of *fimAICDFGH* expression in early stationary growth phase, although statistically significant, is difficult to interpret, and additional experiments will be needed to clarify this point.

Real-time PCR experiments were performed to analyze the effects of Lrp on the expression of the three *fim* transcriptional units. cDNA was synthesized from total RNA extracted from wild-type and *lrp* null mutant cells of *C. rodentium* grown in

TABLE 3. Real-time PCR analysis of *fim* gene expression in wild-type and *lrp* null mutant strains<sup>a</sup>

Gene(s) and medium	Mean expression $\pm$ SD	
	ATCC 51459 (wild type)	EM2 ( <i>lrp</i> )
<i>fimAICDFGH</i>		
Min	1.00 $\pm$ 0.17	0.060 $\pm$ 0.014
Min + leu	2.20 $\pm$ 0.20	N.D.
<i>fimB</i>		
Min	1.00 $\pm$ 0.20	0.90 $\pm$ 0.10
Min + leu	1.20 $\pm$ 0.10	N.D.
<i>fimE</i>		
Min	1.00 $\pm$ 0.15	1.10 $\pm$ 0.26
Min + leu	1.60 $\pm$ 0.22	N.D.

<sup>a</sup> Cells were grown in minimal medium (Min) or minimal medium plus leucine (Min + leu) and collected at the onset of stationary growth phase. Data are presented as arithmetic mean  $\pm$  standard deviation. Data indicate relative levels of transcription compared to the wild-type value in minimal growth conditions for each individual transcriptional unit. N.D., not determined.

minimal medium and collected at the onset of stationary phase. As reported in Table 3, *fimAICDFGH* expression was significantly ( $P < 0.005$ ) higher in wild-type cells than in the *lrp* mutant, while *fimB*- and *fimE*-specific RNA levels did not differ significantly in the two strains. When wild-type cells were grown in leucine-supplemented minimal medium, *fimAICDFGH* and *fimE* expression showed a 2.2-fold ( $P < 0.005$ ) and a 1.6-fold ( $P < 0.005$ ) increase, respectively, with respect to transcription levels observed in the absence of leucine, while *fimB* expression was the same in the two growth conditions ( $P < 0.005$ ) (Table 3).

These results suggest that Lrp is a positive regulator of *fimAICDFGH* expression and that its ligand, leucine, potentiates this positive effect. Lrp does not influence *fimB* expression, while the Lrp-leucine complex, but not Lrp alone, positively affects *fimE* expression. This last observation could also be explained by an increased stability of the *fimE* mRNA due to an effect of leucine on the phase variation mechanism, as previously proposed for *E. coli* (22).

To confirm the transcriptional results in Table 3 and verify the role of Lrp in the formation of the type I pilus, we also performed a yeast agglutination assay (23). This assay is indicative of the presence of type I pili on bacterial cells since their tip is formed by FimH, an adhesin that mediates attachment to mannose-containing receptors, abundantly present on the surface of yeast cells. As shown in Fig. 5, *lrp* mutant cells of *C. rodentium* (Fig. 5A) have a reduced ability to agglutinate *Saccharomyces cerevisiae* cells compared with wild-type cells (Fig. 5B), as evidenced by the smaller number of bacteria-yeast aggregates. This result is in agreement with the transcriptional data summarized in Table 3 and indicate that Lrp has a positive role in the formation of the type I pilus in *C. rodentium*.

**Lrp regulates *fimS* orientation.** In *E. coli* Lrp controls the *fim* phase variation mechanism through binding to three sites in the *fimS* region (19). It is possible that in *C. rodentium* the Lrp role in *fimAICDFGH* is also played through the control of the phase variation switch. To check this hypothesis, we compared by real-time PCR *fimAICDFGH* expression and *fimS* orientation. Real-time PCR experiments were performed on

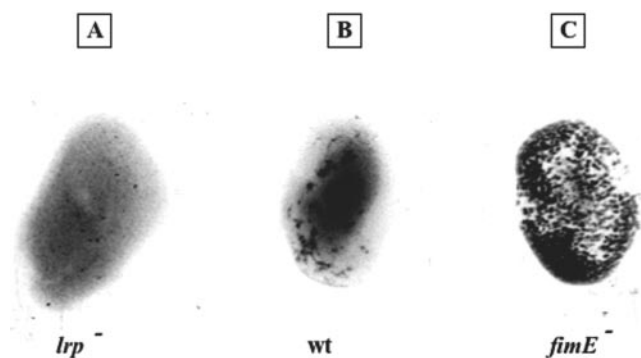


FIG. 5. Yeast agglutination experiments. We mixed 10  $\mu$ l of 3% (wt/vol) *Saccharomyces cerevisiae* yeast cells on a glass slide with the same volume of three bacterial cultures of *Citrobacter rodentium* EM2 (A), wild-type (wt) (B), and EM3 (C) strains at an optical density of 595 nm.

cDNA synthesized from total RNA and chromosomal DNA extracted from wild-type and *lrp* mutant cells of *C. rodentium* harvested at entry into the stationary growth phase. In the case of *fimS*, the synthetic oligonucleotides (ONs and ONa, Table 1) utilized allowed us to monitor only the phase ON orientation, since a computer-assisted search (Primer Express-Software Applied Biosystem) did not identify oligonucleotides to reliably monitor the OFF orientation.

As reported in Table 4, the number of FimS molecules in phase ON was 37-fold ( $P < 0.05$ ) higher in wild-type cells than in *lrp* null mutant cells, indicating that Lrp controls the phase variation by favoring the ON orientation. In the same conditions, the number of *fimAICDFGH* mRNA molecules was 16-fold ( $P < 0.005$ ) higher in the wild type than in the *lrp* mutant (Table 4). The different increase in the number of potentially active promoters (FimS in phase ON) and of synthesized mRNA molecules observed in the presence of Lrp suggests that *fimAICDFGH* expression is not constitutive but somehow negatively regulated.

To test this hypothesis, we constructed a *fimE* null mutant by the same strategy described for the construction of the *lrp* null mutant. Strain EM1 (see above) was transformed with a 1,099-bp PCR product, containing the *cat* cassette flanked by 40 bp homologous to DNA adjacent to *fimE*. Chloramphenicol-resistant clones were the result of a double crossover. Several clones were checked by PCR and Southern blot and one clone, EM3, was selected for further analysis.

Yeast agglutination assays performed with strain EM3 showed that in cells not expressing *fimE* the agglutination activity is stronger and, as a consequence, the number of type

I pili on the cell surface is much higher than on wild-type cells (Fig. 5), suggesting that in *C. rodentium*, as in *E. coli* and other enterobacteria, *fimE* encodes the recombinase that specifically catalyzes the ON-to-OFF switch.

A real-time PCR analysis of *fimAICDFGH* expression was performed on chromosomal DNA and on cDNA synthesized from total RNA extracted from cells of strain EM3 grown in minimal medium. As reported in Table 4, in EM3 the number of FimS molecules in phase ON was 86-fold ( $P < 0.05$ ) higher than in wild-type cells, while the number of *fimAICDFGH* mRNA molecules was 50-fold ( $P < 0.05$ ) higher than in the wild type. Consistent with data derived from the *lrp* mutant, the results obtained with strain EM3 (*fimE*) indicate a different increase in the number of DNA molecules in phase ON and of mRNA molecules synthesized, suggesting that *fimAICDFGH* expression is not constitutive and is instead regulated by a dual control at the levels of both phase variation and transcription.

Although statistically significant, the difference between the number of *fimS* DNA and *fimAICDFGH* mRNA molecules shown in Table 4 could be affected by a different efficiency of the oligonucleotides used to prime the real time PCR amplification. To check the efficiency of our primer pairs, wild-type cells were used to extract chromosomal DNA and total RNA and cDNA were synthesized from the latter. DNA and cDNA were then serially diluted in a range including the dilutions applied in the experiments of Table 4 and used as templates in real-time PCR experiments. Reactions were primed with oligonucleotide pairs ONs and ONa (amplifying *fimS* in the ON orientation; Table 1), *fimA*-rt1 and *fimA*-rt2 (amplifying a coding region of *fimA*; Table 1), and *sig70s* and *sig70a* (amplifying a coding region of the normalizing gene *rpoD*; Table 1). Parallel straight lines with similar slopes ( $-3.70$  for ONs-ONa;  $-3.64$  for *fimA*-rt1-*fimA*-rt2; and  $-3.35$  and  $-3.55$  for *sig70s*-*sig70a* amplifying *rpoD* chromosomal DNA and cDNA, respectively) were obtained with the template dilutions used for all oligonucleotide pairs (data not shown). The slope values were in the range considered optimal (Materials and Methods) and indicated that  $\Delta C_t$  did not vary in different PCR conditions and therefore all oligonucleotide pairs tested were working with the same efficiency.

## DISCUSSION

In enterobacteria, members of the Lrp family of transcriptional factors are global regulators controlling genes involved in amino acid metabolism and transport and in the synthesis of various type of cell appendages. In this work we show that the mouse pathogen *C. rodentium* encodes a structural and functional homologue of Lrp able to complement the Lrp role in the leucine-mediated control of the *ilvIH* operon in *E. coli*. The construction of a *C. rodentium lrp* null mutant allowed us to show that Lrp negatively controls the expression of its own structural gene and to analyze the effect of Lrp on the formation of the type I pilus.

Type I pili, encoded by the *fim* gene system, are the most common virulence factors of uropathogenic bacteria and allow bacterial adhesion to oligosaccharides containing mannose (21). The adhesive properties of the type I pilus are determined by FimH, a lectin-like protein associated with the fibrial tip and encoded by the last gene of the *fimAICDFGH*

TABLE 4. Real-time PCR analysis of *fimS* orientation and expression of *fimAICDFGH*<sup>a</sup>

Strain	Mean relative expression $\pm$ SD	
	<i>fimS</i> -ON	<i>fimAICDFGH</i>
ATCC 51459 (wild type)	1.00 $\pm$ 0.25	1.0 $\pm$ 0.17
EM2 ( <i>lrp</i> )	0.027 $\pm$ 0.016	0.06 $\pm$ 0.014
EM3 ( <i>fimE</i> )	86.3 $\pm$ 14.3	50.3 $\pm$ 6.6

<sup>a</sup> Cells were grown in minimal medium and collected at the onset of stationary growth phase. Data are presented as arithmetic means  $\pm$  standard deviations.



operon. The type I pilus has recently been identified as a virulence factor of the invasive pathogen *Citrobacter freundii* (9). However, although they belong to the same genus, *C. freundii* and *C. rodentium* have *fim* genes with different chromosomal organization and low DNA homology. The *fim* genes of *C. freundii* are instead similar to those of *Salmonella enterica* serovar Typhimurium (9) and, as shown here, the *C. rodentium* ones are instead similar to those of *E. coli*.

Yeast agglutination assays indicated that *lrp* null mutant cells of *C. rodentium* are strongly impaired in the ability to agglutinate yeast cells, showing a positive effect of Lrp on pilus formation. A real-time PCR approach allowed us to observe that the expression of the *fimAICDFGH* operon, but not that of *fimB* and *fimE*, was strongly enhanced by the presence of Lrp, suggesting that the positive role of Lrp on pilus formation is exerted through the control of *fimAICDFGH* expression. When the Lrp ligand, leucine, is present the positive Lrp effect on *fimAICDFGH* is enhanced further.

The positive Lrp control of *fimAICDFGH* expression might either influence the phase variation mechanism by favoring the ON orientation or enhance transcription of the operon. We observed that Lrp strongly favors the ON orientation of the *fimS* switch and that this effect is not mediated by an action of Lrp on the expression of the *fimB* or *fimE* gene, encoding the specific FimS recombinases. Therefore, we propose that the Lrp action on the phase variation mechanism is most probably due to the DNA-bending activity of Lrp (25), which allows the physical interaction of the recombinases bound to the two ends of FimS, as previously proposed for *E. coli* (19). However, *fimAICDFGH* transcription does not appear to be constitutive but rather negatively controlled. Whether this transcriptional level of control is also dependent on Lrp remains to be clarified.

Our real time PCR experiments, performed in growth conditions in which *fimAICDFGH* is highly expressed (Fig. 4), suggested that to an increased number of FimS molecules in the ON orientation did not correspond to an equal increase in the number of *fimAICDFGH*-specific mRNA molecules. The statistically significant difference between the increase in DNA molecules ready to be transcribed and of *fimAICDFGH*-specific mRNA molecules induced us to conclude that not all molecules in the ON orientation were transcribed, as expected from an unregulated, constitutive promoter and, as a consequence, that the *fimAICDFGH* promoter is somehow negatively regulated.

Consistent with this conclusion is the analysis of a mutant that does not produce the recombinase that specifically catalyzes the ON-to-OFF switch. In this mutant the number of FimS molecules in the ON orientation is 86-fold higher than in the isogenic wild type, while the number of *fimAICDFGH*-specific molecules is only 50-fold higher in the mutant. Taken together, these statistically significant differences suggest that, in addition to the Lrp-mediated control of the *fim* switch, *fimAICDFGH* expression is also negatively controlled by a transcriptional mechanism.

Since *C. rodentium* causes a transmissible colonic hyperplasia in mice similar to that induced in humans by enteropathogenic and enterohemorrhagic *E. coli* strains (12), it has recently been selected as an in vivo model system to study human infections (26). The characterization in this model organism of Lrp, a transcriptional regulator that affects a large variety of genes including those coding for potential virulence factors,

opens a new field of investigation in *C. rodentium* pathogenesis. The construction of an *lrp* null mutant will allow the identification of Lrp-controlled virulence factors and a better understanding of the role in the infection process of Lrp and the virulence factors that it controls.

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