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A Nonswarming Mutant of *Proteus mirabilis* Lacks the Lrp Global Transcriptional Regulator

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*Proteus* swarming is the rapid cyclical population migration across surfaces by elongated cells that hyperexpress flagellar and virulence genes. The mini-Tn5 transposon mutant mns2 was isolated as a tight nonswarming mutant that did not elongate or upregulate flagellar and hemolysin genes. Individual cell motility was retained but was reduced. The transposon had inserted in the gene encoding the global transcriptional regulator Lrp (leucine-responsive regulatory protein), expression of which was upregulated in differentiating swarm cells. Swarming was restored to the *lrp* mutant by artificial overexpression of the *flhDC* flagellar regulator master operon. Lrp may be a key component in generating or relaying signals that are required for flagellation and swarming, possibly acting through the *flhDC* operon.

**MATERIALS AND METHODS**

**Mutagenesis and assays of swarming, motility, and differentiation.** Wild-type *P. mirabilis* U6450 was mutagenized by the pUT mini-Tn5 system (13), and mutants resistant to chloramphenicol (80 μg ml⁻¹) were selected. Swarming was assessed on 1.5% LB agar plates. Swarming inhibitor *a*-nitrophenyl-glycerol (100 μg ml⁻¹, Sigma) was added to isolate single colonies. Motility was assessed in 0.3% LB agar. Differentiation was initiated by spreading 200-μl stationary-phase LB cultures (ca. 5.10⁹ ml⁻¹) onto 8-cm-diameter LB agar plates and incubating them at 37°C (25). Growth on seeding plates was assayed by harvesting cells in LB and normalizing *A*₅₄₀ to constant volume. Cell length was assessed by phase-contrast microscopy. Levels of surface FlIC were determined by vortexing of washed cells for 5 min, centrifugation, and trichloroacetic acid precipitation of supernatants, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. Cell-associated hemolysin activity was calculated by normalizing hemoglobin released from erythrocytes (*A*₅₄₀ to *A*₅₄₀) (30). Migrating cells were collected from the swarm front (within 3 mm) of parallel swarm colonies following central inoculation with a stationary-phase LB culture.

**Recombinant DNA and sequencing.** Routine DNA manipulation and electroporation were carried out with *Escherichia coli* XL1Blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (proAB lacI T7)}. Plaque hybridizations and Southern analysis were carried out with digoxigenin (Dig System User's Guide [11a]). DNA fragments were subjected to exonuclease III digestion (Erase-a-Base; Promega), and DNA sequencing was performed with the T7 sequencing kit (Pharmacia). Sequences were analyzed by GCG, version 8 (16).

**mRNA hybridization.** Total RNA was isolated with hot phenol (35). Decaturated RNA samples (10 μg) were separated by agarose gel electrophoresis and transferred onto nitrocellulose filters (Hybond C; Amersham). In each experiment, parallel samples were separated, transferred, and stained with 0.04% methylene blue to confirm equivalent quantities of total RNA in each track. Probe labeling, hybridization, and detection were carried out as previously described (25).

**Nucleotide sequence accession number.** The DNA sequence of *lp* has been submitted to the EMBL database (accession no. Y10417).

**RESULTS**

**Isolation and characterization of the motile nonswarming mutant mns2.** Following mutagenesis of *P. mirabilis* U6450 by the pUT mini-Tn5 system, mutant mns2 was isolated as a nonswarming colony unable to migrate even after prolonged incubation (Fig. 1A). Microscopic examination of cells and inoculation of the mutant into semisolid agar showed that it was motile, although less so than the wild type (Fig. 1B). Growth rates in LB medium appeared unchanged (not shown).

Differentiation in the absence of migration was assessed by seeding of stationary-phase cells at high density onto the entire surface of 1.5% LB agar plates. Cells differentiate synchronously with kinetics similar to that of a swarm cycle, although differentiation is not as extreme as that seen in cells at a...
mutant. Overnight cultures (1 μl) were inoculated onto 1.5% LB agar (A) and into 0.3% LB agar (B) and incubated for 6 h at 37°C. (C) Cell-associated hemolysin activity, with hemoglobin released from erythrocytes (Fig. 2C). In contrast, urease activity remained at 70% of that of the wild type at 3 to 4 h, and protease activity was unaffected (not shown).

A mutation in the lrp global regulator gene. A restriction map of the mutant locus was constructed by Southern hybridization with a probe internal to the transposon, and the locus was cloned into the vector pBluescript SK (Stratagene) as a PstI-SalI fragment, selecting Tn5Cm-encoded chloramphenicol resistance. This generated the plasmid p200PS (Fig. 3A). A fragment of the cloned chromosomal region was then used to probe a λ DashII phage library of partial Sau3A fragments from the wild-type P. mirabilis chromosome (25). Several phage hybridized and were used to assemble a restriction map of the locus (Fig. 3A), and the plasmid p200XP was constructed by subcloning a 2.03-kb fragment of the phage insert (Fig. 3A) into pBluescript SK. Transformation by p200XP restored swarming to the mutant, albeit determining a mildly dendritic pattern with swarm terraces closer together than those of the wild type.

Sequencing of p200XP and of p200PS showed that the transposon had inserted between bases 101 and 102 of a 492-bp, 164-codon open reading frame (ORF) (Fig. 3B). The predicted amino acid sequence has 97% identity with the E. coli leucine-responsive regulatory protein (Lrp), which is highly conserved in members of the Enterobacteriaceae (20). Lrp is a global transcriptional regulator that influences many pathways, including several of amino acid metabolism (13, 36). E. coli lrp mutants grow poorly in the absence of exogenous serine because the biosynthetic gene serD is positively regulated by Lrp (41). This was true of the P. mirabilis mns2 mutant (not shown). Downstream (128 bp) of the Proteus lrp gene, within p200XP, are the first 804 bp of an ORF with 55% identity to ftsK, an E. coli cell division gene that lies similarly 3′ of lrp (8). Proteus ftsK is preceded by potential −35 and −10 promoter components and a potential lex box, an element allowing induction by the SOS response also identified 5′ of E. coli ftsK (34). The loss of swarming cannot be explained by a polar effect upon ftsK, since swarming is restored by the complementing plasmid p200XP, which contains only the predicted first 20% of the ftsK gene (the E. coli protein has 1,330 amino acids). This was confirmed by subsequent complementation with a plasmid (constructed by exonuclease digestion of p200XP) encoding Lrp but only the first 23 amino acids of FtsK (p200xp4 [Fig. 3]) and by the absence of complementation by a plasmid lacking ftsK and in which lrp was missing the last 14 codons (p200xp5 [Fig. 3]).

Upregulation of the lrp transcript during swarm cell differentiation. Northern blots of RNA from wild-type cells with a probe internal to the lrp gene identified major transcripts with sizes of about 650, 750, and 1,100 nucleotides, considerably larger than the 492-bp ORF1, but none large enough to encompass the downstream ftsK gene (Fig. 4A). In E. coli, the start of the lrp transcript has been mapped by primer extension at 267 bp upstream of the ORF (43). The 600-bp sequence upstream of lrp lacks detectable ORFs but also contains no clear 5′ promoter consensus sequences for σ70 or σ32, although there are several poor matches. There is a potential stem-loop structure 65 bp downstream of lrp that might act as a transcription terminator.

Analysis of cultures from seeding plates showed that expression of all of the lrp transcripts is upregulated during swarm cell differentiation (Fig. 4A), following kinetics similar to those
of flic, but reaching a maximum about 30 min earlier. Levels of an rRNA transcript remained constant. A growth curve of the seeding plate culture indicated that this transient lrp induction occurs in the late exponential phase of growth, decreasing again as cells enter the stationary phase (Fig. 4B). Whether this batch growth phase is a determinant of swarm cell differentiation is not clear, but to ensure that the upregulation was not specific to the culture growing after high-density seeding, the result was confirmed with mRNA isolated from cells at the edge of actively swarming colonies. Both the flic and lrp transcript levels peaked in maximally differentiated cells shortly before migration (Fig. 4C [6 h]), with lower levels of transcript in cells of migrating populations (Fig. 4C [4 h]). So, whether in response to growth phase or to other signals, lrp is upregulated in differentiating swarm cells.

Downregulated flagellar and hemolysin gene expression in the lrp mutant. Hemolysin and flagellar gene mRNAs were assayed in the lrp mutant at 4 h in the seeding assay at maxi-
The fliC message concentration was reduced (Fig. 5A) to a low level corresponding to the level of FliC filament protein detected on the surface of these cells (Fig. 2A). The levels of expression of flagellar genes flgM (another class III gene), flgB (class II), and flhDC (class I) were all substantially reduced (Fig. 5A). Levels of hpm transcript encoding hemolysin were strongly reduced, such that radiolabeled message from the mutant was detected by autoradiography only after overexposure of the wild-type mRNA signal. Six randomly cloned U6450 transcribed sequences did not show any reduction in transcript in the lrp mutant (Fig. 5B).

In cells grown in liquid culture, levels of flhDC transcript were also lower in the lrp mutant than in the wild type (Fig. 5C), in agreement with the lower flagellin protein levels seen in overnight cultures (Fig. 2A) and impaired motility in semisolid agar (Fig. 1). This suggests that the influence of Lrp on flagellar gene expression may not be limited to swarm cells, but there remains some doubt, because these conditions do support a low level of differentiation of the wild type (17), and introduction of an E. coli lrp mutation (12) into a motile strain of that species (14) did not impair motility (not shown).

Overexpression of flhDC restores swarming to the lrp mutant. Swarming is a demanding process with a substantial proportion of metabolism given over to the assembly and operation of flagella. It has been argued that the physiological changes in an E. coli lrp mutant result in overall metabolic compromise (7), so it seemed possible that the Proteus lrp mutant was metabolically incapable of supporting hyperflagellation. Flagellar gene expression was artificially induced in the lrp mutant by placing the P. mirabilis flhDC master operon and the preceding 60 bp under the control of the arabinose promoter in the vector pBAD18 (24), from which expression is repressed by glucose and induced by arabinose. Transformants grown on LB agar with 0.2% glucose remained nonswarming, although transformants of the wild type swarmed normally on that medium. However, induction of gene expression by the inclusion of 0.2% arabinose restored swarming in the lrp mutant, both mutant and wild-type transformants swarmed at almost twice the normal rate (Fig. 6A). High levels of surface FliC were also restored in the pBADDC-complemented mutant (Fig. 6B), and the cells became fully elongated (data not shown), but the hemolytic activity of the lrp mutant was not restored (data not shown).

**DISCUSSION**

The mns2 mutant was one of five motile nonswarming mutants among more than 3,500 Cm' transconjugants (the others...
The flagellin from the cells listed above seeded onto LB agar and harvested at 4 h.

containing pBAD

P. mirabilis flhDC

flhDC

lar hierarchy was strongly reduced in the

flhDC

, the operon at the top of the flagellar hierarchy (19, 22),

branch. The effect of Lrp upon hyperflagellation seems likely

indicating that their swarm cell induction follows a different

elongate, or hyperinduce hemolysin toxin. Protease and urease

volved in amino acid biosynthesis and degradation, nitrogen

assimilation, biosynthesis of one-carbon units, peptide transport,

and pilin synthesis (13, 36), repressing expression of some

genes and activating others. It is a small basic protein that

bends DNA and is relatively abundant in the cell, prompting

the proposal that it acts as a chromosome organizer (36, 42).

Expression of lp itself is, in E. coli, higher in poor growth

media and in the stationary growth phase (31).

The Proteus lp mutant was profoundly defective in both

swarming and differentiation, being unable to hyperflagellate,

elongate, or hyperinduce hemolysin toxin. Protease and urease

production were not significantly decreased in the mutant,

indicating that their swarm cell induction follows a different

branch. The effect of Lrp upon hyperflagellation seems likely to

be mediated through the flhDC master regulon, since tran-

scription of this operon and of the genes below it in the flagel-

lar hierarchy was strongly reduced in the lp mutant, and

swarming was fully restored by artificial induction of flhDC

expression. In both Serratia and Proteus, hyperflagellation, cell

elongation, and swarming can be induced by upregulation of

flhDC, the operon at the top of the flagellar hierarchy (19, 22),

indicating that flhDC is a focal point of the mechanistic rela-

tionship between hyperflagellation and inhibition of cell divi-

sion (25) and may be a key control point in swarming. The

restoration of swarming following artificial induction of flhDC

suggests that the lp mutant fails to induce flhDC upregulation

rather than being physiologically incapable of supporting hy-

perflagellation. In keeping with a role for Lrp in the induction

of swarming, lp transcription was upregulated in differenti-

ating swarm cells, peaking prior to swarming migration slightly

earlier than flIC. The reduction in surface flagellin and flhDC

transcription seen in lp cells grown in liquid culture suggests

that Lrp also has some influence over flagellation in Proteus

vegetative cells. Lrp-mediated regulation of flagellation might

become critical during the hyperinduction involved in swarm-

ing. Hemolytic activity was not restored by flhDC overproduc-

tion, implying that despite being coupled to swarm cell differ-

entiation, it is not under flhDC control. Alternatively, Lrp may

be directly required for hemolysin induction.

Lrp may itself positively regulate the flagellar and other

swarming-associated genes, or its effect may be indirect, me-

diated by an Lrp-dependent factor or factors. In E. coli, flhDC

is subject to regulation by the cyclic AMP-receptor protein

CRP and the osmoregulator OmpR (39, 40). H-NS mutants

are also defective in flagellation, and there are possible con-

sensus sequences for regulation by the integration host factor

(11, 39). Like Lrp, the latter proteins alter DNA topology upon

binding, and these proteins are global regulators (21, 28).

Complex transcriptional regulation involving Lrp along with

other master regulators has been reported for osmY and lviH

(32, 33), and the possibility that DNA topology changes allow

multiple layers of gene regulation in response to environmen-

tal or physiological factors is attractive (18, 37). Lrp may act

together with other regulators to integrate a range of signals

into the control of flagellation and swarming.

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