The Lon protease regulates swarming motility and virulence gene expression in Proteus mirabilis

Katy M. Clemmer1 and Philip N. Rather1,2

1Research Service, Veterans Affairs Medical Center, Decatur, GA, USA
2Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA

A mini-Tn5lacZ1 transposon insertion in a gene encoding an orthologue of the Lon protease confered a hyper-swarming phenotype on Proteus mirabilis. The lon mutation increased the accumulation of mRNA for representative class 1 (flhDC), class 2 (fliA) and class 3 (flaA) genes during swarmer cell differentiation. In addition, the stability of the FlhD protein was fourfold higher in the lon::mini-Tn5lacZ1 background. Expression of a single-copy lon::lacZ fusion increased during the swarming cycle and reached peak levels of expression at a point just after swarmer cell differentiation had initiated. In liquid media, a condition normally non-permissive for swarming, the lon::mini-Tn5lacZ1 insertion resulted in motile, highly elongated cells that overexpressed flagellin. Finally, the lon::mini-Tn5lacZ1 mutation was shown to result in increased expression of the hpmBA and zapA virulence genes during swarmer cell differentiation.

INTRODUCTION

In Proteus mirabilis, the ability to swarm on agar surfaces requires vegetative cells to differentiate into elongated swarmer cells (Fraser & Hughes, 1999; Rather, 2005). Swarmer cells assemble into multicellular rafts and migrate across surfaces (Jones et al., 2004). The differentiated swarmer cell represents a transient state and cells de-differentiate back to vegetative cells in a process termed consolidation. This represents one swarming cycle and the repeating cycle of differentiation and de-differentiation results in the formation of characteristic rings that form a bull’s-eye pattern on agar plates (Rauprich et al., 1996).

A central component in the regulation of swarmer cell differentiation in P. mirabilis is the class 1 FlhDC activator of the flagellar gene cascade (Furness et al., 1997). The FlhD2C2 heterotetrameric complex activates class 2 genes in the flagellar cascade, including fliA encoding α2β (αF), which directs RNA polymerase to class 3 promoters, such as the flagellin (flaA) promoter (Claret & Hughes, 2000a; Chilcott & Hughes, 2000; Manos & Belas, 2004). Artificial overexpression of FlhDC from an arabinose-inducible promoter results in a hyper-swarming phenotype (Furness et al., 1997; Hay et al., 1997). In P. mirabilis, the flhDC operon is negatively regulated by the RcsBCD phosphorelay (Clemmer & Rather, 2007) and positively regulated by the Lrp and UmoB proteins (Hay et al., 1997; Dufour et al., 1998). In addition, FlhD2C2 activity is inhibited at the post-transcriptional level by an unknown mechanism that requires DisA, a putative phenylalanine decarboxylase (Stevenson & Rather, 2006). The P. mirabilis FlhD and FlhC proteins are also degraded by an energy-dependent protease in differentiated swarmer cells (Claret & Hughes, 2000b). The protease responsible for this turnover was proposed to be the Lon protease based on increased stability in a heterologous Escherichia coli lon mutant (Claret & Hughes, 2000b). However, prior to this study, the role of Lon in P. mirabilis on the stability of the native FlhD and FlhC proteins has not been addressed. In this study, we report the pleiotropic effects of a lon-null allele on swarming, flagellar gene expression and virulence gene expression.

METHODS

Bacterial strains, plasmids and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Medium for the growth of all strains was modified Luria–Bertani (LB) medium containing 10 g tryptone l−1, 5 g yeast extract l−1 and 5 g NaCl l−1. Agar was added at a concentration of 1.5%. Antibiotics were used at the following concentrations: streptomycin, 25 μg ml−1 for E. coli and 35 μg ml−1 for P. mirabilis; tetracycline, 25 μg ml−1 for E. coli and P. mirabilis; chloramphenicol, 25 μg ml−1 for E. coli and 100 μg ml−1 for P. mirabilis; and kanamycin, 35 μg ml−1 for P. mirabilis. DNA was introduced into P. mirabilis by electroporation as described previously (Rather et al., 1993).

Generation of a mini-Tn5lacZ1 insertional library. A conjugal mating was used to introduce random insertions of mini-Tn5lacZ1 into the P. mirabilis chromosome. Overnight cultures of E. coli SM10/pUT::mini-Tn5lacZ1 and P. mirabilis PM7002 were mixed together in 100 μl aliquots, plated on an LB agar plate and grown for 6 h at 37°C. Cells were then scraped off the plate, resuspended in LB broth and plated on LB agar plates containing tetracycline (15 μg ml−1), kanamycin (20 μg ml−1) and X-Gal (0.15%).

Received 21 November 2007
Accepted 26 March 2008

DOI 10.1099/jmm.0.47778-0

Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong> E. coli</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>Δlac-pro [F’ proAB lacI2 lacZAM15 Tn10]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5α</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SM10 ipir</td>
<td>thi t reu tonA lacY supE recA RP4-2-Tc::Mu, KmR ipir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUT::mini-Tn5lacZ1</td>
<td>Transposon delivery vector</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pKNG101</td>
<td>R6K-derived suicide plasmid, SmR</td>
<td>Kaniga et al. (1991)</td>
</tr>
<tr>
<td>pBC.SK(−)</td>
<td>High-copy-number vector, CmR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Medium-copy-number vector, CmR, TcR</td>
<td>Chang &amp; Cohen (1978)</td>
</tr>
<tr>
<td>pFDC1H</td>
<td>pACYC184::FLAG–FlhD + FlhC–His6</td>
<td>This study</td>
</tr>
<tr>
<td>pPM-lon</td>
<td>pKNG101::2.5 kb BamHI fragment containing lon</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Cloning procedures.

The mini-Tn5lacZ1 insertion in PM2173 was not accompanied by a pUT co-integration as determined by Southern blot analysis and ampicillin sensitivity. The mini-Tn5lacZ1 insertion and flanking upstream DNA was isolated from PM2173 by digestion of chromosomal DNA and ligation of HindIII fragments into pACYC184 and selection for lacI+ XL1-Blue recombinants on X-Gal plates. The sequence of chromosomal DNA adjacent to the transposon insertion was determined using a primer (5’-CCAGATCTCTGATCAAGAGACAG-3’) that read outward from the lacZ (I-end) end of mini-Tn5lacZ1. To create epitope-tagged versions of the FlhD and FlhC proteins, PCR (30 cycles at 50 °C for annealing and 72 °C for extension) was used to amplify the flhDC operon from PM7002 using the following primers: FlhD amino acid, 5’-CGATCGTCTAGATCGGACGGGATGTAAAGAGATG and 5’-CCGATATG-3’.

To clone a wild-type copy of the lon gene, the following primers were used to amplify the lon gene and its native ribosome-binding site by PCR: 5’-GCTAGGATCTCTACGATGAAAAGCTTGAAGC-3’ and 5’-GCTAGGATCTCTATGATCATCAGTACAGC-3’. This fragment was digested with BamHI and cloned into the BamHI site of pKNG101 (Kaniga et al., 1991) to generate pPM-lon. To integrate pPM-lon into the P. mirabilis chromosome as a single copy, E. coli SM10/pPM-lon was subjected to a filter mating with PM2173 and exconjugants with pPM-lon integrated into the chromosome were selected on LB plates containing streptomycin (35 μg ml⁻¹) and tetracycline (15 μg ml⁻¹).

### Northern blot analysis.

Expression of flhDC, flaA, flaA, hmpA and zapA during the swarm cell differentiation cycle was measured by Northern blot analysis of total RNA. Cells were harvested for RNA analysis by plating 150 μl of overnight cultures of PM7002 and PM2173 at an OD₆₀₀ of 1.5 on individual LB agar plates, followed by isolation of cells from plates at hourly intervals. RNA was extracted using a MasterPure RNA Purification kit (Epicentre) following the standard protocol with the addition of a 20 min DNase treatment. Formamide/formaldehyde-denatured RNA was resolved by electrophoresis through a 1.2% agarose/formaldehyde gel and transferred onto nitrocellulose membranes by capillary transfer. Blots were probed with digoxigenin-labelled PCR products corresponding to each gene, which were generated with the following primers: flaA, 5’-GATTCAGTTGCTCCGTAAC-3’ and 5’-CCGGTTTGTGAACATC-3’; flaC, 5’-TTGGACGACCAGTAAAGAAG-3’ and 5’-CAGGATTGGGAGAGATGTTA-3’; flaB, 5’-GACCGCTTTACGAGCAGATGCTT-3’ and 5’-GACGCTTTACGAGCAGATGCTT-3’; hmpA, 5’-GAAACCGTTTGACAGT-3’ and 5’-TTGCTTACCCGTTGTTTTGTTCT-3’; and zapA, 5’-CCGGCTGAGGAGCATTTACACG-3’ and 5’-TGGCTTACAGAAGCAGTT-3’.

### Western blot analysis.

Fla protein levels were assessed in whole-cell lysates of P. mirabilis strains using Western blot analysis. Cells were grown overnight without shaking at 37 °C to an OD₆₀₀ of 0.5. One milliliter of cells was pelleted and lysed in Laemmlı sample buffer by heating at 95 °C for 10 min. Protein samples were separated by SDS-PAGE using a Bio-Rad ReadyGel system and 15% Tris/HCl gels. Identical amounts of protein were loaded for each sample based on pre-quantification on SDS-PAGE gels, followed by Coomassie blue staining. Total protein was electrotransferred to nitrocellulose and probed with rabbit antibodies to P. mirabilis FlaA (kindly provided by Robert Belas, Center of Marine Biotechnology, University of Maryland, MD, USA) at a 1:40 000 dilution, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) at a 1:35 000 dilution. Detection was carried out using ECL Western Blotting Detection Reagents in accordance with standard procedures (Amersham Biosciences). Detection of FLAG-tagged and His₆-tagged proteins was done by Western blot analysis as described above using an anti-FLAG (Sigma) or anti-His₆ (Qiagen) antibody.
RESULTS

A lon::mini-Tn5lacZ1 insertion increases swarming motility

Approximately 80,000 mini-Tn5lacZ1 insertions were screened visually after 12 h growth on LB agar plates for colonies with an enhanced swarming phenotype. One mutant with this phenotype contained mini-Tn5lacZ1 inserted in an ORF that encoded a product with 90% identity to a Lon homologue from Photorhabdus luminescens and 89% identity to Lon from Yersinia pestis. The insertion disrupted this ORF at a position corresponding to aa 219 of the predicted 784 aa protein. Based on the genome sequence of HI4320 (http://www.sanger.ac.uk/Projects/P_mirabilis), the DNA upstream of the putative lon gene encoded an ORF that directed a product with 88% amino acid identity to the ClpX protease subunit, and downstream of lon was a putative hupB gene, encoding a product with 81% amino acid identity to the histone-like Hu-1 protein of E. coli. Therefore, the genetic organization of the region surrounding the P. mirabilis lon gene was identical to that seen in E. coli. In P. mirabilis, the hupB gene was separated by 210 nt from the 3’ end of the lon coding region; therefore, the insertion in lon was not likely to be polar on hupB.

Swarming migration of the PM2173 lon::mini-Tn5lacZ1 mutant was compared with that of the wild-type PM7002. Swarming was initiated at the same time as the wild-type; however, the migration rate of the lon mutant was 50% greater by the first consolidation period (Fig. 1a). At the end of 8 h of migration, the lon mutant had translocated 8.1 cm compared with 4.7 cm for the wild-type PM7002 cells.

Based on previous work demonstrating that a lon mutation in Vibrio parahaemolyticus resulted in constitutive swarmer cell differentiation in liquid medium (Stewart et al., 1997), normally a non-permissive condition, we examined the morphology of PM2173 lon::mini-Tn5lacZ1 in liquid medium. In contrast to wild-type PM7002, which contained undifferentiated cells, the culture of PM2173 contained cells that were highly elongated and very motile (Fig. 1b) and appeared identical to swarmer cells harvested from plates. The elongated cells (greater than 10 μm) represented 15.6% (20/128) of the total cell population in the PM2173 culture. One characteristic of a differentiated swarmer cell is the increased expression of flagellin (Belas, 1994). To obtain further evidence that the elongated cells observed in liquid cultures of the lon mutant were differentiated swarmer cells, the levels of flagellin were assayed in wild-type PM7002 and the PM2173 lon::mini-Tn5lacZ1 mutant in cells grown in liquid and harvested at mid-exponential phase (OD600=0.6). The overall levels of flagellin for the pooled cells were threefold higher in PM2173 cells than in wild-type PM7002 (Fig. 1c). It is important to note that,

---

**Fig. 1.** Effect of the lon::mini-Tn5lacZ1 mutation on swarming. (a) Swarming assays. Cultures of PM7002, PM2173 lon::mini-Tn5lacZ1 and PM2174 lon::mini-Tn5lacZ1, pPM-lon were grown to saturation in LB medium or LB medium containing 35 μg streptomycin ml⁻¹ (for PM2174) with shaking at 37 °C. Cells from each tube were adjusted to an OD600 of 1.5 and duplicate 1 μl spots for each strain were applied to the surface of a fresh LB agar (1.5%) plate without antibiotics. Plates were incubated at 37 °C and migration was measured at 30 min intervals. The reported values represent the means ± SD of duplicate samples for each strain. (b) The cell morphology of the liquid cultures at an OD600 of 0.6 was examined by phase-contrast microscopy. (c) Cells were pelleted at an OD600 of 0.6 for analysis of flagellin (FlaA) expression by Western blot analysis using an anti-FlaA antibody. Identical amounts of protein from cell lysates were loaded in each lane.
as this experiment was done with pooled cells and elongated cells only represented 15.6% of the total cell population, the actual increase in flagellin expression in each individual swarmer cell is likely to be much higher. Therefore, in *P. mirabilis*, as in *V. parahaemolyticus*, loss of the Lon protease allowed swarmer cell differentiation under non-permissive conditions.

To verify that the swarming and cell-elongation phenotypes were due to the *lon* mutation, we introduced pPM-lon, containing the cloned wild-type *lon* gene, back into PM2173. In PM2173/pPM-lon cells (PM2174), the levels of swarming were reduced to wild-type levels (Fig. 1a). For the phenotype of cell elongation in liquid, the presence of pPM-lon also fully complemented the *lon* mutation (Fig. 1b).

**Increased expression of genes in the flagellar cascade in a *lon* mutant background**

To examine the possible basis for the hyper-swarming phenotype of the *lon*::mini-Tn5lacZ1 mutant, the expression of representative class 1, 2 and 3 genes in the flagellar cascade was examined by Northern blot analysis (Fig. 2). Cells were harvested for RNA analysis by plating 150 μl of overnight cultures of PM7002 and PM2173 at an OD600 of 1.5 on individual LB agar plates, followed by isolation of cells from the plates at hourly intervals (T3 indicates 3 h after plating). The accumulation of mRNA for the class 1 master regulator *flhDC* at T3, when swarmer cell differentiation began, was similar in the wild-type PM7002 and PM2173 *lon*::mini-Tn5lacZ1 backgrounds and then declined significantly at T4 and T5 in wild-type cells. In contrast, the decrease in *flhDC* mRNA at T4 and T5 was not observed in the PM2173 mutant. The FlhDC-dependent class 2 gene *fliA* (σ28) and the *fliA*-dependent class 3 *fliA* gene also exhibited significantly enhanced mRNA accumulation at T4 and T5 in the *lon*::mini-Tn5lacZ1 background, when compared with wild-type (Fig. 2).

**FlhD accumulates to higher levels in a *lon* mutant**

Previous studies have indicated that both FlhD and FlhC are rapidly degraded in swarmer cells by an energy-dependent protease (Claret & Hughes, 2000b). To examine the possible role of Lon in the proteolysis of FlhD and FlhC, a FLAG epitope tag (DYKDDDDK) was placed at the N-terminus of FlhD and a His6 tag at the C-terminus of FlhC by PCR (see Methods) and antibodies to the respective tags were used to monitor protein accumulation. In addition, as the results in Fig. 2 indicated that transcription of *flhDC* was increased in the *lon* mutant, the tagged FlhDC proteins were expressed from a heterologous promoter (*E. coli lac*) that is not FlhDC regulated to uncouple transcription from the native promoter and allow changes in protein accumulation to be monitored independently of transcription. *P. mirabilis* PM7002 containing plasmid pFDCH1 with the tagged FlhDC proteins exhibited a hyper-swarming phenotype, indicating that the FLAG–FlhD and FlhC–His6 proteins retained biological function (data not shown). The levels of FLAG–FlhD and FlhC–His6 proteins were examined in wild-type PM7002 and PM2173 *lon*::mini-Tn5lacZ1 by Western blot analysis using anti-FLAG and anti-His antibodies. For these experiments (Fig. 3), cells were harvested from plates at T4, a time point where maximal expression of *lon* was observed based on lacZ fusion analysis (see Fig. 5). The levels of FlhD were clearly higher in the PM2173 *lon*::mini-Tn5lacZ1 background relative to wild-type PM7002 (Fig. 3). However, the level of the FlhC protein was similar in both PM2173 and wild-type PM7002. The half-life of FlhD was determined in both the wild-type and *lon* mutant backgrounds (data not shown). The half-life for FlhD was 8 min in the wild-type background and 32 min in the *lon* mutant background. For the FlhC protein, there was no difference in the wild-type or *lon* mutant backgrounds at 32 min and the levels were unchanged at this time point relative to the cells before inhibition of protein synthesis (data not shown). Therefore, FlhD is much less stable than FlhC and this instability is largely due to degradation by Lon.

**Fig. 2.** Effect of the *lon* mutation on flagellar gene expression. PM7002 and PM2173 were grown to saturation by shaking overnight at 37 °C in LB broth, adjusted to the same optical density and 10 μl aliquots were plated on LB agar plates to generate synchronously differentiating cells. At hourly intervals, cells were harvested from the plates by resuspending in cold LB broth and RNA was prepared from cells at each time point for Northern blot analysis using the indicated probes. Each lane contained 10 μg RNA and all samples were standardized to the same concentration before loading. For all time points, lane 1 represents wild-type PM7002 samples and lane 2 represents samples from PM2173 *lon*::mini-Tn5lacZ1.
Increased virulence gene expression in the lon mutant background

The expression of two virulence genes, zapA encoding an IgA metalloprotease and hpmBA encoding a haemolysin, was examined in fully differentiated swarmer cells (at \( T_6 \)) of the wild-type and lon mutant by Northern blot analysis (Fig. 4). For both transcripts, the levels of expression were at least fivefold higher in the lon mutant background when compared with wild-type.

Lon expression increases in differentiated swarmer cells

In the lon mutant, there was minimal difference in flagellar gene expression in cells that were in the early stages of differentiation (Fig. 2; \( T_3 \) time point), but a larger change was seen in cells that had already differentiated (Fig. 2; \( T_5 \) time point). This indicated that Lon expression might be increased at \( T_5 \) and could contribute to the decrease in flagellar gene expression at this point in swarming. To examine this possibility, we took advantage of the fact that the \( \text{lon}::\text{mini-Tn}5\text{lacZ1} \) insertion was in the correct orientation to create a single-copy \( \text{lon}::\text{lacZ} \) fusion.

Expression of the \( \text{lon}::\text{lacZ} \) fusion was measured at hourly intervals in synchronously differentiating cells of \( P. \) mirabilis. Peak levels of \( \text{lon}::\text{lacZ} \) expression were observed in cells from \( T_2 \) to \( T_3 \), when swarmer cell differentiation had just initiated (Fig. 5). At these time points, the levels of expression were modestly increased (60 % higher) from that at \( T_1 \). At time points when cells began to de-differentiate back to vegetative cells (\( T_7 \)), the levels of \( \text{lon}::\text{lacZ} \) expression dropped back to pre-swarming levels.
DISCUSSION

A transposon insertion within the Lon orthologue of P. mirabilis was identified based on the resulting hyper-swarming phenotype (Fig. 1). The basis for this phenotype was twofold. First, in the lon mutant background, there were higher levels of flhDC expression during swarmer cell differentiation. This allowed cells to maintain higher levels of flagellin expression at T3 and T5, a point when this would normally start to decrease (Fig. 2). Since FlhD₂C₂ also has an important role in swarmer cell differentiation (Furness et al., 1997), the prolonged expression of flhDC and possibly flIA ($\sigma^{28}$) maintains the expression of genes required for swarmer cell differentiation for longer than normal periods. Secondly, FlhD was present in higher amounts (Fig. 3) and had a significantly longer half-life in the lon mutant background. Previous studies examining the stability of P. mirabilis FlhD and FlhC in a heterologous E. coli host have revealed a role for the Lon protease in the degradation of both proteins (Claret & Hughes, 2000b). However, our data indicated that FlhD is the primary substrate of Lon in the native P. mirabilis host.

The prolonged flhDC mRNA accumulation during swarming in a lon mutant background was unexpected. However, as FlhD was stabilized in the lon mutant, the increase in flhDC mRNA accumulation may be due to a positive feedback mechanism from a downstream gene product that is dependent on FlhDC for expression. One candidate for this gene product is flIA ($\sigma^{28}$), whose expression is FlhDC-dependent. Previous studies in E. coli have shown that RNA polymerase containing FlIA can transcribe the flhDC promoter in a positive feedback loop (Clarke & Sperandio, 2005). Similar studies in P. mirabilis have not been reported and the role of FlIA in a feedback loop is speculative at this time. A second possibility is that another activator of flhDC is a substrate of Lon and accumulates in a lon mutant background, leading to prolonged flhDC expression. One candidate for this regulator is the leucine-responsive regulatory protein (Lrp), an activator of flhDC during swarmer cell differentiation (Hay et al., 1997). Lrp is also required for activation of the hpmAB operon (Fraser et al., 2002) and the potentially increased levels of Lrp in the lon mutant during swarmer cell differentiation may explain the higher levels of hmp expression at this time (Fig. 4). A second regulatory protein that may be degraded by Lon is UmoB, a protein of unknown function that is required for the expression of both flhDC and hmpBA (Dufour et al., 1998; Fraser et al., 2002). Further studies will be aimed at determining whether Lrp, UmoB or other proteins are additional substrates of the Lon protease.

ACKNOWLEDGEMENTS

We are grateful to Robert Belas for providing the FlIA antibody. This work was supported by a Merit Review Award from the Department of Veterans Affairs. P. N. R. is the recipient of a Research Career Development Award from the Department of Veterans Affairs.

REFERENCES


