Swarming-coupled expression of the Proteus mirabilis hpmBA haemolysin operon

Gillian M. Fraser, Laurent Claret, Richard Furness, Srishti Gupta and Colin Hughes

Author for correspondence: Colin Hughes. Tel: +44 1223 333732. Fax: +44 1223 333327.
e-mail: cmole.bio.cam.ac.uk

The HpmA haemolysin toxin of Proteus mirabilis is encoded by the hpmBA locus and its production is upregulated co-ordinately with the synthesis and assembly of flagella during differentiation into hyperflagellated swarm cells. Primer extension identified a σ70 promoter upstream of hpmB that was upregulated during swarming. Northern blotting indicated that this promoter region was also required for concomitant transcription of the immediately distal hpmA gene, and that the unstable hpmBA transcript generated a stable hpmA mRNA and an unstable hpmB mRNA. Transcriptional luxAB fusions to the DNA regions 5′ of the hpmB and hpmA genes confirmed that hpmB σ70 promoter activity increased in swarm cells, and that there was no independent hpmA promoter. Increased transcription of the hpmBA operon in swarm cells was dependent upon a 125 bp sequence 5′ of the σ70 promoter −35 hexamer. This sequence spans multiple putative binding sites for the leucine-responsive regulatory protein (Lrp), and band-shift assays with purified Lrp confirmed the presence of at least two such sites. The influence on hpmBA expression of the key swarming positive regulators FlhD2C2 (encoded by the flagellar master operon), Lrp, and the membrane-located upregulator of the master operon, UmoB, was examined. Overexpression of each of these regulators moderately increased hpmBA transcription in wild-type P. mirabilis, and the hpmBA operon was not expressed in any of the flhDC, lrp or umoB mutants. Expression in the mutants was not recovered by cross-complementation, i.e. by overexpression of FlhD2C2, Lrp or UmoB. Expression of the zapA protease virulence gene, which like hpmBA is also upregulated in swarm cells, did not require Lrp, but like flhDC it was upregulated by UmoB. The results indicate intersecting pathways of control linking virulence gene expression and swarm cell differentiation.

Keywords: flagella, LRP, toxin, virulence gene expression

INTRODUCTION

Proteus mirabilis undergoes swarming, multicellular behaviour in which differentiated long aseptate hyperflagellated swarm cells migrate over surfaces (Fraser et al., 2000). During swarming, P. mirabilis co-ordinately upregulates expression of the flagellar gene hierarchy and production of several virulence factors (Allison et al., 1992; Rozalski et al., 1997), including the haemolysin HpmA (Welch, 1987; Uphoff & Welch, 1990; Senior & Hughes, 1987), urease (Mobley et al., 1995), and Zap protease (Walker et al., 1999). In particular, HpmA production is severely reduced in non-swarming flagellar gene mutants (Gygi et al., 1995, 1997), suggesting that biogenesis of flagella and expression of the hpmBA haemolysin operon are coupled.

Several transcriptional regulators of swarming differentiation have been identified, including the FlhD2C2 activator complex encoded by the flagellar hierarchy master operon flhDC (Furness et al., 1997; Claret & Hughes, 2000a, b), the leucine-responsive regulatory protein (Lrp) (Hay et al., 1997), and the cell envelope Umo proteins, especially UmoB (Dufour et al., 1998). It seems that these proteins might act as part of a regulatory network that integrates a number of stimuli.
and co-regulates virulence and flagellar gene expression (Fraser & Hughes, 1999). While signals that govern expression of the flagellar gene hierarchy are assumed to funnel through flhDC, this need not be the case for the co-regulated virulence genes. In particular, Lrp appears to regulate both flbDC and hpmBA, as a non-swarming lrp::Tn5 mutant is non-haemolytic even when its hyperflagellation and swarming is recovered by overexpression of flhDC (Hay et al., 1997). In this report, we characterize hpmBA transcription, and investigate the influence of the swarm regulators Lrp, FlhD, C, and UmoB upon expression of hpmBA and also zapA.

**METHODS**

**Bacterial strains, recombinant DNA techniques and sequencing.** The Proteus mirabilis lrp::Tn5 (Hay et al., 1997) and umoB (Dufour et al., 1998) strains are derivatives of wild-type P. mirabilis U6450 (Allison & Hughes, 1991). Bacteria were grown at 37°C in Luria–Bertani (LB) broth or on LB agar supplemented with ampicillin (100 µg ml⁻¹), chloramphenicol (40 µg ml⁻¹) or spectinomycin (50 µg ml⁻¹), when necessary. Plasmids were introduced by electroporation. Swarming differentiation on seeding plates (Gygi et al., 1995) was initiated by spreading 200 µl stationary-phase LB cultures (approx. 5 × 10⁸ cells ml⁻¹) onto 8 cm diameter LB agar plates. Plasmid DNA was manipulated by standard techniques (Ausubel et al., 1988; Sambrook et al., 1989) and maintained in Escherichia coli XL-Blue (recA1 ΔlacIq ΔaraD13975 lysA1 ∆(M15Tn10); Stratagene). Plasmids are listed in Table 1. DNA sequence was obtained from double-stranded pBluescript II templates by the chain-terminating method (Sanger et al., 1977), using T7 DNA polymerase (Pharmacia), and was analysed using GCG software (Devereux et al., 1984). Database searches were performed at the NCBI using the BLAST network service (Altschul et al., 1990).

**Northern blot analysis.** Total cellular RNA was isolated using the hot phenol method (Koronakis & Hughes, 1988). Formamide/formaldehyde-denatured RNA (10 µg) was resolved by electrophoresis through 1:2% agarose formaldehyde gels (Sambrook et al., 1989) and transferred onto nitrocellulose membranes (Hybond-C; Amersham) by vacuum blotting (Pharmacia LKB). Duplicate blots were stained with methylene blue to ensure that rRNA levels in the samples were equivalent (not shown). Probes were a 1.5 kb Xhol–EcoRV fragment internal to hpmBA, a 0.8 kb AvaI fragment internal to flbDC, a 0.9 kb BamHI–HindIII fragment internal to lrp and a 1.1 kb XbaI–Xhol fragment internal to zapA. Probes were labelled with [32P]dATP (Amersham) by random priming (Feinberg & Vogelstein, 1984), and hybridization and detection were carried out as described by Gygi et al. (1993).

**Promoter mapping by primer extension.** Oligonucleotides A1 (5'-CGCCCCGAGGTGAAGTTAACG-3', complementary to nucleotides 15 to 38 of hpmA), B1 (5'-CAGCCATTAGTGTTAATAAACG-3', complementary to nucleotides 16 to 38 of hpmB), and B2 (5'-CCAGTCTCTTTATTG-3', complementary to nucleotides -304 to -321 5' of hpmB) were 5' end-labelled with [32P]dATP (Amersham) by T4 polynucleotide kinase. Primer extension reactions were performed as described by Dufour et al. (1998).

**Luciferase reporter assays.** Transcriptional fusion assays were performed as previously described (Gygi et al., 1995). P. mirabilis wild-type and the flbDCΩ strains carrying derivatives of the reporter plasmid pQF120 (Ronald et al., 1990) containing transcriptional fusions of putative hpmBA promoter regions to the luxAB genes of Vibrio fischeri were monitored in a Bio-Orbit 1253 single-tube luminometer in the presence of 0.02% (v/v) dodecanal (Aldrich). The baseline luciferase activities of wild-type cells carrying the pQF120 parental vector were subtracted from the activities produced by cells containing hpmBA-luxAB fusions. Each data collection point was replicated five times and the mean and standard error were calculated.

**Purification of N-terminally His-tagged Lrp protein.** The lrp gene was PCR-amplified with Pfu Polymerase (Fromega) from P. mirabilis U6450 chromosomal DNA using the mutagenizing oligonucleotide primers Lrp1 (5'-GAAAGAGAGTATACAT-

---

Table 1. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript IKS</td>
<td>Amp' expression vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGF74</td>
<td>pBluescript IKS PstI/KpnI hpmBA locus</td>
<td>This study</td>
</tr>
<tr>
<td>pGF72</td>
<td>pBluescript IKS ΔhpmBhpmA KpnI fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pQF120</td>
<td>Luciferase transcriptional fusion vector</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX712</td>
<td>pQF120 with 712 bp PstI/Xhol 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX415</td>
<td>pQF120 with 415 bp PCR1/Xhol 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX227</td>
<td>pQF120 with 227 bp PCR2/Xhol 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX183</td>
<td>pQF120 with 183 bp PCR3/Xhol 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX125</td>
<td>pQF120 with 125 bp Dral/Xhol 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX413</td>
<td>pQF120 with 413 bp Dral/Dral 5' hpmB insert</td>
<td>This study</td>
</tr>
<tr>
<td>pLUX430</td>
<td>pQF120 with 230 bp EcoRV/Dral 5' hpmA insert</td>
<td>This study</td>
</tr>
<tr>
<td>pET15b</td>
<td>Amp' T7 expression vector, 6 x His fusion</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>pET15b-lrp</td>
<td>pet15b lrp (lrp amplified using Lrp1 and Lrp2)</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD18</td>
<td>Amp' arabinose-inducible expression vector</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pBADflbDC</td>
<td>pBAD18 expressing flbDC</td>
<td>Hay et al. (1997)</td>
</tr>
<tr>
<td>pBADlrp</td>
<td>pBAD18lrp (lrp amplified using Lrp1 and Lrp2)</td>
<td>This study</td>
</tr>
<tr>
<td>pBSumoB</td>
<td>pBluescript IKS expressing umoB</td>
<td>Dufour et al. (1998)</td>
</tr>
</tbody>
</table>
ATGATTGATAT-3' (NdeI site) and Lrp2 (5'-GCACCTAATCATTAGGTTAGCGGTG-3'; BamHI site). The PCR fragment was inserted into pET15b (Novagen). N-His-Lrp was produced in E. coli BL21(DE3) (Studier & Moffatt, 1986), by induction at mid-exponential phase with 1 mM IPTG for 3 h. Protein purification was carried out under non-denaturing conditions using His-Bind Quick 900 columns (Novagen) as described by the manufacturer.

**Gel retardation assays.** DNA fragments were PCR-amplified with Fnu Polymerase (Promega) using pGF74 as a template. The downstream oligonucleotide primer B1 (5'-CAGGTTTATAGTTGAATAAAA-3') is internal to the hpmB gene. The upstream oligonucleotide primers hpm hpm Acc (5'-GATTGGTAGTTGCCCAGTTCTC-3') and hpm Clal (5'-CGTTTATTGATCGATAATTACTAAAAAG-3') were used to amplify the 239 bp fragment spanning nucleotides 473 to 711 and the 183 bp fragment from nucleotide 529 to 711 (see Fig. 4), respectively. PCR products were digested with XbaI, dephosphorylated with calf intestinal phosphatase and labelled using T4 polynucleotide kinase and [γ-32P]pATP. The control 157 bp labelled DNA fragment was obtained as described by Clarèt & Hughes (2000b). The 183 bp, 239 bp and 157 bp fragments were purified on a polyacrylamide gel as described by Sambrook and 157 bp fragments were purified on a polyacrylamide gel as described by Sambrook et al. (1989). Labelled fragments (0.02 pmol) in 10 µl buffer A (20 mM Tris/HCl pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 20%, v/v, glycerol, 100 µg BSA ml−1) were incubated with 0–30 ng purified N-His-Lrp. After 20 min incubation at room temperature, samples were run on a 6% polyacrylamide gel (2 h, 130 V, room temperature) containing 0.5× TBE; the gel was dried and autoradiographed.

**Generation of the P. mirabilis flhDC null mutant.** A 250 bp fragment between nucleotides 62 and 312 (HinP11 and HincII sites, respectively) of the 351 bp P. mirabilis flhD gene was removed and replaced by the 2 kbp spectinomycin resistance cassette (Ω) cassette (Fellay et al., 1987), creating the plasmid pRF103. The approximately 1 kbp flhDΩ locus of pRF103 was inserted into the suicide vector pG704 (Miller & Mekalanos, 1988). Conjugational transfer of the suicide plasmid from E. coli SM10::pZIP to P. mirabilis U6450, and isolation of flhDCΩ mutants, were carried out as described by Swihart & Welch (1990). Colony blots were probed with an approximately 1.4 kbp BamHI fragment of pG704 and an approximately 2 kbp HincII fragment of the Ω cassette.

**RESULTS**

**Identification of a swarming-regulated σ^70 promoter 5' of hpmBA**

DNA sequence analysis of the hpmBA operon has previously identified a putative σ^70 promoter 78 bp 5' of the proximal hpmB gene and a second weak match to the σ^70 promoter consensus 104 bp 5' of the distal hpmA gene, in the hpmB coding region (Uphoff & Welch, 1990). We identified a third potential promoter sequence (TAAA-N₁₅-GTACGAGGTTACGCGTGTTAGCGGTG-3'; BamHI site) and Lrp2 (5'-GCACCTAATCATTAGGTTAGCGGTG-3'; BamHI site). The PCR fragment was inserted into pET15b (Novagen). N-His-Lrp was produced in E. coli BL21(DE3) (Studier & Moffatt, 1986), by induction at mid-exponential phase with 1 mM IPTG for 3 h. Protein purification was carried out under non-denaturing conditions using His-Bind Quick 900 columns (Novagen) as described by the manufacturer.

A single extension product terminating 70 bp 5' of hpmB and 8 bp 3' of the −10 hexamer of the putative hpmB σ^70 promoter was detected in the swarm-cell RNA but not in that from the vegetative cells (Fig. 1a). No extension product was evident immediately 3' of the putative σ^70 promoter, and this was verified by carrying out primer extension with an oligonucleotide primer complementary to sequence 5' of the σ^70 consensus (not shown). Several swarm-cell-specific primer extension products terminated in the region 5' of hpmA (Fig. 1b), but none was compatible with transcription initiation from the putative hpmA σ^70 promoter. These data indicate that the σ^70 promoter 5' of the hpmB gene is the only promoter active and upregulated during swarming. They suggest that the hpmA mRNA might arise from processing of a bicistronic hpmBA transcript.

**Swarming-coupled transcription of hpmA requires the hpmB promoter region**

The primer extension data indicate that hpmBA might be transcribed as an operon. To establish whether the hpmB promoter region is indeed essential for the high-level transcription of hpmA during swarming, hpmA RNA was assayed following expression of the hpmBA operon with and without the hpmB 5' region. The plasmids pGF74, carrying hpmBA, preceded by 725 bp encompassing the hpmB promoter region, and pGF72, carrying hpmBA preceded by a 5' truncated hpmB allele lacking the hpmB promoter region and nucleotides 1 to 612 of hpmB, were introduced into wild-type P. mirabilis cells in parallel with the parental pBluescript vector. Total cellular RNA was prepared from differentiated swarm cells carrying the plasmids and Northern blots were probed with a Clal fragment internal to hpmA (Fig. 2). In cells carrying pGF74, the amount of hpmA mRNA was about 20-fold higher than that in cells carrying the pBluescript vector control, i.e. in which hpmA mRNA is produced by transcription of the chromosomal hpmBA locus. The amount of hpmA mRNA in cells carrying pGF72, encoding the 5'-truncated hpmBA determinant, was comparable to that in the control cells. There was therefore no swarming-regulated transcription of the plasmid-encoded hpmA in the absence of a cis hpmB promoter region. These data further support the hypothesis that hpmBA is transcribed as an operon from a swarming-regulated promoter 5' of hpmB.

**The hpmBA transcript yields a stable hpmA mRNA**

Previous Northern blot analysis of hpmA mRNA detected a major 5.2 kb transcript and a minor 6.9 kb transcript, both upregulated during swarming differentiation (Allison et al., 1992). The primer extension data presented here (Fig. 1) suggest that hpmB mRNA is also abundant, as expected from expression of an operon. To assess the size and abundance of the hpmBA transcript,
Fig. 1. Primer extension mapping of mRNA termini 5' of (a) hpmB and (b) hpmA. Wild-type vegetative (v) cells and differentiated swarm (s) cells were isolated from LB agar plates at 1-5 and 4 h after seeding, respectively. T, G, C and A indicate the DNA sequence lanes. (a) The hpmB transcript start site (+1) lies 8 bp 3' of a putative promoter which is compared with the consensus 70 promoter sequence. (b) Several transcript termini (*) were detected in the 284-base region 5' of hpmA. The previously suggested (Uphoff & Welch, 1990) -35 and -10 hexamers 104 bp 5' of the putative hpmA 70 promoter are boxed.

Fig. 2. Northern hybridization of a hpmA probe to total cellular RNA isolated from wild-type differentiated swarm cells carrying pBluescript (-), pGF74 (hpmBA) or pGF72 (ΔhpmBA). A 281- to 6583-base RNA ladder (Promega) was used to determine transcript size. Fluorographs were photographed using a Kodak DC-40 camera and transcripts were quantified using Kodak Digital Science 1D software.

total cellular RNA was prepared from wild-type P. mirabilis cells isolated from LB agar plates after 2, 3 and 4 h seeding differentiation, and Northern blot analysis was carried out using probes internal to hpmB and hpmA (Fig. 3). After a 4 h exposure of the fluorogram, an approximately 5 kb hpmA transcript was detected that was strongly induced during differentiation (Fig. 3, left-hand panel). However, no hpmBA or hpmB transcripts were visible. Only after a 240 h exposure of the fluorogram was an approximately 6-9 kb hpmBA transcript detected by the hpmB probe (Fig. 3, right-hand panel). This transcript was, as expected, also recognized by the hpmA probe (not shown). The hpmBA transcript was visible in the 2 h and 3 h samples, but was barely visible in the 4 h sample isolated from differentiated swarm cells. Degradation products of the hpmBA and hpmB transcripts, seen only after long exposure, were detected by the hpmB probe as a heterogeneous population migrating between about 0.2 and 6.9 kb, although most of the products appeared to be about 1.7 kb and smaller. This suggests that the hpmBA transcript might be cleaved to produce a stable approximately 5 kb hpmA transcript and a highly unstable approximately 1.7 kb hpmB transcript. Extensive attempts were made to generate stable hpmB/A and hpmBA' transcripts by...
deleting the \(hpmB\)–\(hpmA\) intergenic region and various segments of the \(hpmBA\) coding region such that predicted RNase cleavage sites were removed. We were unable to isolate any stable mRNA containing \(hpmB\) (not shown), suggesting that the degradation is not simple and might involve endonucleolytic cleavage of the \(hpmB\) mRNA at multiple sites.

Swarming-coupled \(hpmBA\) transcription requires regulatory sequences upstream of the \(hpmB\) \(\sigma^70\) promoter

To identify elements that might be involved in the transcriptional regulation of the \(P.\ mirabilis\ hpmBA\) operon during swarming, the DNA sequence of the 1195 bp region \(5'\) of \(hpmB\) was determined (GenBank accession number AJ250100). A 384 nt ORF was identified 697 bp \(5'\) of the \(hpmB\) initiation codon. The ORF encodes a putative 128 aa polypeptide with 85% and 48% identity, respectively, to the MutT nucleoside triphosphatases of \(E.\ coli\) and \(Proteus\ vulgaris\) (Akiyama et al., 1987; Kamath & Yanofsky, 1993). Several putative Lrp-binding sequences (Cui et al., 1995) were evident in the \(mutT\)–\(hpmB\) intergenic region (Fig. 4), which seems significant as a non-swarming \(P.\ mirabilis\) mutant unable to hyper-produce haemolysin or flagella has been reported to have a lesion in the \(lrp\) gene (Hay et al., 1997). Each of the putative Lrp-binding sequences \(5'\) of \(hpmB\) has four mismatches to the 15 bp consensus derived by Cui et al. (1995), i.e. approximately 73% identity.

To assess the elements required for swarming-coupled expression of \(hpmBA\), transcriptional fusions of regions \(5'\) of \(hpmB\) and of \(hpmA\) were made to the \(lux\)AB luciferase genes of \(Vibrio\ fischeri\). Two derivatives of the reporter plasmid pQF120 (Ronald et al., 1990) were initially constructed (Fig. 5a), one with the entire 712 bp non-coding region \(5'\) of \(hpmB\) (pLUX712), the second (pLUX125) with a truncated 125 bp region containing the \(hpmB\) \(\sigma^70\) promoter but lacking the \(\sigma^{28}\) consensus and three of the four putative Lrp-binding sites. These plasmids were introduced into wild-type \(P.\ mirabilis\) and its derivative non-swarming \(flhDC\) null mutant, which was constructed by deleting a 250 bp internal fragment of \(flhD\) and replacing it with the spectinomycin resistance omega (\(\Omega\)) cassette (see Methods).

Luciferase activity was monitored over the swarm cell differentiation cycle. In wild-type cells carrying pLUX712, activity was strongly upregulated, with peak activity in differentiated cells being about ninefold higher than in swarm cells carrying pLUX125 (Fig. 5a, b). There seemed to be no substantial plasmid copy number titration of positive or negative regulatory factors. Wild-type \(P.\ mirabilis\) carrying pLUX712 retained wild-type haemolytic activity over the differentiation cycle, suggesting that the presence of multiple copies of the \(hpmB\) upstream region did not disrupt regulation of the chromosomal \(hpmBA\). In the \(flhDC\) mutant, which grew like the wild-type but does not differentiate into swarm cells, peak luciferase activities in cells carrying either pLUX712 or pLUX125 were approximately 10-fold lower than that observed in wild-type cells carrying pLUX712 (Fig. 5a). The peak activity produced by pLUX125 in the wild-type was not significantly different to those of the \(flhDC\) mutant carrying either plasmid, and may reflect transcription from the \(hpmB\) promoter in the absence of a response to differentiation signals. The data suggest that regulatory sequences \(5'\) of the \(hpmB\) promoter are required for full transcriptional upregulation in response to swarming signals.

Three derivative reporter fusions, pLUX415, pLUX227 and pLUX183, contained truncations of the 712 bp \(hpmB\) upstream region (Fig. 5a). The largest, pLUX415, contained a \(5'\) truncation to 415 bp, retaining the putative \(\sigma^{28}\) consensus sequence, the four putative-Lrp-binding sites and the \(hpmB\) \(\sigma^70\) promoter. In wild-type cells it produced a peak luciferase activity comparable to pLUX712 (Fig. 5a), suggesting that the sequences required for full transcriptional upregulation during swarming are within this 415 bp region. Further \(5'\) truncation to 227 bp removed the potential \(\sigma^{28}\) –35 consensus and the putative Lrp1 site to generate pLUX227, which in the wild-type generated a peak luciferase activity about 30% of that produced by pLUX425 and pLUX712 (Fig. 5a).

Fig. 3. Northern hybridization of \(hpmA\) and \(hpmB\) probes to total cellular RNA isolated from differentiating wild-type cells collected 2, 3, and 4 h after seeding onto LB agar. The positions of the \(hpmA\) and \(hpmBA\) transcripts are indicated and \(hpmB\) degradation products are bracketed. Fluorography was carried out for 4 h (left panel) and 240 h (right panel). Transcript size was determined using a 281- to 6583-base RNA ladder (Promega).
Fig. 4. DNA sequence of the mutT–hpmB intergenic region showing the hpmB transcription start site, the putative σ70 and σ28 promoter sequences, and four putative Lrp-binding sites (Lrp 1–4).

Fig. 5. (a) Transcriptional fusions of the non-coding region 5' of hpmBA to luxAB. A 712 bp region 5' of hpmB encompassing putative promoter sequences (black boxes, σ70 and σ28) and four putative Lrp-binding sites (arrows, 1–4) was inserted 5' of luxAB in the reporter plasmid pQF120 (Ronald et al., 1990), creating pLUX712. Deletion derivatives were constructed by PCR (PCR1, PCR2 and PCR3) and/or digestion with restriction endonucleases: D, DraI; P, PstI; X, XhoI. Peak luciferase activities in relative light units (RLU) of wild-type P. mirabilis (WT) and the flhDC null mutant (flhDCΩ) carrying the transcriptional fusions (pLUX) are shown on the right. (b) Luciferase activity over the swarm-cell differentiation cycle of wild-type cells carrying pLUX712. Cells were isolated from seeded agar plates at hourly intervals between 2 and 7 h post-inoculation. Peak swarm cell differentiation was at 5 h.

Nevertheless, pLUX227 peak activity in wild-type was about sixfold higher than that in the flhDC mutant, indicating that the hpmB promoter on pLUX227 was still substantially upregulated in response to differentiation. Further truncation of the hpmB promoter region to 183 bp in pLUX183 removed the putative Lrp1 and Lrp2 sites and caused severe reduction in the promoter's activity, such that wild-type and flhDC cells carrying pLUX183 produced comparably low peak luciferase activities (Fig. 5a). In addition to these hpmB σ70 and σ28 promoter fusions, a luxAB transcriptional fusion was constructed containing a 413 bp region encompassing the hpmB σ28 consensus but not the σ70 promoter (pLUXD413). Peak luciferase activities in the wild-type and the flhDC mutant carrying pLUXD413 were comparably low (Fig. 5a), in agreement with the primer extension data (Fig. 1a, b), which indicated that the putative σ28 consensus is not an active promoter. A second transcriptional reporter plasmid containing the 230 bp region immediately 5' of hpmA (pLUXA230, not shown) was also constructed; it generated very low activities in wild-type and flhDC mutant cells, again indicating that there is probably not an independently regulated promoter immediately 5' of hpmA

Multiple Lrp binding to the hpmB promoter region

The fusion analysis suggested that sequences 5' of the hpmB σ70 promoter are needed for the transcriptional upregulation of the hpmBA operon during swarming. In particular, a region spanning two putative Lrp-binding sites (Lrp1 and Lrp2, Fig. 4) appears to be essential. To
establish if Lrp does indeed bind to this hpmB promoter region, interaction was assessed in band-shift experiments. Soluble N-terminally His-tagged Lrp protein was purified under non-denaturing conditions on a nickel affinity cartridge. It was incubated with a 183 bp DNA region spanning nucleotides 529 to 711 (Fig. 4) encompassing the putative Lrp sites 2, 3 and 4. Incubation was carried out in the presence of a 1000-fold excess of poly(dI-dC) non-specific competitor DNA. We estimated the concentration of His-Lrp needed for half saturation of the labelled DNA in repeated band-shift assays. For the smaller fragment (–123 to +60) this was 17 nM, for the larger fragment (–179 to +60) 5 nM. Fig. 6 shows that migration of the shorter fragment was retarded by increasing amounts of His-Lrp, indicating the formation of a nucleoprotein complex, while the larger fragment generated two retarded bands, indicating formation of two different complexes. Interactions of His-Lrp with a still larger fragment, the 239 bp region 5’ to 711, respectively (see Fig. 3, nucleotides 529 to 711 and 473 to 711, respectively) were incubated with 0–30 ng purified His-Lrp protein. A control was performed with a 157 bp DNA fragment carrying the upstream promoter region of the P. mirabilis flhB gene (Claret & Hughes, 2000b). These data indicate that His-Lrp binds specifically to both the 183 bp and 239 bp fragments, but there may be multiple interactions in each case; the data show that the 239 bp fragment contains at least one more site than the 183 bp fragment.

**Fig. 6.** Band-shift analysis of Lrp binding to the regulatory region 5’ of hpmBA. End-labelled DNA fragments of a 183 bp and a 239 bp region 5’ of hpmB encompassing Lrp sites 2, 3 and 4, respectively (see Fig. 3, nucleotides 529 to 711 and 473 to 711, respectively) were incubated with 0–30 ng purified His-Lrp protein. A control was performed with a labelled DNA fragment containing the 157 bp 5’ of the P. mirabilis flhB flagella gene (Claret & Hughes, 2000b). Samples were electrophoresed through a 6% polyacrylamide gel and DNA bands were visualized by fluorography.

Multifactorial regulation of virulence factors by FlhD,C, Lrp and UmoB

The cytosolic DNA-binding proteins Lrp and FlhD,C, and the UmoB cell envelope protein, have been identified as strong positive regulators of swarming differentiation (Hay et al., 1997; Furness et al., 1997; Claret & Hughes, 2000a; Dufour et al., 1998; Fraser & Hughes, 1999). Their influence on the swarming-coupled transcription of hpmBA was investigated by Northern blot analysis of mRNA isolated from differentiated wild-type swarm cells and the lrp::Tn5, umoBΩ, and flhDCΩ mutants carrying pBAD (–), pBAD/ flhDC (flhDC), pBAD/ lrp (lrp) or pBAD/ umoB (umoB). Cells were collected 4 h after seeding onto LB agar. Transcript size was determined using a 281- to 6583-base RNA ladder (Promega). Fluorographs were photographed using a Kodak DC-40 camera and transcripts were quantified using Kodak Digital Science 1D software.

**Fig. 7.** Northern hybridization of hpmA (left column), zapA (middle column) and flhDC (right column) probes to total cellular RNA isolated from differentiated wild-type swarm cells and the lrp::Tn5, umoBΩ, and flhDCΩ mutants carrying pBAD (–), pBAD/ flhDC (flhDC), pBAD/ lrp (lrp) or pBAD/ umoB (umoB). Cells were collected 4 h after seeding onto LB agar. Transcript size was determined using a 281- to 6583-base RNA ladder (Promega). Fluorographs were photographed using a Kodak DC-40 camera and transcripts were quantified using Kodak Digital Science 1D software.
transcription of the flhDC flagellar master operon is upregulated by the overexpression of UmoB (Dufour et al., 1998), and the swarming and cell elongation defects of the lrp mutant are recovered by overexpression of FlhDC.C2 (Hay et al., 1997). To investigate the possibility that hpmA transcription could be increased by similar overexpression of FlhDC.C2, Lrp or UmoB, the recombinant expression plasmids pBADflhDC (Hay et al., 1997), pBADlrp and pBSumoB (Dufour et al., 1998) were introduced in parallel into the wild-type strain and the flhDC, umoB and lrp mutants. Northern blot analysis was again carried out to hpmA and, as controls, to flhDC and lrp. Overproduction of FlhDC.C2, Lrp and UmoB in the wild-type increased hpmA and flhDC mRNA levels by three- to fivefold (Fig. 7), and lrp transcription was also slightly increased (not shown). However, transcription of hpmA was not recovered in the flhDC, umoB and lrp mutants, suggesting that regulation of haemolysin expression during swarming is controlled by a network of regulators and not by a single pathway. Transcription of flhDC was partially recovered in the lrp mutant overexpressing UmoB (Fig. 7).

In addition to the upregulation of hpmBA expression during swarming, several other virulence factors are hyper-produced, e.g. the Zap protease (Wassif et al., 1995). To discover if hpmBA and the zapA protease gene were similarly regulated, parallel Northern blots using a probe internal to zapA were also carried out (Fig. 7). As with hpmA, zapA mRNA could not be detected in the flhDC and umoB regulator mutants; however, zapA transcription was only slightly reduced in the lrp mutant. Transcription of zapA could be increased in the flhDC and lrp mutants by overexpression of UmoB, suggesting that zapA might be directly regulated by UmoB.

**DISCUSSION**

The production of several virulence proteins is upregulated during the Proteus swarm cycle. The synthesis and secretion of HpmA haemolysin is modulated in parallel with expression of the flagellar gene hierarchy, with haemolytic activity approximately 20-fold higher in hyper-flagellated swarm cells than that in vegetative cells (Allison et al., 1992). Coordinate regulation of virulence gene expression and flagellar biogenesis has been observed in other enterobacteria. In Salmonella enterica serovar Typhimurium, expression of the hilA activator of invasion gene transcription is upregulated by FlIZ, a poorly characterized flagellar protein that positively regulates class 2 flagellar genes (Lucas et al., 2000; Iyoda et al., 2001). In Serratia liquefaciens and Yersinia enterocolitica, the genes encoding phosphohosphate are transcribed as part of the flagellar regulon from promoters recognized by the flagellar-specific sigma factor σ28 (Givskov et al., 1995; Schmiel et al., 2000). While a putative σ28 promoter sequence has been identified S’ of the P. mirabilis hpmB gene, our primer extension analyses indicated that hpmB is not transcribed from this promoter, but rather from a σ70 promoter sequence 78 bp S’ of hpmB that is substantially upregulated in differentiated swarm cells. It has been reported (Ide et al., 1999) that active σ28 promoters in S. typhimurium and E. coli feature an extended –35 motif that is not present in sequences that are otherwise homologous to the consensus described by Helmann & Chamberlin (1987). The revised consensus is TAAAGTTT-N6-GCCGATAAA compared to the old consensus TAAAN13-GCCGATAAA. In agreement with our experimental analyses, the suggested σ28 promoter S’ of hpmB does not have the extended –35 sequence. Although several transcript extension products were detected immediately S’ of hpmA, none was compatible with initiation from the putative σ28 promoter sequence identified by Uphoff & Welch (1990). The distance between the most abundant extension product and the putative hpmA σ28 –10 hexamer was 12 bp, larger than the typical 5–9 bp. No promoter consensus sequences were found S’ of any of the hpmA primer extension products, and it might be that these mRNA termini result from endonucleolytic cleavage of a bicistronic hpmBA operon transcript originating from the swarm-upregulated hpmB promoter. This view was strengthened by Northern blot analysis of hpmA mRNA isolated from cells carrying, in trans, either the full-length hpmBA determinant or a S’ truncated hpmBA lacking the hpmB promoter region, which indicated that there was no independently regulated promoter immediately S’ of hpmA.

During differentiation, the steady-state levels of hpmA mRNA were considerably higher than the levels of hpmB mRNA. This was somewhat unexpected, as the primer extension data suggest that the hpmB transcript is abundant. However, abundance of the hpmB CDNA extension product does not necessarily reflect the stability of the hpmBA messenger, as a successful primer extension would only require hpmB mRNA fragments containing the first 200 bases. When considered together, the primer extension and Northern blot data indicate that an unstable full-length hpmBA transcript is processed to produce a stable hpmA mRNA and an unstable hpmB mRNA. Similar processing of polycistronic transcripts has been widely observed: e.g. the E. coli fisZ and malEF transcripts, the Salmonella histidine transport operon, and the extensively studied Rhodobacter capsulatus psf operon (Cam et al., 1996; Newbury et al., 1987; Stern et al., 1988; Klug, 1993). In the case of the R. capsulatus psfQBA LMX transcript, which encodes proteins involved in photosynthesis, processing of the mRNA produces transcript segments with different stabilities and this subsequently influences translation and the stoichiometry of the proteins in the photosynthetic complexes (Klug, 1993). Similarly, processing of the P. mirabilis hpmBA transcript might be one of the mechanisms that regulates the ratio of the HpmA haemolysin and its cognate exporter/activator protein, HpmB. These proteins are highly similar to ShlA and ShlB of Serratia marcescens. ShlB has been shown to form pores in artificial lipid bilayer membranes, and it is thought to form a channel in the bacterial outer membrane through which the ShlA haemolysin is exported (Konninger et al., 1999). It seems unlikely that
the HpmB (ShB) exporter and HpmA (ShA) haemolysin would be required in equimolar amounts, and limiting the production of the pore-forming outer-membrane exporter while producing large amounts of exported haemolysin might bring maximal benefits to the bacterial cell.

As hpmBA is most likely transcribed as an operon, we investigated the role of sequences upstream of hpmB in the modulation of its transcription during the swarm cycle. Analysis of luxAB transcripational fusions to the non-coding region upstream of hpmB showed that this 697 bp region can be truncated to 415 bp without affecting maximal promoter activity in swarm cells. This 415 bp region encompasses four putative consensus sequences for Lrp (Fig. 4, Lrp1–Lrp4). Further truncation of the hpmB upstream region, removing the Lrp1 site, reduced maximal promoter activity to 50%, and additional removal of the Lrp2 site abolished upregulation of promoter activity during swarming. This suggests that while the putative Lrp1 site enhances, but is not essential for, upregulation of the hpmB operon promoter, the region spanning the Lrp2 site is absolutely required. Binding of Lrp to the hpmB upstream region was confirmed in vitro band-shift experiments, and the data indicate that there are at least two Lrp-binding sites in the 239 bp region encompassing the putative Lrp2, Lrp3 and Lrp4 consensus sequences, and at least one binding site in the 183 bp region that includes the Lrp3 and Lrp4 sequences. There may be further Lrp-binding sites in the larger 415 bp region which carries all four of the putative Lrp consensus sequences, as this fragment formed a very large nucleoprotein complex that did not migrate into the native polyacrylamide gel. Previously characterized examples of Lrp binding to promoter regions (Gazeau et al., 1994; Cui et al., 1995; Rhee et al., 1996) show that Lrp often binds at multiple sites, and that binding sometimes shows a degree of cooperativity. Therefore, we cannot exclude the possibility that the 183 bp hpmB upstream fragment contains more than one Lrp-binding site, and the 239 bp region contains more than two sites. Our results suggest that Lrp acts directly to activate the transcription of this operon. As the Lrp-binding sites 2, 3 and 4 upstream of hpmB are within 30 bp of each other, it seems possible that they bind Lrp cooperatively.

Lrp is only one component of a regulatory network that also includes the FlhD$_2$C$_2$ and UmoB proteins, both of which have key roles in the control of flagellar biogenesis during swarming (Furness et al., 1997; Dufour et al., 1998). Less is known about their involvement in regulating the production of virulence factors such as the HpmA haemolysin. We investigated the influence of Lrp, FlhD$_2$C$_2$ and UmoB on the transcription of hpmBA during the swarm cycle and found that they were all essential, as the hpmA transcript was not detected in any of the regulator mutants. In wild-type Proteus mirabilis, the peak level of hpmA transcript could be moderately increased by overexpression of lrp, flhDC and umoB, and while hpmA expression could be recovered in the regulator mutants by direct complementation (not shown), it was not restored by ‘cross-complementation’ by the other regulators, e.g. by overexpression of flhDC in the lrp null mutant, which overcomes the cell elongation and swarming defects of this mutant but does not restore haemolytic activity (Hay et al., 1997). This suggests that while Lrp, FlhD$_2$C$_2$ and UmoB can positively influence transcription of hpmBA, there is not a single pathway that controls haemolysin expression during swarming. Rather, regulation of hpmBA appears to be complex and multifactorial. The present study and previously published data suggest that Lrp directly regulates transcription of hpmBA while FlhD$_2$C$_2$ and UmoB could be acting indirectly. In parallel with our studies of hpmBA expression, we investigated briefly the effects of Lrp, FlhD$_2$C$_2$ and UmoB on transcription of the zapA protease gene. Unlike the HpmA haemolysin, which is maximally produced in actively swarming cells, peak production of the Zap protease occurs slightly later in the swarm cycle, in differentiated cells that are about to enter the consolidation phase (Walker et al., 1999). We found that zapA regulation is markedly different to that of hpmBA, as Lrp is not required for zapA upregulation during swarming; instead, zapA appears to be primarily regulated by UmoB.

The data presented outline several components of hpmBA regulation, including differential mRNA stability, and indicate direct and indirect roles for several proteins in upregulating the single $\sigma^{26}$ operon promoter during swarming differentiation. Taken with the initial characterization of zapA expression, the data strengthen a view of swarming as a major physiological shift, involving a complex network of regulation.

REFERENCES


Received 13 February 2002; revised 26 March 2002; accepted 27 March 2002.