Regulation of bacterial motility in response to low pH in *Escherichia coli*: the role of H-NS protein

Olga A. Soutourina,† Evelyne Krin, Christine Laurent-Winter, Florence Hommais, Antoine Danchin and Philippe N. Bertin‡

The effect of detrimental conditions on bacterial motility in *Escherichia coli* was investigated. Expression profiling of mutant *E. coli* strains by DNA arrays and analysis of phenotypic traits demonstrated that motility and low-pH resistance are coordinately regulated. Analysis of transcriptional fusions suggests that bacterial motility in response to an acidic environment is mediated via the control by H-NS of *flhDC* expression. Moreover, the results suggested that the presence of an extended mRNA 5’ end and DNA topology are required in this process. Finally, the presence of a similar regulatory region in several Gram-negative bacteria implies that this mechanism is largely conserved.

Keywords: acidic pH, osmolarity, DNA supercoiling, DNA array

INTRODUCTION

Complex cellular responses are often controlled by regulatory networks in which transcription factors regulate the expression of a diverse set of target genes. In eukaryotes such complex systems are implicated in cell differentiation into specialized tissues and in the maintenance of tissue homeostasis (Arnold & Winter, 1998; Kawakami et al., 2000; Relaix & Buckingham, 1999). In prokaryotes, multiple regulatory networks are usually organized in a similar way to those in higher organisms, with master regulatory genes at the top of the hierarchy, ensuring adequate responses and/or transformation into another stable cellular state to fine tune cellular metabolism. As an example, RpoS is a central element that governs the expression of bacterial stress- or stationary-phase-induced genes (Hengge-Aronis, 1999). Studies of genetic circuits such as those involved in bacteriophage infection, chemotaxis or the cell division cycle in prokaryotes have also provided important insight into the understanding of regulatory networks (Huang, 1999).

Bacterial flagellum biosynthesis is under the control of the *flhDC* master operon, which governs motility and chemotaxis, as well as differentiation into swarming cells in enterobacteria. Moreover, this operon ensures global communication between flagellar genes and external factors, as well as cell division (Aizawa & Kubori, 1998). The complex motility and chemotaxis system in *Escherichia coli* includes nearly 50 genes organized in an ordered cascade in which the expression of a gene located at a given level requires the transcription of another one at a higher level (Macnab, 1996). This system is subject to a complex regulation by multiple environmental factors and regulatory proteins. For example, flagellum biosynthesis is sensitive to catabolite repression (Adler & Templeton, 1967; Silverman & Simon, 1974; Yokota & Gots, 1970) and is inhibited by stressful environmental conditions, such as increased temperature and high osmolarity (Adler & Templeton, 1967; Li et al., 1993). Moreover, numerous mutations in stress-related genes, such as those encoding heat-shock proteins, membrane components or DNA replication initiation factors, are known to affect motility (Farr et al., 1989; Kitamura et al., 1994; Mizushima et al., 1995, 1997; Shi et al., 1992, 1993a) by repressing transcription of the *flhDC* master operon (Mizushima et al., 1995, 1997; Shi et al., 1993b). Unlike *flhDC* transcriptional control by cAMP–CAP complex (Soutourina et al., 1999), the mechanism by which stress-related conditions affect master operon expression remains still largely unknown.

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Abbreviation: CAT, chloramphenicol acetyltransferase.
H-NS is a nucleoid-associated protein known to be involved in the control of motility in E. coli (Bertin et al., 1994) and in Salmonella typhimurium (Hinton et al., 1992). This protein positively controls the master flagellar operon but the mechanism of this regulation remains unclear (Kutsukake, 1997; Soutourina et al., 1999). In the present study we demonstrated that flagellar gene expression is inhibited under low-pH conditions and that this regulation of the flhDC master operon may be dependent on the H-NS protein. For the first time, we provide evidence that the 5' end of mRNA plays a crucial role in flhDC expression in response to environmental factors.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria–Bertani (LB), tryptone, M9 or M63 media (all media as in Miller, 1992), supplemented as indicated with 0.1% (w/v) Casamino acids, 0.4% (w/v) sodium succinate, 0.4% (w/v) glycerol or 0.4% (w/v) glucose as a carbon source. Tryptone swarm plates containing 1% Bacto-tryptone, 0.5% NaCl and 0.3% Bacto-agar were used to test bacterial motility as previously described (Bertin et al., 1999), except for plates at pH 4.6, which contained 0.5% Bacto-agar. Metabolism of β-glucosides was tested on MacConkey indicator plates with 1% salcin as a carbon source. When required, antibiotics were added at the following concentrations: ampicillin, 50 µg ml⁻¹; and kanamycin, 20 µg ml⁻¹. All experiments were performed in accordance with the European regulation requirements concerning the contents of Genetically Modified Organisms of Group I (agreement no. 2735).

Chloramphenicol acetyltransferase (CAT) assay. Strains were grown in tryptone medium supplemented with sodium succinate or M9 medium supplemented with Casamino acids and glycerol at pH 7.0 or 4.6 to an OD₆₀₀ of 0.15 to 0.3. CAT activity was measured with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) on cell extracts as previously described (Soutourina et al., 1999).

Resistance to low pH. Strains were grown to stationary phase overnight in M9 medium, pH 5.5, supplemented with glucose and Casamino acids. Acidic stress was analysed in M9 medium at pH 2.5 supplemented with 0.012% glutamate as previously described (Hommais et al., 2001).

Two-dimensional gel electrophoresis. Strains were grown in M9 medium supplemented with Casamino acids and glycerol at pH 7.0 or 4.6 to an OD₆₀₀ of 0.7. Total protein extracts and two-dimensional gel electrophoresis were carried out as previously described (Hommais et al., 2001; Laurent-Winter et al., 1997).

Expression profiling. Bacterial cells were grown in M63 minimal medium supplemented with glucose (Miller, 1992) to an OD₆₀₀ of 0.6. Handling of RNA, cDNA synthesis from 10 µg RNA, hybridization on DNA arrays (Panorama E. coli gene arrays from Sigma-GenoSys Biotechnologies) and data analysis were performed as previously described (Hommais et al., 2001). Briefly, hybridization probes were generated from 10 µg RNA following standard cDNA synthesis using [32P]dCTP (7.4 × 10⁷ to 1 × 10⁸ Bq mmol⁻¹, New England Nuclear), AMV reverse transcriptase (Roche) and E. coli labelling primers (Sigma-Genosys). The prehybridization and hybridization were carried out according to the manufacturer's recommendations with some modifications (Hommais et al., 2001). Blots were exposed to PhosphorImager screen (Molecular Dynamics) and were then scanned on a 445SI PhosphorImager. The intensity of each dot was measured with the XDOTSREADER software (Cose) and analysed using an Excel spreadsheet.

In vitro transcription assays. In vitro transcription experiments were performed with pDIA546 containing the entire flhDC regulatory region as previously described (Soutourina et al., 1999). Plasmid pDIA546 was restricted by EcoRI for 2 h at 37°C and used as linearized template for in vitro transcription.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>Wild-type</td>
<td>CGSC 6300</td>
</tr>
<tr>
<td>PS2209</td>
<td>Wild-type</td>
<td>Bertin et al. (1994)</td>
</tr>
<tr>
<td>PS2652</td>
<td>PS2209 hns-1001</td>
<td>Bertin et al. (1994)</td>
</tr>
<tr>
<td>FB8</td>
<td>Wild-type</td>
<td>Bruni et al. (1977)</td>
</tr>
<tr>
<td>BE1410</td>
<td>FB8 hns-1001</td>
<td>Laurent-Winter et al.(1997)</td>
</tr>
<tr>
<td>BE2120</td>
<td>hns-1001 carrying a mot° suppressor mutation</td>
<td>This study</td>
</tr>
<tr>
<td>BE2121</td>
<td>hns-1001 carrying a mot° suppressor mutation</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDIA28</td>
<td>pKK232-8-derivative carrying the flhDC promoter (nt – 213 to +78)</td>
<td>Soutourina et al. (1999)</td>
</tr>
<tr>
<td>pDIA545</td>
<td>pKK232-8 derivative carrying the flhDC regulatory region (nt – 213 to +205)</td>
<td>Soutourina et al. (1999)</td>
</tr>
<tr>
<td>pDIA346</td>
<td>pJCD01 derivative carrying the flhDC regulatory region (nt – 213 to +202)</td>
<td>Soutourina et al. (1999)</td>
</tr>
<tr>
<td>pDIA359</td>
<td>pKK232-8-derivative carrying the flhC promoter region</td>
<td>Soutourina et al. (1999)</td>
</tr>
<tr>
<td>pPM61</td>
<td>ColE1 derivative multicopy expression vector carrying the flhDC operon under its native promoter</td>
<td>Bartlett et al. (1988)</td>
</tr>
</tbody>
</table>
RESULTS

Isolation and characterization of suppressor mutations

The comparative analysis of expression profiles in E. coli wild-type and hns strains (Hommais et al., 2001) revealed two major alterations in the hns background: on the one hand, a decrease in flagellar gene expression, and on the other hand, an increase in the acidic pH resistance gene expression, suggesting that these two H-NS-controlled phenotypes may be related to each other. To test this possible link, we isolated and characterized in an hns background suppressor mutations with regard to motility. Several spontaneous mutants showing H-NS-independent swarming were isolated at a frequency of about $10^{-9}$ by incubating hns strains on semi-solid agar plates at $30^\circ C$ for 24–48 h, and two of them were purified. Attempts to locate these suppressor mutations by genetic techniques were unsuccessful, which suggests that they may involve more than one gene, e.g. an

Table 2. Effect of suppressor mutations on various H-NS-related phenotypes

<table>
<thead>
<tr>
<th>Strain (phenotype)</th>
<th>Mucoidy*</th>
<th>$\beta$-Glucoside utilization†</th>
<th>Motility‡</th>
<th>Survival (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB8 (wild-type)</td>
<td>—</td>
<td>—</td>
<td>30 + 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BE1410 (hns)</td>
<td>+</td>
<td>+</td>
<td>3 + 1</td>
<td>24 + 4</td>
</tr>
<tr>
<td>BE2120 (mor*)</td>
<td>+</td>
<td>+</td>
<td>18 + 2</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>BE2121 (mor*)</td>
<td>+</td>
<td>+</td>
<td>21 + 2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* +, Mucoid phenotype observed on LB medium; —, no mucoid phenotype.
† +, Ability to metabolize $\beta$-glucoside revealed by the appearance of red colonies on MacConkey-salicyl agar plates; —, lack of ability to metabolize $\beta$-glucoside, indicated by white colonies.
‡ Diameter of the swarming ring (in mm) after 15 h at 30°C. Data are the mean values ± standard deviations of three independent experiments.
§ Percentage survival is calculated as $100 \times$ number of c.f.u. per ml remaining after low-pH treatment divided by the initial c.f.u. per ml at time zero. Data are the mean values of three independent experiments.

Table 3. Expression profiling by DNA arrays of various E. coli strains

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression level* in strain (phenotype):</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FB8 (wild-type)</td>
<td>BE1410 (hns)</td>
</tr>
<tr>
<td>$\beta$gB</td>
<td>0.80</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$gD</td>
<td>0.67</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$gG</td>
<td>0.75</td>
<td>0.29</td>
</tr>
<tr>
<td>$\beta$gK</td>
<td>1.02</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$gl</td>
<td>1.02</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$hA</td>
<td>0.72</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$hC</td>
<td>7.04</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$hD</td>
<td>1.57</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$hI</td>
<td>1.10</td>
<td>0.12</td>
</tr>
<tr>
<td>$\beta$hK</td>
<td>0.83</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$gB</td>
<td>0.57</td>
<td>16.98</td>
</tr>
<tr>
<td>$\beta$gB</td>
<td>0.43</td>
<td>15.16</td>
</tr>
<tr>
<td>$\beta$gC</td>
<td>0.26</td>
<td>8.91</td>
</tr>
<tr>
<td>$\beta$gX</td>
<td>—</td>
<td>3.48</td>
</tr>
<tr>
<td>gdeA</td>
<td>0.36</td>
<td>10.67</td>
</tr>
<tr>
<td>gdeB</td>
<td>—</td>
<td>9.42</td>
</tr>
<tr>
<td>gdeD</td>
<td>—</td>
<td>3.17</td>
</tr>
</tbody>
</table>

* Expression level (arbitrary units) was measured as previously described (Hommais et al., 2001). —, Below the background level.
essential locus with an additional compensatory mutation. The impossibility of transferring these mutations to other strains by P1 transduction or of obtaining strains with similar phenotype by transposon insertion further supports the possible existence of distant mutations. The presence of such multiple genetic alterations could be explained by the high frequency of spontaneous mutation observed in \textit{hns} strains (Lejeune & Danchin, 1990). A similar failure to characterize suppressor mutations involved in the thermoregulation of motility has been reported in \textit{Yersinia enterocolitica} (Rohde \textit{et al.}, 1994).

To investigate the effect of the suppressor mutations on the physiology of \textit{E. coli}, we tested various H-NS-related phenotypes in strains BE2120 and BE2121. Like the original \textit{hns} mutant, both strains remained mucoid and able to use salicin as a carbon source (Table 2). In contrast, the mutations reversed, as expected, the loss of motility on semi-solid medium. Surprisingly, the suppressor mutants also showed a strong susceptibility to low pH, similar to that of the wild-type strain (Table 2).

To address the mechanism underlying the possible link between loss of motility and low pH resistance, expression profiling was performed using DNA arrays. As seen in Table 3, the major differences concerned the expression of genes involved in flagellum biosynthesis and in low pH resistance. Indeed, the transcript level of many flagellar genes measured in suppressor strains BE2120 and BE2121 was close to that in the wild-type strain. More importantly, a strong reduction in the expression of the \textit{gad} and \textit{hde} genes involved in resistance to low pH (Hommais \textit{et al.}, 2001) was observed in these mutants in comparison with the \textit{hns} strain (Table 3). These results are consistent with the alteration of motility and acid resistance we observed (Table 2) and support a coordinate regulation by H-NS of both processes in \textit{E. coli}.

The analysis of expression profiles of the suppressor mutants did not allow us to identify an altered expression of genes known to be involved in the control of motility, i.e. CsrA- or HdfR-encoding genes (Ko & Park, 2000; Romeo, 1998; Wei \textit{et al.}, 2001). Furthermore, both genes were not mutated in the suppressor strains (data not shown). Finally, spontaneous mutants were also obtained in an \textit{hns stpA} double mutant context (data not shown), suggesting that the motility reversion process we observed is independent of the presence of StpA, in agreement with our recent data (Bertin \textit{et al.}, 2001). Taken together, these observations suggest that these proteins do not play any role in the H-NS-regulated control of motility and low pH resistance.

*Regulation of motility under acidic pH conditions*

In \textit{E. coli}, the optimum pH for motility and chemotaxis is close to that for growth (Adler, 1973; Adler & Templeton, 1967). However, the control of bacterial motility by acidic pH, which can reflect the growth conditions frequently encountered by enterobacteria inside their host (Mahan \textit{et al.}, 1996), has not yet been well documented. To investigate the direct effect of low pH on motility, we tested the swarming behaviour of wild-type \textit{E. coli} on semi-solid plates at pH 7.0 and at pH 4.6. Plates were incubated for 13–15 h at 30 °C. (B) Protein extracts of wild-type strain MG1655 grown at pH 7.0 and at pH 4.6 were resolved by two-dimensional electrophoresis and silver stained. Only the region in the vicinity of Flic is shown. Flic is indicated by an arrow. To facilitate the comparison, NusA and PtsI are indicated as landmarks.
Role of H-NS in *flhDC* regulation by acidic pH

neutral pH. It has been proposed that disintegration of flagella into subunits occurs at acidic pH (Stocker & Campbell, 1959; Weibull, 1948). However, to prevent unnecessary energy consumption—the cost to cell of flagellar synthesis is about 2% of total biosynthetic energy expenditure (Macnab, 1996)–it can be assumed that detrimental conditions such as acidic pH repress expression of flagellar genes. To test this hypothesis, we measured the expression level of the flagellin-encoding gene using a *flIC-cat* transcriptional fusion under neutral and acidic pH conditions. A sevenfold decrease in *flIC* transcription was observed at acidic as compared to neutral pH: CAT activities of 530 ± 30 and 68 ± 8 units were measured at pH 7.0 and pH 4.6, respectively (1 unit corresponds to 1 µmol chloramphenicol acetylated per min per µg protein).

**Regulation of the *flhDC* expression by low pH**

The *flhDC* master operon, located at the top of the flagellum biosynthesis cascade, constitutes the major target for regulatory proteins, such as H-NS and cAMP-CAP (Soutourina et al., 1999). To determine whether the control by acidic pH affected bacterial motility by downregulating the expression of the master operon, the motility of an *E. coli* wild-type strain overexpressing the *flhDC* operon (Table 1) was assayed under low-pH conditions. FlhDC overproduction from plasmid pPM61 resulted in a partial restoration of the motility defect (data not shown), suggesting that low pH affects the motility via the master regulator, as observed with some other environmental factors (Shi et al., 1993b). This hypothesis was tested with CAT transcriptional fusions carrying either the *flhDC* promoter region alone or the extended regulatory region that includes the mRNA untranslated 5′ end (Fig. 2). Our previous studies (Soutourina et al., 1999) have demonstrated a crucial role for the *flhDC* 5′ end in the positive control of the flagellar master operon by H-NS. In the wild-type strain, similar values were obtained under neutral and low-pH conditions from the transcriptional fusion carrying the promoter region alone on plasmid pDIA528. In contrast, a more than threefold decrease in CAT activity was measured under acidic pH in comparison with neutral pH from the transcriptional fusion carrying the entire *flhDC* regulatory region on plasmid pDIA545. A similar reduction in *flhDC* expression has been previously measured in *E. coli* expression has been previously measured in *E. coli* expressing in the presence of an extended regulatory region that includes the mRNA end region. Such a reduction in *flhDC* expression was observed in the presence of 200 µM novobiocin activity from the *flhDC–lacZ* fusion (Soutourina et al., 1999). Moreover, a comparative analysis of the plasmid content revealed no difference between wild-type and *hns* strains (data not shown). This suggests that the reduced level of *cat* activity measured in the *hns* strain did not result from any effect of the mutation on the plasmid copy number of pKK232-8 derivatives, in accordance with the results obtained with plasmid pGR71 carrying the same origin of replication (Bertin et al., 1992). More importantly, no further reduction in transcription was observed in the *hns* mutant under low pH (Fig. 2), indicating that the effects of low pH and H-NS deficiency are not additive. This suggests that the control of bacterial motility in response to acidic pH is mediated by the H-NS protein. These results are in accordance with the coordinate regulation of motility and low pH response by H-NS protein observed in suppressor mutants (Tables 2 and 3).

One possibility that could explain the role of H-NS in the control of gene expression is its implication in DNA topology (Higgins et al., 1988; Dorman et al., 2001). Moreover, some environmental factors or drugs that are known to inhibit bacterial motility also affect DNA supercoiling, e.g. high salt concentration or novobiocin (Anderson & Bauer, 1978; Goldstein & Drlica, 1984; Higgins et al., 1988; Shi et al., 1993b). The overexpression of DNA gyrase subunits resulting in a partial restoration of motility in an *hns* mutant (data not shown). Furthermore, in the presence of novobiocin, a DNA gyrase inhibitor decreasing DNA supercoiling, we observed a more than twofold decrease in *flhDC* activity from the *flhDC* transcriptional fusion containing the entire regulatory region, similar to that obtained in the presence of an *hns* mutation or at low pH (Fig. 2). Finally, as compared to the wild-type strain, which was non-motile in the presence of novobiocin (28 and 4 mm swarming ring diameter in the absence and presence of 200 µM novobiocin, respectively), a significant restoration of motility was observed in the presence of plasmid pPM61 overexpressing the *flhDC* operon (10 mm swarming ring diameter).
pH regulates flagellar gene expression in
Nevertheless, our results provide evidence that acidic
Campbell, 1959; Weibull, 1948) suggests that these
exposed to damage caused by acidic pH (Stocker &
control by acidic pH (Adler, 1973; Adler & Templeton,
To date, only limited data are available on the motility
DISCUSSION
To further investigate the effect of DNA topology on
flhDC expression, we performed in vitro flhDC
transcription assays. Supercoiled (1) and linearized plasmid
pDIA546 (2) were incubated with RNA polymerase. Samples
were subjected to electrophoresis on 7% polyacrylamide
sequencing gel. The flhDC transcript originating from the flhDC
promoter (271 nt) and that from the RNA-I promoter located
on the same plasmid (108 nt) used as a control are indicated by

Fig. 3. Effect of plasmid DNA supercoiling on in vitro flhDC
transcription assays. Supercoiled (1) and linearized plasmid
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on the same plasmid (108 nt) used as a control are indicated by

To further investigate the effect of DNA topology on
flhDC expression, we performed in vitro transcription
experiments with either supercoiled or linearized plasmid
pDIA546 carrying the entire flhDC regulatory region. A severe reduction in the flhDC transcription level was observed when linearized plasmid was used as a template (Fig. 3). This suggests that variations in DNA topology involving the 5’ end of the flhDC operon may play an important role in the transcriptional control of the flagellar master operon by H-NS in response to low pH.

To date, only limited data are available on the motility
control by acidic pH (Adler, 1973; Adler & Templeton,
1967; Bowra & Dilworth, 1981). The fact that bacterial
flagella, with other extracellular organelles, are directly
exposed to damage caused by acidic pH (Stocker &
Campbell, 1959; Weibull, 1948) suggests that these
structures might be disintegrated under these conditions.
Nevertheless, our results provide evidence that acidic
pH regulates flagellar gene expression in E. coli by a
transcriptional control on the flhDC master operon

(Figs 1 and 2). These results are consistent with general
energy consumption considerations under detrimental
conditions, flagellum biosynthesis being extremely expen-
sive for the cell (Macnab, 1996), and also with the
necessity to close the proton entrance during flagellum
motor functioning by reducing the biosynthesis of this
structure. Moreover, we demonstrated here a remark-
able parallelism in the regulation of motility by the H-
NS protein and low pH (Tables 2 and 3, Fig. 2). In
particular, we showed that the H-NS protein might play
a central role in the pH-mediated control of bacterial
flagellum biosynthesis (Fig. 2). The control of flagellar
master operon expression in response to low pH (Fig. 2)
or high osmolarity (O.S., unpublished) requires an
extended flhDC mRNA 5’ untranslated region. The
comparative analysis of the upstream sequence of flhDC
homologous operons in enterobacteria revealed the
presence of a similar domain in many Gram-negative
cells. Furthermore, a long 5’ untranslated region has
also been identified in the master flagellar regulatory
gene of polarly flagellated bacteria of the genera Vibrio
and Pseudomonas (Soutourina et al., 2001). Taken

Together, these results suggest that the mechanism that
controls expression of flagellar master regulatory genes
in response to environmental factors may be largely
conserved among Gram-negative bacteria.

Despite the similarities between E. coli and S. typhi-
murium, some differences have been reported in the
regulation of motility in these two organisms. These
include the autogenous and global control by H-NS on
flhDC master operon expression (Kutsukake, 1997) and
the initiation of flhDC transcription (Soutourina et al.,
1999; Yanagihara et al., 1999). On the other hand, some
specificities may also exist in the response of these
bacteria to acidic pH (Lin et al., 1995), including
the PhoPQ-mediated acid tolerance response (ATR)
(Bearson et al., 1998). As the synthesis of H-NS protein
has been shown to be unaffected by low pH (Adams et al.,
2001), it has been recently suggested that H-NS is not
involved in the control of flagellar biosynthesis in S.
typhimurium under the acidic conditions tested. Similarly,
in E. coli, we observed no alteration in bns
expression at acidic pH. However, flhDC transcription
measurement (Fig. 2) and characterization of suppressive
mutants (Table 3) demonstrated the role of H-NS protein in motility control under low-pH conditions in
E. coli. Nevertheless, these observations do not exclude
the possibility that other regulators may participate in
this process.

The presence of a 5’ untranslated mRNA region has
been usually associated with post-transcriptional regu-
lation mechanisms. For example, such regions are
responsible for either transcriptional anti-termination
and RNA processing or translational control of
threonyl-tRNA synthetase genes in E. coli and Bacillus
subtilis (Condon et al., 1997; Putzer et al., 1995;
Sacerdot et al., 1998). As compared to the wild-type
strain, an up to threefold decrease in flhDC activity
was observed in an bns strain from both transcriptional
and translational fusions (Soutourina et al., 1999). More-
over, a similar reduction in the level of \textit{flhDC} mRNA was measured in the \textit{hns} mutant as compared to the wild-type in RT-PCR experiments and this reduction did not result from an effect of the H-NS protein on the \textit{flhDC} mRNA stability (Soutourina, 2001). Finally, \textit{in vivo} activity measurements from a transcriptional fusion containing the extended \textit{flhDC} regulatory region (Fig. 2) demonstrated that H-NS, acidic pH and DNA supercoiling affect the \textit{flhDC} expression at the level of transcription initiation.

Despite numerous studies on the control of bacterial motility by environmental factors, the molecular basis of this process remains largely unknown. In \textit{E. coli}, the H-NS protein affects the expression of many genes involved in the cellular response to environmental changes, including those required for acidic pH resistance (Hommais \textit{et al.}, 2001). Although the mechanism by which H-NS controls gene expression remains the subject of debate (Williams & Rimsky, 1997), an alteration of plasmid and chromosomal DNA supercoiling has been demonstrated \textit{in vivo} in an \textit{hns} mutant (Mojica & Higgins, 1997). Moreover, the involvement of DNA supercoiling has been proposed, for example, to explain the regulation by H-NS of osmotically regulated genes (Higgins \textit{et al.}, 1988), stringently controlled bacterial promoters (Johansson \textit{et al.}, 2000) or virulence gene expression in \textit{Shigella flexneri} (Dorman \textit{et al.}, 2001). On the other hand, various environmental conditions are also known to affect the level of DNA supercoiling, even though a cause-and-effect relationship has not yet been established (Higgins \textit{et al.}, 1988; Tse-Dinh \textit{et al.}, 1997). It has been proposed that a direct effect of environmental signals on promoter architecture, and then transcription, through influencing the interaction of architectural proteins with DNA, might be an important concept in understanding the environmental regulation of gene expression in bacteria (Jordi \textit{et al.}, 1997). Similarly, different environmental cues might influence the action of H-NS by changing the structure of regulatory regions, the ability of H-NS to bind to DNA target and/or the conformation or the oligomerization state of H-NS. We did not observe any alteration in the level of \textit{hns} gene expression or in the isoform composition of the H-NS protein under low pH or in the presence of DNA gyrase inhibitor (data not shown). In contrast, \textit{flhDC} expression could be modulated by local alteration of DNA topology, resulting from interactions between H-NS and the regulatory region. Several observations argue in favour of this hypothesis. First, the alteration of swarming properties in presence of novobiocin, a DNA gyrase inhibitor, or in strains overproducing DNA gyrase or mutated in its structural gene suggests the existence of a critical DNA supercoiling level for normal motility in \textit{E. coli} (see Results) (Shi \textit{et al.}, 1993b; O.S., unpublished). Second, we observed a severe reduction in \textit{flhDC} expression when linearized rather than supercoiled plasmid was used in \textit{in vitro} transcription assay (Fig. 3). Third, the involvement of the extended \textit{flhDC} regulatory region in the control of the master operon by H-NS, acidic pH and novobiocin suggests a strong correlation between these regulatory processes (Fig. 2). Finally, further support is provided by the partial restoration of motility in the \textit{hns} mutant by overexpression of DNA gyrase subunit gene \textit{gyrB} (data not shown), and the alteration of topoisomerase distribution of plasmids carrying the entire \textit{flhDC} regulatory region in the presence of chloroquine phosphate as an intercalating agent (Soutourina, 2001). Taken together, these data suggest that the H-NS-mediated effect on motility may be at least in part explained by an alteration in the level of DNA topology of the \textit{flhDC} regulatory region. They are consistent with the recent demonstration that a 339 bp DNA fragment having a bent structure can strongly affect the level of plasmid DNA supercoiling (Rohde \textit{et al.}, 1999) and suggest that the regulatory region of the flagellar master operon may play a crucial role for an adequate control by the H-NS protein and environmental factors.

The control of bacterial motility via the \textit{flhDC} operon includes several participants at multiple regulatory levels, e.g. transcription initiation control by cAMP–CAP complex and H-NS (Soutourina \textit{et al.}, 1999), mRNA stability control by 	extit{CsrA} (Wei \textit{et al.}, 2001) in \textit{E. coli}, or 	extit{FlhDC} protein degradation by Lon protease in \textit{Proteus mirabilis} (Claret & Hughes, 2000). Our results extend the knowledge of the regulation of the flagellar system and represent an important step toward the understanding of complex mechanisms governing bacterial motility in response to environmental challenges.

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