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

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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.

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 agar plates with 1% salicin as a carbon source. When required, antibiotics were added at the following concentration: ampicillin, 50 μg ml⁻¹, and kanamycin, 20 μg ml⁻¹. All experiments were performed in accordance with the European regulation requirements concerning the contained use 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 OD_{600} 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 OD_{600} 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 OD_{600} 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 [α-³³P]dCTP (7·4 × 10¹³–1·1 × 10¹⁴ 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 *Eco*RI for 2 h at 37 °C and used as linearized template for *in vitro* transcription.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strain		
MG1655	Wild-type	CGSC 6300
PS2209	Wild-type	Bertin *et al.* (1994)
PS2652	PS2209 *hns-1001*	Bertin *et al.* (1994)
FB8	Wild-type	Bruni *et al.* (1977)
BE1410	FB8 *hns-1001*	Laurent-Winter *et al.* (1997)
BE2120	*hns-1001* carrying a *mot*⁺ suppressor mutation	This study
BE2121	*hns-1001* carrying a *mot*⁺ suppressor mutation	This study
Plasmid		
pDIA528	pKK232-8-derivative carrying the *flhDC* promoter (nt −213 to +78)	Soutourina *et al.* (1999)
pDIA545	pKK232-8 derivative carrying the *flhDC* regulatory region (nt −213 to +205)	Soutourina *et al.* (1999)
pDIA546	pJCD01 derivative carrying the *flhDC* regulatory region (nt −213 to +202)	Soutourina *et al.* (1999)
pDIA559	pKK232-8-derivative carrying the *fliC* promoter region	Soutourina *et al.* (1999)
pPM61	ColE1 derivative multicopy expression vector carrying the *flhDC* operon under its native promoter	Bartlett *et al.* (1988)

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 °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

Strain (phenotype)	Mucoidy*	β-Glucoside utilization†	Motility‡	Survival (%)§
FB8 (wild-type)	−	−	30＋3	<0·001
BE1410 (*hns*)	+	+	3＋1	24＋4
BE2120 (*mot*⁺)	+	+	18＋2	<0·005
BE2121 (*mot*⁺)	+	+	21＋2	<0·001

* ＋, Mucoid phenotype observed on LB medium; −, no mucoid phenotype.

† ＋, Ability to metabolize β-glucoside revealed by the appearance of red colonies on MacConkey-salicin agar plates; −, lack of ability to metabolize β-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 × 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

Gene	Expression level* in strain (phenotype):				Function
	FB8 (wild-type)	BE1410 (*hns*)	BE2120 (*mot*⁺)	BE2121 (*mot*⁺)	
					Flagellum biosynthesis
flgB	0·80	−	0·38	1·36	Proximal rod protein
flgD	0·67	−	0·61	0·86	Basal body rod modification protein
flgG	0·75	0·29	0·61	1·32	Distal rod protein
flgK	1·02	−	0·80	1·04	Hook-associated protein 1
flgL	1·02	−	0·44	1·34	Hook-associated protein 3
fliA	0·72	−	0·30	0·75	Flagella-specific sigma factor
fliC	7·04	−	0·26	0·41	Flagellin
fliD	1·57	−	0·44	1·66	Hook-associated protein 2
fliJ	1·10	0·12	1·01	0·95	Export for some substrates
fliK	0·83	−	0·66	1·39	Hook length control protein
					Acid pH resistance
gadA	0·57	16·98	2·76	2·03	Glutamate decarboxylase α
gadB	0·43	15·16	2·33	1·83	Glutamate decarboxylase β
gadC	0·26	8·91	1·21	0·39	Putative amino acid antiporter
gadX	−	3·48	2·83	0·83	Regulatory protein
hdeA	0·36	10·67	2·75	0·66	Putative chaperone-like protein involved in low pH resistance
hdeB	−	9·42	2·08	0·37	Involved in low pH resistance
hdeD	−	3·17	0·40	0·57	Unknown function

* 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 *hns* strains (Lejeune & Danchin, 1990). A similar failure to characterize suppressor mutations involved in the thermoregulation of motility has been reported in *Yersinia enterocolitica* (Rohde *et al.*, 1994).

To investigate the effect of the suppressor mutations on the physiology of *E. coli*, we tested various H-NS-related phenotypes in strains BE2120 and BE2121. Like the original *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 *gad* and *hde* genes involved in resistance to low pH (Hommais *et al.*, 2001) was observed in these mutants in comparison with the *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 *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 *et al.*, 2001). Furthermore, both genes were not mutated in the suppressor strains (data not shown). Finally, spontaneous mutants were also obtained in an *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 *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 *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
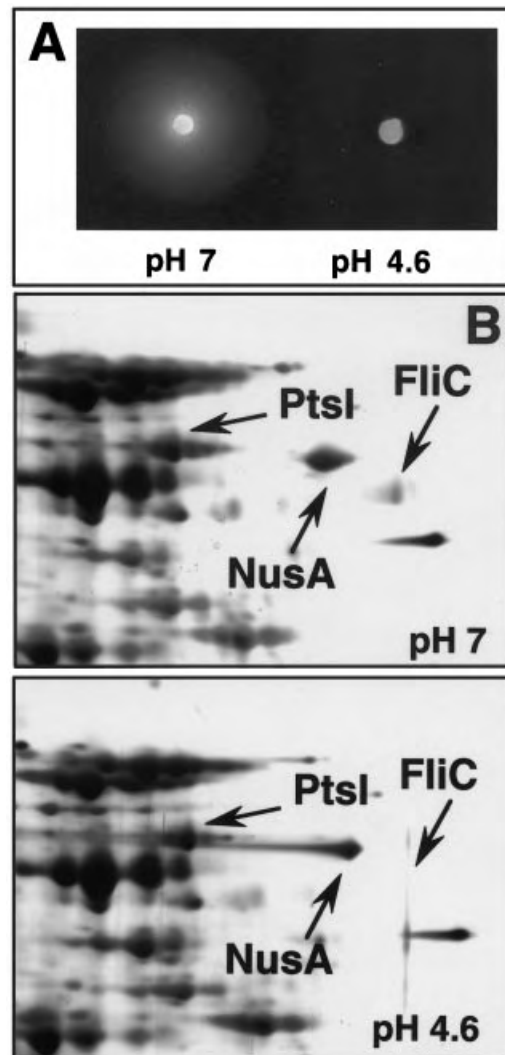

Fig. 1. Effect of acidic pH on motility and flagellin synthesis. (A) Motility of the *E. coli* wild-type strain was assayed on semi-solid medium plates at pH 7·0 and at pH 4·6. Plates were incubated for 13–15 h at 30 °C. (B) Protein extracts of wild-type strain MG1655 grown at pH 7·0 and at pH 4·6 were 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.

inside their host (Mahan *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 *E. coli* on semi-solid plates at neutral and acidic pH. As seen in Fig. 1(A), a loss of motility was observed under low pH conditions. To determine whether this alteration in swarming behaviour resulted from a lack of flagella, we analysed the flagellin content by two-dimensional gel electrophoresis. As seen in Fig. 1(B), the spot corresponding to the FliC protein on our *E. coli* two-dimensional protein map (Hommais *et al.*, 2001) was undetectable in the protein extract of bacteria grown at low pH by comparison with those grown at
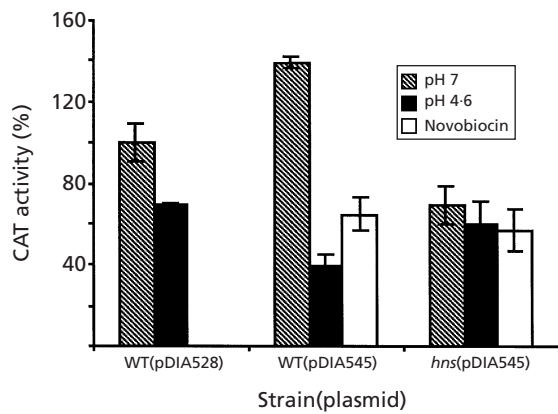
Fig. 2. Effect of low pH and novobiocin on *flhDC* transcription. The *flhDC–cat* transcriptional fusion activity was measured in exponential growth phase in M9 medium supplemented with 0·1 % (w/v) Casamino acids and 0·4 % (w/v) glycerol at pH 7 and pH 4·6 or in the presence of 200 µM novobiocin. The data are the mean values from three independent assays. WT, wild-type; pDIA528, plasmid carrying the *flhDC* promoter; pDIA545, plasmid carrying the entire *flhDC* regulatory region.

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* strains subjected to various stresses, such as high osmolarity or increased temperature (Shi *et al.*, 1993b), supporting a role for the *flhDC* 5′ end in the control of flagellar genes in response to acidic pH.

Both low pH and *hns* mutation resulted in an up to threefold decrease in *flhDC–cat* activity in the presence of the 5′ end region. Such a reduction in *flhDC* expression has been previously observed in *hns* strains carrying a chromosomal *flhDC–lacZ* transcriptional 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 subunit GyrB resulted 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).
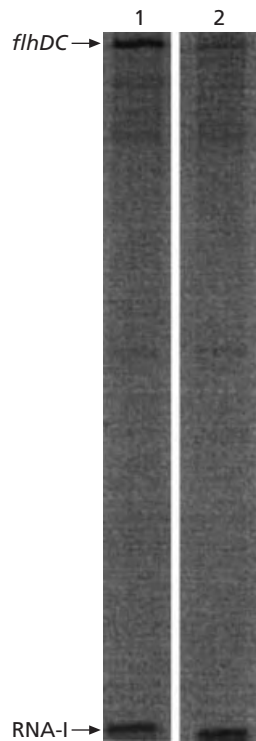
Fig. 3. Effect of plasmid DNA supercoiling on *in vitro* *flhDC* transcription assays. Supercoiled (1) and linearized plasmid pDIA546 (2) were incubated with RNA polymerase. Samples were subjected to electrophoresis on 7 % polyacrylamide sequencing gel. The *flhDC* transcript originating from the *flhDC* promoter (271 nt) and that from the RNA-I promoter located on the same plasmid (108 nt) used as a control are indicated by arrows.

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.

DISCUSSION

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 expensive 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 remarkable 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 bacteria. 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. typhimurium*, 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 *hns* 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 regulation 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 *hns* strain from both transcriptional and translational fusions (Soutourina *et al.*, 1999). More-

over, a similar reduction in the level of *flhDC* mRNA was measured in the *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 *flhDC* mRNA stability (Soutourina, 2001). Finally, *in vivo* activity measurements from a transcriptional fusion containing the extended *flhDC* regulatory region (Fig. 2) demonstrated that H-NS, acidic pH and DNA super-coiling affect the *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 *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 re-sistance (Hommais *et al.*, 2001). Although the mech-anism by which H-NS controls gene expression remains the subject of debate (Williams & Rimsky, 1997), an alteration of plasmid and chromosomal DNA super-coiling has been demonstrated *in vivo* in an *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 *et al.*, 1988), stringently controlled bacterial promoters (Johansson *et al.*, 2000) or virulence gene expression in *Shigella flexneri* (Dorman *et al.*, 2001). On the other hand, various environmental condi-tions are also known to affect the level of DNA supercoiling, even though a cause-and-effect relation-ship has not yet been established (Higgins *et al.*, 1988; Tse-Dinh *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 *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 *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, *flhDC* expression could be modu-lated 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 *E. coli* (see Results) (Shi *et al.*, 1993b; O.S., unpublished). Second, we observed a severe reduction in *flhDC* expression when linearized rather than supercoiled plasmid was used in *in vitro* transcription assay (Fig. 3). Third, the involvement of the extended *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 *hns* mutant by overexpression of DNA gyrase subunit gene *gyrB* (data not shown), and the alteration of topoisomer distribution of plasmids carrying the entire *flhDC* regulatory region in the presence of chloroquine phosphate as an intercalating agent (Sou-tourina, 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 *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 *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 environ-mental factors.

The control of bacterial motility via the *flhDC* operon includes several participants at multiple regulatory levels, e.g. transcription initiation control by cAMP–CAP complex and H-NS (Soutourina *et al.*, 1999), mRNA stability control by CsrA (Wei *et al.*, 2001) in *E. coli*, or FlhDC protein degradation by Lon protease in *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 bac-terial motility in response to environmental challenges.

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