The regulation of Enzyme IIA\textsuperscript{Glc} expression controls adenylate cyclase activity in 
\textit{Escherichia coli}

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INTRODUCTION

In enterobacteria, the histone-like nucleoid-structuring (H-NS) protein is one of the most abundant DNA-binding proteins involved in the organization of the bacterial chromosome (Bertin et al., 2001). Numerous phenotypes have been associated with \textit{hns} mutations, resulting from a modification in the expression of several genes. Most of them are regulated by environmental parameters, such as pH, osmolarity and temperature, or are known to be involved in bacterial virulence (Atlung & Ingmer, 1997; Laurent-Winter et al., 1997; Hommais et al., 2001).

It has been demonstrated that the H-NS protein interacts with other regulatory systems. For example, the leucine-responsive regulatory protein Lrp regulates the transcription of a number of genes by binding DNA at a specific site located upstream from the transcription start site of the genes (Calvo & Matthews, 1994). Some of these target genes and the Lrp structural gene itself are regulated by the H-NS protein (Levinthal et al., 1994; Oshima et al., 1995). The H-NS protein also possesses common targets with the cAMP-catabolite activator protein (CAP) complex: \textit{nmpC} and \textit{malT} genes (Coll et al., 1994; Johansson et al., 1998) and \textit{pap, bgI, mccABCDE} and \textit{flhDC} operons (Forsman et al., 1996; Gonzalez-Pastor et al., 1995; Schnetz & Wang, 1996; Soutourina et al., 1999). In contrast to its role in the control of \textit{flhDC} expression (Bertin et al., 1994; Soutourina et al., 1999), the cAMP-CAP complex acts as an H-NS protein antirepressor in the regulation of \textit{pap} and \textit{bgI} operons (Forsman et al., 1996; Schnetz & Wang, 1996).

The cAMP-CAP complex, which controls the expression of a multitude of genes or operons, has been characterized for its role in catabolite repression. Indeed, high glucose levels reduce Enzyme IIA\textsuperscript{Glc} phosphorylation, which decreases adenylate cyclase activity and cAMP concentration. This results in a repressed synthesis of the enzymes needed for the catabolism of alternative carbon sources. It is now known that CAP is also involved in the expression of genes needed for adaptation to changes in growth conditions. Moreover, CAP regulates the synthesis of some membrane components, numerous proteins involved in various stresses and some regulator-encoding genes. Finally, it is worth

\begin{flushleft}
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mentioning that many CAP-regulated genes are also controlled by other transcription factors (for a review see Busby & Kolb, 1996).

It has been suggested that the cellular CAP concentration might be somewhat reduced in hns strains (Johansson et al., 1998). As it has been demonstrated that the activity of CAP is mainly modulated by the intracellular level of cAMP (Roy et al., 1983; Kolb et al., 1993), we wanted to know whether the H-NS protein could be involved in the control of the intracellular cyclic AMP (cAMP) concentration. In the present paper, we show that the H-NS protein plays a role in this process by acting on crr gene expression. The effect on the crr-specific P2 promoter was mainly observable in the absence of CAP and, although the H-NS protein has been considered as a transcriptional repressor, our results constitute a new example of a positive effect of this regulatory protein on bacterial physiology. Moreover, we show for the first time that crr expression is regulated and that a twofold reduction in the global amount of Enzyme IIA^hns is sufficient to significantly decrease adenylate cyclase activity and the cAMP level.

METHODS

Bacterial strains, plasmids and growth conditions. The *Escherichia coli* K-12 derivative strains and the plasmids used in this work are listed in Table 1. Mutations hns-1001 and hns-118 were introduced by P1 transduction as described previously (Bertin et al., 1994). For reasons of incompatibility with the kanamycin resistance marker of the resident plasmid, the hns-118 mutation was used instead of the hns-1001 mutation in hns-lacos experiments. All strains were grown at 37 °C in M63 medium (Miller, 1972), supplemented with glucose (0.4%), thiamine (5 µg ml⁻¹) and Casamino acids (0.1%). When required, kanamycin and ampicillin were added at 25 and 100 µg ml⁻¹, respectively. Plasmid pDIA3350 was constructed by insertion of the *SalI–BamHI* fragment of pDIA3328, corresponding to the full phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) operon with promoters P0, P1 and P2, into plasmid pDIA3240 (De Reuse et al., 1986). 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).

**cAMP assay.** Total cAMP production was determined by a radioimmunological assay from at least four samples of exponentially growing cells as described by Guidi-Rontani et al. (1981) and Crasnier et al. (1994).

**In vitro adenylate cyclase assay.** Exponentially growing cell cultures were centrifuged at 9000 g for 10 min at 20 °C. The pellet, corresponding to 0.5 g bacteria, was resuspended in 3 ml 25 mM Tris/HCl, 10 mM MgCl₂, pH 8.3, (Tris-Mg buffer) to obtain a 20 mg ml⁻¹ protein concentration. Bacteria were then broken with the FastPrep System and FastProtein Blue (Bio-101) and centrifuged at 13000 g at 4 °C. Protein (0.4–0.8 mg) of the bacterial supernatant was added to 1.2 ml of assay mixture (125 mM Tris/HCl, pH 8.3, 50 mM MgCl₂, 5 mM dithiothreitol, 5 mM ATP) and 468 µl Tris-Mg buffer. During incubation at 28 °C, several samples of 1 ml were taken between 0 and 60 min and heated for 5 min at 100 °C, according to the method of Joseph et al. (1982). The cAMP synthesized was quantified with the cAMP[¹²⁵I]-RIA kit (NEN).

**β-Galactosidase assay.** β-Galactosidase activity was determined by the method of Miller (1992) on exponentially growing cells. The assay was performed on more than three samples from at least two independent cultures.

**Determination of the phosphorylation state of Enzyme IIA^hns.** The phosphorylation state of Enzyme IIA^hns was determined with a Western blotting experiment with anti-IIA^hns antibodies (kindly provided by P. Postma, University of Amsterdam, The Netherlands) on 20 µl protein extracts from strains grown to an OD₅₆₀ of precisely 0–400 (Takahashi et al., 1998). Immunoblots were scanned with a JX-330 Sharp scanner and quantified using PDI software, PDQuest, based on a SUN computer system.

### Table 1. *E. coli* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP2101</td>
<td>F⁻ xyl ΔlacX74 argH1</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BE1522</td>
<td>F⁻ xyl ΔlacX74 argH1 hns-1001::Tn5seq1</td>
<td>This study</td>
</tr>
<tr>
<td>TP2139</td>
<td>F⁻ xyl Δcrr39 ΔlacX74 argH1 ileA</td>
<td>Roy et al. (1983)</td>
</tr>
<tr>
<td>BE1420</td>
<td>F⁻ xyl Δcrr39 ΔlacX74 argH1 hns-1001::Tn5seq1</td>
<td>This study</td>
</tr>
<tr>
<td>BE1421</td>
<td>F⁻ xyl Δcrr39 ΔlacX74 argH1 ileA hns-118::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDIA3226</td>
<td>ptsH-ptsL-lacZ protein fusion</td>
<td>De Reuse &amp; Danchin (1988)</td>
</tr>
<tr>
<td>pDIA3238</td>
<td>ptsH-pts-crr-lacZ protein fusion</td>
<td>De Reuse &amp; Danchin (1988)</td>
</tr>
<tr>
<td>pDIA3247</td>
<td>ptsH-lacZ protein fusion</td>
<td>De Reuse &amp; Danchin (1988)</td>
</tr>
<tr>
<td>pDIA3241</td>
<td>ptsH-lacZ operon fusion</td>
<td>De Reuse &amp; Danchin (1988)</td>
</tr>
<tr>
<td>pDIA3242</td>
<td>ptsH-pts-lacZ operon fusion</td>
<td>De Reuse &amp; Danchin (1988)</td>
</tr>
<tr>
<td>pDIA3350</td>
<td>ptsH-pts-crr-lacZ operon fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pDIA4705</td>
<td>pBR322 derivative carrying crr under anti-Te' promoter</td>
<td>Zeng et al. (1992)</td>
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</table>
RESULTS
Effect of crp and/or hns mutations on cAMP level
To determine whether the H-NS protein modulates cAMP synthesis, the total cAMP level was measured in exponentially growing cells of wild-type TP2101 and isogenic strains deficient for the H-NS protein (BE1522), CAP (TP2139) or both regulators (BE1420), by using a radioimmunological method. As expected (Potter et al., 1974; Joseph et al., 1982), the presence of a crp mutation in TP2139 resulted in an important increase in cAMP concentration (more than 300-fold). In contrast, no significant difference was observed in the hns strain BE1522 (Table 2). Moreover, compared to the strain carrying the sole crp mutation, the presence of both crp and hns mutations in BE1420 resulted in a more than sixfold reduction in the cAMP concentration (Table 2). This result was confirmed by a cAMP excretion test on MacConkey maltose medium (data not shown). This suggests that the repression by the cAMP-CAP complex seems to be epistatic with regard to the activation by the H-NS protein. However, at least in a crp background, the H-NS protein affects the level of cAMP.

Table 2. cAMP levels in H-NS protein-deficient and/or CAP-deficient strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Total cAMP [pmol (mg protein)^-1]</th>
</tr>
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<tbody>
<tr>
<td>TP2101</td>
<td>Wild-type</td>
<td>314</td>
</tr>
<tr>
<td>BE1322</td>
<td>hns</td>
<td>415</td>
</tr>
<tr>
<td>TP2139</td>
<td>crp</td>
<td>97,625</td>
</tr>
<tr>
<td>BE1420</td>
<td>crp hns</td>
<td>15,069</td>
</tr>
</tbody>
</table>

Data are the mean values of four samples from two independent cultures that differed by less than 20%.

Positive effect of the H-NS protein on the activity and the synthesis of adenylate cyclase in a crp strain
Adenylate cyclase plays a major role in the control of cAMP level in bacterial cells. This enzyme is known to be regulated by CAP at the level of its activity and at the transcriptional level (Aiba, 1985; Inada et al., 1996; Takahashi et al., 1998).

Adenylate cyclase activity was measured with an in vitro assay on exponentially growing cells. A more than 30-fold increase in enzyme activity was measured in the crp strain TP2139 [12,600 pmol cAMP (mg protein)^-1 min^-1] compared to the wild-type TP2101 [400 pmol cAMP (mg protein)^-1 min^-1], in accordance with the results of others (Rephaeli & Saier, 1976; Joseph et al., 1982). In contrast, an eightfold decrease in activity was measured in the crp hns double mutant BE1420 [1,600 pmol cAMP (mg protein)^-1 min^-1] compared to the crp strain. These results are in agreement with the total cAMP level measured in bacterial cultures (Table 2).

To investigate the role of the H-NS protein on the transcription of the adenylate cyclase encoding gene, we measured the activity of a cyaA- lacZ operon fusion from plasmid pDIA1973 (Roy et al., 1988). Compared to the wild-type TP2101 [6,320 Miller units (mg protein)^-1], a moderate increase in β-galactosidase activity was observed in the crp strain TP2139 (Table 3), in agreement with the data of others (Kawamukai et al., 1985). In contrast, in the crp hns double mutant BE1421 (Table 3), a more than twofold decrease in β-galactosidase activity was observed compared to that in the crp strain.

Regulation of adenylate cyclase activity by the H-NS protein via the Enzyme IIA^Glc level
Adenylate cyclase activity is known to be regulated by the PTS via Enzyme IIA^Glc and the cAMP-CAP complex (Levy et al., 1990; Saier et al., 1996; Reddy & Kamireddi,...
The HPr protein could also be involved in this process (Peterkofsky et al., 1995). Although the three PTS genes ptsH, ptsI and crr, which encode the proteins HPr, Enzyme I and Enzyme IIA\textsuperscript{Glc}, respectively, are considered to be part of a single operon, transcription is known to be initiated from three distinct promoters, P0, P1 and P2 (Fig. 1) (De Reuse & Danchin, 1988; De Reuse et al., 1992). As the decrease in cyA-lacZ activity does not seem to be sufficient to explain the strong decrease in adenylate cyclase activity measured in the crp bns double mutant BE1421 (see above), the expression level of ptsH, ptsI and crr was analysed using protein and operon fusions (De Reuse & Danchin, 1988). In the crp strain, the lack of H-NS protein had only a minor effect on the expression of both ptsH-lacZ and ptsI-lacZ fusions, which are exclusively under the control of promoters P0 and P1, i.e. a 1.3-fold decrease and 1.3-fold increase, respectively (Table 3). In contrast, crr expression, which is under the control of the three promoters, showed a 3.7-fold and twofold decrease in the \(\beta\)-galactosidase activity of the crr-lacZ transcriptional and protein fusion, respectively, in the crp bns double mutant BE1421 compared to the crp strain (Table 3). This suggests that the H-NS protein exerts a positive and specific effect on crr transcription from the P2 promoter.

The Enzyme IIA\textsuperscript{Glc} is known to be phosphorylated by the phosphorylated HPr protein (HPr-P), which is itself phosphorylated by Enzyme I-P (Postma & Lengeler, 1985). Phosphorylated Enzyme IIA\textsuperscript{Glc} is known to activate adenylate cyclase while the unphosphorylated form has no effect on enzyme activity (Den Blaauwen & Postma, 1985; Crasnier & Danchin, 1990; Reddy & Kamireddi, 1998; Takahashi et al., 1998). Moreover, it has been supposed that the global regulation of adenylate cyclase activity depends on the sole phosphorylation state of Enzyme IIA\textsuperscript{Glc}. Indeed, the major effect of the cAMP-CAP complex on adenylate cyclase activity results from the decrease in Enzyme IIA\textsuperscript{Glc} phosphorylation (Saier et al., 1996; Crasnier-Mednansky et al., 1997; Reddy & Kamireddi, 1998; Takahashi et al., 1998). To determine the phosphorylated state of Enzyme IIA\textsuperscript{Glc} in the different strains, a Western blotting experiment was performed with anti-IIA\textsuperscript{Glc} antibodies on protein extracts from strains grown to an OD\textsubscript{600} of 0.4-0.6 for 100 min. This is representative of two experiments performed with 20 \(\mu\)l protein extract from two independent cultures at an OD\textsubscript{600} of precisely 0.400.

To discover whether the reduced amount of Enzyme IIA\textsuperscript{Glc} measured in the crp bns strain (Fig. 2) was sufficient to explain the decrease in adenylate cyclase activity, we determined this activity in the double mutant BE1420 containing plasmid pDIA4705 carrying the crr gene. The presence of this plasmid in a \(\Delta\)crr strain has been shown to restore the wild-type cAMP level and glucose transport (Zeng et al., 1992). The activity measured in the crp bns mutant containing plasmid pDIA4705, 11400 pmol cAMP (mg protein)\textsuperscript{-1} min\textsuperscript{-1}, was similar to that obtained in the TP2139 crp strain [12600 pmol cAMP (mg protein)\textsuperscript{-1} min\textsuperscript{-1}]. This is consistent with the Western blotting experiment which showed that a similar amount of Enzyme IIA\textsuperscript{Glc} was present in both TP2139 and BE1420(pDIA4705) (data not shown). This demonstrated that the variation in adenylate cyclase activity resulting from an bns mutation depends on the reduced amount of Enzyme IIA\textsuperscript{Glc} present in the cells rather than the effect the H-NS protein on cyA gene expression (Table 2).

**DISCUSSION**

In *E. coli*, adenylate cyclase activity is known to be very low in a wild-type genetic context. Indeed, Enzyme IIA\textsuperscript{Glc}, which is the major activator of this activity, is mainly in an unphosphorylated form due to the presence
of a high concentration of cAMP-CAP complex (Saier et al., 1996; Crasnier-Mednansky et al., 1997; Reddy & Kamireddi, 1998; Takahashi et al., 1998). Only the phosphorylated form of the enzyme plays a role as activator. This phosphorylation results from a transfer of the phosphoryl group from phosphoenolpyruvate to Enzyme IIA^{Glc} via Enzyme I and HPr (Postma & Lengeler, 1985). The three PTS genes ptsH, ptsI and crr, which encode the proteins HPr, Enzyme I and Enzyme IIA^{Gle}, respectively, belong to a single operon. However, transcription is initiated from three distinct promoters (Fig. 1). Both ptsH and ptsI depend on P0 and P1 promoters, while crr is controlled by the three promoters, P2 being responsible for 80% of total crr mRNA synthesis (De Reuse & Danchin, 1988; De Reuse et al., 1992). Transcriptional regulation by glucose, the cAMP-CAP complex and the global repressor McIc has been observed on promoter P0 (De Reuse et al., 1992; Ryu & Garges, 1994; Tanaka et al., 1999), while promoter P1 is known to be regulated by the fructose repressor FruR (Ryu et al., 1995). However, no regulatory mechanism has been shown to affect promoter P2.

Our results demonstrate, for the first time, that crr is regulated at the transcriptional level. This regulation only affects the P2-specific promoter and has almost no effect on the two other promoters of the PTS operon. The H-NS protein controls the expression of numerous genes involved in bacterial adaptation to environmental changes (Hommais et al., 2001) and it is generally considered as a transcriptional repressor. Although the mechanism, which could be indirect, remains to be determined, our results constitute a new example of the positive effect of this regulatory protein on bacterial physiology. Recently, the existence of a complex between HhA and the H-NS protein has been shown to be involved in the regulation of the haemolysin operon in Escherichia coli (Nieto et al., 2000). Moreover, an hha mutation results in a fivefold decrease in Enzyme IIA^{Gle} in rich medium under high osmolarity conditions (Balsalobre et al., 1999). Taken together, these observations may suggest an interaction of both proteins in the regulation of crr expression. However, in contrast to the H-NS protein (Table 3 and Fig. 2), the effect of HhA on Enzyme IIA^{Gle} was only observed in conditions of high osmolarity, suggesting that the two regulatory proteins affect crr expression by a different mechanism. Finally, the repression by the cAMP-CAP complex is predominant with regard to the activation by the H-NS protein. Indeed, in the presence of the cAMP-CAP complex, only a small fraction of Enzyme IIA^{Gle} was phosphorylated (Fig. 2). This suggests that adenylate cyclase has its lowest activity in the wild-type strain, which could explain that a twofold alteration in the amount of Enzyme IIA^{Gle} in bns strains has no major effect on adenylate cyclase activation.

We also showed that, despite a large excess of Enzyme IIA^{Gle} in the cell [about 15000 Enzyme IIA^{Gle} phosphorylated molecules in comparison with 15–50 adenylate cyclase molecules (Yang & Epstein, 1983; Mitchell et al., 1987)], a twofold variation in its accumulation level resulted in an eightfold decrease in adenylate cyclase activity. In a glucose-rich medium, the phosphorylated form of Enzyme IIA^{Gle} interacts preferentially with the glucose permease (Enzyme IIBC^{Gle}) to allow the entry of glucose into the cell. This suggests that the affinity of phosphorylated Enzyme IIA^{Gle} is much lower for adenylate cyclase, or for the putative intermediary that could activate it (Saier et al., 1996), than for glucose permease. Depending on environmental conditions, adenylate cyclase may be regulated either by the amount or by the phosphorylation of Enzyme IIA^{Gle}.

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