Type 1 Fimbriae, a Colonization Factor of Uropathogenic Escherichia coli, Are Controlled by the Metabolic Sensor CRP-cAMP

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Abstract

Type 1 fimbriae are a crucial factor for the virulence of uropathogenic Escherichia coli during the first steps of infection by mediating adhesion to epithelial cells. They are also required for the consequent colonization of the tissues and for invasion of the uroepithelium. Here, we studied the role of the specialized signal transduction system CRP-cAMP in the regulation of type 1 fimbriae. Although initially discovered by regulating carbohydrate metabolism, the CRP-cAMP complex controls a major regulatory network in Gram-negative bacteria, including a broad subset of genes spread into different functional categories of the cell. Our results indicate that CRP-cAMP plays a dual role in type 1 fimbriae, affecting both the phase variation process and fimA promoter activity, with an overall repressive outcome on fimbriation. The dissection of the regulatory pathway let us conclude that CRP-cAMP negatively affects FimB-mediated recombination by an indirect mechanism that requires DNA gyrase activity. Moreover, the underlying studies revealed that CRP-cAMP controls the expression of another global regulator in Gram-negative bacteria, the leucine-responsive protein Lrp. CRP-cAMP-mediated repression is limiting the switch from the non-fimbriated to the fimbriated state. Consistently, a drop in the intracellular concentration of cAMP due to altered physiological conditions (e.g. growth in presence of glucose) increases the percentage of fimbriated cells in the bacterial population. We also provide evidence that the repression of type 1 fimbriae by CRP-cAMP occurs during fast growth conditions (logarithmic phase) and is alleviated during slow growth (stationary phase), which is consistent with an involvement of type 1 fimbriae in the adaptation to stress conditions by promoting biofilm growth or entry into host cells. Our work suggests that the metabolic sensor CRP-cAMP plays a role in coupling the expression of type 1 fimbriae to environmental conditions, thereby also affecting subsequent attachment and colonization of host tissues.

Introduction

Bacteria have the ability to rapidly adapt to changes in the environment, a feature that is important for survival and multiplication both during colonization of host organisms and in the environment. An efficient adaptation implies the ability to sense external parameters and to transduce the perceived signals to cellular regulators, which then incite adaptive changes in the physiology of the cell. One form of signal transduction occurs via cytoplasmatic secondary messenger systems, so-called alarmones, which can mediate a rapid response. Alarmones are low molecular mass, non-proteinaceous, enzymatically synthesized compounds. Several modified nucleotides have been described to execute this function in bacteria, among them the 3',5'-cyclic adenosine monophosphate (cAMP). cAMP is a ubiquitous molecule found in both prokaryotes and eukaryotes. In bacteria, the activity of cAMP was initially thought to be restricted to its role in catabolite repression [1]. However, there is evidence for an extended role of cAMP as sensory signal involved in global gene regulation in bacteria [2–5]. The level of intracellular cAMP is modulated by several environmental factors [6–8]. The cellular target for cAMP-signaling is the cAMP receptor protein (CRP). Dimeric CRP in complex with one molecule of cAMP exhibits DNA-binding activity to sites located near promoter regions [9]. Thereby, CRP-cAMP acts as a global regulator of gene expression by controlling the expression of almost 200 operons in E. coli [10–12].

Type 1 fimbriae mediate attachment to both biotic and abiotic surfaces and are involved in the early stages of biofilm formation [13,14]. In E. coli, type 1 fimbriae play a crucial role during urinary tract infections by mediating adhesion to mannose-containing receptors on the uroepithelium and promoting the formation of intracellular bacterial communities [15–17]. Those
Author Summary

Attachment of bacteria to the surface of host tissues is a crucial initial step in the establishment of bacterial infections. This process is mediated by adhesins, such as the type 1 fimbriae of Escherichia coli, which play a key role during urinary tract infections by mediating adhesion to the uroepithelium. The expression of type 1 fimbriae is finely regulated according to environmental signals and is under phase variation control, which determines the percentage of fimbriated cells in the population. In this report, we show that the expression of type 1 fimbriae is repressed by a metabolic sensor of the cell, the global regulatory complex CRP-cAMP. We demonstrate that CRP-cAMP affects the switching outcome by selectively inhibiting the recombination process in one direction only, resulting in a lower percentage of fimbriated cells. Such a switch to the non-fimbriated state after successful adhesion might be advantageous in the urinary tract, where the immune mechanisms of the host favor the removal of bacteria expressing immunogenic surface structures. Understanding the regulatory networks that govern regulation of virulence and colonization factors is both of basic interest and might help to develop novel strategies to treat bacterial infections.

adhesins are encoded by the fim determinant composed of two independent transcription units coding for the recombinases FimE and FimB, and a polycistronic operon encoding the structural components (FimA, FimF, FimG, and FimH) and a pilus assembly system (FimC and FimD) [18,19]. Phase variable expression of the fim operon is associated with the inversion of a 314-bp chromosomal region, flanked by two 9-bp inverted repeats, that contains the fimL promoter [20,21]. When the invertible element is in the so-called ON orientation, the promoter is directed towards the structural fim genes, thus allowing transcription, whereas transcription is abolished in the inverted OFF orientation. The inversion process is catalyzed by FimB and FimE, two members of the tyrosine site-specific recombinase family [22,23].

Several regulators are involved in the fine modulation of the expression of type 1 fimbriae by environmental conditions [24,25]. A proper supercoiling state of the DNA and the presence of accessory proteins, such as the DNA binding proteins Lrp and IHF, are essential features that affect the recombination process and determine whether the fim operon is repressed or not [26–29]. Other regulators such as RpoS, ppGpp, NanR and NagC modulate type 1 fimbriation mostly by altering the expression of the recombinases that catalyze the recombination event [30–32]. Moreover, the global regulator H-NS has been shown to affect type 1 fimbrialization both by regulating the expression of the recombinases and by directly interacting with the fim invertible element [33,34]. Effects on the expression of type 1 fimbriae in cya derivatives of E. coli K-12, which are defective in cAMP synthesis, were reported earlier [35]. However, different strains responded divergently upon addition of exogenous cAMP in static cultures and it was not clarified at what level the reported effects were operating. In this work, we show that CRP-cAMP represses type 1 fimbrialization. The dissection of the mechanism underlying the observed phenomenon demonstrated that CRP-cAMP indirectly represses FimB-mediated recombination during the phase variation process. In contrast to many other regulators of the phase variation of type 1 fimbriae described, CRP-cAMP affects phase variation independently of the levels of the recombinases. We propose a novel model by which CRP-cAMP controls the type 1 fimbriation state in the bacterial population by affecting DNA gyrase activity. In addition, our studies led to the new discovery that Lrp expression in E. coli is under the control of the CRP-cAMP complex.

Results/Discussion

Type 1 fimbriation in E. coli is enhanced by the lack of the CRP-cAMP regulatory complex

For a successful colonization of hosts by bacteria, it is crucial that the expression of bacterial surface structures, which mediate the interaction with the host tissues, is finely regulated. In E. coli, the CRP-cAMP complex has been shown to regulate the production of several of those surface structures, such as flagella or P-fimbriae [36–39]. Using a cta deletion mutant derivative of the extensively studied uropathogenic E. coli (UPEC) isolate J96, we further characterized the role of CRP-cAMP in the modulation of the expression of those colonization factors. Confirming previous data, the CRP-cAMP deficient derivatives were non-motile and had lost the ability to cause mannose-resistant haemagglutination (MRHA) (data not shown). Agglutination tests using specific antisera against the Pap and Prs fimbriae, adhesins that mediate MRHA, confirmed that the expression of those fimbriae is strictly dependent on the presence of functional CRP-cAMP in the cell (data not shown).

J96, as most of the UPEC isolates, also expresses type 1 fimbriae, which are essential for the adherence and invasion of the bladder uroepithelium. The expression of type 1 fimbriae can be detected by mannose-sensitive yeast agglutination (MSYA), which attests the ability of type 1 fimbriated bacteria to bind to mannoses-containing receptors on the surface of yeast cells. A clear stimulation in the ability to cause MSYA was observed in the J96crp strain as compared with wt when growing in various culture media (LB, TBA, CFA, and TSA; data not shown). Semi-quantitative MSYA, using serially diluted LB cultures, corroborated these results: agglutination of yeast cells was observed with a higher dilution of the J96crp cell suspension (4-fold, i.e. containing 8-times less bacterial cells) as compared to wt (Table 1). These results indicate that the deficiency in CRP-cAMP caused a substantial increase in the expression of type 1 fimbriae on the cell surface.

In UPEC, a regulatory crosstalk between fimbrial operons occurs, which also affects the expression of type 1 fimbriae. It is known that the UPEC-specific regulators PapB, SfaB, and FocB, which are involved in the regulation of P-related, S-related, and F1C-related fimbriae, respectively, have the ability to repress the expression of type 1 fimbriae [40–42]. It has been described that

| Strain          | Genotype | MSYA | crp |
|-----------------|----------|------|-----|
| J96             | fim<sub>op</sub> | 1/2  | 1/8 |
| VL751/pACYC184  | fim<sup>-</sup> | n.d. | n.d. |
| VL751/pSH2      | fim<sub>op</sub> | 1/32 | 1/128 |
| MG1655          | fim<sub>MG1655</sub> | 1/4  | 1/8  |

Bacterial cultures of the indicated strains (wt and crp) were grown in LB medium overnight at 37°C with vigorous shaking. The origin of the fim determinant present in each strain is indicated. The numeric values indicate the highest dilution of the bacterial culture that agglutinated yeast cells. n.d. = no MSYA detected.

References:

1. Author Summary
2. Results/Discussion
3. Table 1. Semi-quantitative MSYA in wt and crp derivatives of different bacterial strains

The table shows the semi-quantitative MSYA of different bacterial strains under phase variation control, which determines the percentage of fimbriated cells in the population. In this report, we show that the expression of type 1 fimbriae is repressed by a metabolic sensor of the cell, the global regulatory complex CRP-cAMP. We demonstrate that CRP-cAMP affects the switching outcome by selectively inhibiting the recombination process in one direction only, resulting in a lower percentage of fimbriated cells. Such a switch to the non-fimbriated state after successful adhesion might be advantageous in the urinary tract, where the immune mechanisms of the host favor the removal of bacteria expressing immunogenic surface structures. Understanding the regulatory networks that govern regulation of virulence and colonization factors is both of basic interest and might help to develop novel strategies to treat bacterial infections.
CRP-cAMP is essential for the expression of the operon encoding the P-related fimbriae and the PapB regulator [39]. Therefore, a possible explanation for the observed increase in type 1 fimbriation in the csp derivative could be the lack of expression of UPEC-specific repressors of type 1 fimbriae such as PapB. To test this, MSYA experiments were performed using wt and csp derivatives of an E. coli K-12 strain, which lacks such regulators. The V1751 strain (mutant in the chromosomal fim gene cluster and consequently defective in type 1 fimbriation), carrying the entire fim determinant of the J96 strain on the pACYC184-based plasmid pSH2 was used. csp derivatives of this commensal strain expressing the fimJ determinant of the J96 strain had an enhanced agglutination ability as compared with the wt strain (Table 1). As expected, strains carrying pACYC184 did not agglutinate yeast cells. Furthermore, type 1 fimbriation expression was also monitored in the K-12 strain MG1655 (fim+) and its csp derivative, giving the same result (Table 1). As a control, in all MSYA assays described, the agglutination could be effectively blocked by the addition of mannoligos (data not shown). Since the up-regulation of type 1 fimbriation was observed in both UPEC and commensals isolates, our results exclude the possibility that the CRP-cAMP effect strictly requires any UPEC-specific factor or is solely due to regulatory crosstalk between fimbrial operons, although we can not rule out a possible contribution to the regulation in pathogenic isolates.

CRP-cAMP plays a dual role in the transcriptional regulation of type 1 fimbriae

The fimA gene encodes the major subunit of the type 1 fimbriae and its expression is phase variable. Transcriptional studies were performed using two lineages derived from AAEc198A and AAEc374A strains, which carry the same fimA-lacZ operon fusion in the chromosome. The AAEc374A derivatives CBP374 (wt) and CBP375 (Acrp) are phase variation deficient due to mutations in the fimB and fimE genes (encoding the site-specific recombinases) and have the invertible element locked in the ON orientation. Such strains were used to monitor the transcriptional activity of the fimA promoter. The AAEc198A derivatives CBP198 (wt) and CBP199 (Acrp) are phase variation proficient and consequently, the fimA expression monitored using these strains integrates both the percentage of fim-expressing cells (phase variation) and the activity of the fimA promoter.

Using strains CBP198 and CBP199, a clear fimA up-regulation was observed in the csp background as compared with wt (Fig. 1A), indicating that CRP-cAMP represses type 1 fimbriation at the transcriptional level, consistent with the agglutination data (Table 1). On the other hand, when using CBP374 and CBP375 strains (Fig. 1B), a significantly lower fimA promoter activity was detected in the csp mutant, thereby suggesting that CRP-cAMP stimulates fimA promoter activity itself. Having in consideration that CRP activity is strictly dependent on its co-factor cAMP, in-frame cya deletion mutant strains (cAMP-deficient strains) were created and compared with isogenic csp and wt strains. The effect of the cya mutation on fimA expression resembled the one observed in the csp strain (Fig. 1A and 1B). Moreover, restoration of CRP-cAMP activity in csp and cya derivatives by using either the low copy number plasmid pCBP68 (pLG338-csp) or external addition of cAMP, respectively, restored fimA expression to wt levels (Fig. 1A and 1B). Taken together, we may conclude that CRP-cAMP has a dual role in the transcriptional expression of type 1 fimbriae: i) to stimulate transcription of the fimA promoter in phase-ON-cells and, ii) to repress the overall type 1 fimbriation expression. A response in the expression of type 1 fimbriae upon addition of exogenous cAMP was reported in static cultures of different cya derivative strains [35]. However, the response was divergent in different bacterial strains. The physiological heterogeneity in static cultures might be the cause of the strain-dependent variation detected. Our experiments using shaken cultures growing under uniformly aerated conditions gave identical results with all the strains used. Moreover, an increased fimA transcriptional expression in a CRP-cAMP deficient strain could be extracted from a microarray dataset on the effect of the csp mutation on the global pattern of expression in E. coli [10], consistent with our results.

To further probe into the dual role of CRP-cAMP in fimA expression, the effect of rapid restoration of CRP activity by addition of cAMP to cya strains was studied. When using early log phase cultures of the phase variation proficient strain CBP198 and its cya counterpart (Fig. 1C), the exposure to cAMP during one hour period did not significantly alter the expression of fimA when compared to the expression in control cultures (no addition of cAMP). However, a significant rapid alteration in expression (p = 0.036 after 20 minutes) as a response to the addition of cAMP was detected when using the phase variation deficient strains CBP374 (wt) and CMM374 (ΔcyA) (Fig. 1D). The differences in the kinetic of the response suggest that the dual role of CRP-cAMP on fimA expression might be achieved by distinct mechanisms: a direct stimulation of the fimA promoter activity and an indirect role in the overall negative effect of CRP-cAMP on type 1 fimA expression.

CRP-cAMP affects phase variation of type 1 fimbriae

So far, we have described that CRP-cAMP deficiency causes: i) a higher degree of type 1 fimbriation, ii) an increase in the expression of type 1 fimbriae in phase variation proficient strains, and iii) a reduction in fimA promoter activity in phase variation deficient strains. These results suggest that the percentage of fimbriated cells (ON-cells) in both csp and cya mutant strains is elevated when compared to wt, causing the overall increase in fimA expression. To test this prediction, the percentage of ON-cells in the population was monitored by plating cultures of the phase variable fimA-lacZ reporter strains on indicator plates containing X-gal. As predicted, a significant increase in the percentage of ON-cells in both the csp and cya mutant strains was observed (Fig. 2A). A quantitative PCR based method was validated (see Materials and Methods and Fig. S1) and used to detect and quantify the subpopulations having the invertible element either in the ON or in the OFF orientation [40]. Consistent with the results obtained from indicator plates, a significantly higher percentage of ON-cells was found for CMM198 (ΔcyA) as compared to CBP198 (wt) (Fig. 2B). Moreover, the cya deficiency was chemically complemented by addition of exogenous cAMP in the culture medium. The effect of CRP-cAMP deficiency in the switching between the ON and OFF orientation was further corroborated when in vivo switching frequencies were measured (see below). Similar results were obtained when using the reporterless and type 1 fimbriation-proficient strain MG1655 and its cya mutant derivative (Fig. 2B), thereby excluding the possibility that the lacZ donor DNA sequence present in the reporter strains might affect the CRP-mediated effect on phase variation. Moreover, when derivatives of the uropathogenic isolate J96 were used, a significant increase in the percentage of ON-cells was detected in csp derivatives. While most of the cells in the wt population were in the OFF orientation under the culture conditions used, a subpopulation of cells with the invertible element in the ON orientation was clearly detected in the mutant derivative (Fig. 2C).

To confirm these results, the level of fimA transcript in cultures of J96 and its derivative J96csp were quantified by Northern blot analysis (Fig. 2D). A 2.4-fold increase in fimA transcript was
detected in J96crp as compared with wt, corroborating the results obtained when using fimA-lacZ reporter strains (Fig. 1A).

The drop in fimA expression in CRP-cAMP deficient strains carrying mutations in fimB fimE (Fig. 1B) was assumed to indicate a stimulatory effect of CRP-cAMP on fimA promoter activity. However, it could also be a consequence of an alteration of the percentage of ON-cells by the action of some alternative FimB/FimE-like recombinase, as described for several E. coli strains [43]. As depicted in Fig. 2E, OFF-cells were not observed in cultures of strains CBP374 (wt), CBP375 (Δcrp), and CMM374 (Δcya), thereby ruling out the involvement of alternative recombinases, which is consistent with the fact that no genes for such enzymes are detected in the MG1655 genome [43]. Taken together, our results suggest that CRP-cAMP acts on the phase variation process by causing a decrease in the percentage of fimbriated cells in the population.

Physiological implications of the CRP-cAMP–mediated regulation of type 1 fimbriation

Type 1 fimbriation is growth phase dependent [31,32]. The fimA expression profile throughout the growth curve was studied using the strains CBP189 (wt) and CBP199 (Δcrp). As previously described, fimA expression in wt cultures was low in the early growth stages, increased in the middle of the logarithmic phase, and stayed constantly high throughout stationary phase (Fig. 3A). In contrast, fimA expression in the crp mutant peaked during early logarithmic phase and then dropped down to almost wt levels during late-logarithmic phase. Consistent with the transcriptional data, a larger difference in the semi-quantitative phenotypic determination of type 1 fimbriae expression (MSYA) was observed with mid-log phase cultures of the wt and crp derivatives of MG1655 (1/2 versus 1/8, respectively) when compared to stationary phase cultures (1/4 versus 1/8, Table 1). The analysis of fimA expression through the growth curve suggests that CRP-cAMP represses type 1 fimbriation in actively growing cells, while during growth arrest, the repression is released and other global regulators such as RpoS and ppGpp assume the control [31,32]. This finding is also in agreement with the described growth phase-dependent levels of CRP-cAMP in the cell. As assessed by Northern blot analysis, crp transcriptional expression is high in early exponential phase and significantly reduced in stationary phase [44].

A well-described factor that alters the intracellular levels of CRP-cAMP is carbon source availability, e.g. the presence of glucose causes a significant reduction [6,7]. The effect of glucose on the expression of type 1 fimbriae was monitored. A modest but
significant increase in the percentage of \( \text{fimA} \)-expressing cells could be observed when CBP198 (wt) cultures were grown in M9-glucose medium compared with cultures grown in M9-glycerol (Fig. 3B). The stimulatory effect of the presence of glucose on transcriptional expression of type 1 fimbriae was also observed by microarray analysis on the effect of glucose in the general expression pattern in \( E. \) coli [45].

CRP-cAMP deficient strains have a significant growth defect compared to the wt (i.e: 89 and 34 minutes generation time in LB for CBP199 and CBP198, respectively), which might raise the question whether the increased type 1 expression in the \( \Delta \text{crp} \) strains is merely due to the growth alterations. However, growth in media that significantly increases the growth rate of the \( \Delta \text{crp} \) strain, i.e. LB medium containing glucose (32 and 48 minutes generation time for CBP198 and CBP199, respectively), did not alter the difference in the expression of type 1 fimbriae between the wt and the \( \Delta \text{crp} \) strains (data not shown), suggesting that the CRP specific effect on type 1 fimbriae expression is not coupled to the growth rate.

**CRP-cAMP affects the FimB-mediated OFF to ON switch both in vivo and in vitro**

The reported increase in the percentage of ON-cells in the CRP-cAMP deficient strains could be achieved either by stimulating the OFF to ON inversion (exclusively catalyzed by FimB) or by causing the opposite effect on the ON to OFF inversion (mainly catalyzed by FimE). To further dissect the role of CRP-cAMP in the recombination event, the percentage of ON-cells in wt and \( \Delta \text{cyA} \) derivative strains expressing either FimB (AAEC370A, \( \Delta \text{fimE} \)) or FimE (AAEC261A, \( \Delta \text{fimB} \)) was determined (Fig. 4A). In the FimB proficient strains (\( \Delta \text{fimE} \)), a significant increase in the percentage of ON-cells was detected in the strain lacking CRP-cAMP (16% in \( \Delta \text{cyA} \) versus 4% in wt). However, in FimE proficient strains (\( \Delta \text{fimB} \)), consistent with published results [46], all cells were in the OFF orientation independently of the presence or absence of the CRP-cAMP complex. These results suggest that CRP-cAMP is directly or indirectly affecting the FimB-mediated inversion. To corroborate these data, \( in \) \textit{vitro} recombination assays were performed using template plasmids as recombination substrate in bacterial extracts of \( \Delta \text{cyA} \) and \( \Delta \text{cyA}^{+} \) strains overexpressing either FimB or FimE. The induction of the synthesis of the recombinases in cultures of \( \Delta \text{cyA} \) and \( \Delta \text{cyA}^{+} \) strains provided apparently identical amounts of the enzymes in the extracts of both strains as determined by Coomassie-stained SDS-PAGE (Fig. S2). When FimB-mediated OFF to ON inversion was monitored (Fig. 4B), recombination occurred with both \( \Delta \text{cyA} \) and wt extracts in the presence of FimB. However, a remarkable 3-fold higher percentage (\( p = 0.003 \)) of invertible fragments in the

![Figure 2](https://example.com/figure2.jpg)

**Figure 2. The percentage of fimbriated cells in the population is increased in \( \Delta \text{crp} \) and \( \Delta \text{cyA} \) strains.** (A) The percentage of \( \text{fimA} \)-expressing cells in presence (white bar) or absence (black bars) of 5 mM cAMP was determined by the indicator plate assay (see Materials and Methods) using mid-log phase cultures of the strains CBP198 (wt), CBP199 (\( \Delta \text{crp} \)) and CMM198 (\( \Delta \text{cyA} \)). Mean values and standard deviations from three independent experiments are shown. (B) Quantification of the percentage of ON-cells in bacterial populations by a PCR-based assay. Cultures of wt and \( \Delta \text{cyA} \) derivatives of strains CBP198 and MG1655 were grown to mid-log phase in presence (white bars) or absence (black bars) of 5 mM CAMP. Mean values and standard deviations of three independent experiments are shown. (C) ON-OFF diagnostic of mid-log phase cultures of the J96 strain and its \( \Delta \text{crp} \) derivative; the arrowhead highlights the fragment corresponding to ON-cells detected in the J96\( \Delta \text{crp} \) samples. (D) Northern hybridization of total RNA extracted from mid-log cultures from strains J96 (wt), J96\( \Delta \text{crp} \) (\( \Delta \text{crp} \)), VL751 (\( \Delta \text{fim} \)), and AAG42 (\( \Delta \text{lrp} \)) with specific probes for \( \text{fimA} \), \( \text{fimB} \), \( \text{lrp} \), and 16S rRNA as indicated. (E) ON-OFF diagnostic of duplicated cultures of the phase variation deficient strains CBP374 (wt), CBP375 (\( \Delta \text{crp} \)), and CMM374 (\( \Delta \text{cyA} \)). A control showing the band pattern of an OFF population was included for comparison. The pictures in panels C and E are electronically inverted images of ethidium bromide stained acrylamide gels.

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A significant effect was observed in the ON to OFF switching and generation in wt and mutant, respectively, while no fimB expression using plasmid pPKL9, which contains the fimB experiments were performed under conditions of constitutive FimB recombinase. To further test this hypothesis, absence of CRP-cAMP is not strictly dependent on the levels of both extracts, suggest that the enhanced OFF to ON switching in MG1655 strains (Fig. 2D and data not shown), the switching rate was significantly increased in strain CBP199 (crp
deficient strain as shown). The percentage of ON-cells in cultures of J96 derivatives CRP-cAMP proficient and deficient genetic backgrounds (data not shown). Altogether, our results both in vivo and in vitro indicate that the CRP-CAMP complex has a negative effect on the switching process independently of the intracellular concentration of the FimB recombinase.

CRP-cAMP represses type 1 fimbriation by an indirect mechanism

Two possible mechanisms by which CRP-cAMP affects the FimB-mediated switch should be considered: either CRP-cAMP can directly interact with the invertible DNA fragment repressing the OFF to ON switch, or the effect of CRP-cAMP may be indirect.

The slow response when adding exogenous cAMP to CMM198 cultures (Fig. 1C) suggested that the role of CRP-cAMP in the regulation of the phase variation occurs by an indirect mechanism. Nevertheless, to establish whether CRP-cAMP might also be directly involved in the switching process, in vitro recombination assays were performed using extracts of the cya strain while restoring CRP-cAMP activity by addition of increasing amounts of cAMP (Fig. 5A). No obvious alteration in the FimB-mediated switch was detected, strongly suggesting that CRP-cAMP does not directly interact with the nucleoprotein complex that is the substrate for the FimB recombinase. Accordingly, no effect was observed in the outcome of in vitro recombination assays when purified CRP was added to extracts obtained from a ctp strain (data not shown).

Simultaneously, the possible binding of CRP-cAMP to various DNA fragments spanning different regions of the fim determinant was tested (Fig. S3A). No strong CRP binding was detected to any of the DNA fragments tested. At most, a low affinity binding was detected in case of the fragment containing the fimA promoter (PCR7; Fig. S3B). However, when DNAse I footprinting analysis of this putative CRP binding site was performed, no binding was observed (data not shown). It has been reported that CRP-cAMP might bind to many low affinity binding sites along the E. coli chromosome [47]. Although it is possible that such low affinity CRP binding site(s) may exist in the fimA promoter region, our experimental evidence (Fig. 5A) suggested that binding is not required for the phase variation control. A possible involvement of the putative CRP binding site(s) in the positive control of the fimA promoter activity (Fig. 1B) will be further studied.

Inhibition of DNA gyrase activity mimics the effect of CRP-cAMP on type 1 fimbrial phase variation

Recently, it has been shown that inhibiting the DNA gyrase promotes the FimB-mediated inversion from OFF to ON and therefore it was concluded that DNA supercoiling determines the directionality of the FimB-mediated recombination [29,48]. DNA gyrase is an enzyme that catalyzes ATP-dependent DNA breakage, strand passage and rejoining of double-stranded DNA [for a recent review see Nollmann et al. [49]]. DNA gyrase is involved in the regulation of DNA topology, but also in other processes such as replication or illegitimate recombination [50,51]. Remarkably, it has been described that CRP-cAMP modulates the expression of the gyrA gene encoding the DNA gyrase. In ctp deficient strains, low levels of gyrA expression and DNA gyrase activity, monitored as alterations in the topology of plasmid DNA, were detected [52]. One may hypothesize that the CRP-cAMP mediated effect on the FimB-recombination process could directly result from the low levels of DNA gyrase activity detected in ctp deficient strains. To test this hypothesis, the effect of inhibiting the DNA gyrase in vivo was analyzed in both wt (CBP198) and cya (CMM198) strains compared with wt, yielding a 50% higher percentage of ON-cells.Comparable results were obtained when using MG1653 derivative strains (data not shown). Altogether, our results both in vivo and in vitro indicate that the CRP-CAMP complex has a negative effect on the switching process independently of the intracellular concentration of the FimB recombinase.

Figure 3. Type 1 fimbriae expression profile in different growth conditions. (A) fimA expression was determined by measuring β-galactosidase activity at various optical densities from cultures of the fimA-lexA reporter strains CBP198 (wt, black bars) and CBP199 (Δcrp, white bars) in LB medium at 37°C. (B) Quantification of the percentage of fimA-expressing cells in the population of strain CBP198 (wt) on indicator plates. Cultures were grown to mid-log phase at 37°C in M9 minimal medium containing either glycerol (glyc.) or glucose (gluc.) as a carbon source. Mean values and standard deviations from three independent experiments are shown.

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ON orientation was detected in the extract from the cya strain when compared with wt extracts. On the other hand, FimE-mediated inversion from the ON to the OFF state did not seem to be affected by a mutation in the cya gene (Fig. 4C). The FimB recombinase can also catalyze the switch from ON to OFF. However, no effect of CRP-cAMP on the FimB-mediated ON to OFF inversion was detected when in vitro recombination assays with DNA template in the ON orientation were performed (data not shown). Altogether, our in vitro studies corroborate the results obtained in vivo and suggest that the CRP-cAMP complex specifically affects the FimB-mediated recombination event from the OFF to the ON orientation. Supporting this conclusion, in vivo switching frequency estimations indicated that the OFF to ON switching rate was significantly increased in strain CBP199 (Δcrp) as compared with CBP198 (wt) (1.1×10^{-6} and 1.4×10^{-6} per cell and generation in wt and mutant, respectively), while no significant effect was observed in the ON to OFF switching (1.0×10^{-6} and 1.6×10^{-6} per cell and generation in wt and mutant, respectively). Also supporting our results, it was reported that the FimB-mediated switching frequency from OFF to ON is 3-fold higher in the presence of glucose (i.e. reduced intracellular levels of CRP-cAMP) than in the presence of glycerol [24].

Although higher expression of fimB was observed in ctp derivatives as compared to wt counterparts in both J96 and MG1653 strains (Fig. 2D and data not shown), the in vitro data, where the recombinases were overexpressed to the same degree in both extracts, suggest that the enhanced OFF to ON switching in absence of CRP-cAMP is not strictly dependent on the levels of the FimB recombinase. To further test this hypothesis, in vivo experiments were performed under conditions of constitutive fimB expression using plasmid pPKL9, which contains the fimB gene under the control of the tet promoter (Fig. 4E). Control experiments by Northern blot analyses verified that the fimB expression levels from plasmid pPKL9 were essentially identical in CRP-cAMP proficient and deficient genetic backgrounds (data not shown). The percentage of ON-cells in cultures of J96 derivatives constitutively expressing fimB (carrying plasmid pPKL9) was significantly elevated in the CRP-cAMP deficient strain as
Addition of increasing amounts of novobiocin (DNA gyrase inhibitor) in wt cultures caused a concomitant increase in fimA expression, consistent with previously reported data [29,48]. Remarkably, the fimA expression level was essentially unaltered by addition of novobiocin in cultures of the cya mutant strain. In agreement with the hypothesis proposed, the novobiocin-mediated inhibition of the DNA gyrase caused an increase in the percentage of ON-cells in the wt strain, but not in the cya derivative (Fig. 5C, upper panel). Results that further corroborated our hypothesis were obtained by inducing overexpression of DNA gyrase from cloned gyrAB genes in the cya strain CMM198. Both repression of fimA expression and reduction in the percentage of ON-cells was observed (Fig. 4A). Moreover, when fimE mutant derivatives were used, thus only reflecting FimB-mediated inversion, an identical response to novobiocin was observed, indicating that the recombination process that was responsive to gyrase inhibition in vivo is FimB-specific (Fig. 5C, lower panel). To rule out the possibility that the lacZ2A sequences present in the fimA-lacZ2A fusion might cause alterations in the regional DNA supercoiling and consequently affect the phase variation, similar experiments were performed using reporterless derivatives of strains MG1655 and J96. Similar results were obtained: i) an increase in the percentage of ON-cells in the wt strains was observed after addition of increasing novobiocin concentration (5-fold and 2-fold increase with the highest concentration of novobiocin tested in MG1655 and J96 strains, respectively), ii) the level of ON-cells was not altered by novobiocin treatment in the CRP-cAMP deficient derivatives, and iii) the percentage of ON-cells in the wt achieved by novobiocin treatment was similar to the level detected in the CRP-cAMP deficient derivatives (data no shown). It is noteworthy that in all approaches (Fig. 5B and 5C) the presence of novobiocin in sub-inhibitory concentrations did not significantly alter the expression of fimA in the cya mutant strains, which is in agreement with a low DNA gyrase activity in the CRP-cAMP deficient background as a result of the low expression of the gyrC gene [52]. To corroborate the in vivo results obtained, in vitro analyses were performed where increasing amounts of novobiocin were added to the wt strain extract. A progressive increase in the OFF to ON switching efficiency in vitro was observed (Fig. 5D), consistent with our in vivo data and with previously reported data [29,48]. Moreover, the unaltered fimE expression in the cya mutant strain by addition of novobiocin, together with the in vitro switching data, indicates that the fimA promoter is indifferent to changes in the DNA gyrase activity, in agreement with previous data [48]. The fact that inhibition of the DNA gyrase activity in vitro stimulated the FimB-mediated recombination suggests an active role of the DNA gyrase during the recombination process itself. Altogether, our data provide evidence that the induction of type 1 fimbriae in vivo is CRP-cAMP dependent.

Figure 4. The CRP-cAMP regulatory complex affects the FimB-mediated OFF to ON switch. (A) Determination of the percentage of ON-cells in streptococci expressing either FimB (AAEC370A, fimB+ fimE) or FimE (AAEC261A, fimB fimE+). The percentage of ON-cells in the cultures (x%) is indicated as mean values and standard deviations in brackets of three independent experiments. (B) In vitro OFF to ON recombination assay in bacterial extracts either containing FimB or being recombinase free. Bacterial extracts were obtained from strain NEC026 (fim, cya+) and its isogenic cya mutant CMM026, transformed with either an inducible fimB expression plasmid (pB378, fimB+) or the vector control (pET111, fimB+). Extracts were mixed with the template plasmid pJL-2 (fim invertible element in the ON orientation). The orientation of the plasmid-encoded fim invertible element was determined after 3 h incubation by using the PCR-based assay (see Materials and Methods). Results are provided in the bar diagram as percentage of invertible elements in the ON orientation. The picture in the right part of the Figure illustrates an ethidium bromide stained gel from one of the experiments used to obtain the data shown. (C) In vitro ON to OFF recombination assay in bacterial extracts either containing FimE or being recombinase free. A similar assay as in B was performed. In this case, bacterial extracts were obtained from strain NEC026 (wt) or its isogenic cya mutant CMM026, transformed with either an inducible fimE expression plasmid (pB382, fimE+) or the vector control (pET111, fimE+). Extracts were mixed with the template plasmid pMM36 (fim invertible element in the ON orientation) and analyzed as in B. In both B and C, mean values and standard deviations from four independent experiments are shown. (D) Effect of CRP-cAMP deficiency in the percentage of ON-cells in cultures of strain J96 and its cya derivative carrying either the plasmid pPKL9 (constitutive fimB expression, fimB OE+) or the vector control (pBR322, fimB OE−). The percentage of ON-cells was quantified from cultures of two independent clones. In A and D, the image corresponds to the upper half of an ethidium bromide stained gel.

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fimbriation detected in the CRP-cAMP deficient strains is a process mediated by the alteration in DNA gyrase activity and therefore can be mimicked by the specific inhibition of this enzymatic activity by novobiocin. Moreover, our in vitro results comparing OFF to ON switching between wt and cya mutant using the same DNA template for both extracts (Fig. 4B and Fig. 5D) demonstrated that the CRP-cAMP effect on phase variation is not merely dependent on the initial supercoiling state of the fim invertible element.

Lack of CRP-cAMP results in increased Lrp levels
Recombination at the fim invertible element requires Lrp, a DNA bending protein that directly binds to specific sites within the invertible element and stimulates DNA inversion [26]. Kelly et al. [29] demonstrated that this binding activity of Lrp is required to promote the FimB-mediated OFF to ON directionality observed when the DNA gyrase was inhibited. The Lrp levels were determined by immunoblot analysis of cya+ and cya strains (Fig. 6A) and the Lrp content detected was several fold higher in the cya strains than in the cya+ strains. These results suggest a possible link between the CRP and Lrp regulons. Interestingly, a direct demonstration of CRP dependent regulation of Lrp expression has not been done, although two putative CRP sites have been predicted in the promoter region of the lrp gene [53], suggesting a possible direct regulation by CRP-cAMP. Additionally, CRP-cAMP could act indirectly by positively regulating GadE, which represses lrp expression [54,55]. Transcriptional studies have been performed by Northern blot analysis of RNA from derivatives of MG1655 and J96 (Fig. 2D). An increase in the level of the lrp transcript in the cya derivatives was detected as compared with wt (2.5 and 1.7-fold in MG1655 and J96 derivatives, respectively), suggesting a role for CRP-cAMP in the control of lrp transcription.
although an additional regulation at the posttranscriptional level can not be ruled out. Further studies would be required to characterize the CRP-cAMP dependent regulation of Lrp expression.

Interestingly, when the levels of intracellular Lrp were monitored in the same cultures as in Fig. 5B, again a differential effect of the inhibition of the DNA gyrase was observed (Fig. 6B). In a wt strain, the Lrp levels were strongly elevated at the highest concentration of novobiocin, where the amount of Lrp was apparently identical to the amount detected in the cya strain in absence of novobiocin, which again might be explained by the low DNA gyrase activity detected in the CRP-cAMP deficient background [52]. We tested whether increased levels of Lrp by itself would cause an alteration in the FimB-mediated switching process. Our results clearly indicate that Lrp overexpression per se did not result in any significant changes in the percentage of ON-cells when no alteration in DNA gyrase activity was induced (Fig. 6C).

Concluding remarks

The expression of type 1 fimbriae implies the allocation of an important part of the asset of the bacterial cell for the production of those proteinaceous appendages, as considerable amounts of energy and amino acids are needed for their synthesis. A tightly regulated expression of such organelles is therefore expected. Considering the important metabolic effort performed by the bacterial cells committed to be fimbriated, regulation by phase variation can be seen as a selective advantage for the bacterial population, in addition of providing phenotypical heterogeneity in an otherwise genetically homogeneous population. In previous works, we have shown that the expression of type 1 fimbriae is stimulated when intracellular levels of the stringent response alarmone ppGpp are raised [52,56]. The level of ppGpp in the cell increases under amino acid starvation and energy stress [57]. In this report, the role of CRP-cAMP, a regulatory complex that is associated with the energy state of the cell, has been included in the extensive list of regulatory networks controlling type 1 fimbriation.

Interestingly, many of the global regulators that affect type 1 fimbriae expression, such as H-NS, RpoS, Lrp, and now CRP-cAMP, have been shown to interplay among each other, thereby orchestrating gene regulation cascades in response to the growth conditions [58]. From our studies, we can conclude that CRP-cAMP is a major regulator of fimbriation during the exponential growth phase (Fig. 3A) and is required to maintain the growth expression profile of type 1 fimbriae. We dissected the initial observation that the cya derivatives of J96 showed an increased ability to agglutinate yeast cells and conclude that CRP-cAMP represses type 1 fimbriation, as schematically shown in Fig. 7, by the recently described mechanism of switching directionality established by the activity of the DNA gyrase and the presence of Lrp [29], thereby affecting FimB-mediated OFF to ON switching. Remarkably, CRP-cAMP inhibited FimB-mediated recombination at a template plasmid isolated from a cya background, indicating that the regulatory effect does not merely depend on the supercoiling status of the DNA and thereby suggesting an active role of the DNA gyrase in the OFF to ON recombination event. Interestingly, CRP-cAMP has a dual effect on type 1 fimbriation by repressing phase variation and promoting promoter activity. Further studies will be required for fully understanding the underlying mechanisms by which CRP-cAMP affects both levels of regulation of type 1 fimbriation.

In Salmonella, cya mutants are avirulent in a mouse model [59] and it has been reported that the cya and the cya genes are strongly repressed during infection of macrophages [60]. Moreover, it has been observed that DNA becomes more relaxed when bacteria are growing in certain intracellular environments and consequently the expression of those genes that are required for intracellular survival is induced [61]. Therefore, CRP-cAMP might be involved in controlling Salmonella virulence in a pathway that includes DNA supercoiling and the sensing of environmental conditions as previously proposed [62].

It is well described that CRP and cAMP levels are affected by environmental conditions such as glucose availability and osmolarity [6,7]. Interestingly, such environmental conditions also affect DNA topology in E. coli in a DNA gyrase dependent manner [63,64]. The link between CRP-cAMP mediated regulation of gene expression and DNA gyrase activity might represent a specialized signal transduction pathway that senses the metabolic and energetic status of the cell. It can not be ruled out that in this regulatory pathway others factors such as the FIS protein might be involved. FIS has been proposed...
Figure 7. Schematic model of action of the CRP-cAMP complex on the regulation of type 1 fimbriation. The integration of different environmental signals modifies the levels of the CRP-cAMP complex which affects the phase variation of type 1 fimbriae by altering the FimB-mediated directionality of the OFF to ON recombination event. Based on our results, a model is proposed where a stimulation of the DNA gyrase activity by CRP-cAMP [52] would repress the FimB-mediated recombination from the OFF to the ON orientation by a mechanism that requires the presence of Lrp [29]. In this work, a repressing effect of CRP-cAMP on the expression of Lrp and a stimulatory effect of CRP-cAMP on fimA promoter activity have also been described. Green arrows indicate stimulatory effects, whereas red lines indicate repressing effects.

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earlier as a metabolic sensor involved in the homeostatic control of DNA supercoiling. Interestingly, CRP-cAMP modulates fis expression [65]. Supporting this model, a correlation between the sensitivity to catabolite repression and to gyrase inhibitors has been observed earlier for different metabolic operons. Hence, inhibition of DNA gyrase activity represses the expression of several CRP-cAMP sensitive genes (three maltose operons, the lactose and galactose operons, and the tryptophanase gene), whereas CRP-cAMP independent genes such as threonine and tryptophan operons were insensitive to DNA gyrase inhibitors [66,67].

The physiological relevance of the CRP-cAMP-mediated signaling pathway controlling type 1 fimbriation needs to be further explored. Nevertheless, the modulation of type 1 fimbriation mediated by glucose availability may provide a possible explanation to the reported observation that diabetic patients commonly are highly susceptible to urinary tract infections [68]. The presence of an unusual high concentration of glucosides in the urine of those patients may cause a reduction in the intracellular level of CRP-cAMP in the bacterial cell. This could result in a concomitant increase in type 1 fimbriation which, in turn, would raise the probability of colonization of the urinary tract.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

All strains and plasmids used in this study are listed in Table 2. Strains were grown to mid-log phase (corresponding to an \(OD_{600nm}\) of around 0.5) with vigorous shaking (200 rpm) at 37°C in Luria Bertani (LB) medium [69] or in M9 medium [70] supplemented with either 0.4% glycerol or 0.4% glucose, unless otherwise stated. For mannose-resistant haemagglutination (MRHA), bacteria were grown in CFA (1% casaminoacids, 0.1% yeast extract, 0.05% MgSO_4, 0.005% MnCl_2). For mannose-sensitive yeast agglutination (MSYA), bacteria were grown in different culture media: TBA (1% trypton, 0.5% NaCl, 1.5% agar), TSA (1.5% trypticase peptone, 0.5% phytone peptone, 0.5% NaCl, 1.5% agar) and CFA. When necessary, the following antibiotics were used: tetracycline (12.5 \(\mu\)g ml\(^{-1}\)), carbencillin (50 \(\mu\)g ml\(^{-1}\)), kanamycin (25 \(\mu\)g ml\(^{-1}\)) and chloramphenicol (15 \(\mu\)g ml\(^{-1}\)). When indicated, cyclic AMP was added in a final concentration of 5 mM. To study type 1 fimbriae expression, cultures of the different strains were always inoculated using colonies showing an OFF (non-fimbriated) phenotype. When fimA-lac\(_Z\) fusion derivatives were used, OFF-colonies could be identified on X-gal plates (white colonies). In reporterless strains (MG1655 and J96 derivatives), the fimbriation state of the inoculum was estimated from the colony morphology, since ON-colonies are small and convex, while OFF-colonies are large and flat, as described by Blomfield et al. [71].

Genetic techniques

Standard molecular manipulations were performed according to Sambrook and Russel [70]. The \(cya\) deletion mutant (\(Δ21–259\)) and \(Δcya::Kmr\) deletion mutant (\(Δ21–259::Kmr\)) were created by allelic exchange as described by Link et al. [72]. The deletion mutant and \(Km\) deletion mutant were verified by PCR amplification using primers cya-A and cya-D, and cya-up and cya-D, respectively. Gene alleles were introduced by phage P1-mediated transduction [73] using the following donor strains; BEU742 for \(Δcya::Tcr\), SS5357 for \(ΔΔcya::Tcr\) and CMM2 for \(Δczy::Kmr\). Derivatives \(cya^+\) and \(Δcya\) were initially selected by colony size and confirmed by PCR using primers CRP1 and CRP3. The plasmid pPAG6 was constructed by cloning a PCR-amplified fragment spanning the \(hp\) gene between the EcoRI-SmaI sites of pBAD30. The PCR fragment was generated using the primers lrp-1 and 64 and MG1655 as template. All primers used are specified in Table S1.

Mannose-resistant haemagglutination (MRHA)

Bacteria were grown on CFA agar plates. Bacterial cell suspension in PBS containing 3% (w/v) mannose (methyl α-D-mannopyranoside, Sigma) were prepared and adjusted to an \(OD_{600nm}\) of 5. MRHA was tested using suspensions from human (agglutination-positive with P-fimbriae) and dog (agglutination-positive with Prs-fimbriae) erythrocytes (8%, v/v) in PBS, giving identical results. The erythrocytes and bacterial suspensions were mixed in proportion 1:1 (v/v) on a glass-slide. Presence of aggregates was considered as agglutination positive.

Fimbrial antisera agglutination

Bacteria were grown on CFA agar plates. Bacterial colonies were mixed with 10 µl of 20× diluted antisera. Two types of antisera were used, pPAP5 and pPAP60 (originally obtained against P and Prs fimbriae, respectively) that strongly cross-react and mediate agglutination through both types of fimbriae [74]. Presence of aggregates was considered as agglutination positive.

Mannose-sensitive yeast agglutination (MSYA)

Bacteria were grown on LB plates overnight at 37°C, washed in PBS and resuspended to an \(OD_{600nm}\) of 5. Yeast cells (\(Saccharomyces cerevisiae\)) were washed and resuspended in PBS to an \(OD_{600nm}\) of 5. The suspensions were mixed in a 1:1 (v/v) ratio on a glass-slide placed on ice. After 30 min, the presence of aggregates as sign for

Environmental Signals
(glucose, osmolarity...)

CRP-cAMP

DNA Gyrase Activity

FimB

Phase Variation

CRP-cAMP Regulation of Type 1 Fimbriae

FimA promoter activity

ON

OFF

Figure 7. Schematic model of action of the CRP-cAMP complex on the regulation of type 1 fimbriation. The integration of different environmental signals modifies the levels of the CRP-cAMP complex which affects the phase variation of type 1 fimbriae by altering the FimB-mediated directionality of the OFF to ON recombination event. Based on our results, a model is proposed where a stimulation of the DNA gyrase activity by CRP-cAMP [52] would repress the FimB-mediated recombination from the OFF to the ON orientation by a mechanism that requires the presence of Lrp [29]. In this work, a repressing effect of CRP-cAMP on the expression of Lrp and a stimulatory effect of CRP-cAMP on fimA promoter activity have also been described. Green arrows indicate stimulatory effects, whereas red lines indicate repressing effects.

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Table 2. Strains and plasmids used in this study

| Strains | Relevant Phenotype | Reference or Source |
|---------|-------------------|---------------------|
| J96    | Clinical isolate  | [81]                |
| BEU805 | J96Δcrp39, Tc<sup>+</sup> | This study          |
| CMM96  | J96Δγα:Km<sup>+</sup> | This study          |
| MG1655 | Δλ::F<sup>+</sup> fim<sup>+</sup> | [82]                |
| BEU742 | M182 Δcrp39, Tc<sup>-</sup> | [7]                 |
| S55357 | Δλ::Tc<sup>-</sup> | S.A. Short          |
| AACE198A | MG1655 ΔlacZYA fimA-lacZYA | [26]               |
| AACE374A | MG1655 ΔlacZYA fimA-lacZYA fimB-am6 fimE-am18, phase locked ON | [26]               |
| AAG42  | AACE198A Δλ::F<sup>+</sup> Tc<sup>-</sup> | This study          |
| CBP198 | AACE198A, Tc<sup>-</sup> | This study          |
| CBP199 | AACE198A Δcrp39, Tc<sup>-</sup> | This study          |
| CMM198 | Δγα, Tc<sup>-</sup> | This study          |
| CMM74  | AACE374A, Tc<sup>-</sup> | This study          |
| CMM75  | AACE374A Δγα, Tc<sup>-</sup> | This study          |
| AACE261A | MG1655 ΔlacZYA fimB-lacZYA | [26]               |
| CMM261A | MG1655 ΔlacZYA fimA-lacZYA fimE-am18 | This study          |
| AACE370A | MG1655 ΔlacZYA fimA-lacZYA fimE-am18 | This study          |
| CMM370A | Δγα::Km<sup>-</sup> | This study          |
| CMM1   | MG1655 Δγα::Km<sup>-</sup> | This study          |
| CMM2   | MG1655 Δγα::Km<sup>-</sup> | This study          |
| NEC026 | BL21 (DE3) ΔfimB-H | [79]                |
| CMM026 | NCO026 Δγα::Km<sup>-</sup> | This study          |
| VL751  | CH50 ara Δacr-pro) rpsL thi fim | [21]                |
| CBP751 | VL751 Δcrp39, Tc<sup>-</sup> | This study          |

Plasmids

| plG338 | cloning vector, Tc<sup>-</sup>, Km<sup>-</sup> | [83]                |
| plG339 | cloning vector, Tc<sup>-</sup>, Km<sup>-</sup> | [83]                |
| pACYC184 | cloning vector, Tc<sup>-</sup>, Cm<sup>-</sup> | [84]                |
| pBR322 | cloning vector, Cb<sup>-</sup>, Tc<sup>-</sup> | [85]                |
| pBAD30 | expression vector, P<sub>ara</sub> promoter, Cb<sup>-</sup> | [86]                |
| pSH2  | fim-cluster from J96 cloned into pACYC184, Cm<sup>-</sup> | [19]                |
| pCBP68 | wt crp allele cloned into pLG338, Km<sup>-</sup> | [7]                 |
| pLG339-CRP | wt crp allele cloned into pLG339, Km<sup>-</sup> | [87]                |
| pET11  | T7 promoter expression vector, Cb<sup>-</sup> | [88]                |
| pBI378 | fim<sup>-</sup> under T7 promoter cloned into pET11 | [79]                |
| pBI382 | fim<sup>-</sup> under T7 promoter cloned into pET11 | [79]                |
| pMM36  | fim invertible element cloned into pACYC184 derivative, Cm<sup>-</sup> (switch ON) | [89]                |
| pJL-2  | fim invertible element cloned into pACYC184 derivative, Cm<sup>-</sup> (switch OFF) | [89]                |
| pPKL9  | fim<sup>-</sup> under tet promoter cloned into pBR322, Cb<sup>-</sup> | [18]                |
| pAA6   | lrp under ara promoter cloned into pBAD30, Cb<sup>-</sup> | This study          |
| pCA24N- gyrA | gyr<sup>-</sup> under T5-lac promoter cloned into pCA24N, Cm<sup>-</sup> | [90]                |
| pRSFDuet-gyrB | gyr<sup>-</sup> under T7-lac promoter cloned into pRSFDuet-1, Km<sup>-</sup> | [90]                |

agglutination was assessed and scored as + (weak) or ++ (strong). Semi-quantitative analysis was performed as described before [32].

Motility assay

Overnight cultures were diluted in LB and adjusted to an OD<sub>600nm</sub> of 1.0 and 4 μl were put on semi-solid LB medium containing 0.3% agar and incubated at 37°C.

Measurement of β-galactosidase activity

The β-galactosidase assay was performed as described by Miller [75]. Data presented represent average values and standard deviations of duplicate measurements from at least three independent experiments.

Determination of the percentage of fimA-expressing cells on indicator plates

To monitor the orientation of the fim invertible element in strains carrying a chromosomal fimA-lacZYA fusion, indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were used as previously described [24]. Cells having the fim invertible element in the ON orientation give rise to blue colonies, whereas those cells having it in the OFF orientation exhibit white color.

Determination of the switch orientation and quantification of the percentage of ON-cells using a PCR based assay

The orientation of the invertible DNA fragment can be determined using a molecular approach described previously [32,40]. In brief, a 602-bp DNA fragment containing the fim invertible element was PCR amplified using primers 2535 and 3137, Hin<sup>I</sup> restricted and analyzed on TBE-acrylamide-gels. Depending on the orientation of the fim invertible element, this method generates different sized fragments (404 and 118 bp when in the ON orientation, 402 and 200 bp when in the OFF orientation).

Quantification of the percentage of ON-cells in a specific sample was performed as described by Aberg et al. [32]. To prove the reliability of this method, calibration experiments were performed in triplicate using templates from strain CBP374 (a 100% ON-cells sample) and CBP198 (representing the OFF-cells samples). Both strains were grown to the same optical density and mixed such that the fraction of ON-cells in the template varied from 0 to 100%. PCR amplification, Hin<sup>I</sup> digestion and gel electrophoresis were performed as described above. The applicability of this method to quantify the percentage of ON-cells in bacterial populations was evidenced by a linear correlation [regression coefficient (R<sup>2</sup>) of 0.998] between the percentage of ON-cells in the sample and the intensity of the bands (Fig. S1). Since the invertible element in CBP198 is not completely in the OFF orientation, the intensity derived from ON-cells in the sample was performed as described by Aberg et al. [32]. To prove the reliability of this method, calibration experiments were performed in triplicate using templates from strain CBP374 (a 100% ON-cells sample) and CBP198 (representing the OFF-cells samples). Both strains were grown to the same optical density and mixed such that the fraction of ON-cells in the template varied from 0 to 100%. PCR amplification, Hin<sup>I</sup> digestion and gel electrophoresis were performed as described above. The applicability of this method to quantify the percentage of ON-cells in bacterial populations was evidenced by a linear correlation [regression coefficient (R<sup>2</sup>) of 0.998] between the percentage of ON-cells in the sample and the intensity of the bands (Fig. S1). Since the invertible element in CBP198 is not completely in the OFF orientation, the intensity derived from ON-cells in the “100%-OFF” sample was subtracted from all intensity values.

RNA isolation and Northern analysis

Total RNA was extracted from mid-log cultures using the hot-phenol method [76]. Contaminating DNA was removed by DNase I (Roche Diagnostics) treatment for 1 h at 37°C, followed by RNA cleanup using the RNeasy Mini kit (Qiagen). For Northern analysis, 20 μg of total RNA were separated on a formaldehyde:agarose gel and transferred to Hybond-N membranes (Amersham) by capillary blotting as described [77]. The membrane was hybridized with 32P-labeled DNA-fragments corresponding to the coding sequences of fimA, fimB, lrp, and m4, which were PCR

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generated using the primer pairs fimA-RT1&2, fimB-RT1&2, lrp-RT1&2 and 16S-RT1&2, respectively. After hybridization overnight at 32°C, membranes were subsequently washed in 1× SSC-0.1% SDS for 15 min at room temperature and in 0.1× SSC-0.1% SDS for 15 min at 52°C. Autoradiograms were obtained using StoragePhosphor screens (Molecular Dynamics), which were scanned using the Storm Imaging System (Molecular Dynamics).

Determination of FimB and FimE switching frequencies in vivo
FimB and FimE-promoted switching frequencies were measured as previously described [24].

In vitro recombination assay
To perform FimB in vitro recombination assays, bacterial extracts were obtained from cultures of the fim mutant strain NEC026 and its isogenic cya mutant strain harboring the plasmid pIB378 (fimB gene under the control of an IPTG inducible promoter in pET11). fimB expression was induced with 0.4 mM IPTG after the cultures grown in minimal MOPS [78] at 28°C had reached an OD600nm of 0.15. Cells were harvested after 24 h of incubation at 37°C and processed as described [40,79]. As control, extracts lacking FimB were obtained from cultures of the same strains carrying the pET11 plasmid. To perform FimE in vitro recombination assays, bacterial extracts were obtained from cultures of the strain NEC026 and its isogenic cya mutant strain transformed with the plasmid pIB382 (fimE gene under the control of an IPTG inducible promoter in pET11). Cultures were manipulated as described above. The in vitro recombination assay was performed as described [40,79]. The resulting orientation of the invertible element was analyzed after 3 h incubation at 37°C using the PCR-based method described above.

Immunoblotting
Whole cell extracts from bacterial cultures were separated by SDS-PAGE as described by Laemmli [80] using 15% polyacrylamide gels. Samples were transferred to PVDF membranes using a semidy blotting apparatus. After blocking the membranes overnight in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk, membranes were incubated for 1 h at room temperature with 2,000× diluted PapA-specific antiserum [38] in TBS-T containing 5% skimmed milk. After 3, 15 min washes in TBS-T and the membranes were incubated for 1 h with 20,000× diluted anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Dianova, Hamburg, Germany). After further washing, membranes were developed using the enhanced chemiluminescence (ECL+) kit (GE Healthcare) and analyzed on a Chemidoc System (BioRad) equipped with the QuantityOne® Software for quantification.

Statistical analysis
Differences between average values were tested for significance by performing an unpaired, two-sided Student’s t-test. The levels of significance of the resulting p values are reported by the following symbols: * = p<0.05; ** = p<0.01; *** = p<0.001 and n.s. = non-significant.

Supporting Information
Figure S1 Validation of the PCR based assay used for quantifying the percentage of cells in the population with the invertible element in the ON-orientation. Suspensions of CBP198 (OFF-template) and CBP374 (ON-template) cells were mixed in the indicated ratios (0–100% ON-template). The HinL restriction pattern of the PCR amplified fragment containing the fim invertible element is shown. The size (in base pairs) of the different diagnostic ON and OFF fragments is indicated. Below each lane, the results of quantification and calculation of the percentage of ON-cells in the population in each sample according to the described method are presented as mean values ± standard deviation of three independent calibration experiments.

Figure S2 Electrophoretic analysis of protein extracts used for the in vitro recombination assays. Extracts from NEC026 (wt) and CMM026 (gyr) strains carrying the plasmids pET11 (vector control), pIB378 (pET11 carrying the fimB gene) and pIB382 (pET11 carrying fimE) were obtained as described in materials and methods. Proteins were separated by SDS-15% PAGE and Coomassie stained. The bands representing the induced FimB and FimE (right margin) and the molecular mass of relevant protein markers (left margin) are indicated.

Figure S3 DNA-binding pattern of CRP at the fim regulatory regions. A. Schematic representation of the fim determinant and position of the promoters (P) of fimB, fimE, and cyaA. Black arrowheads represent the inverted repeats flanking the invertible element. The relative positions of the PCR fragments used for the gel mobility shift are depicted below. B. Gel mobility shift assay of purified CRP protein (5 μM) and various PCR-amplified DNA fragments (PRC1-5, and PCR7, here shown using OFF-cells as template for amplification). In all panels, samples correspond to: lane 1: no protein (CAMP present); lane 2: CRP protein with 20 mM cAMP (active form); lane 3: CRP protein without cAMP (inactive form). Identical result was obtained when PCR7 fragment was obtained from ON-cells as template. Recombinant CRP protein was purified from strain pp6/pHA7 essentially as described (Zhang et al., 1991). The PCR amplification and the mobility shift assay were performed as described (Xia et al., 2000). - Zhang, X.P., Gunasekera, A., Ebright, Y.W., and Ebright, R.H. (1991) J. Biolom. Struct. Dyn. 9: 463-473. - Xia, Y., Gally, D., Forsman-Semb, K., and Uhlin, B.E. (2000) EMBO J. 19: 1450-1457.

Figure S4 Effect of gyrA and gyrB overexpression on the expression of type 1 fimbriae in a CRP-cAMP deficient strain. A. fimA expression from strain CMM198 (gyr) carrying the plasmids pCA24N-gyrA and pRSFDuet-gyrB, which carry the gyrA and the gyrB genes, respectively, under IPTG-inducible promoters, was monitored in either the absence (−) or the presence (+) of IPTG. Bacterial cultures were grown in LB medium to mid-log phase. IPTG was added at a final concentration of 0.015 mM (condition that did not cause any deleterious effect on the bacterial growth). Mean values and standard deviations from three independent experiments are shown. B. ON-OFF diagnostic of two of the bacterial cultures used in A. The panel depicts an electronically inverted image of the upper half of acrylamide gel after ethidium bromide staining; the arrowhead highlights the fragment corresponding to ON-cells.

Table S1 Oligonucleotides used in this study.

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