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CadC-Mediated Activation of the *cadBA* Promoter in *Escherichia coli*

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Key Words
pH · CadC · Signal transduction · Transcriptional activator · H-NS · Oligomerisation

Abstract
The transcriptional activator CadC in *Escherichia coli*, a member of the ToxR-like proteins, activates transcription of the *cadBA* operon encoding the lysine decarboxylase CadA and the lysine-cadaverine antiporter CadB. *cadBA* is induced under conditions of acidic external pH and exogenous lysine; anoxic conditions raise the expression level up to 10 times. To characterize the binding mechanism of CadC, procedures for the purification of this membrane-integrated protein and its reconstitution into proteoliposomes were established. The binding sites of CadC upstream of the *cadBA* promoter region were determined by in vitro DNaseI protection analysis. Two regions were protected during DNaseI digestion, one from –144 to –112 bp, designated Cad1, and another one from –89 to –59 bp, designated Cad2. Binding of purified CadC to Cad1 and Cad2 was further characterized by DNA-binding assays, indicating that CadC was able to bind to both DNA fragments. Genetic analysis with promoter-*lacZ* fusions confirmed that both sites, Cad1 and Cad2, are essential for activation of *cadBA* transcription. Moreover, these experiments revealed that binding of H-NS upstream of the CadC-binding sites is necessary for repression of *cadBA* expression at neutral pH and under aerobic conditions. Based on these results, a model for transcriptional regulation of the *cadBA* operon is proposed, according to which H-NS is involved in the formation of a repression complex under non-inducing conditions. This complex is dissolved by binding of CadC to Cad1 under inducing conditions. Upon binding of CadC to Cad2 *cadBA* expression is activated.

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Introduction

At acidic external pH several genes are induced to support the survival of *Escherichia coli* under this unfavorable condition. Among these genes are those for the degradative amino acid decarboxylase systems *adi, gad* and *cad*.

The Cad system of *E. coli* consists of the cytoplasmic protein CadA and the integral membrane proteins CadB and CadC. CadA is a lysine decarboxylase catalyzing the decarboxylation of lysine to cadaverine. It is suggested that the generated cadaverine is excreted by the lysine-cadaverine antiporter CadB, which concomitantly mediates the uptake of lysine from the medium [Auger et al., 1989; Soksawatmaekhin et al., 2004]. CadB is probably anchored with 12 transmembrane helices in the cytoplasmic membrane, displaying similarity to the ornithine-putrescine-antiporter PotE [Auger et al., 1989; Soksawat-

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Table 1. In vivo activities of CadC derivatives in *E. coli* EP314

Plasmid	β-Galactosidase activity, units			
	pH 7.6		pH 5.8	
	0 m*M* lysine	10 m*M* lysine	0 m*M* lysine	10 m*M* lysine
pT-*cadC*	4 ± 1	15 ± 5	9 ± 3	287 ± 24
pT-*cadC-his*$_6$	73 ± 7	933 ± 56	87 ± 11	1,211 ± 77
pQE30-*cadC*	24 ± 2	29 ± 8	30 ± 5	27 ± 5
pET16b-*cadC*	4 ± 2	12 ± 3	9 ± 2	394 ± 22

E. coli EP314, harboring a chromosomal *cadA-lacZ* fusion and a non-functional *cadC*, was transformed with plasmids pT-*cadC*, pT-*cadC-His*$_6$, pQE30-*cadC* or pET-16b-*cadC*. Cells were grown in minimal medium at the corresponding pH and the lysine concentration as indicated. At OD_{600} ~1 cells were harvested by centrifugation, and β-galactosidase activity was determined [Miller, 1972] and is given in Miller units. The data presented represent average values obtained in at least three independent experiments (mean ± SD).

maekhin et al., 2004]. *cadA* and *cadB* are organized in one operon, localized at 93.7 min on the *E. coli* chromosome [Meng and Bennett, 1992a]. The *cadBA* operon is under the control of the P_{Cad} promoter. Expression of the *cadBA* operon is induced by external acidification, and the simultaneous presence of lysine in the medium. *cadBA* expression is inhibited by the end products of lysine decarboxylation, cadaverine [Neely et al., 1994] and CO_2 [Takayama et al., 1994]. Under anaerobic conditions the expression level is almost 10 times higher than under aerobic conditions [Sabo et al., 1974]. CadC is the positive regulator of *cadBA* expression [Watson et al., 1992].

The *cadC* gene lies upstream of the *cadBA* operon and encodes a 58-kDa inner membrane protein. CadC, a member of the ToxR-like transcriptional activators [Miller et al., 1987], consists of a cytoplasmic N-terminal domain (amino acids 1–158), a single transmembrane domain (amino acids 159–187), and a periplasmic C-terminal domain (amino acids 188–512) [Dell et al., 1994; Watson et al., 1992]. The cytoplasmic domain shows sequence similarity to the RO_{II} subgroup of DNA-binding domains of response regulators like PhoP from *Bacillus subtilis*, VirG from *Agrobacterium tumefaciens* or OmpR from *E. coli* [Watson et al., 1992]. In contrast to OmpR-like transcriptional regulators, signal transduction in CadC functions without any chemical modification, e.g. phosphorylation. Based on CadC derivatives with altered sensing properties due to single amino acid changes within the periplasmic domain, it is suggested that this domain is the signal input domain and that pH and lysine are sensed independent of each other [Dell et al., 1994]. Thus, CadC and all other ToxR-like proteins represent the simplest form of a stimulus-response mechanism in bacteria. However, not so much is known about the biochemical properties of these exceptional proteins, which comprise sensory and regulatory properties in a single polypeptide. Besides the transcriptional activator CadC, two other proteins, LysP and H-NS, were identified to affect *cadBA* expression. Both were found to be involved in the negative regulation of *cadBA* expression under non-inducing conditions [Neely et al., 1994; Shi et al., 1993].

Earlier, the transcriptional start point of the *cadBA* operon was localized [Neely and Olson, 1996], and Meng and Bennett [1992b] detected a region upstream of the *cadBA* operon that is important for the activation of the *cadBA* transcription. Here, we present a model about the activation mechanism of *cadBA* expression by CadC which integrates all known and new genetic and biochemical results.

Results

Properties of His-Tagged CadC Derivatives in vivo

To test the in vivo sensing and signal transduction capacity of various CadC derivatives, *E. coli* EP314 was used as reporter strain, which lacks a functional chromosomal *cadC* gene and carries a *cadA-lacZ* fusion gene on the chromosome. Upon transformation of this strain with plasmids carrying *cadC* derivatives, the inducible expression of the *cadBA* operon was probed by measuring β-galactosidase activity (table 1). EP314/pT-*cadC,* producing wild-type CadC, expressed *cadA-lacZ* only at pH 5.8 and in the presence of lysine as reported for chromosomal-

encoded CadC [Meng and Bennett, 1992b]. EP314/pT-*cadC-His$_6$* producing a CadC derivative with a C-terminal His-tag supported *cadA* expression at both pH 5.8 and pH 7.6, while lysine dependency was still preserved. Furthermore, β-galactosidase activities were about 4- to 5-fold higher than the activities determined for the wild-type control. EP314/pQE30-*cadC*, expressing a CadC derivative containing six His residues attached directly to the N-terminus prevented *cadBA* expression for unknown reasons. Finally, the separation of the N-terminal His-tag from the first methionine in CadC by a linker of 9 amino acids (SGHIEGRH), including the Factor Xa cleavage site, resulted in a CadC derivative, encoded by pET16b-*cadC*, that induced *cadA-lacZ* expression only at low external pH and in the presence of lysine as wild-type CadC.

Purification and Reconstitution of His$_{10}$-CadC

Based on in vivo complementation and overexpression studies, His$_{10}$-CadC was used for the in vitro characterization. The protein was overproduced, and inverted membrane vesicles were prepared. The best solubilization efficiency was achieved with lauryldimethylamine oxide (LDAO) in the presence of 0.15 *M* NaCl. Optimal purification results were achieved when binding of the protein to Ni^{2+}-NTA agarose was performed in the presence of 30 m*M* imidazole and 0.6 *M* NaCl. In a typical experiment 1.2 mg His$_{10}$-CadC was obtained from 40 mg membrane proteins. As judged from a silver-stained gel, the purity of His$_{10}$-CadC was approximately 95%. Reconstitution was carried out using the detergent-mediated method as described [Rigaud et al., 1995; Jung et al., 1997]. Purified His$_{10}$-CadC was mixed with *E. coli* phospholipids at a ratio of lipid:protein of 25:1. The efficiency of His$_{10}$-CadC reconstitution was determined to be 75%. Because CadC contains large hydrophilic domains on either side of the membrane, the expected orientation of His$_{10}$-CadC will be 50:50 (outside:inside) in proteoliposomes.

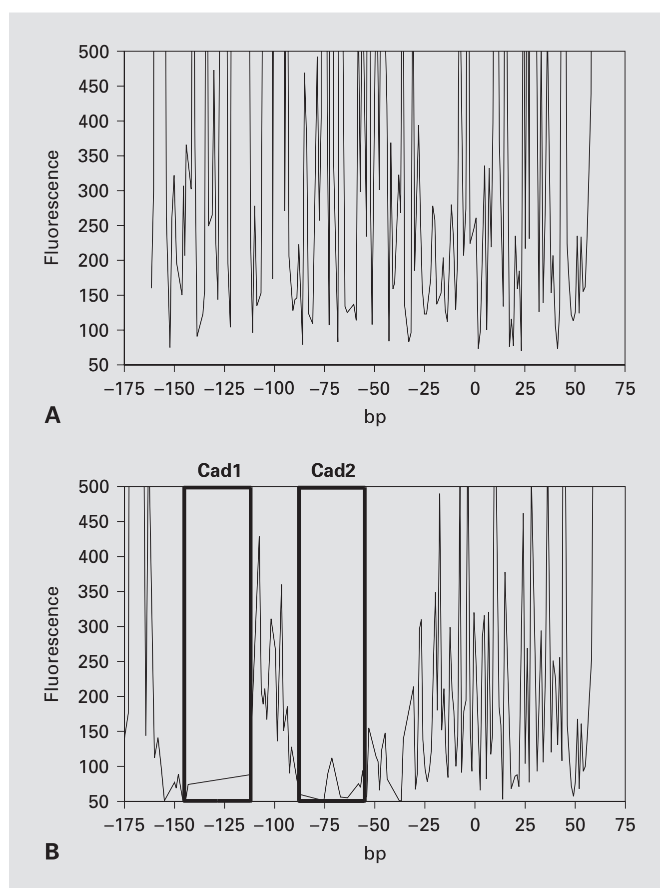


Fig. 1. DNaseI footprinting of purified CadC on the *cadBA* promoter region. A DNA fragment corresponding to base pairs –200 to +72 of the promoter region, labeled with fluorescein at the 5′ end of the top strand was subjected to DNaseI footprinting as described in Experimental Procedures. The chart shows the restriction pattern of the *cadBA* promoter fragment in the absence (**A**) or presence (**B**) of 3.75 μ*M* purified, reconstituted His$_{10}$-CadC protein. Regions protected by CadC are indicated by the boxes labeled 'Cad1' (bp –144 to –112 relative to the transcriptional start point) and 'Cad2' (bp –89 to –59).

Determination of CadC-Binding Sites on the cadBA *Promoter by DNaseI Protection Analysis*

For identification of the CadC-binding site(s), in vitro DNaseI protection analysis with the purified reconstituted His$_{10}$-CadC was performed. Analysis of the digestion fragments was carried out on a sequence analyzer as described by Sandaltzopoulos et al. [1994], in this case an ABI Prism 377. For this purpose, the *cadBA* promoter region was amplified by polymerase chain reaction (PCR) using a fluorescein-labeled oligonucleotide as sense primer which results in a double-stranded DNA fragment with one fluorescein-labeled strand at the 5′ end. This fragment was incubated with various concentrations of His$_{10}$-CadC followed by DNaseI digestion. Analysis of the resulting fragments revealed two gaps indicating two sites that were protected by His$_{10}$-CadC (fig. 1). The first gap comprised base pairs (bp) from position –144 to –112, relative to the transcriptional start point of the *cadBA* operon, designated as Cad1. A second gap appeared, however it was difficult to determine its exact length from the footprint analysis (fig. 1B). The region between bp –89 to –59 was maximal protected, but also the sequence more

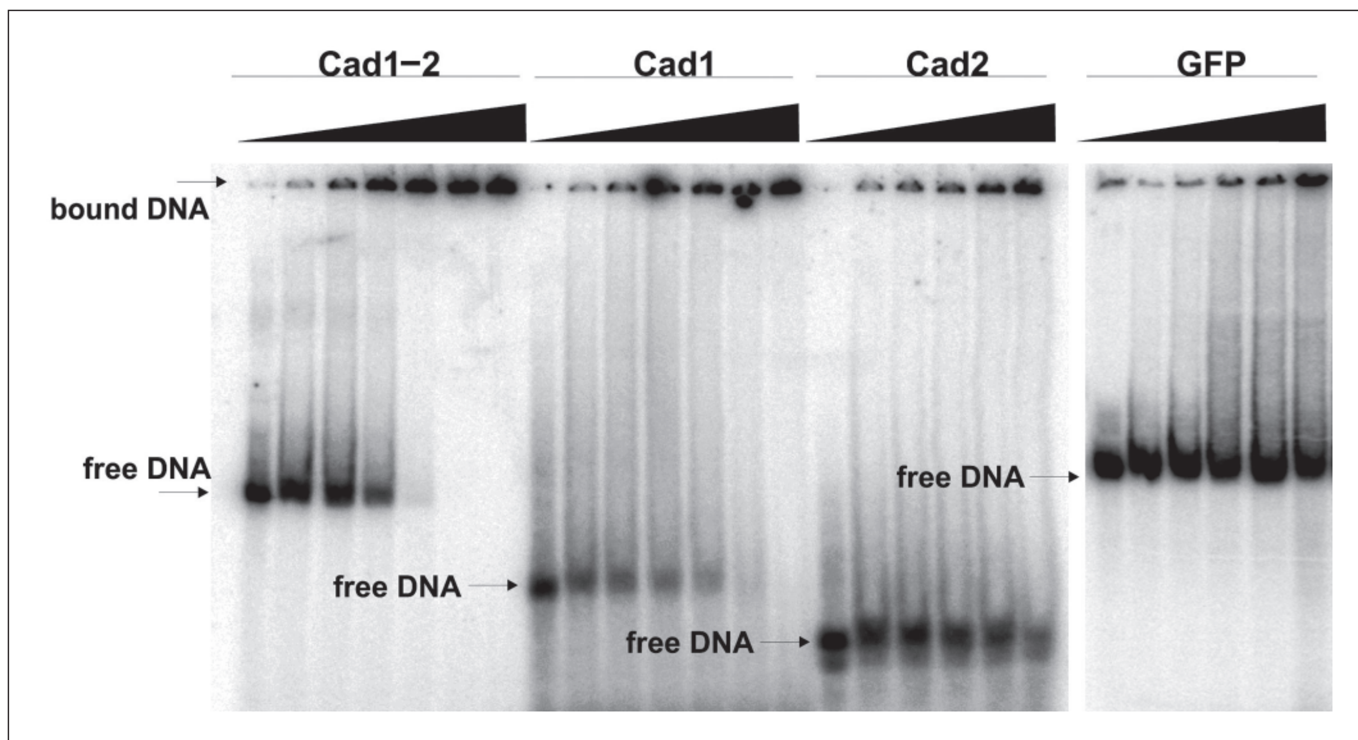


Fig. 2. Binding of purified CadC to the *cadBA* upstream region. Radiolabeled DNA fragments Cad1, representing the region from bp –150 to bp –112 of the *cadBA* upstream region, Cad2, representing the bp –89 to bp –59 region, and Cad1–2, representing the region from bp –150 to bp –59, were incubated with purified, reconstituted His_{10}-CadC of increasing concentrations (0, 0.1, 0.5, 1, 2 and 3 μ*M*), as indicated by black triangles. A DNA fragment representing the sequence of the *gfp* gene was used as control to test unspecific binding. The samples were incubated at 37°C for 10 min, and subsequently resolved in a 5% native polyacrylamide gel. Protein-DNA complexes remained in the wells, whereas unbound DNA ran into the gel according to size.

downstream (up to bp –30) appeared to be protected relative to the negative control. However, binding of CadC up to bp –30 would cover the –35 recognition site of the RNA polymerase which, for a transcriptional activation process, is unlikely. Furthermore, earlier in vivo methylation results [Meng and Bennett 1992a, b] did not support CadC-binding beyond bp –48. Hence the second CadC-binding site (dedicated as Cad2) was defined as the region of bp –89 to –59.

Analysis of CadC-Binding Properties to the cadBA *Promoter*

To test the DNA-binding properties of purified His_{10}-CadC and to characterize the binding site of CadC, the protein was analyzed by an electrophoretic mobility shift assay [Fried and Crothers, 1981] using fragments Cad1 (representing the DNA sequence of bp –150 to bp –112 relative to the transcriptional start point), Cad2 (bp –89 to bp –59) and Cad1–2 (bp –150 to bp –59) of the *cadBA* upstream region (fig. 2). As control, the binding of CadC to a DNA fragment representing the sequence of the *gfp* gene was analyzed. DNA fragments were incubated with increasing amounts of His_{10}-CadC reconstituted into proteoliposomes. Because CadC was tested in proteoliposomes, the protein could not enter the gel, and DNA-protein complexes were retained in the wells whereas unbound DNA ran into the gel according to its size. All three DNA fragments, Cad1, Cad2 and Cad1–2, were bound by purified His_{10}-CadC. A slightly lower affinity was determined for Cad2 in comparison to Cad1 and Cad1–2 (fig. 2). Experiments were also done with proteoliposomes extruded in buffer containing 10 m*M* lysine (co-activator of *cadBA* expression in vivo) or 1 m*M* cadaverine (inhibitor of *cadBA* expression in vivo). Both substances did not affect the affinity of CadC to the tested DNA fragments (data not shown).

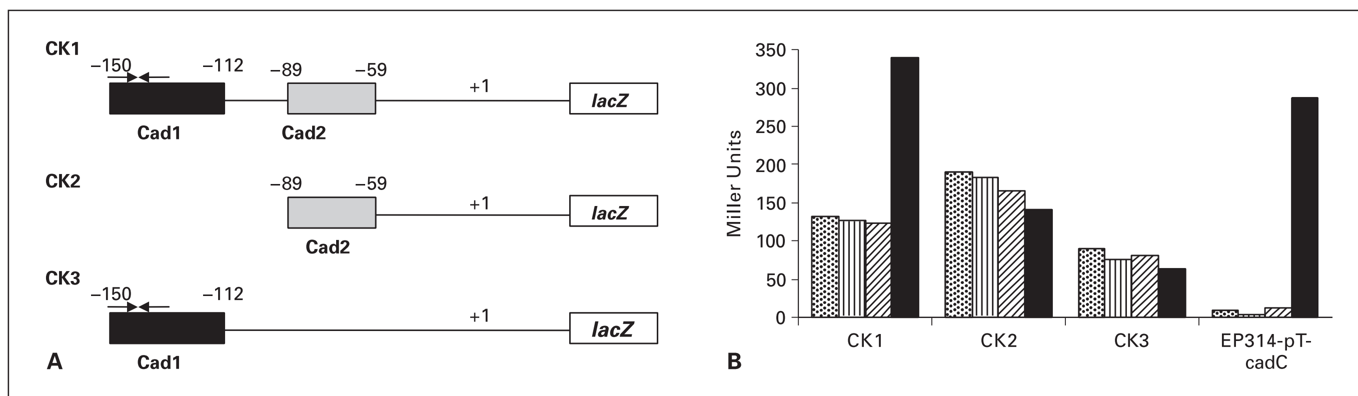


Fig. 3. In vivo analysis of CadC-binding sites. **A** Fusions were made by cloning PCR products containing portions of the *cadBA* promoter in front of a promoterless *lacZ* gene in plasmid pRS415. Subsequently, fusions were transferred to the chromosome of *E. coli* MG1655-Δ*lacZ* with the λRZ5-phage, resulting in strains CK1, CK2 and CK3, which are schematically presented. **B** Cells were cultivated in medium at pH 7.6, 0 m*M* lysine (▩), pH 5.8, 0 m*M* lysine (▥), pH 7.6, 10 m*M* lysine (▨), or pH 5.8, 10 m*M* lysine (■), and β-galactosidase activities were determined by the method described and are given in Miller units [Miller, 1972]. *E. coli* EP314 with a chromosomal wild-type promoter was transformed with plasmid pT-*cadC* and used as control.

Because of the buffering of the gel system at pH 7.5, the influence of pH on CadC binding could not be tested with electrophoretic mobility shift assays (EMSAs). Therefore, filter assays were performed in reaction buffers at pH 7.5 or pH 5.8. The results were essentially the same as those obtained by EMSAs. Significant differences in the affinity of CadC to the corresponding DNA fragments incubated at pH 7.5 or pH 5.8 were not observed (data not shown).

The overall affinity of CadC to the corresponding DNA fragments was found to be low. This became especially evident when binding of CadC to a DNA fragment comprising the *gfp* gene was tested (fig. 2). The determined affinity was only 5–10 times lower than those for the specific DNA fragments. From the literature differences in the order of 10^2–10^3 times between specific and unspecific DNA are known for transcriptional activators. Unexpectedly neither lysine nor protons affected the affinity of CadC to the DNA.

In vivo Analysis of the cadBA *Upstream Region*

The existence of two binding sites was further tested by in vivo studies. Vector pRS415 [Simons et al., 1987] was used as a reporter vector in which Cad1–2, Cad1 and Cad2 were fused to the promoterless *lacZ* gene of *E. coli*. Plasmid pRS415-Cad1–2 contained the *cadBA* upstream region from bp –150 to bp +72 (relative to the transcriptional start point), pRS415-ΔCad1 the *cadBA* upstream region from bp –89 to bp +72, and pRS415-ΔCad2 the *cadBA* upstream region from bp –150 to bp +72, but the Cad2 site (bp –89 to bp –59) was replaced with a random 30 bp DNA sequence.

All three operon *lacZ* fusions were transferred on the chromosome of *E. coli* MG1655-Δ*lacZ* using the λRZ-5-phage [Jones and Gunsalus, 1987], obtaining *E. coli* strains CK1 (Cad1–2), CK2 (ΔCad1) and CK3 (ΔCad2; fig. 3A) These strains were tested for pH and lysine-dependent *cadBA* expression. Only strain CK1, containing both CadC-binding sites, exhibited a wild-type- like expression pattern, as *cadBA* expression was significantly induced at pH 5.8, and in the presence of 10 m*M* lysine. Strains CK2 and CK3 were unable to induce *cadBA* expression in the presence of lysine at acidic pH (fig. 3B). It should be noted that in comparison to strain *E. coli* EP314/pT-*cadC*, all CK strains were characterized by an increased expression of the operon under non-inducing conditions at either pH 7.6 or pH 5.8 in the absence of lysine.

H-NS Acts as a Negative Regulator of cadBA *Expression under Non-Inducing Conditions*

The striking difference in strains EP314/pT-*cadC* and the CK strains in regard to *cadBA* expression under non-inducing conditions was investigated in more detail. Strain EP314/pT-*cadC* contains the *exa-1*::Mu dI*1734* allele resulting in a *cadA-lacZ* fusion. Whereas this strain exhibited only a basal level of *cadBA* expression under

non-inducing conditions, expression of this operon was approximately 10 times increased in the strains which comprised a definite length of the *cadBA* upstream region. These results suggested that an additional region located upstream of the CadC-binding sites is required for repression of *cadBA* under non-inducing conditions. A logic candidate for a repressor would be H-NS. H-NS is a small chromosome-associated protein found in enterobacteria and is involved in the modulation of a large number of genes in *E. coli* [Atlung and Ingmer, 1997]. Earlier, Shi et al. [1993] reported a significant increase in *cadBA* expression at neutral pH in *hns* deletion mutants. To test the hypothesis of a H-NS-mediated transcriptional silencing from a site upstream of the CadC-binding sites, DNA fragments of various lengths comprising sequences of the *cadBA* upstream region were fused to a promoterless *lacZ* gene and cloned into plasmid pRS551. The fusions were designated as $P_{Cad}150$, $P_{Cad}181$, $P_{Cad}221$, $P_{Cad}260$, $P_{Cad}290$ and $P_{Cad}600$, depending on their length relative to the transcriptional start point of the *cadBA* operon (fig. 4A). The plasmids were transformed into *E. coli* strains MC4100 (wild-type) and PD32 *(hns$^-$)*, and cells were grown at inducing pH 5.8 or non-inducing pH 7.6. Under both conditions, the fusions supported at least a 5–10 times stronger *cadBA* expression in the *hns* mutant PD32 than in the wild-type MC4100. In the wild-type strain MC4100, fusions $P_{Cad}150$ to $P_{Cad}260$ exhibited activities at neutral pH that were about 30% relative to their activities at acidic pH, but importantly, with fusions $P_{Cad}290$ and $P_{Cad}600$ a significant decrease in the activities was observed at non-inducing neutral pH (fig. 4B). In contrast, prolongation of the upstream region was without any effect in the *hns* mutant PD32. Fusion $P_{Cad}600$ exhibited the same high activities as $P_{Cad}150$. While in strain MC4100 fusion $P_{Cad}150$ produced 32% β-galactosidase activity under non-inducing conditions (relative to 100% activity under inducing conditions), and β-galactosidase activity of fusion $P_{Cad}600$ dropped to 8%, activities determined in strain PD32 remained high for all tested fusions (fig. 4B). These results clearly indicate that H-NS binding upstream of the CadC-binding sites causes a transcriptional silencing of the *cadBA*-operon at neutral external pH.

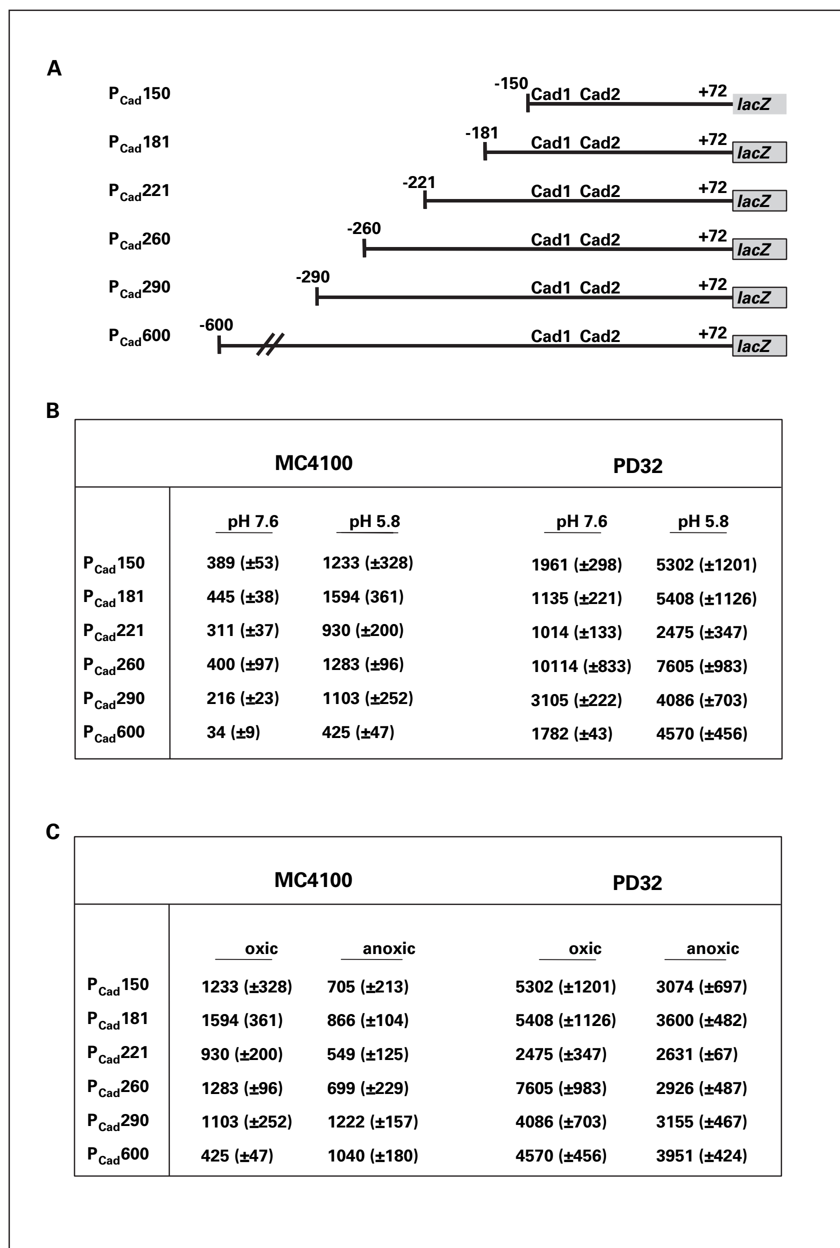
Reams et al. [1997] reported that a *cadA-lacZ* fusion in an *hns* mutant was de-repressed under aerobic conditions to approximately the same level as observed during anaerobic growth. Therefore, the same strains as described above were used to determine reporter activity in cells grown under aerobic and anaerobic conditions. Again, in strain PD32 lacking a functional *hns*, activities were generally much higher than in wild-type strain MC4100 (fig. 4C). Remarkably, in the wild-type strain MC4100 a significant drop in reporter activity was observed for the fusions $P_{Cad}290$ and $P_{Cad}600$ under aerobic conditions, while this effect was missing in strain PD32. These results suggest that repression of *cadBA* under aerobic conditions follows the same mechanism as the repression at neutral pH. The unusually low activities of *E. coli* MC 4100 under anaerobic conditions might be related to a slow growth rate. The reason for the high activities caused by fusion $P_{Cad}260$ in strain PD32 is unknown.

Computer-Assisted Analysis of the cadBA *Promoter Region*

It is well known that H-NS binds preferentially to intrinsically curved DNA. By computer-assisted analysis of the *cadBA* upstream region using the BendIt algorithm [Goodsell and Dickerson, 1994], several potential H-NS-binding sites at the *cadBA* upstream region with curvatures of >5° were found. The sites are shown in figure 5, and are designated from H-NS 1 to H-NS 5. A significant drop in reporter activity was found between fusions $P_{Cad}260$ to $P_{Cad}290$ unraveling H-NS 2 as site for H-NS-mediated transcriptional silencing of *cadBA*. The further decrease in reporter activities between fusion sites $P_{Cad}290$ to $P_{Cad}600$ indicated another H-NS-binding site upstream of H-NS 2, which could be H-NS 1, though its location inside the *cadC* structural gene seems to be unusual. It is interesting to note that H-NS 4 and H-NS 5 overlap with the CadC-binding sites Cad1 and Cad2.

Activation of cadBA *Transcription Is Strictly Dependent on CadC*

To test if *cadBA* expression under non-inducing conditions is still dependent on CadC, an *hns*, *cadC* double mutant was constructed (PD32-Δ*cadC*). Strains PD32 and PD32-Δ*cadC* were tested for lysine decarboxylase activity of CadA, whereby CadA activity reflected the ability of a strain to activate *cadBA* expression. Both strains were cultivated under inducing or non-inducing conditions. While strain PD32, as expected, resulted in CadA activities independent of the growth conditions, almost no activities were detectable in strain PD32-Δ*cadC* (table 2). This result clearly indicated that *cadBA* expression is strictly dependent on CadC.



B

	MC4100		PD32	
	pH 7.6	pH 5.8	pH 7.6	pH 5.8
P_{Cad}150	389 (±53)	1233 (±328)	1961 (±298)	5302 (±1201)
P_{Cad}181	445 (±38)	1594 (361)	1135 (±221)	5408 (±1126)
P_{Cad}221	311 (±37)	930 (±200)	1014 (±133)	2475 (±347)
P_{Cad}260	400 (±97)	1283 (±96)	10114 (±833)	7605 (±983)
P_{Cad}290	216 (±23)	1103 (±252)	3105 (±222)	4086 (±703)
P_{Cad}600	34 (±9)	425 (±47)	1782 (±43)	4570 (±456)

C

	MC4100		PD32	
	oxic	anoxic	oxic	anoxic
P_{Cad}150	1233 (±328)	705 (±213)	5302 (±1201)	3074 (±697)
P_{Cad}181	1594 (361)	866 (±104)	5408 (±1126)	3600 (±482)
P_{Cad}221	930 (±200)	549 (±125)	2475 (±347)	2631 (±67)
P_{Cad}260	1283 (±96)	699 (±229)	7605 (±983)	2926 (±487)
P_{Cad}290	1103 (±252)	1222 (±157)	4086 (±703)	3155 (±467)
P_{Cad}600	425 (±47)	1040 (±180)	4570 (±456)	3951 (±424)

Fig. 4. Activity of various-length *cadBA-lacZ* operon fusions. **A** Schematic representation of P_{Cad}-*lacZ* fusions. Fusions were made by cloning PCR products containing portions of the *cadBA* promoter in front of a promoterless *lacZ* gene in plasmid pRS551. Cells of MC4100, representing wild-type *E. coli,* and PD32, harboring a nonfunctional *hns*, were transformed with the plasmids. **B**, **C** Cells were cultivated at pH 7.6 under aerobic conditions (pH 7.6), at pH 5.8 under aerobic conditions (oxic, pH 5.8) or at pH 5.8 under anaerobic conditions (anoxic). When cells reached the mid-logarithmic growth phase, β-galactosidase activities were measured as described by Miller [1972] and are given in Miller units. The data presented represent average values obtained in at least three independent experiments and the standard deviations are included in parentheses.

Discussion

CadC is an unusual transcriptional activator, because it is an integral membrane protein. In order to characterize this protein in vitro, it was necessary to establish efficient solubilization and purification protocols. Several plasmids encoding CadC derivatives with the His-tag [Ljungquist et al., 1989] at various positions were constructed. The derivative with a 6-His tag at the C-terminus was only poorly overproduced. The low overproduction was probably due to difficulties transporting six charged His residues over the cytoplasmic membrane. CadC derivatives containing a His-tag directly linked to the N-terminus (His_6-CadC) or linked by 9 amino acids to the N-terminus (His_{10}-CadC) exhibited a satisfactory overproduction. In addition, these CadC derivatives were tested for complementation of a Δ*cadC* strain. For this purpose, *E. coli* EP314 was used as a reporter strain which also contains a chromosomal *cadA-lacZ* fusion. Derivative CadC-His_6 activated *cadBA* expression in a lysine-

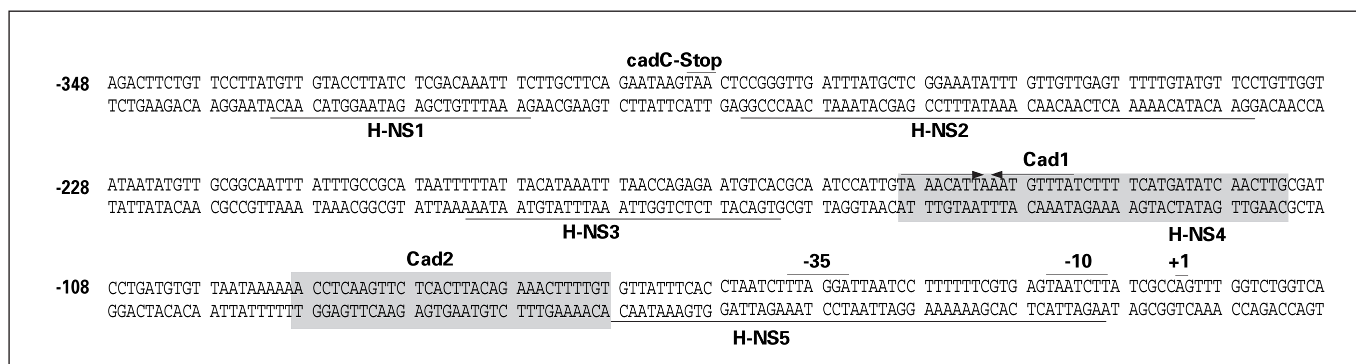


Fig. 5. The *cadBA* upstream region. Genetic, biochemical and computer-assisted data are summarized. The grey shaded regions mark the CadC-binding sites Cad1 and Cad2. Black lines represent regions with a proposed curvature of more than 5°, a prerequisite for H-NS binding as they were obtained by a computer-based analysis of DNA curvature. The positions of the –35/–10 region, the transcriptional start of *cadB* and the stop codon of *cadC* are indicated.

Table 2. CadC-dependent lysine decarboxylase activity (mean ± SD)

E. coli strain	Specific lysine decarboxylase activity, mmol/min · mg protein	
	non-inducing condition	inducing condition
MC4100	0.05 ± 0.01	0.52 ± 0.04
PD32	0.72 ± 0.12	0.84 ± 0.09
PD32-Δ*cadC*	0.09 ± 0.04	0.03 ± 0.02

Activity of the lysine decarboxylase CadA was used as measure of *cadBA* activation in different strains. Cells of *E. coli* MC4100, PD32 and PD32-Δ*cadC* were cultivated under inducing or non-inducing conditions, and specific activities of lysine decarboxylase were determined as described [Lemonnier and Lane, 1998] and are given in U/mg protein (1 U = 1 mmol cadaverine/min).

dependent manner, but independent of external pH, and the expression level was much higher compared to wild-type CadC. These results are in agreement with earlier findings according to which single amino acid substitutions close to the C-terminus of CadC abolished the pH-sensing properties of CadC [Dell et al., 1994]. In contrast, a His-tag at the N-terminus led to a CadC derivative that failed to activate *cadBA* expression under all conditions. Presumably an N-terminal His-tag sterically hinders the binding of the helix-turn-helix motif to the *cadBA* promoter. Finally, extension of the N-terminus of CadC by 10 His residues that were separated by a linker resulted in a CadC derivative that exhibited almost wild-type activity in β-galactosidase assays. By using metal-chelate affinity chromatography, His_{10}-CadC was purified to 95% homogeneity, and the protein was subsequently reconstituted in *E. coli* phospholipids.

The purified CadC in proteoliposomes was used to characterize the CadC-binding site in vitro by DNaseI protection and further binding assays. All results indicate the existence of two binding sites for CadC, one, designated as Cad1, comprising base pairs from –144 to –112, and a second, designated as Cad2, comprising base pairs from –89 to –59. These results specify the earlier finding of Meng and Bennett [1992b], who located the site essential for *cadBA* induction between bp –150 and –48. While Cad1 contains the inverted repeat sequence TAAACAT-T-A-AATGTTTA, Cad2 lacks either an inverted repeat or a palindromic sequence. Generally, the homology between Cad1 and Cad2 is low. When probing Cad1 and Cad2 by DNA-binding assays, the affinity of CadC to Cad1 was slightly higher than to Cad2. Remarkably, the observed binding efficiency of CadC to both sites was rather low. There are experimental difficulties inherent in the work with a membrane-integrated transcriptional regulator that might account for this low binding efficiency: (i) reconstituted CadC remained on top of the gel, therefore it was impossible to determine how many CadC molecules bind to one DNA-fragment; (ii) based on the membrane topology of CadC, an 50:50 (inside:outside) orientation of CadC in proteoliposomes is expected implying that maximal 50% of all molecules have an outside-oriented DNA-binding domain, and (iii) the molecular mechanism of how CadC is able to respond to pH and/or lysine is unknown thus far.

Binding of CadC to Cad1 and Cad2 was also tested at low pH, and/or the simultaneous presence of either the co-activator lysine or the inhibitor cadaverine. None of these parameters significantly altered the affinity of CadC to the DNA-binding sites. Since CadC functions as a transcriptional activator in vivo with lysine and protons as co-activators, the in vitro results indicate that either the test system is not sensitive enough or the activation mechanism is more complex. Despite the difficulties inherent in the test system mentioned above, we do not feel that the discrepancy relies on the in vitro test systems used. Rather we prefer a model according to which another protein is involved in CadC activation. The lysine permease LysP is a good, but so far a less well-understood candidate for such an interacting protein; its effect on *cadBA* expression has already been shown [Neely et al., 1994]. In addition, kinetic studies of *cadBA* activation indicated a fast response when cells were exposed sequentially to low pH and lysine, whereas the simultaneous exposure to both stimuli resulted in a delay of expression [Neely and Olsen, 1996]. The latter studies reveal that even more components might be involved in *cadBA* regulation.

Importantly, in vivo analyses confirmed that two sites, Cad1 and Cad2, are necessary for the activation of *cadBA* transcription. In addition, H-NS-mediated transcriptional silencing was observed. When the *cadBA* promoter region was prolonged to bp –290 or bp –600 (fusions $P_{Cad}290$ and $P_{Cad}600$), reporter activity was significantly decreased at neutral pH and also under aerobic conditions in the wild-type strain MC4100, but not in the *hns* mutant PD32. These results clearly indicate a function of H-NS as a repressor.

Molecular models for H-NS-mediated repression vary with respect to the position of H-NS binding to the DNA. When H-NS-binding sites overlap with the –35/–10 region, transcription is repressed by preventing the binding of RNA polymerase to the promoter. Such a mechanism has been demonstrated for the repression of *rrnB* in *E. coli* [Tippner et al., 1994] or *virB* in *Shigella flexneri* [Tobe et al., 1993]. Alternatively, it is also known that H-NS can exert its regulatory effect far upstream of the transcriptional start site. Examples for this mechanism are the repression of *proU* in *Salmonella enterica* [Owen-Hughes et al., 1992], or *toxT* in *Vibrio cholerae* [Nye et al., 2000]. For these genes it is proposed that H-NS binding generates locally constrained super-coiling of the DNA that specifically silences the promoter. Alternatively, binding of H-NS might decrease the rate of open complex formation.

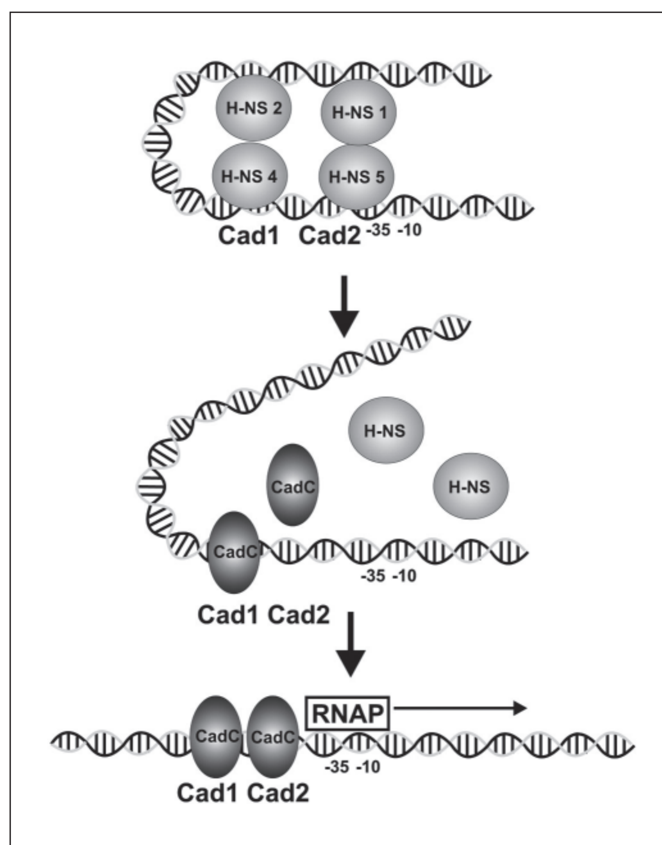


Fig. 6. CadC-mediated activation of the *cadBA* promoter in *E. coli*. Under non-inducing conditions, H-NS establishes a repressor complex by binding to the four sites H-NS 1, H-NS 2, H-NS 4 and H-NS 5 within the *cadBA* promoter region resulting in looping of the DNA and occupation of the –35/–10-binding region for RNA polymerase. Binding of CadC to Cad1 dissolves the repressor complex, and the H-NS molecules are released. Subsequently, RNA polymerase can bind to the non-occupied –35/–10 site, and upon stimulation by binding of CadC to Cad2 transcription of the *cadBA* operon is activated.

Our genetic data provide evidence for two H-NS-binding sites far away from the transcriptional start site (H-NS 1 and H-NS 2). In addition, computational analysis revealed three further sites that would overlap with the Cad1 and Cad2 sites, and with the –35/–10 region. In analogy to H-NS-mediated repression of the *virF* virulence gene promoter in *S. flexneri* [Falconi et al., 2001], we propose a model according to which H-NS molecules bound to the sites H-NS 1 and H-NS 2 interact with H-NS molecules bound to H-NS 4 and H-NS 5 resulting in a looping of the intervening DNA (fig. 6). Upon binding of CadC to Cad1 and Cad2, this repression complex would be dissolved allowing the RNA-polymerase to bind

to the *cadBA* promoter and start transcription. It has to be emphasized here that binding of H-NS to the H-NS#4 and H-NS#5 sites is only supported by the computational analysis thus far. The verification of these sites requires further experimentation.

Experiments with *S. flexneri* indicated an activation of expression of the virulence gene activator *virF* by the protein FIS and a repression by H-NS [Falconi et al., 2001]. In *S. flexneri* strains lacking *hns*, FIS is no longer required to activate *virF* expression. These results indicate that the only function of FIS is the elimination of the H-NS repression complex. In contrast, experiments with the *E. coli hns*, *cadC* double mutant revealed that CadC has a dual function by eliminating the H-NS repression complex and activating transcription. Binding of CadC to Cad2 might be responsible for the activation of transcription because strain CK2, harboring only the Cad2 site, exhibited a higher *cadBA* expression than CK3, harboring only the Cad1 site. Moreover, CadC had a slightly higher affinity to Cad1 than to Cad2 in in vitro EMSA experiments, suggesting that binding of CadC to the *cadBA* promoter is initiated and stabilized by binding to Cad1.

The effect of H-NS was tested with respect to the pH and oxygen-dependent expression of *cadBA*. In the wild-type strain MC4100, the fusions $P_{Cad}150$ to $P_{Cad}260$ could not be repressed by H-NS, due to the lack of the H-NS1 and H-NS2 sites. Remarkably, β-galactosidase activities in these strains were significantly higher at pH 5.8 than at pH 7.6 indicating that CadC still responds to environmental pH. In contrast, β-galactosidase activities were not further increased under anaerobic conditions. These results indicate that (i) CadC is not an oxygen sensor, and (ii) anaerobically increased *cadBA* expression is dependent on H-NS, which causes partial repression under aerobic conditions. Since H-NS binding depends on the topology of DNA, and anaerobiosis is known to alter DNA topology [Cortassa and Aon, 1993], such H-NS-mediated regulation would explain the variable expression levels of *cadBA* under different environmental conditions.

Experimental Procedures

Materials

[γ-^{32}P]ATP was purchased from Amersham Biosciences. All restriction enzymes and T4 DNA ligase were from New England Biolabs. *Taq* DNA polymerase was from Invitrogen. Synthetic oligonucleotide primers were obtained from Invitrogen or IBA. DNaseI was from Promega. Goat anti-(rabbit IgG) alkaline phosphatase conjugate was purchased from Biomol. Ni^{2+}-NTA resin, monoclonal anti-His antibody, and vector pQE30 were purchased from Qiagen, and pET16b from Novagen. Purified *E. coli* lipids were purchased from Avanti Polar Lipids. SM-2 Bio-Beads were from BioRad. Detergents were obtained from Calbiochem and Anatrace. All other materials were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids

Strains and plasmids are listed in table 3. *E. coli* JM109 was used as carrier for the plasmids described and for expression of *cadC* from the T5-promoter. *E. coli* BL21(DE3)/pLysS was used for expression of *cadC* from the T7-promoter. *E. coli* EP314 carries a *cadA-lacZ* fusion gene and is *cadC*$^-$. This strain was used to determine in vivo signal transduction. Strain PD32-Δ*cadC* was created by transducing the *cadC::kan* allele from strain W3110-Δ*cadC* into strain PD32 via P1 transduction. Plasmid pT-*cadC,* encoding wild-type CadC, was obtained by cloning of *cadC* into vector pT7-5 [Tabor and Richardson, 1985]. Plasmid pT-*cadC-His*$_6$, encoding CadC-His$_6$, was obtained by cloning of *cadC-His*$_6$ into vector pT7-5. In plasmid pET-16b-*cadC, cadC* was cloned into vector pET-16b. In all three plasmids expression of *cadC* is under the control of the T7 promoter. In plasmid pQE30-*cadC, cadC* was cloned into vector pQE30, in which expression of *cadC* is under the control of the T5 promoter.

DNA Sequencing

Constructs were verified by sequencing the length of the PCR-generated segment through the ligation junctions in double-stranded plasmid DNA using the dideoxynucleotide termination method [Sanger et al., 1992].

Overproduction of CadC Derivatives

Overproduction of CadC derivatives was tested with the following *E. coli strains*: BL21(DE3)/pLysS/pT-*cadC-His*$_6$, and JM109/pQE30-*cadC*; BL21(DE3)/pLysS/pET-16b-*cadC*. Each strain was grown in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl) with 100 μg/ml ampicillin at 37°C for 4 h. Expression of *cadC* derivatives was induced with 0.5 m*M* IPTG.

Preparation of Inverted Membrane Vesicles

E. coli BL21(DE3)/pLysS transformed with plasmid pET-16b-*cadC* was grown aerobically at 37°C in LB complex medium supplemented with ampicillin (100 μg/ml) and induced with 0.5 m*M* IPTG. Cells were harvested at an absorbance at 600 nm of approximately 1.8. Inverted membrane vesicles were prepared by passage of cells through a High Pressure Cell Disrupter (Constant Systems) and washed twice in EDTA-containing buffer of low ionic strength [Siebers and Altendorf, 1988], except that the buffer was changed from HEPES/Tris to Tris/HCl. The washed membrane vesicles were homogenized in 50 m*M* Tris/HCl, pH 7.5, 10% glycerol (v/v), 2 m*M* dithiothreitol (DTT), adjusted to 10 mg/ml protein and stored at –80°C until use.

Purification and Reconstitution of His$_{10}$*-CadC*

To an aliquot (typically 4 ml) of the washed membrane vesicles (10 mg/ml), 0.15 *M* NaCl (final concentration) was added and, while stirring on ice, membrane proteins were extracted with LDAO which was added stepwise until a final concentration of 2% (v/v) was reached. After stirring on ice for 30 min, this solubilization mixture was centrifuged for 45 min at 264,000 *g* for 45 min

Table 3. Strains and plasmids used in this study

Strain	Relevant genotype	Source or reference
BL21(DE3)-pLysS	F^- *ompT* r^-_B m^-_B	Studier and Moffatt [1986]
EP314	F^- IN(*rrnD-rrnE*) Δ(*lacIOPZYA*) *exa*-1::Mu*DI1734*(*Km lac*) *cadC1::Tn10*	Neely et al. [1994]
W3110-Δ*cadC*	F^- IN(*rrnD-rrnE*) *cadC::Kan*	Soksawatmaekhin et al. [2004]
MG1655	K12 Reference strain	Blattner et al. [1997]
MG1655-Δ*lacZ*	MG1655 *lacZ::Tet*	K. Jahreis (personal gift)
CK1	MG1655-λ*lacZ* (λ*RZ5-Cad1-2*)	This work
CK2	MG1655-λ*lacZ* (λ*RZ5-ΔCad1*)	This work
CK3	MG1655-λ*lacZ* (λ*RZ5-ΔCad2*)	This work
MC4100	F^- *araD139 (argF-lac)U169 rpsL150 relA#flb-5301 ptsF25 deoC1*	Casadaban and Cohen [1979]
PD32	MC4100 *hns::amp*	Dersch et al. [1993]
PD32-Δ*cadC*	PD32 *cadC::Kan*	This work
pET-16b	Expression vector, Ap^r	Novagen
pET-16b-*cadC*	*cadC* in pET-16b	This work
pQE30	Expression vector, Ap^r	Qiagen
pQE30-*cadC*	*cadC* in pQE30	This work
pT7-5	Expression vector, Ap^r	Tabor and Richardson [1985]
pT-*cadC*	*cadC* in pT7-5	This work
pUC19	Cloning vector, Ap^r	Yanisch-Perron et al. [1985]
pRS415	Promoter test vector, Ap^r	Simons et al. [1987]
pRS415-Cad1-2	pRS415::*cadBA* promoter region (–150 to +72)	This work
pRS415-ΔCad1	pRS415::*cadBA* promoter region (–89 to +72)	This work
pRS415-ΔCad2	pRS415::*cadBA* promoter region (–150 to +72; bp –89 to –59 replaced)	This work
pRS551	Promoter test vector, Ap^r, Kan^r	Simons et al. [1987]
pRS551-P_{Cad}150	pRS551::*cadBA*-promoter region (–150 to +72)	This work
pRS551-P_{Cad}181	pRS551::*cadBA*-promoter region (–181 to +72)	This work
pRS551-P_{Cad}221	pRS551::*cadBA*-promoter region (–221 to +72)	This work
pRS551-P_{Cad}260	pRS551::*cadBA*-promoter region (–260 to +72)	This work
pRS551-P_{Cad}290	pRS551::*cadBA*-promoter region (–290 to +72)	This work
pRS551-P_{Cad}600	pRS551::*cadBA*-promoter region (–600 to +72)	This work

in a Beckmann TL100 centrifuge. The supernatant fraction containing the solubilized His_{10}-CadC was purified by affinity chromatography in batch with 0.4 ml of Ni^{2+}-NTA resin in the following manner. The resin was pre-equilibrated with 10 bed volumes of washing buffer (50 m*M* Tris/HCl, pH 7.5, 10% (v/v) glycerol, 10 m*M* β-mercaptoethanol, 0.6 *M* NaCl, 30 m*M* imidazole, 0.2% (v/v) LDAO). Binding of His_{10}-CadC was carried out in batch by incubation of the solubilized proteins and the Ni^{2+}-NTA resin in buffer containing 30 m*M* imidazole and 0.6 *M* NaCl at 4°C for 30 min. The protein/resin complex was washed 3 times with washing buffer. Bound His_{10}-CadC was eluted with elution buffer (50 m*M* Tris/HCl, pH 7.5, 10% (v/v) glycerol, 10 m*M* β-mercaptoethanol, 0.6 *M* NaCl, 250 m*M* imidazole, 0.2% (v/v) LDAO). Purified His_{10}-CadC was reconstituted into *E. coli* phospholipids essentially as described [Rigaud et al., 1995; Jung et al., 1997]. Liposomes were solubilized with 0.47% (v/v) Triton X-100. Then His_{10}-CadC in elution buffer was added. The mixture was stirred at room temperature for 10 min. The final ratio of phospholipids to protein was kept at 25 (w/w). Bio-Beads were used to remove the detergent. Bio-Beads were thoroughly rinsed with methanol and buffer [Holloway, 1973] and stored in 50 m*M* Tris/HCl, pH 7.5, 10% (v/v) glycerol until use at 4°C. In order to remove the detergents, Bio-Beads at a bead to detergent ratio of 5 (w/w) were added and the mixture was kept under gentle stirring at 4°C. After incubation overnight, additional Bio-Beads were added to remove residual detergent. After further incubation for 3 h the turbid solution was pipetted off and centrifuged for 1 h at 372,000 *g*. The pellet was resuspended in 50 m*M* Tris/HCl, pH 7.5, 10% (v/v) glycerol, 2 m*M* DTT. Proteoliposomes were either used instantly or stored in liquid nitrogen. The efficiency of reconstitution was calculated from the amount of protein obtained after ultracentrifugation of the proteoliposomes.

Immunological Analysis

For Western blot analysis inverted membrane vesicles or proteoliposomes were resuspended in an equal volume of two-times concentrated SDS-sample buffer. Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted, immunoblots were probed with monoclonal anti-His antibodies, and immunodetection was performed using a goat anti-(rabbit IgG) alkaline phosphatase antibody according to Jung et al. [1997].

Probing Signal Transduction in vivo

Signal transduction activity of different CadC derivates in vivo was probed with *E. coli* strain EP314 transformed with plasmids described. Cells were grown overnight in LB medium. Cells were diluted 1:100 in minimal medium [Neidhardt et al., 1974], adjusted to pH 7.6 with NaOH and buffered with 100 m*M* MOPS (MM 7.6), or adjusted to pH 5.8 with HCl and buffered with 100 m*M* MES (MM 5.8), in the presence or absence of 10 m*M* lysine. Cells were grown at 32°C to mid-logarithmic growth phase and harvested by centrifugation. The cell pellet was resuspended in sodium phosphate buffer (60 m*M* Na phosphate buffer, pH 7.0, 10 m*M* KCl, 1 m*M* $MgSO_4$, 39 m*M* β-mercaptoethanol). An aliquot of this suspension was used to determine the optical density at an absorbance of 600 nm, and another aliquot was used for determination of β-galactosidase activity after cells were permeabilized with chloroform and SDS. β-Galactosidase activity was determined from at least three different experiments and is given in Miller units calculated as described by Miller [1972].

The functionality of P_{Cad}-promoter derivatives in *E. coli* strains CK1, CK2 and CK3 was tested as described above.

For determination of H-NS-mediated repression of P_{Cad}-promoter derivatives, plasmids pRS551-P_{Cad}150, pRS551-P_{Cad}181, pRS551-P_{Cad}221, pRS551-P_{Cad}260, pRS551-P_{Cad}290, and pRS551-P_{Cad}600 were transformed into the *E. coli* strains MC4100 and PD32. Cells were grown overnight in LB medium. Cells were diluted 1:100 in LB medium, adjusted to pH 5.8 or pH 7.6 by addition of 100 m*M* Na-phosphate buffer. Cells were cultivated at the pH indicated under aerobic or anaerobic conditions to mid-logarithmic growth phase, and β-galactosidase activity was measured as described above.

DNaseI Protection Analysis

DNaseI protection analysis was performed by the method described by Sandaltzopoulos [1994]. The *cadBA* upstream region was amplified by PCR using the oligonucleotide 5-Fam-CAD (5′-TTTATTTGCCGCATAATTTTTATT-3′) as sense primer and CAD-anti as antisense primer. 5-Fam-CAD was labeled at its 5′-end with the 5-isomer of fluorescein (5-Fam). 75 or 150 n*M* of the DNA fragment was incubated with the 2- to 50-fold amount of reconstituted His_{10}-CadC in the appropriate buffer (50 m*M* Tris/HCl, pH 8.0, 100 m*M* KCl, 12.5 m*M* $MgCl_2$, 1 m*M* EDTA, 10% (v/v) glycerol, 1 m*M* DTT) in a final volume of 50 μl at room temperature for 10 min. After that, samples were incubated with 50 μl $MgCl_2$/$CaCl_2$ solution (10 m*M* $MgCl_2$, 5 m*M* $CaCl_2$) for another minute. Cleavage reactions were performed with 0.25 U DNaseI at room temperature for 1 min. The reaction was stopped by adding 90 μl stop solution (200 m*M* NaCl, 1% (w/v) SDS, 30 m*M* EDTA, 125 μg/ml yeast tRNA). The DNA fragments were purified by phenol/chloroform extraction followed by ethanol precipitation or by using the Qiagen 'Nucleotide Removal Kit'. Finally, fragments were analyzed with an ABI PRISM 377 DNA sequencer.

Electrophoretic Mobility Shift Assays

EMSA was performed by the method described [Fried and Crothers, 1981]. DNA fragments were obtained in the following way: fragment Cad1 by annealing the oligonucleotides Cad1-sense (5′-TGTAAACATTAAATGTTTATCTTTTCATGATATCAACTTGCGAT-3′), and Cad1-antisense (5′-ATCGCAAGTTGATATCATGAAAAGATAAACATTTAATGTTTACA-3′), fragment Cad2 by annealing Cad2-sense (5′-AAACCTCAAGTTCTCACTTACAGAAACTTT-3′) and Cad2-antisense (5′-AAAGTTTCTGTAAGTGAGAACTTGAGGTTT-3′), fragment Cad1–2 by PCR, using oligonucleotides Cad1-sense and Cad2-antisense as primers and genomic DNA of *E. coli* MG1655 as template. Cad1–2 was purified by a 'QIAquick PCR-Purification Kit' (Qiagen). Labeling of DNA was carried out with 10 U T4-polynucleotide kinase and 10 μCi [γ^{32}-P]ATP in the appropriate buffer (50 m*M* Tris/HCl (pH 8.2), 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, 5 m*M* DTT, 0.1 m*M* spermidine) at 37°C for 1 h. Subsequently, the labeled DNA was purified using a 'Nucleotide Removal Kit' (Qiagen). DNA fragments with a radioactivity of 50,000 counts were incubated with different concentrations of reconstituted or solubilized His_{10}-CadC in buffer (50 m*M* Tris/HCl (pH 7.5), 50 m*M* KCl, 2 m*M* $MgCl_2$, 10% glycerol (v/v), 1 m*M* DTT) with a final volume of 20 μl. In experiments in which the influence of lysine (10 m*M*) or cadaverine (1 m*M*) was tested, stock solutions of these compounds were added, and subsequently proteoliposomes were extruded (13 times) through a 50-nm polycarbonate filter. Binding reactions were performed at 37°C for 10 min, and samples were run on a 5% polyacrylamide non-denaturing gel.

Filter Assays

DNA-binding assays using filters were performed as follows: 50,000 counts of each radiolabeled DNA fragments Cad1, Cad2 and Cad1–2 were incubated with various concentrations (0.1–1.5 μ*M*) of reconstituted or solubilized His_{10}-CadC in binding buffer pH 7.5 (50 m*M* Tris/HCl (pH 7.5), 50 m*M* KCl, 2 m*M* $MgCl_2$, 10% glycerol (v/v), 1 m*M* DTT) or binding buffer pH 5.8 (50 m*M* MES (pH 5.8), 50 m*M* KCl, 2 m*M* $MgCl_2$, 10% glycerol (v/v), 1 m*M* DTT) in a final volume of 50 μl. Binding was performed at 37°C for 10 min, and then samples were transferred to a 25-mm nitrocellulose filter (Schleicher & Schuell), filtered by applying vacuum and subsequently washed with 350 μl of the corresponding binding buffer. The amount of protein-DNA complex on the filter was analyzed by measuring Cerenkov radiation in a scintillation counter (Packard).

Construction of Strains CK1, CK2 and CK3

Strains CK1, CK2 and CK3 were constructed as follows. The *cadBA* promoter sequence from bp –150 to bp +72 ('Cad1–2') was amplified by PCR using oligonucleotides Cad1-*Eco*RI-sense (5′-GGAATTCTTGTAAACATTAAATGTTTATCTT-3′) and Cad-*Bam*HI-anti (5′-CGGGATCCCATGCTCTTCTCCTAATTTCATTT-3′). The *cadBA* promoter sequence from bp –89 to bp +72 ('ΔCad1') was amplified by PCR using oligonucleotides Cad2-*Eco*RI-sense (5′-GGAATTCAACCTCAAGTTCTCACTTACAGAA-3′) and Cad-*BamH*I-anti. The *cadBA* promoter sequence from bp –150 to bp +72, with a replacement of the Cad2-binding site from bp –89 to bp –59 ('ΔCad2') was obtained by ligating three DNA fragments: the first fragment was amplified by PCR using primers Cad1-*Eco*RI-sense and Cad2-Del-*Xho*I-anti (5′-TTCTCGAGTTTTATTAACACATCAGGATCGCA-3′); the second one was obtained by annealing primers Cad2-Replace-sense (5′-AACTCTAGAACGTAGCTTCAGCCCATATCCTCGAGTCC-3′) and Cad2-Replace-anti (5′-GGACTCGAGGATATGGGCAGAAGCTACGTCTAGAGTT-3′), and the third fragment was created by PCR using primers Cad2-Del-*Xba*I-sense (5′-AATCTAGAGTGTTATTTCACCTAATCTTTAGGATT-3′) and Cad-*Bam*HI-anti. The first fragment was restricted with *Xho*I, the second with *Xho*I and *Xba*I,

and the third one with *Xba*I. All three fragments were ligated using T4-Ligase from NEB, according to the manufacturers protocol. Fragments 'Cad1–2', 'Cad1' and 'Cad2' were fused to a promoterless *lacZ* by cloning these fragments into pRS415 using *Eco*RI and *Bam*HI. Subsequently, promoter-*lacZ* fusions were transferred on the chromosome of *E. coli* MG1655-*lacZ* as described by Jones and Gunsalus [1987], using phage λRZ-5.

*Construction of Plasmid-Encoded Promoter-*lacZ *Fusions*

Fusions were constructed by PCR amplification of different *cadBA* promoter derivatives, using the oligonucleotides Cad1-*Eco*-RI-sense (5′-GGAATTCTTGTAAACATTAAATGTTTATCTT-3′); P_{Cad}181-sense (5′-AGAATTCAAATTTAACCAGAGAATG-3′); P_{Cad}221-sense (5′-AGAATTCATGTTGCGGCAATTTATT-3′); P_{Cad}260-sense (5′-AGAATTCTATTTGTTGTTGAGTTTT-3′); P_{Cad}290-sense (5′-AGAATTCGTAACTCCGGGTTGATTT-3′), and P_{Cad}600-sense (5′-AGAATTCCCGGAATTGAACAAC-ATG-3′) as sense primers, and Cad-*Bam*HI-anti (5′-CGGGAT-CCCATGCTCTTCTCCTAATTTCATTT-3′) as antisense primer. PCR fragments were cloned into pRS551, using *Eco*RI and *Bam*HI. Plasmids were transformed into strains MC4100 and PD32.

Curvature Prediction

Curvature predictions were performed using the BEND-IT program (http://www.icgeb.org/dna/bend_it.html), which utilizes the BEND algorithm [Goodsell and Dickerson, 1994] to predict the degree of curvature per helical turn (10.5 bp). Fragments containing motifs with a known intrinsic curvature generally give a result of 5–25° per helical turn using this algorithm, whereas those containing straight motifs are predicted to have less than 5° curvature per helical turn.

Lysine Decarboxylase Assay

Specific activity of the lysine decarboxylase CadA was measured in *E. coli* strains MC4100, PD32 and PD32-Δ*cadC* as described. Cells were cultivated under aerobic conditions in LB-medium, buffered to pH 5.8 or pH 7.6 by addition of 100 m*M* Na-phosphate buffer, to mid-logarithmic growth phase, centrifuged and resuspended in 1 ml Ldc buffer (100 m*M* Na-acetate, pH 6.0, 1 m*M* EDTA, 0.1 m*M* pyridoxal phosphate, 10 m*M* 2-mercaptoethanol, 10% (w/v) glycerol). Lysozyme was added to 0.1 mg/ml and the mixture was incubated at 0°C for 30 min. The cells were broken by sonication and the lysate centrifuged at 16,100 *g* for 30 min. Activity of lysine decarboxylase in the cell-free extracts was measured as described [Lemonnier and Lane, 1998]. One unit is defined as 1 mmol cadaverine produced per minute.

Analytical Procedures

Protein was assayed by the method described by Peterson [1977] using bovine serum albumin as standard. Proteins were separated by SDS-PAGE [Laemmli, 1970] using 12.5% acrylamide gels. Silver staining was performed as described [Blum et al., 1987].

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