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Silencing and Activation of ClyA Cytotoxin Expression in *Escherichia coli*

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Cytolysin A (ClyA) is a pore-forming cytotoxic protein encoded by the *clyA* gene of *Escherichia coli* K-12. Genetic analysis suggested that *clyA* is silenced by the nucleoid protein H-NS. Purified H-NS protein showed preferential binding to *clyA* sequences in the promoter region, as evidenced by DNase I footprinting and gel mobility shift assays. Transcriptional derepression and activation of a chromosomal *clyA*::*luxAB* operon fusion were seen under conditions of H-NS deficiency and SlyA overproduction, respectively. In H-NS-deficient bacteria neither the absence nor the overproduction of SlyA affected the derepressed ClyA expression any further. Therefore, we suggest that overproduction of SlyA in *hns*$^+$ *E. coli* derepresses *clyA* transcription by counteracting H-NS. The cyclic AMP receptor protein (CRP) was required for ClyA expression, and it interacted with a predicted, albeit suboptimal, CRP binding site in the *clyA* upstream region. Site-specific alterations of the CRP binding site to match the consensus resulted in substantially higher levels of ClyA expression, while alterations that were predicted to reduce CRP binding reduced ClyA expression. During anaerobic growth the fumarate and nitrate reduction regulator (FNR) was important for ClyA expression, and the *clyA* gene could be activated by overexpression of FNR. A major *clyA* transcript having its 5′ end (+1) located 72 bp upstream of the translational start codon and 61 bp downstream of the CRP-FNR binding site was detected in the absence of H-NS. The *clyA* promoter was characterized as a class I promoter that could be transcriptionally activated by CRP and/or FNR. According to DNA bending analyses, the *clyA* promoter region has high intrinsic curvature. We suggest that it represents a regulatory region which is particularly susceptible to H-NS silencing, and its features are discussed in relation to regulation of other silenced operons.

Bacteria having the ability to infect animals and humans are often capable of expressing virulence factors that can be of fundamental importance for the interactions that occur between the microorganism and the host. Molecular genetic analyes of different virulence determinants of enterobacteria encoding, e.g., cytotoxic substances, specific adhesins, and invasion proteins, have demonstrated that pathogenic isolates have complex gene systems that appear to be regulated in response to environmental growth conditions around the bacteria (36). Both enteropathogenic and uropathogenic isolates of *Escherichia coli* have become good model systems for this research. From analyses of genes controlling expression of fimbrial adhesins and invasiveness it was earlier shown that histone-like bacterial proteins are important for the regulation of virulence factors (20). The nucleoid-associated protein H-NS is known to influence the regulation of many genes in *E. coli*, and it appears that H-NS may cause silencing of many different operons (1). Even bacteria belonging to the normal flora presumably need to have their genes "tuned" to fit the environmental conditions within the host. Such regulation may be even more crucial for commensal organisms.

Cytolysin A (ClyA) is a 34-kDa cytolytic protein encoded by the *clyA* gene (also referred to as *sheA* and *hlyE* [13, 21]) located at 26.5 min on the *E. coli* K-12 chromosome. X-ray crystallography has shown that ClyA has unusual structural features and does not resemble any previously studied cytotoxin (59). We demonstrated recently that highly purified ClyA protein from *E. coli* K-12 causes lysis of mammalian cells by pore formation in a Ca^{2+}-independent fashion (40) and apoptosis in murine-derived macrophage-like cells (30). It is interesting that the gene encoding this potentially toxic protein is found in *E. coli* K-12, which is considered to be nonpathogenic. In fact, it appears that most nonpathogenic strains of *E. coli* carry this gene and have the capacity to express cytotoxicity (39). Evidently, there is strict regulation of the *clyA* gene since it is phenotypically silent in *E. coli* K-12 under many tested laboratory conditions (39). The *clyA* gene is derepressed in H-NS-deficient *E. coli* strains (58; J. M. Gómez-Gómez, J. Blazquez, F. Baquero, and J. L. Martinez, Letter, Mol. Microbiol **19:**909–910, 1996; Y. Mizunoe and B. E. Uhlin, Abstr. 34th Intersci. Conf. Antimicrob. Agents Chemother., p. 63, 1994), and strains overexpressing SlyA and MprA (13, 34, 35, 41). SlyA and MprA belong to a family of proteins thought to regulate diverse physiological processes in bacterial pathogens (57). A direct interaction between purified His-$SlyA_{EC}$ and the DNA upstream of the *clyA*-coding region was suggested from results obtained by band shift assays (41). In addition, ClyA expression is activated in *E. coli* K-12 by the expression of HlyX from *Actinobacillus pleuropneumoniae* (21). HlyX has 73% identical amino acid sequence compared with the oxygen-responsive transcriptional regulator, FNR, which binds to a putative FNR binding site in the *clyA* upstream region (21). Furthermore, it was recently shown that altered FNR proteins, similarly to HlyX, could activate the expression of *clyA* (43); i.e., minor alterations in a gene encoding a global regulator have a profound effect on the production of cytotoxic factors like ClyA. Because of the potential to express such a host-damaging product, the *clyA* gene represents a novel class of genes not previously characterized in commensal bacteria. In

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TABLE 1. Bacterial strains used in this work

Strain	Genotype or relevant characteristics	Reference or source
DH5α	*endA1 hsdR17* (r_K m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* [ϕ80(Δ*lacZ*)M15]	23
SY327(λ*pir*)	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56* (λ*pir*)	38
MC1061	*araD139* Δ(*ara*, *leu*) 7697 Δ*lac*X74 *galU galK hsr hsm*$^+$ *strA*	11
BEU616	MC1061 *hns*::*cat* (Cm^r)	This work
M182	Δ(*lacIPOZY*) X74 *galK galU strA*	11
M182 *crp*	M182 Δ*crp*	9
M182 *fnr*	M182 *fnr*::*tet* (Tc^r)	22
M182 *crp fnr*	M182 Δ*crp fnr*::*tet* (Tc^r)	2
JON31	M182 *hns*::*cat* (Cm^r)	This work
JON32	M182 Δ*crp hns*::*cat* (Cm^r)	This work
BEU701	M182 *fnr*::*tet hns*::*cat* (Tc^r Cm^r)	This work
BEU705	M182 Δ*crp fnr*::*tet hns*::*cat* (Tc^r Cm^r)	This work
MC4100	*araD139* Δ(*lac*)*U169 strA thi*	10
BSN26	MC4100 *trp*::*tet* (Tc^r)	26
BSN27	MC4100 *trp*::*tet* Δ*hns* (Tc^r)	26
JON33	BSN26 *clyA*::*luxAB*	This work
JON34	BSN27 *clyA*::*luxAB*	This work
MWK6	BSN27 Δ*slyA*	This work
MWK10	MWK6 *clyA*::*luxAB*	This work
MWK11	MC4100 with a consensus CRP binding site in the *clyA* promoter region[a]	This work

[a] 5′-AAATGTGATCTAGATCACATTT-3′

the present paper we present data from experiments aimed at elucidating features about the strict regulation of *clyA*.

MATERIALS AND METHODS

Bacterial strains and culture media. The relevant genotypes of strains and plasmids used in this work are listed in Tables 1 and 2, respectively. The strains were grown in LB broth (4) with vigorous shaking or on LB broth solidified with 1.5% (wt/vol) agar. Blood agar plates were 5% horse erythrocytes solidified with 1% (wt/vol) Columbia agar base (Oxoid Ltd.). Antibiotic selection for pFZY1-derived plasmids was carried out using carbenicillin (25 $\mu g \cdot ml^{-1}$). In other cases the growth medium was supplemented with carbenicillin (50 $\mu g \cdot ml^{-1}$), kanamycin (50 $\mu g \cdot ml^{-1}$), chloramphenicol (10 $\mu g \cdot ml^{-1}$), or tetracycline (15 $\mu g \cdot ml^{-1}$). Anaerobic growth conditions were achieved by using the Anaero*Gen* compact atmosphere generation system of Oxoid Ltd., following the instructions of the manufacturer.

Genetic techniques. Standard procedures were used in all general molecular applications (46). Generalized bacteriophage P1 transduction was performed as described by Willets et al. (61). Sequencing oligonucleotides were made on an Applied Biosystems 394 synthesizer or obtained from DNA Technology, Aarhus, Denmark. Dideoxy sequencing was carried out using a T7 sequencing kit (Pharmacia Biotech) according to the specifications of the manufacturer, using pYMZ62 as the template. For general purposes DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase and an ABI PRISM 377 DNA sequencer. Site-specific alterations of DNA sequences were obtained by using the QuickChange site-directed mutagenesis kit of Stratagene, following the instructions of the manufacturer. The desired mutations were always placed in the middle of the primer with approximately 15 bases of correct sequence on each side.

Plasmid and strain constructions. The construct pYMZ83 was made by ligation of a 0.4-kb *Eco*RI-*Bgl*II restriction fragment from pYMZ80 into *Eco*RI-*Bam*HI-digested pFZY1, resulting in a *clyA*::*lacZ* transcriptional fusion having its 5′ end 290 bp upstream of the *clyA* start codon and its fusion junction 76 bp into the *clyA* coding sequence. The plasmid pMWK4 was constructed by ligating a blunt end-generated *Sma*I-*Bgl*II restriction fragment from pYMZ80, containing the DNA 290 bp upstream of and down to 76 bp within the *clyA* coding sequence, into *Eco*RV-digested pCH257 suicide vector, using *E. coli* SY327(λ*pir*) as the host strain. The plasmid pMWK4 was integrated into the chromosome of MC1061 by a single recombination event between the 366-bp *clyA* sequence in our construct and the corresponding region of *clyA* on the chromosome. The resulting strain was designated MWK2. BSN26, BSN27, and MWK6 were transduced with P1 grown on MWK2 (*clyA*::*luxAB*), and transductants were isolated by selection for chloramphenicol resistance, resulting in the strains JON33, JON34, and MWK10, respectively. The plasmid pMWK24 was constructed by using the PCR-based strategy described above and the primers crp5 (5′-CATTAAACATTGTGTGATATTTATCATATT-3′) and crp6 (5′-AATATGATAAATATCACACAATGTTTAATG-3′), with pYMZ81 as the template. The same approach was utilized, with pYMZ81 as the template, to construct pMWK28 (primers crp7 [5′-CATTAAACATTGTTTAATATTTATCATATT-3′] and crp8 [5′-AATATGATAAATATTAAACAATGTTTAATG-3′], pMWK29 (primers crp9 [5′-TGACATTAAACATTGTCTAATATTTATCATATTAAT-3′] and crp10 [5′-ATTAATATGATAAATATTAGACAATGTTTAATGTCA-3′], pMWK31 (primers a-10 [5′-TCCCGCCCGGCTAACCACGAACTAGATGAAGTAAAA-3′] and b-10 [5′-TTTTACTTCATCTAGTTCGTGGTTAGCCGGGCGGGA-3′], and pMWK9 (primers crp1 [5′-CATTGTTTGATATAGATCACATTTATAGAAATAAAGAC-3′] and crp2 [5′-GTCTTTATTTCTATAAATGTGATCTATATCAAACAATG-3′]. The plasmid pMWK10 was constructed by the same method, with the primers crp3 (5′-GACATTAAACAAAATGTGATCTAGATCACATTTATAG-3′) and crp4 (5′-CTATAAATGTGATCTAGATCACATTTTGTTTAATGTC-3′) and pMWK9 as the template. A *Bbr*PI-*Bgl*II promoter fragment containing the consensus cyclic AMP (cAMP) receptor protein (CRP) site (5′-AAATGTGATCTAGATCACATTT-3′) (see reference 16 and references therein) was cloned into the corresponding sites of the construct pJON78 to generate pMWK45. The derivative pJON78 is a 3.5-kb subclone of the *clyA* locus in the suicide donor plasmid pKO3. Using the derivative pMWK45, the CRP consensus site was introduced onto the chromosome of MC4100, as previously described (31), to generate the strain MWK11. Strain BEU616 (*hns*::*cat*) was constructed by transduction of MC1063 with P1 grown on a derivative of JC7623 carrying an *hns*::*cat* allele in the chromosome. JC7623 is *recC22 recB21 sbcB15 sbc201* (24). MC1063 is MC1061 with *trp*::Tn*10* (11). BEU701 and BEU705 were constructed by transduction of M182 *fnr* and M182 *crp fnr*, respectively, with P1 grown on BEU616. To construct a 429-bp in-frame deletion mutant of the *slyA* coding region, we used a PCR-based strategy (see

TABLE 2. Plasmids used in this work

Plasmid	Relevant characteristics	Reference or source
pCH257	Suicide vector, Cm^r	17
pFZY1	Mini-F transcriptional *lacZ* fusion vector, Cb^r	28
pKO3	Gene replacement vector, Cm^r	31
pLG339	Cloning vector, Tc^r Km^r	54
pSL1180	Cloning vector, Cb^r	7
pYMZ62	3.5-kb subclone of the *clyA* locus in pUC18	This laboratory
pYMZ80	1.6-kb subclone of the *clyA* locus in pUC18	This laboratory
pYMZ81	Same as plasmid pYMZ80, but with the 1.6-kb *clyA* locus in the opposite orientation	This laboratory
pYMZ83	Transcriptional *clyA*::*lacZ* fusion in pFZY1	This work
pMWK4	*clyA*::*luxAB* in pCH257	This work
pMWK9	pYMZ81 with altered CRP binding site: 5′-TTGTTTGATATAGATCACATTT-3	This work
pMWK10	pYMZ81 with altered CRP binding site: 5′-AAATGTGATCTAGATCACATTT-3′	This work
pMWK24	pYMZ81 with altered CRP binding site: 5′-TTGTGTGATATTTATCATATTA-3′	This work
pMWK28	pYMZ81 with altered CRP binding site: 5′-TTGTTTAATATTTATCATATTA-3′	This work
pMWK29	pYMZ81 with altered CRP binding site: 5′-TTGTCTAATATTTATCATATTA-3′	This work
pMWK31	pYMZ81 with altered *clyA* −10 sequence: TATGAAT→CACGAAC	This work
pMWK32	Subclone of the *clyA* locus from position −349 to −50 in pGEM-T Easy	This work
pMWK33	Subclone of the *clyA* locus from position −250 to +50 in pGEM-T Easy	This work
pMWK34	Subclone of the *clyA* locus from position −150 to +150 in pGEM-T Easy	This work
pMWK35	Subclone of the *clyA* locus from position −50 to +250 in pGEM-T Easy	This work
pMWK36	Subclone of the *clyA* locus from position +50 to +349 in pGEM-T Easy	This work
pMWK37	Subclone of the *clyA* locus from position +150 to +449 in pGEM-T Easy	This work
pMWK45	pJON78 with altered CRP binding site: 5′-AAATGTGATCTAGATCACATTT-3′	This work
pJON22	2.1-kb subclone of the $slyA_{EC}$ locus in pACYC177	This laboratory
pJON78	3.5-kb subclone of the *clyA* locus in pKO3	This laboratory
pMOJ2	*slyA* in-frame deletion in pKO3	This work
pDW300	*crp* gene in pLG339, formerly known as pLG339/CRP	2, 60
pGS24	*fnr* gene in pBR322	50
pGS215	FNR-V208R;S212G;G216K	53
pGS297	FNR-V208R;E209D	52

above) and DH5α as the host strain: By using the primers SKO1 (5′-AATTAT AAGGAGATGGAATTCGAATCGCCACTAGGT-3′), SKO2 (5′-ACCTAGT GGCGATTCGAATTCCATCTCCTTATAATT-3′), SKO3 (5′-ATTGAGTTA CAGGCCGAATTCTGAAATGAAGGGGGC-3′), and SKO4 (5′-GCCCCCT TCATTTCAGAATTCGGCCTGTAACTCAAT-3′), and pJON22 as template, two new *Eco*RI restriction sites (underlined sequences) were introduced in the *slyA* gene, at bp 4 to 9 and bp 433 to 438, respectively. The resulting plasmid clone, designated pMWK11, was subsequently digested with *Eco*RI and religated to generate pMWK12. To facilitate cloning into pKO3, a 1.5-kb *Pst*I fragment of pMWK12, encompassing the constructed in-frame deletion, was cloned into *Pst*I-digested pSL1180 cloning vector, resulting in the plasmid pMWK13, which was used as an intermediate. A 1.5-kb *Pml*I-*Bam*HI fragment of pMWK13, containing the *slyA* in-frame deletion, was cloned into *Sma*I-*Bam*HI-digested pKO3, resulting in the plasmid pMOJ2. This construct, containing the *slyA* in-frame deletion, was introduced into BSN27 as previously described (31) to generate MWK6.

ClyA expression assays. Lytic activity towards erythrocytes was scored by a clearance zone on blood agar plates after 16 to 17 h of incubation at 37°C or by quantification of the release of hemoglobin from erythrocytes as described below. Bacteria were grown to late logarithmic phase and diluted to 8.0×10^6 cells ml^{-1} in 1× phosphate-buffered saline. Fifty microliters of bacterial suspension was mixed with 50 μl of a horse erythrocyte suspension in 1× phosphate-buffered saline in a 96-well microtiter plate and incubated for 120 min at 37°C prior to determination of the release of hemoglobin, as described previously (47). β-Galactosidase activity was measured as previously described (36), with compensation for the number of plasmid-free cells (51). Luciferase assays were performed as described earlier (42).

Sodium dodecyl sulfate-PAGE and immunoblot analysis. For determination of the ClyA and SlyA protein content in cells, bacterial samples grown at 37°C were harvested at late logarithmic phase, or from agar plates after 16 to 17 h of incubation, prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) as previously described (29). Western immunoblotting was performed using an antiserum raised against ClyA as described previously (40) or using an antiserum raised against SlyA as described below at final dilutions of 1:1,000 and alkaline phosphatase-conjugated secondary antibody at a final dilution of 1:3,000. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting detection system of Amersham Pharmacia Biotech, following the instructions of the manufacturer. Rabbit anti-SlyA antibodies were raised against His-SlyA which had been purified as described previously (41). An antiserum taken 8 weeks after the fourth injection was affinity purified as previously described (56).

RNA isolation and primer extension. Total RNA was isolated from late logarithmic phase cultures, which had been grown in LB broth, using the hot-phenol method (63). Primer extension analysis was carried out as follows. Oligomers were 5′ end labeled using polynucleotide kinase and [γ-^{32}P]ATP. A molar excess of the primer was annealed to 5 μg of total RNA in 8 μl of an annealing buffer (50 mM Tris-HCl [pH 8.3], 60 mM NaCl, 10 mM dithiothreitol [DTT]). Samples were heated for 5 min at 80°C and subsequently chilled on ice for 5 min. Eight microliters of extension mixture (25 mM Tris-HCl [pH 8.3], 30 mM NaCl, 15 mM $MgCl_2$, 1.25 mM DTT, a 1 mM concentration of each deoxynucleoside triphosphate) was added together with 3 U of avian myeloblastosis virus reverse transcriptase, and samples were incubated at 42°C for 60 min. The oligonucleotides used were cct1 (5′-CCGTTTTATCTGCAACGATTTCAGTC-3′) and cct4 (5′-GGAGGCTGCCTGTGAATACTCCTGTTTAAAGCGACTTAAC-3′). The extension products were analyzed by electrophoresis on 6% polyacrylamide-urea gels.

DNA bending analysis. Overlapping 300-bp DNA fragments of the *clyA* locus were generated by PCR using pYMZ62 as a template and the following oligonucleotide primers from the *clyA* locus (see Fig. 2): for fragment a, −349 (5′-GCGGAAAAGTCACAATTTCG-3′) and −50b (5′-TTAATATGATAAA TATCAAA-3′); for fragment b, −250 (5′-CCAGCAGATCAATACTGATT-3′) and +50b (5′-ATAAATTGTAATGAAACTCC-3′); for fragment c, −150 (5′-ACGCTCATCCAGCAGAAATG-3′) and +150b (5′-AAGATCTAATGCTCC ATCTG-3′); for fragment d, −50a (5′-ATAGAAATAAAGACATTGAC-3′) and +250 (5′-CGGAGGCTGCCTGTGAATAC-3′); for fragment e, +50a (5′-TATATTTAAAGAGGCGAATG-3′) and +349 (5′-ATTGCGTCGCAACACC ACAC-3′); and for fragment f, +150a (5′-TTATAATAAATATCTCGATC-3′) and +449 (5′-TTCGTGATGCCGTCATCCAG-3′). The generated PCR fragments were cloned utilizing the pGEM-T Easy Vector System (Promega), as specified by the manufacturer, resulting in the constructs pMWK32 (position −349 to −50 [base pairs] relative to the clyA transcriptional start point), pMWK33 (−250 to +50), pMWK34 (−150 to +150), pMWK35 (−50 to +250), pMWK36 (+50 to +349), and pMWK37 (+150 to +449). These constructs were digested with *Eco*RI and used for DNA bending analysis.

The fragments were run on 6% polyacrylamide–bis-acrylamide gels (30:0.8, vol/vol) in a 90 mM TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM disodium EDTA, pH 8.3) at 6 mA for 10 h at 5°C, room temperature, and 37°C, and subsequently stained with ethidium bromide. The migration lengths were measured and compared to a 1 Kb PLUS DNA Ladder molecular size standard (GibcoBRL). The ratio between the observed migration length (M_o) and the expected migration length (M_e) was plotted against the center position of the DNA fragment.

Gel mobility shift assay. Band shift assays were performed with DNA fragments from the plasmid pYMZ80 obtained by digestion with *Dra*I and *Eco*RI. Purified H-NS protein was obtained from a strain carrying an expression plasmid (B. Sondén and B. E. Uhlin, unpublished data). DNA at a final concentration of approximately 36 nM was mixed with H-NS at a final concentration of 0.8 to 12 μM in buffer B (25 mM HEPES [pH 7.5], 0.1 mM EDTA, 5 mM DTT, 10% glycerol) in a total volume of 10 μl. The samples contained 100 ng of poly(dI-dC), and KCl was added to 50 mM. The reactions were incubated for 15 min at 26°C and then resolved by nondenaturing PAGE in a 6% gel using TBE running buffer. The gel was subsequently stained with ethidium bromide.

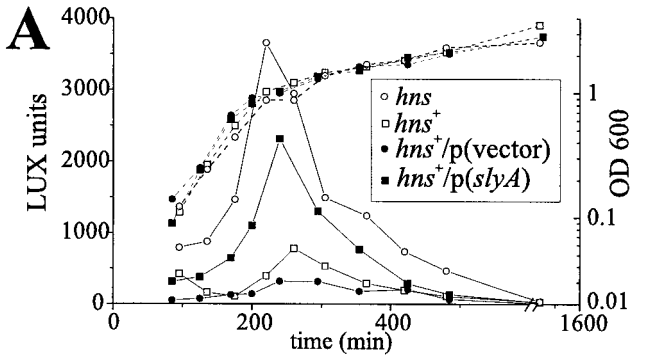
DNase I footprint analysis. DNase I footprint analysis with CRP was carried out essentially as described previously (19). The DNA fragments were obtained by PCR of the strain MC1061, with the primers cct1 and umu1 (5′-AATATTT GTCGCTGC-3′) and the latter primer had been labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Reactions were carried out in a total volume of 50 μl. CRP was purified essentially as described previously (64), with the exception that CRP was precipitated with $(NH_4)_2SO_4$ to obtain CRP free from cAMP instead of removing the cAMP by chromatography. Samples of CRP (final concentration, 4.7 to 38 nM) and/or RNA polymerase (22 to 87 nM) were added to approximately 10 ng of DNA in buffer B plus 50 mM KCl. When included, cAMP was added to a final concentration of 20 mM. Fifty nanograms of DNase I and $MgCl_2$ to a final concentration of 5 mM were added to start the digestion. After 120 s (90 s for samples without protein) the reactions were stopped by the addition of 12 μl of stop mix (0.25 mM EDTA, 1.5 M NaCl, oyster glycogen [1.5 mg ml^{-1}]). The samples were then phenol extracted, ethanol precipitated, and analyzed on 6% polyacrylamide-urea gels.

DNase I footprint analysis with H-NS was carried out essentially as described previously (19). The DNA fragments were obtained by PCR of the plasmid pYMZ62, with the primers p73 (5′-GAATGTCTTTCTGGGCGG-3′) and umu1, labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Reactions were carried out in a total volume of 50 μl. Samples of H-NS (final concentration, 4.8 to 9.6 μM) were added to approximately 30 ng of DNA in buffer B plus 50 mM KCl. Fifty nanograms of DNase I and $MgCl_2$ at a final concentration of 5 mM was added to start the digestion. After 120 s (90 s for samples without protein) the reactions were stopped by the addition of 12 μl of stop mix (defined above). The samples were then phenol extracted, ethanol precipitated, and analyzed on 6% polyacrylamide-urea gels.

Computer projection of DNA curvature. Projections of calculated DNA curvature were obtained by using the BEND program of the DNASTAR software package, which uses a wedge model to predict the helix trajectory. The dinucleotide bending angles used were according to published data (5).

RESULTS

Effects of H-NS deficiency and SlyA overproduction on *clyA* transcription as monitored by a chromosomal *clyA*::*luxAB* operon fusion. To determine at which level H-NS affects the expression of *clyA*, and to quantitatively monitor the transcription of *clyA* in *hns* mutant and SlyA-overproducing strains during different growth phases, we used the strains JON33 and JON34, which have a transcriptional *clyA*::*luxAB* fusion at the site of the *clyA* locus (see Materials and Methods). We investigated the transcription of *clyA* in isogenic *hns* wild-type and mutant strains (JON33 and JON34) by monitoring the expression of the chromosomal *clyA*::*luxAB* fusion throughout the growth cycle. As shown in Fig. 1, the luciferase activity of the *hns* strain JON34 peaked in late logarithmic phase and showed a more-than-fourfold increase in activity compared with the *hns*$^+$ strain. In parallel we studied the activation of *clyA* by SlyA by assaying the expression of the chromosomal *clyA*::*luxAB* fusion throughout the growth cycle, using JON33 (*hns*$^+$) as the host strain. As shown in Fig. 1, the luciferase activity of the SlyA-overproducing strain JON33/pJON22 peaked in late logarithmic phase and showed a more-than-fivefold increase in activity compared with the vector control strain JON33/pACYC177. Similar results were obtained when expression was monitored at the translational level using contact hemolysis assays with erythrocytes (see Materials and Methods; data not shown). These results were in accordance with the observation of a peak ClyA activity in samples taken from MprA- or SlyA-overproducing strains at late logarithmic phase (13, 34). Therefore, we conclude that the expression of *clyA* is mainly controlled at the transcriptional level and that H-NS is responsible for silencing the transcription of *clyA*. Furthermore, upon re-



ATCGAAACCGCAGATGGAGCATTAGATC

FIG. 1. A. Effects of H-NS deficiency and SlyA overproduction on the transcription of *clyA* throughout the growth curve. (A) Expression of luciferase activity from a chromosomal *clyA*::*luxAB* fusion in the strains JON33 (*hns*$^+$) (□), JON34 (*hns*) (○), JON33/pACYC177 (vector control) (●), and JON33/pJON22 p(SlyA) (■). The expressed luciferase activity was quantified by the luciferase assay (see Materials and Methods), and LUX units were displayed as millivolts/(milliliters × optical density at 600 nm). The growth curves are indicated with dotted lines. (B) DNA sequence of the *clyA* promoter region down to 76 bp into the *clyA* coding sequence, which is the position of the *lux* and *lac* fusion junctions. The positions of the transcriptional initiation point (+1) and putative regulatory elements, i.e., binding sites for CRP and FNR, Shine-Dalgarno (S.D.) sequence, and −10 and −35 boxes are shown. The mutational alterations in the plasmid pMWK31 are shown in parentheses below the −10 region sequence.

lief of this silencing (*hns* mutants and SlyA-overproducing strains), the highest expression of the chromosomal *clyA*::*luxAB* fusion occurred in the late logarithmic phase.

Site of initiation of derepressed *clyA* transcription and promoter analysis in *hns* mutant *E. coli*. Analysis of *clyA* transcription by Northern blot hybridization suggested that it is a monocistronic operon (41). In order to further localize the *clyA* promoter active in the absence of H-NS, the *clyA* transcript was assayed by primer extension analysis. RNA was extracted from the *hns* strain, BEU616, that expresses phenotypically detectable levels of the ClyA protein. The *clyA* primer extension resulted in a distinct product that should represent one major transcript with the 5′ end 72 nucleotides upstream of the ATG translational start codon of the *clyA* structural gene (data not shown). Therefore, we concluded that the observed *clyA* transcriptional start point (+1) in the *hns* mutant strain was the same as in strains in which the *clyA* gene was activated by the cloned *slyA* locus (34). To functionally assess the predicted −10 promoter box (TATGAAT) (Fig. 1B), we introduced site-specific alterations in the *clyA* upstream sequence, using a PCR-based strategy (see Materials and Methods) and the plasmid pYMZ81 as the template. This plasmid contains the *clyA* sequence cloned in the opposite orientation to the promoter of the vector, the gene thus being controlled by its native promoter region only. The resulting construct (plasmid pMWK31), with the −10 sequence changed to (CACGAAC), had lost its promoter activity according to the in vivo tests. As shown below, DH5α harboring pMWK31 showed a lack of expression of ClyA protein and cytolytic activity compared with DH5α/pYMZ81. Thus, the predicted −10 promoter box is important for *clyA* expression, and we conclude that this analysis localised the promoter sequences.

H-NS shows preferential interaction in vitro with *clyA* sequences. To examine whether there is a direct interaction between H-NS and the *clyA* locus, electrophoretic mobility shift assays were performed as described in Materials and Methods, using purified H-NS protein and *clyA* DNA. An initial indication of preferential binding of H-NS to *clyA* DNA (fragments 3 and 5) was observed using *Eco*RI-*Dra*I-digested pYMZ80 as the target DNA (Fig. 2B). It was also observed that one of the vector DNA fragments in this experiment (fragment 1) shifted in the presence of H-NS, which is consistent with previous findings of H-NS interaction with the plasmid carried *bla* promoter region (33, 65). DNase I footprinting assays showed that H-NS interacted preferentially with two regions of the *clyA* promoter region (Fig. 2C). The H-NS protein interacted both in the downstream region of the promoter (designated I in Fig. 2C) and in the upstream region (designated II in Fig. 2C). These findings support a model where H-NS directly interacts with, and negatively affects, *clyA* transcription.

SlyA is not required for *clyA* expression in *hns* mutant *E. coli*. Since SlyA, when overexpressed, activates the expression of ClyA, we wanted to investigate the requirement of SlyA for derepression of *clyA* in the absence of H-NS. We introduced (see Materials and Methods) a *slyA* in-frame deletion into the *slyA* locus of the *hns* strain BSN27, resulting in the strain MWK6, and a *clyA*::*luxAB* fusion into the *clyA* locus of MWK6, resulting in the strain MWK10. We found that there was no significant difference in luciferase activity throughout the growth curve in strain JON34 compared with MWK10 (data not shown), indicating that SlyA is not required for *clyA* expression in H-NS mutant strains. We also found that BSN27 and MWK6 showed similar ClyA activity on blood agar (Table 3) and quantified lytic activity using the erythrocyte assay (data not shown). This was in accordance with overproduction of SlyA in the *hns clyA*::*luxAB* strain JON34/pJON22 (data not shown), in the *hns* strain JON31/pJON22, and in the *hns slyA* strain MWK6/pJON22 (Table 3), which did not result in an additive effect on ClyA expression compared with the control strains JON34/pACYC177, JON31/pACYC177, and MWK6/pACYC177. We therefore concluded that SlyA was not essential for *clyA* expression when H-NS was absent.

The *clyA* promoter is dependent on CRP for efficient expression. Analysis of the *clyA* promoter region revealed a potential CRP binding site (5′-TTGTTTGATATTTATCATATTA-3′) that matched the consensus in 13 out of 22 bases. The CRP binding site partly overlapped with a previously identified FNR binding site (21). To investigate the requirement of CRP for the transcription of *clyA*, a *cat* (Cm^r) gene block was introduced into the *hns* locus of the strains M182 and M182 *crp*, resulting in the strains JON31 and JON32, respectively (see Materials and Methods). The low-copy (one to two copies per chromosome) *clyA*::*lacZ* reporter system pYMZ83 (based on the mini-F vector pFZY1) was used in these strains to study the level of *clyA* expression (Fig. 3). JON31 showed a more-than-sixfold-greater expression of β-galactosidase activity than

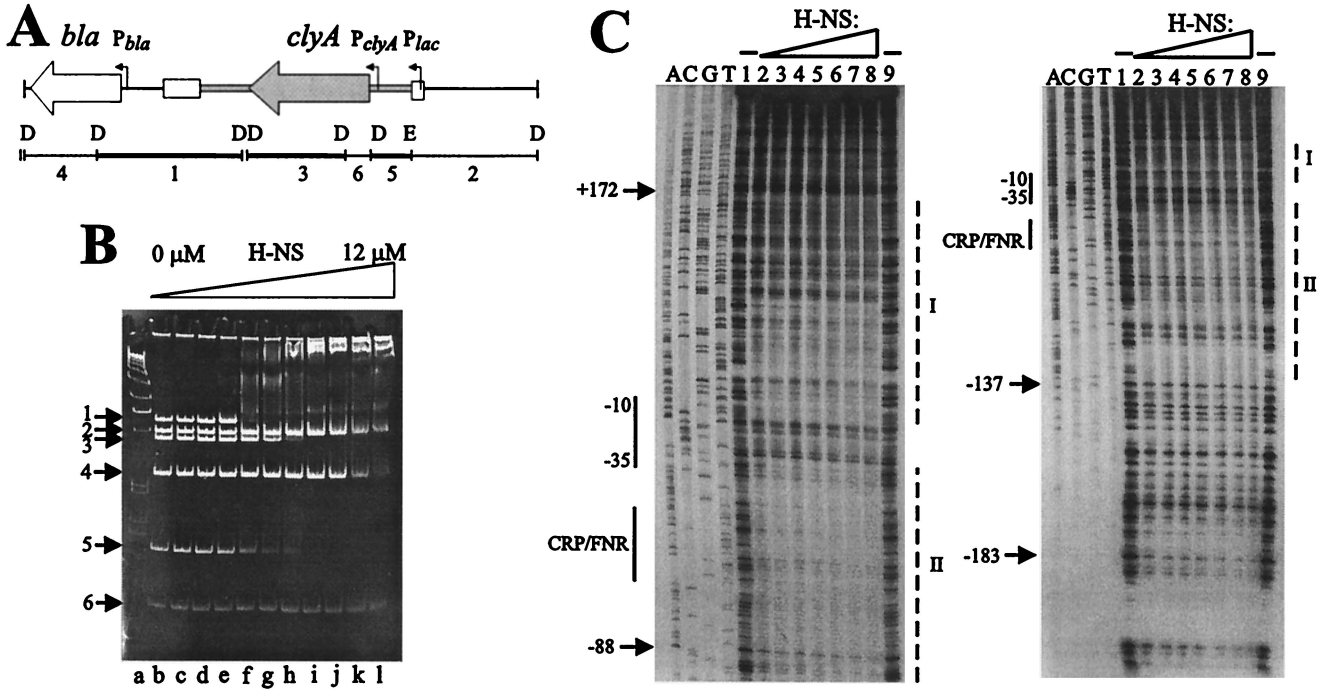


FIG. 2. Binding of purified H-NS to *clyA*. (A) Schematic drawing of the plasmid pYMZ80. Relevant features, positions of the restriction endonuclease sites (D, *Dra*I; E, *Eco*RI), and the extent of the resulting restriction endonuclease fragments used in gel shift assays with H-NS are indicated. Fragments bound preferentially by H-NS are indicated by thick black horizontal bars, and fragments showing no specific shift are indicated by thin black horizontal bars. Cloned chromosomal DNA encompassing the *clyA* locus is indicated in grey. (B) Gel shift assay of the *clyA* gene with purified H-NS protein. The different *Dra*I and *Eco*RI restriction fragments generated from pYMZ80 are indicated within the figure. Lanes: a, DNA ladder; b, no protein; c, 0.8 μM H-NS; d, 1.7 μM; e, 2.5 μM; f, 3.4 μM; g, 4.2 μM; h, 5.0 μM; i, 6.7 μM; j, 8.4 μM, k, 10 μM; 1, 12 μM. (C) DNase I footprint assay of the *clyA* promoter region with H-NS. Addition of H-NS was as indicated at the top. Lanes 1 and 9 show samples without any H-NS added and lanes 2 to 8 show samples with increasing amounts (4.8, 5.6, 6.4, 7.2, 8.0, 8.8, and 9.6 μM) of H-NS added. The positions of the *clyA* promoter (−10 and −35 regions) and the CRP-FNR site are shown by solid lines along the left side. The two main regions of H-NS interaction (labelled I and II) are shown by dashed lines along the right.

JON32, which expressed the same low levels as the *crp*$^+$ *hns*$^+$ strains (even lower β-galactosidase activity was observed in *hns*$^+$ *crp* strains). This was consistent with a much reduced cellular level of ClyA protein in the *hns crp* double mutant strain, as evidenced by Western immunoblotting (data not shown) and a substantially reduced lysis of erythrocytes in agar (Table 3). In addition, the results with the *clyA*::*lacZ* fusion indicated that the regulatory DNA sequences required for control of *clyA* transcription are present within the region spanning from 290 bp upstream of the *clyA* coding sequence to 76 bp into the *clyA* structural gene (the operon fusion junction). The reduced ClyA activity of JON32 could be restored by the reintroduction of CRP on a plasmid (pDW300). Thus, we concluded that CRP is required for derepression of *clyA* in *hns* strains. To investigate whether CRP is also required for the SlyA-mediated relief of H-NS silencing, we introduced the plasmid pJON22 (encoding SlyA) into the *crp* mutant and wildtype *E. coli* strains, M182 *crp* and M182, respectively. As shown in Table 3, overexpression of SlyA resulted in a strong cytolytic phenotype in M182, but only a weak cytolytic phenotype in M182 *crp*. This suggested that CRP is important for activation of ClyA expression by SlyA. The absence of CRP did not affect the level of SlyA protein, which was similar in M182/pJON22 and M182 *crp*/pJON22 as evidenced by western immunoblotting (data not shown), using an antiserum raised against SlyA (see Materials and Methods). Therefore, we concluded that the *clyA* promoter is dependent on CRP for efficient *clyA* expression.

CRP interaction at the *clyA* promoter in vitro. The involvement of CRP in the regulation of *clyA* gene expression and the presence of a potential CRP binding site in the *clyA* upstream region suggested a direct interaction of CRP with the *clyA* promoter. To investigate whether CRP could directly bind to the *clyA* promoter region, gel mobility shift assays and DNase I footprint analysis with purified CRP and the *clyA* DNA were carried out as described in Materials and Methods. A weak interaction between CRP and the *clyA* promoter was indicated by results from gel shift assays (data not shown). In the footprint analysis weakly footprinted regions were obtained only when CRP and RNA polymerase were both present. A region of protection from position −53 to −72, which encompasses the putative CRP binding site, was caused by CRP in the presence of cAMP and RNA polymerase (Fig. 4). Apparent hypersensitivity at positions −55 and −64 was caused by CRP in the presence of cAMP and RNA polymerase and by RNA polymerase at positions −21 and −22, which is similar to the hypersensitive sites −24, −25, and −54, noted in footprint analysis with FNR, HlyX, and RNA polymerase (21). Additional hypersensitive sites were found at position −98 with RNA polymerase (diminished by the addition of CRP and cAMP), at positions +35 and +36 with RNA polymerase plus CRP and cAMP, and at position +63 with RNA polymerase (diminished by the addition of CRP and cAMP). Evidently CRP interacted in a cAMP-dependent manner with the postulated suboptimal binding site. Based on the above observations we suggest a direct role for CRP in the positive regulation of *clyA* expression.

An altered CRP site in the *clyA* promoter results in altered expression of ClyA protein. Since the potential CRP binding site in the *clyA* upstream region shows only partial homology

TABLE 3. Expression of ClyA as evidenced by lysis[a] of erythrocytes in agar

E. coli strain	Relevant characteristic(s)	ClyA expression under the following conditions	
		Aerobic	Anaerobic
BSN26	Wild type	−	−
BSN27	*hns*	++	++
MWK6	*hns slyA*	++	++
JON31	*hns*	++	++
JON32	*hns crp*	(+)	(+)
BEU701	*hns fnr*	++	++
BEU705	*hns crp fnr*	(+)	(+)
JON32/pDW300	*hns crp*/p(CRP)	++	++
JON32/pLG339	*hns crp*	(+)	(+)
BEU701/pDW300	*hns fnr*/p(CRP)	++	++
BEU701/pLG339	*hns fnr*	++	++
BEU701/pGS24	*hns fnr*/p(FNR)	++	++
BEU701/pGS215	*hns fnr*/p(FNR-V208R;S212G; G216K)	++	++
BEU701/pGS297	*hns fnr*/p(FNR-V208R;E209D)	++	++
BEU701/pBR322	*hns fnr*	++	++
BEU705/pDW300	*hns crp fnr*/p(CRP)	++	++
BEU705/pLG339	*hns crp fnr*	(+)	(+)
BEU705/pGS24	*hns crp fnr*/p(FNR)	(+)	+
BEU705/pGS215	*hns crp fnr*/p(FNR-V208R; S212G;G216K)	(+)	+
BEU705/pGS297	*hns crp fnr*/p(FNR-V208R; E209D)	(+)	+
BEU705/pBR322	*hns crp fnr*	(+)	(+)
M182/pJON22	Wild type/p(SlyA)	++	++
M182/pACYC177	Wild type	−	−
M182 *crp*/pJON22	*crp*/p(SlyA)	(+)	(+)
M182 *crp*/pACYC177	*crp*	−	−
JON31/pJON22	*hns*/p(SlyA)	++	++
JON31/pACYC177	*hns*	++	++
MWK6/pJON22	*hns slyA*/p(SlyA)	++	++
MWK6/pACYC177	*hns slyA*	++	++

[a] Lysis of erythrocytes was scored on blood agar plates as ++, lysis around individual colonies; +, lysis beyond the edge of the bacterial cell mass only; (+), weak lysis in the center of the bacterial cell mass only; or −, no lysis.

(13 out of 22 bases) with the proposed consensus sequence, (5′AAATGTGATCTAGATCACATTT-3′) (16), we wanted to investigate whether the sequence features of this site are relevant for the regulation of ClyA expression. We therefore introduced site-specific changes in the *clyA* upstream sequence of the plasmid pYMZ81 (see Materials and Methods) (Fig. 5A). Site-specific alterations in the upstream pentamer of the potential CRP site resulted in the plasmid clones pMWK24 (TGTGA), pMWK28 (TTTAA), and pMWK29 (TCTAA). In addition, we substituted four positions in the predicted CRP binding site, resulting in the plasmid pMWK9, having an altered CRP site (5′-TTGTTTGATATAGATCACATTT-3′) which matches the consensus in 17 out of 22 bases. The construct pMWK10 contains an altered CRP site (5′-AAATGTG ATCTAGATCACATTT-3′) that perfectly matches the consensus. We subsequently quantified the cytolytic activity of different *E. coli* strains carrying these constructs by using the erythrocyte assay (Fig. 5B) and by monitoring the cellular levels of ClyA protein with Western immunoblotting (Fig. 5C). Compared with DH5α/pYMZ81, substantial decreases in cellular ClyA protein and cytolytic activity were exhibited by DH5α/pMWK28 and DH5α/pMWK29. This is consistent with previous findings (25) which suggested that alterations in the upstream pentamer (TGTGA) at position two (G→C) and at position four (G→A) abolish CRP binding. DH5α/pMWK24, which has an improved CRP site (T→G) at position two in the upstream pentamer, showed an increased cellular level of ClyA protein and a stronger cytolytic activity than DH5α/pYMZ81. An even greater increase in cellular ClyA protein and cytolytic activity was exhibited by DH5α carrying pMWK9 or pMWK10. That the *clyA* expression was CRP-dependent in these cases was confirmed by tests with a *crp* mutant strain. Only a low, barely detectable level of cellular ClyA protein and cytolytic activity in the *crp* strain M182 *crp* carrying pMWK9, pMWK10, and pYMZ81 was observed (data not shown). We also constructed a strain with the CRP consensus DNA binding site in the *clyA* promoter region on the chromosome of the hns^+ strain MC4100. The resulting derivative (strain MWK11) showed a strong hemolytic phenotype on blood agar plates, and there was a high level of ClyA protein in the cells detected by Western blot analysis (Fig. 6). These findings support a model where CRP is involved in expression of ClyA. We concluded that the sequence features of the CRP binding site are important for the positive role of CRP in the regulation of *clyA* expression.

The interaction by CRP with typical binding sites may cause local bending of the DNA, which may affect the curvature properties of a nearby promoter. We noted that the *clyA* promoter region is rich in A-T base pairs (73.1% for the 186-bp region upstream of the start codon) and the region shows features typical of curved DNA. We studied the potential DNA bending properties by using overlapping DNA fragments in a gel migration analysis and by computer projection (see Materials and Methods). The results confirmed that, in particular, the region containing the 5′ end of *clyA* may be intrinsically curved (Fig. 7).

Involvement of both CRP and FNR in regulation of *clyA* expression during anaerobic growth conditions. It was previously reported that overproduction of the FNR homolog HlyX of *A. pleuropneumoniae* in anaerobically grown *E. coli* K-12 results in binding to the FNR site and activation of *clyA* expression, while overproduction of the *E. coli* FNR protein results in less efficient activation (21). The requirement of CRP and FNR for anaerobic *clyA* expression was subsequently investigated. The low-copy *clyA*::*lacZ* reporter system pYMZ83 was used in different strains to study the level of *clyA* expres-

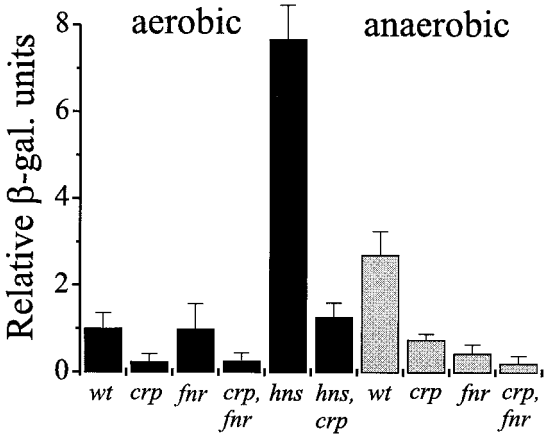


FIG. 3. CRP-dependent transcription of *clyA*. Shown is the quantification of *clyA* transcription from a *clyA*::*lacZ* reporter system on the plasmid pYMZ83 in the following strains grown under aerobic (solid black bars) and anaerobic (grey bars) conditions for 16 to 17 h at 37°C on LB agar: *wt*, M182; *crp*, M182 *crp*; *fnr*, M182 *fnr*; *crp fnr*, M182 *crp fnr*; *hns*, JON31; and *hns crp*, JON32. β-Galactosidase (β-gal.) activity was measured as described in Materials and Methods. A relative β-galactosidase activity of 1.0 equals the activity of the wild-type strain, M182/pYMZ83, under aerobic growth conditions (350 Miller units). Error bars indicate standard errors of the means from three separate experiments.

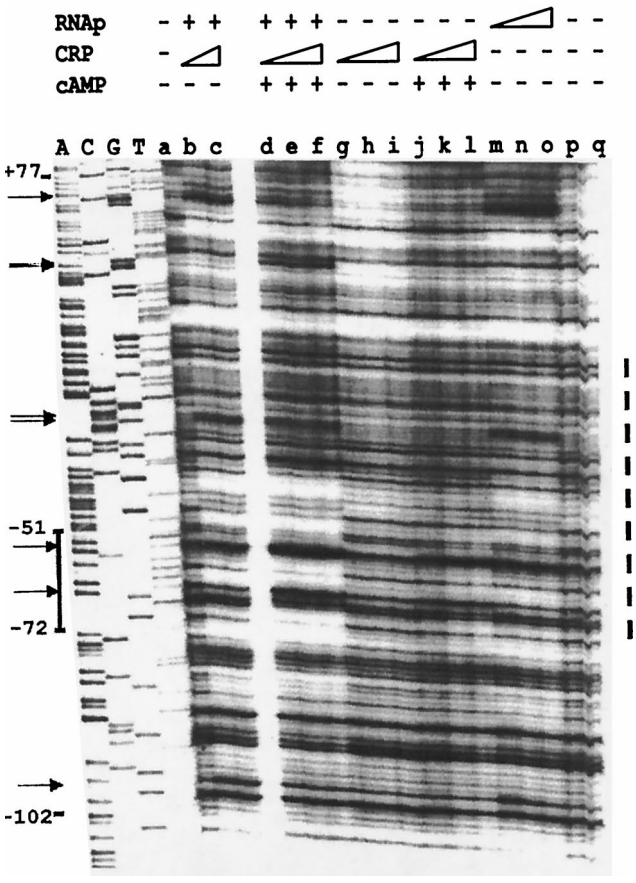
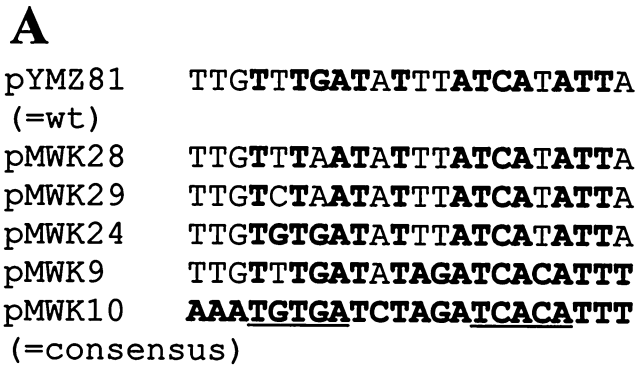


FIG. 4. Binding of purified CRP to *clyA*. DNase I footprint assay of the *clyA* promoter region with CRP and RNA polymerase. The additions of CRP, RNA polymerase (RNAp), and cAMP were as indicated at the top. The extent of the DNA fragment used is shown by the indicated positions (base pairs). The position of the putative CRP binding site is shown by a solid line along the left side. The approximate region of interaction with RNA polymerase is shown by a dashed line along the right. Hypersensitive sites are indicated with arrows. When included, cAMP was added at a concentration of 20 mM. Lanes: a, no protein; b, CRP (4.7 nM) plus RNA polymerase (0.5 U); c, CRP (9.4 nM) plus RNA polymerase (22 nM); d, CRP (4.7 nM) plus cAMP plus RNA polymerase (22 nM); e, CRP (9.4 nM) plus cAMP plus RNA polymerase (22 nM); f, CRP (19 nM) plus cAMP plus RNA polymerase (22 nM); g, CRP (9.4 nM); h, CRP (19 nM); i, CRP (38 nM); j, CRP (9.4 nM) plus cAMP; k, CRP (19 nM) plus cAMP; l, CRP (38 nM) plus cAMP; m, RNA polymerase (22 nM); n, RNA polymerase (44 nM); o, RNA polymerase (87 nM); p, no protein; q, no protein.

sion (Fig. 3), and a *cat* (Cm^r) gene block was introduced into the *hns* locus of the strains M182 *fnr* and M182 *crp fnr*, resulting in the strains BEU701 and BEU705, respectively (see Materials and Methods). During anaerobic growth a clearly reduced β-galactosidase activity was observed in both *fnr* and *crp* strains (most reduced in the *crp fnr* double mutant strain) (Fig. 3), suggesting an involvement of both CRP and FNR in the transcriptional regulation of *clyA* expression. As evidenced by the lysis of erythrocytes in agar (Table 3), the absence of CRP, but not of FNR, reduced the level of *clyA* derepression in anaerobically grown *hns* strains. The attenuated lytic activity of BEU705 could be restored both with and without the presence of oxygen by the introduction of CRP on a plasmid (pDW300), and to some extent, under anaerobic conditions only, by the introduction of FNR (plasmid pGS24) or altered FNR proteins having CRP binding specificities (plasmids pGS215 and pGS297). We concluded that the *clyA* promoter is dependent on CRP also during anoxic growth conditions and that FNR to some extent can complement the requirement for CRP during anaerobic growth only.

DISCUSSION

Silencing of *clyA* by H-NS and relief of silencing by SlyA. The *clyA* locus in the *E. coli* K-12 chromosome does not seem to be expressed under most laboratory growth conditions, and our present evidence established that the gene is subject to silencing by H-NS. The *slyA* and *mprA* genes have been shown to activate the expression of *clyA* when present in multiple copies (13, 34, 35, 41). In the present work we demonstrate that the major *clyA* transcript in the *hns* strain BEU616 has a 5′ end (+1) located 72 nucleotides upstream of the start of the



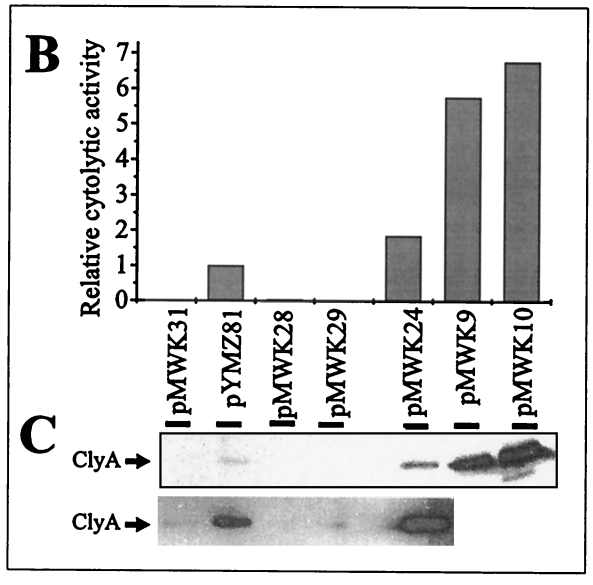


FIG. 5. Effect of site-specific alterations in the CRP DNA site and −10 sequence of the *clyA* promoter on *clyA* expression from various plasmids in DH5α. The construct pMWK31 contains an altered −10 promoter box (TATG AAT→CACGAAC). The strains were grown to late logarithmic phase and treated as described in Materials and Methods. (A) Sequences of the CRP binding sites in wild-type and mutant *clyA* clones. The consensus CRP binding site (see reference 16 and references therein) and positions showing identity to the consensus are shown in boldface type. Pentamers referred to in the text are underlined. (B) Cytolytic activity of the different strains towards erythrocytes. The cytolytic activity was measured as described in Materials and Methods, and the activity of the strain DH5α/pYMZ81 was arbitrarily set to 1.0. (C) Determination of ClyA protein content in the different strains by Western immunoblotting using a ClyA-specific antiserum (see Materials and Methods). Strains were grown in LB broth to late logarithmic phase. Approximately 10^7 bacteria were used for the extract loaded in each lane. The lower panel shows a prolonged exposure, and the ClyA reactive band is indicated with an arrow.

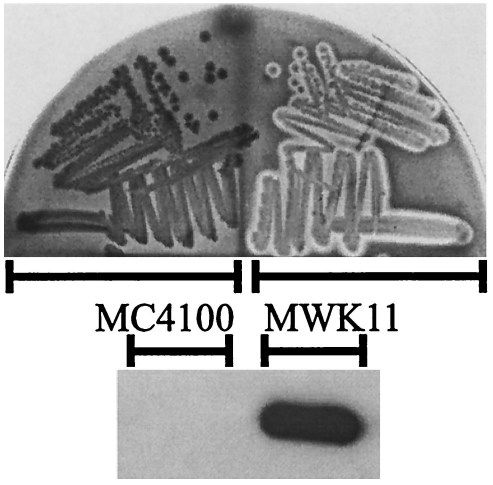


FIG. 6. Effect of alterations in the CRP binding site in the *clyA* promoter region on the chromosome to match the consensus sequence (5′-AAATGTGA TCTAGATCACATTT-3′). (Upper panel) MC4100 and MWK11 on a blood agar plate after incubation at 37°C for 17 h. (Lower panel) Detection of ClyA protein content in MC4100 and MWK11 by Western immunoblotting using a ClyA-specific antiserum (see Materials and Methods). Strains were grown in LB broth to late logarithmic phase. Approximately 10^7 bacteria were used for the extract loaded in each lane.

clyA coding sequence. Thus, upon relief of the H-NS silencing, either in *hns* mutants (this work), or by overproduction of SlyA (34), the same promoter appeared to be active. Site-specific alterations of the putative −10 *clyA* promoter box (TATGAA T→CACGAAC) resulted in a significantly decreased expression of ClyA, establishing that this particular promoter is crucial for ClyA expression.

When H-NS acts as a silencer or repressor it binds to AT-rich, curved sequences and thereby blocks transcription of the gene in question (6). The DNA of the *clyA* promoter (the 186 bp immediately upstream of the *clyA* start codon) is notably A-T rich (73.1%). Computer bend predictions of the *clyA* locus suggested sharp bends both in the promoter and in the structural gene, and DNA bending analysis of the *clyA* promoter showed that it contains intrinsic curvature (Fig. 7). By studying the interaction with purified H-NS and the *clyA* gene in vitro using electrophoretic mobility shift and DNase I footprint assays (Fig. 2), it could be concluded that H-NS binds preferentially to DNA fragments upstream and downstream of the *clyA* transcriptional start point (+1). The protection of the *clyA* −10 and −35 regions was less pronounced than for surrounding sequences, something that has also been seen with the promoter for the *proU* operon, encoding a glycine betaine transport system (33). Hence, it appears that the very low level of *clyA* expression in *E. coli* K-12 strains is due to a direct interaction of H-NS with the *clyA* locus. By monitoring the expression from a chromosomal *clyA*::*luxAB* fusion, we observed that the highest *clyA* transcription coincided with the late logarithmic phase in both H-NS mutants and SlyA-overproducing strains (Fig. 1), which was consistent with previous observations with ClyA activity in MprA and SlyA-overproducing strains (13, 34). SlyA was not essential for *clyA* expression in an *hns* strain background, since the *hns slyA* strain MWK6

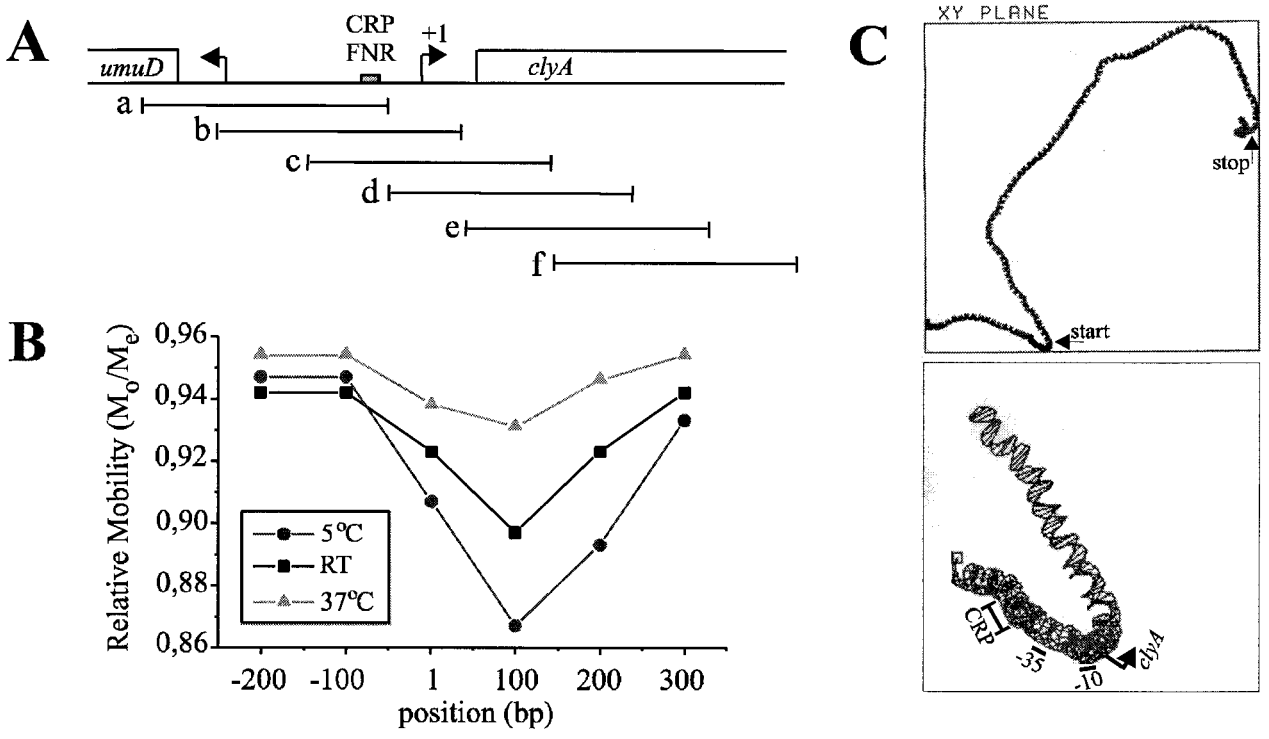


FIG. 7. (A) Schematic drawing of the *umuD clyA* intercistronic region. The DNA site for CRP-FNR is shown as a grey box, and transcriptional start points for *clyA* and *umuD* are indicated by horizontal arrows. The extents of the overlapping DNA fragments generated by PCR, which were used in DNA bending analysis experiments, are shown as horizontal lines. (B) Mapping of the center of the sequence directed bend. M_os as distances from the top of the gel were divided by the M_e for each fragment. The M_o/M_e value was plotted against the center position (in base pairs) of the DNA fragment. RT, room temperature. (C) The upper panel shows a computer projection analysis of 1,402 bp from the *clyA* DNA region, which includes the 912-bp *clyA* coding sequence with 372 bp upstream of the *clyA* start codon and 118 bp downstream of the *clyA* stop codon. The positions of the translational start and stop codons of *clyA* are indicated by arrows. The lower panel shows an enlargement of the predicted curvature pattern in the *clyA* promoter region from position 200 to 400. The positions of the predicted CRP binding site (−72 to −51), the −35 sequence (−35 to −30), the −10 sequence (−12 to −6), and the transcriptional start point (+1) are indicated.

and the *hns slyA clyA*::*luxAB* strain MWK10 did not show a reduced ClyA expression. In addition, the overexpression of SlyA in the *hns* strain JON31/pJON22, the *hns clyA*::*luxAB* strain JON34/pJON22, and the *hns slyA* strain MWK6/pJON22, did not result in a further elevation of ClyA expression. Based on our findings, demonstrating no absolute requirement of SlyA for ClyA expression, but rather a copy number effect, we suggest that SlyA may not be involved specifically in the natural regulation of *clyA*. The observed regulatory effects on *clyA* with SlyA (and likely also MprA) may well be of a more general nature, e.g., competing with H-NS binding at the *clyA* locus. It has been suggested that SlyA-related proteins play key roles in the global regulation of diverse aspects of bacterial physiology (57). It has also been implied that rather than being a classical transcriptional activator, MprA may act like some histone-like *E. coli* proteins, modulating the transcription of specific promoters by locally altering DNA topology (14). In *Salmonella*, SlyA was demonstrated to regulate the expression of multiple proteins during stationary phase and during infection of macrophages (8), but the role of SlyA in *E. coli* is not yet understood. When present in multiple copies in *E. coli* K-12, the cloned *slyA* locus affected the expression of more than 50 proteins according to analyses using two-dimensional PAGE (39). This indicates that SlyA may not be specifically linked with the regulation of the H-NS-silenced *clyA* locus.

The *clyA* promoter is dependent on CRP for efficient expression. In addition to the strict control exerted by H-NS, the *clyA* locus appeared to be controlled by the global regulatory protein CRP. According to our data CRP is required for efficient ClyA expression. A much reduced transcription of *clyA* in *hns crp* double mutants compared with *hns* mutants was evident by using a transcriptional *clyA*::*lacZ* fusion, and in line with these findings a substantial decrease in cellular ClyA protein and cytolytic activity was observed. The relief of H-NS silencing by SlyA was also much less efficient in the absence of CRP, since only a very weak cytolytic activity could be detected in CRP-deficient strains overexpressing SlyA (Table 3). Results from DNase I footprint experiments were consistent with the idea that the role of CRP in ClyA expression is to directly interact with the *clyA* promoter region (Fig. 4). Further evidence supporting the model that CRP is involved in the expression of ClyA was obtained by altering the sequence of the potential CRP binding site in the *clyA* upstream region, both located on the plasmid and on the chromosome (Fig. 5 and 6). By altering the DNA site for CRP to reduce its similarity to the consensus, ClyA expression was significantly lowered. In contrast, the altered CRP binding site that matched the consensus more closely resulted in substantially increased ClyA expression in crp^+ but not *crp* strains. These findings supported a model in which the *clyA* promoter is dependent on CRP for efficient expression, and where the predicted DNA site for CRP is important for this regulation.

Anaerobic regulation of *clyA* involves both CRP and FNR. Results from experiments using a low-copy plasmid-borne *clyA* promoter-*lacZ* fusion were consistent with the idea that both CRP and FNR are involved in the transcriptional regulation of *clyA* under anaerobic growth conditions (Fig. 3). Our findings suggest that FNR and CRP bind to the same sequence in the *clyA* promoter. There are other examples of binding of FNR and CRP to the same site (48). Evidently, CRP, and not FNR, was required for the expression of ClyA in *hns* mutants under anoxic conditions, although it appeared that FNR could partly complement CRP. We also observed that the *clyA-lacZ* fusion in the wild-type strain (M182) was expressed at a higher level (more than twofold) during anaerobic growth, suggesting that the *clyA* locus may be less repressed in the absence than in the presence of oxygen. It was shown previously that when expressed in anaerobically grown *E. coli* K-12, the FNR homolog HlyX of *A. pleuropneumoniae* and, although much less effective, FNR, are able to activate ClyA expression, presumably by binding to the FNR binding site in the *clyA* upstream region (21).

The *clyA* promoter: an H-NS silenced class I promoter. Unlike the situation in eukaryotes, where gene expression is thought to be generally repressed by packaging of the DNA into nucleosomes, the DNA of prokaryotes is generally considered to be available for transcription at all times (55). There are, however, certain prokaryotic gene loci that are apparently not expressed under tested growth conditions. Such loci are referred to as cryptic, and some of them are efficiently silenced by the nucleoid-associated protein H-NS (3, 62). The *clyA* locus is an interesting new example of H-NS-silenced operons. The locus has some features in common with the cryptic β-glucoside (*bgl*) operon of *E. coli*, which is thought to be kept in a silenced state by a repressing nucleoprotein complex consisting of H-NS and other cellular factors. The complex renders the *bgl* promoter inaccessible to RNA polymerase and CRP (49). Silencing of the *bgl* operon is relieved by various mutations, including (i) mutations in *hns* (initially termed *bglY*) (12, 20) and in genes encoding the subunits of DNA gyrase (15), (ii) integration of insertion elements in *cis* to the promoter (44), and (iii) deletion of either one of the silencer sequences (32). These mutations may all, directly or indirectly, affect the locked conformation of the upstream region so that they allow more-efficient transcription at the *bgl* promoter. In addition, point mutations that improve the CRP binding site within the *bgl* promoter, resulting in CRP binding with higher affinity, cause activated *bgl* transcription (45). It appears that *clyA*, similar to the *bgl* operon, has a weak promoter and no classical operator site. The presence of a −10 promoter box (TATGAAT) centered at −9, a −35 sequence (TTGACG) centered at −32.5, and binding sites for CRP and FNR centered at −61.5 suggested that this *clyA* promoter is a class I promoter that could be transcriptionally activated by CRP or FNR. Another case of an H-NS silenced operon in which CRP has a positive role is the *pap* fimbrial adhesin determinant found in uropathogenic *E. coli* (19). However, transcription of *pap* is independent of CRP activation in the *E. coli* K-12 mutant lacking H-NS (18). That finding led to the suggestion of a new role for CRP: it can mediate its positive regulatory function by alleviating transcriptional silencing. In contrast to the situation found in the *clyA* locus, the binding site for CRP in *pap* is located relatively far from the promoters and the protein-DNA interaction there is rather clearly shown by in vitro footprint analysis (19). It is possible that CRP may also alleviate the action of H-NS in the case of *clyA*, e.g., by altering the local DNA conformation and/or by interfering with its DNA binding. An indication of such a role was obtained when the CRP site on the chromosome was altered to perfectly match the consensus, resulting in derepression of the *clyA* gene. However, the results were also consistent with the suggestion that CRP directly interacted with the RNA polymerase. The genetic evidence suggested a positive role for CRP both in the absence of H-NS and during SlyA overproduction. The suboptimal design of its binding site in the *clyA* DNA evidently did not allow for any efficient CRP-mediated alleviation of H-NS silencing, but there was a need for additional factors. The tight control of *clyA* transcription in wild-type *E. coli* during laboratory cultivation is intriguing, and it remains to be seen if there are different pathways for induction of its expression. For example, it will be of interest to consider whether or not CRP may act in direct cooperation with other factors under some

conditions. It is also possible that expression of ClyA could be initiated at some stage during an infection process, as has been shown for the *bgl* operon (27). Considering the potent cytotoxic properties of this cytolysin, it appears reasonable that it would be strictly regulated, especially in nonpathogenic strains.

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