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NOTES

A *cka-gfp* Transcriptional Fusion Reveals that the Colicin K Activity Gene Is Induced in Only 3 Percent of the Population

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In prokaryotes, only a few examples of differential gene expression in cell populations have been described. Colicin production in natural populations of *Escherichia coli*, while providing a competitive advantage in the natural habitat, also leads to lysis of the toxin-producing cell. Colicin K synthesis has been found to be induced due to an increase in ppGpp (I. Kuhar, J. P. van Putten, D. Žgur-Bertok, W. Gaastra, and B. J. Jordi, Mol. Microbiol. 41:207-216). Using two transcriptional fusions, *cka-gfp* and *cki-gfp*, we show that at the single-cell level, the colicin K activity gene *cka* is expressed in only 3% of the bacterial population upon induction by nutrient starvation. In contrast, the immunity gene *cki* is expressed in the large majority of the cells. Expression of the *cka-gfp* fusion in a *lexA*-defective strain and in a *relA spoT* mutant strain indicates that differential expression of *cka* is established primarily at the level of transcription.

Colicins are plasmid-encoded bacteriocins, synthesized by and active against cells of *Escherichia coli* and sometimes related species such as *Shigella* and *Salmonella* spp. Colicin-producing strains are found with high frequency among natural isolates, and they have been implicated in intraspecies population dynamics (4, 13). Many other bacterial species also produce bacteriocins to defend or invade an ecological niche (3, 11). Colicin K production is encoded by three genes: a gene encoding the colicin activity protein; the immunity gene, encoding the immunity protein, which protects the producing strain; and a lysis gene, encoding the lysis protein (1). Colicins kill sensitive cells by one of several mechanisms: channel formation in the plasma membrane, nuclease activity, and degradation or inhibition of cell wall peptidoglycan.

Colicin K belongs to the group of pore-forming colicins which destroy the electrochemical potential of the cytoplasmic membrane. The genes *cka*, encoding colicin activity, *cki*, encoding immunity, and *ckl*, encoding lysis, have been described previously on pColK-K49 (12) and pColK-K235 (13). In the colicin K gene cluster, as in clusters of other pore-forming colicins, the activity and lysis genes are transcribed from a common promoter while the immunity gene is downstream from the activity gene with opposite transcriptional polarity. Previously, it was demonstrated that colicin K synthesis is induced primarily by an increase in ppGpp due to nutrient depletion (8). Bacteria respond to nutritional stress by adjustment in gene expression and physiological activities, collectively termed the stringent response. Guanosine 3′,5′-bispyrophosphate (ppGpp) is the effector of this global response (15). Recently, it has been postulated that ppGpp indirectly regulates translation of colicin K mRNA (9).

A number of colicins are released semispecifically, by cell lysis. To prevent excessive lysis, the colicin-encoding genes should be differentially expressed so that, under inducing conditions, only a part of the population expresses the activity and lysis genes. In contrast, all or the large majority should constitutively express the immunity gene.

Green fluorescent protein (GFP) produces a strong green fluorescence when excited by blue light without any exogenously added substrate or cofactor (2) and is a powerful tool for monitoring gene expression and protein localization at the single-cell level. To observe expression of the colicin K activity and immunity protein genes at the single-cell level through the growth cycle, transcriptional fusions of the *cka* and *cki* promoters and the promoterless *gfp* gene were prepared on the natural colicin K-encoding plasmid pColK-K235.

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium with aeration at 37°C with the appropriate antibiotics.

Construction of the *cka-gfp* gene fusion. The promoterless *gfp* gene, which is part of a gene cassette on plasmid pAG408 (16), was cloned into plasmid pColK-K235 in several steps (Fig. 1). Since pColK-K235 carries no selectable markers, first the Ap^r gene from plasmid pUC19 was introduced into pColK-K235. For this purpose, pUC19 was digested with *Taq*I, and the two *Taq*I fragments encoding the Ap^r gene were cloned into the *Cla*I site of plasmid pColK-K235, producing pKCT1. Subsequently, pKCT1 was cut with *Eco*RI, and the longer,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
MC4100	*araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 ptsF25 deoC1*	10
MC4100-1	MC4100 resistant to colicin K	This work
DH5α	*thi-1 relA hsdR17 lac*	A. Francky
AB1133	Sensitive to all colicins	B. Bachman
RO98	MC4100 *relA251*::*kan spoT*::*cat*	10
RW118	*thr-1 araD139* Δ*(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE thi-1 sulA211*	R. Woodgate
RW542	RW118 *lexA51* (Def)	R. Woodgate
Plasmids		
pColK-K235	Encoding colicin K	14
pAG408	Promoter-probe *gfp* minitransposon suicide delivery system	16
pUC19	Ap^r cloning vector	17
pKCT1	*Taq*I fragments with pUC19 Ap^r gene cloned into pColK-K235	This work
pKCT2	*cka cki ckl* Ap^r and *Kpn*I restriction site	This work
pKCT3	pKCT2 with *cka-gfp*	This work
pKCT4	pColK-K235 *cki-gfp*	This work
pIK471	*cka-lacZ*	8

approximately 7 kb *Eco*RI fragment encoding the *cka* gene without the 5′ end, *cki*, *ckl*, the pColK-K235 replication region, and the Ap^r gene was isolated. The 2.1-kb *Eco*RI fragment, with the *cka* promoter region and the *cka* 5′ end, was used as a template for a PCR with two primers, K1P (5′-TCGAAT TCA TGCGTCTTGCCTGGTATA-3′), designed on the basis of the *cka* promoter region, and K2A (5′-TTGAATTC**GGTA CC**GTCATAACAATA-3′), corresponding to colicin K nucleotides 338 to 331 (12) and carrying a *Kpn*I site (boldfaced). The PCR-amplified 2.1-kb *Eco*RI fragment with the *Kpn*I site was subsequently ligated to the 7-kb *Eco*RI fragment of pColK-K235 described above, generating pKCT2. Nucleotide sequencing was performed to confirm that no base changes had occurred during PCR amplification. Finally, plasmid pKCT3 was prepared by cutting the single pKTC2 *Kpn*I site and ligating with the *Kpn*I cassette carrying the promoterless *gfp* region.

Construction of the *cki-gfp* gene fusion. The *cki-gfp* fusion was prepared using the minitransposon suicide delivery plasmid pAG408 (17). Conjugal transfer of pAG408 to DH5α carrying pColK-K235 was performed by filtration of exponential-phase donor and recipient strains, at a ratio of 1:4, through a 0.22-μm-pore-size Millipore filter. The filters were then incubated at 37°C for 24 h, and cultures were transferred to fresh prewarmed liquid LB medium supplemented with gentamycin, kanamycin, and nalidixic acid to select for transconjugants carrying the *gfp* minitransposon. To obtain pColK-K235 plasmids with the inserted minitransposon, plasmid DNA was isolated from the overnight transconjugant culture and used to transform strain MC4100, again selecting for resistances encoded by the minitransposon system. Transformants which carried the *gfp* gene downstream of a plasmid pColK-K235 promoter exhibited fluorescence when observed under long-wave UV light. To determine the site of insertion of the *gfp* minitransposon in the fluorescent clones, DNA sequencing was performed with primer G1 (5′-GAATTGGGACAACTCCAG TG-3′), specific for *gfp*. Thus, plasmid pKCT4, carrying the *cki-gfp* fusion, was isolated with *gfp* inserted at nucleotide 1953 (12).

A random chromosomal *gfp* transcriptional fusion exhibiting strong fluorescence throughout the growth cycle, also obtained with the minitransposon system described above, was used as a positive-control strain.

***cka* is fully expressed in the stationary phase in 3% of the bacterial population.** On the basis of the β-galactosidase activity of a *cka-lacZ* fusion and immunoblot experiments to detect native colicin K, it was previously shown that colicin K synthesis is increased approximately 20-fold in the stationary phase of growth when nutrients are depleted and intracellular concentrations of ppGpp increase (8, 9). To resolve how these results relate to expression in individual cells, strain MC4100 carrying pCKT3, with a *cka-gfp* transcriptional fusion, was grown with aeration at 37°C. Samples were removed at intervals, and 400 μg of chloramphenicol/ml was immediately added to block protein synthesis. Flow cytometry was performed with a FACScalibur (Becton Dickinson, Oxford, United Kingdom) equipped with a 15-mW, air-cooled argon-ion laser as the excitation light source (488 nm). Prior to microscopy, cells were permitted to attach to 0.1% (wt/vol) poly-L-lysine (Sigma)-coated glass slides. Fluorescence in single cells was detected by using a Zeiss Axiovert 135 M microscope, equipped with an excitation filter at 450 to 490 nm and with emission at wavelengths of 515 to 565 nm, and by bright-field microscopy.

Based on fluorescence of the *cka-gfp* fusion, no expression was observed in the lag or early-exponential phase. The first fluorescent cells were detected in the late-exponential phase, when approximately 1% of the bacterial population exhibited fluorescence. In stationary phase, fluorescence was observed in approximately 3% of the cells analyzed (Fig. 2 and Table 2). No further increase in the number of fluorescent cells was observed in the late-stationary phase, 24 h after inoculation (data not shown).

Even though it is generally accepted that colicin production is induced in only a part of the population (5), our investigation shows for the first time, at the single-cell level, that the colicin activity gene, *cka*, encoding colicin K, is induced in only a fraction of the population.

***cka* is expressed in the large majority of LexA-defective cells.**

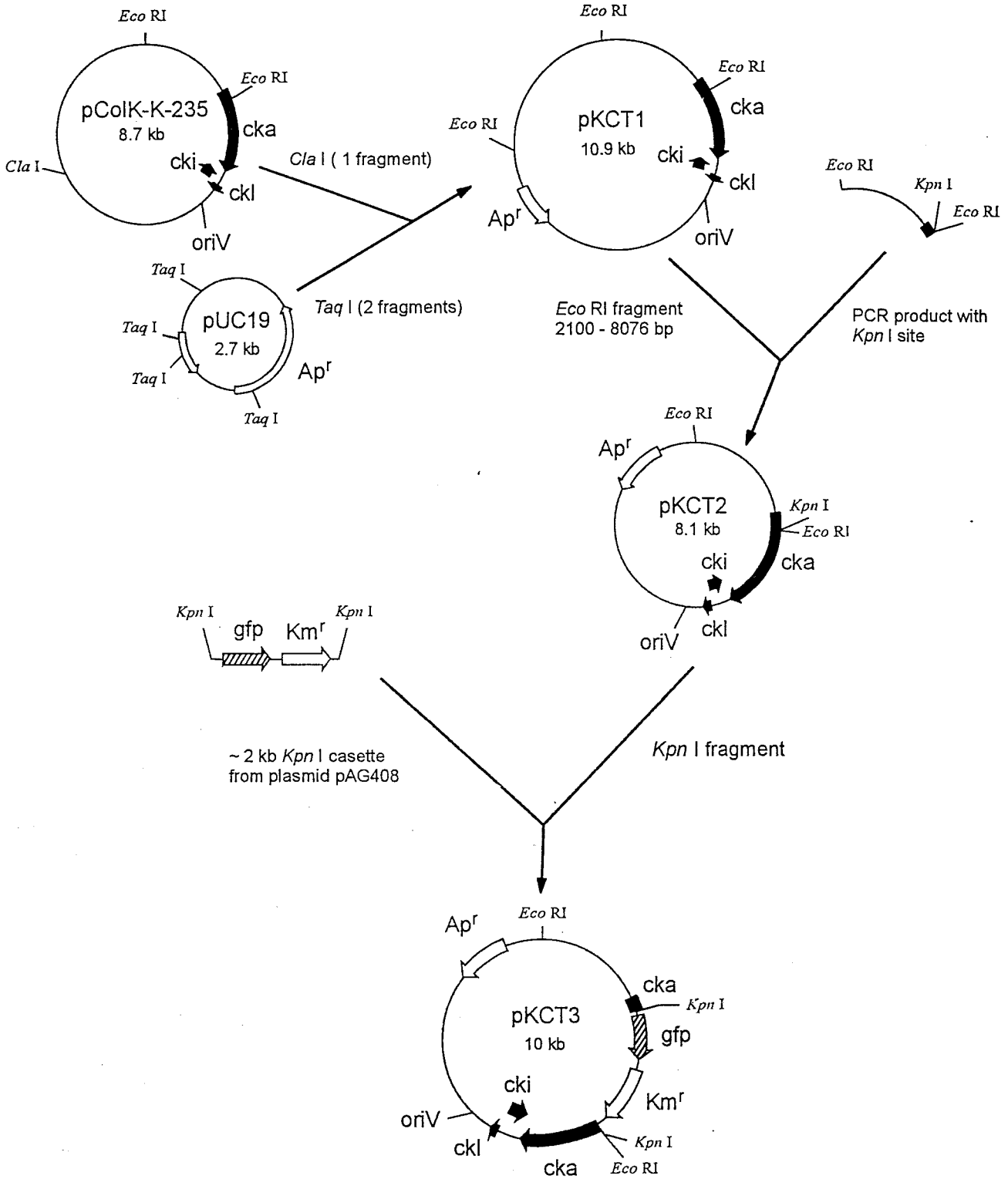

FIG. 1. Strategy for construction of plasmid pKCT4 with the *cka-gfp* fusion. Arrows indicate transcription polarity. Cleavage sites used for cloning are designated.

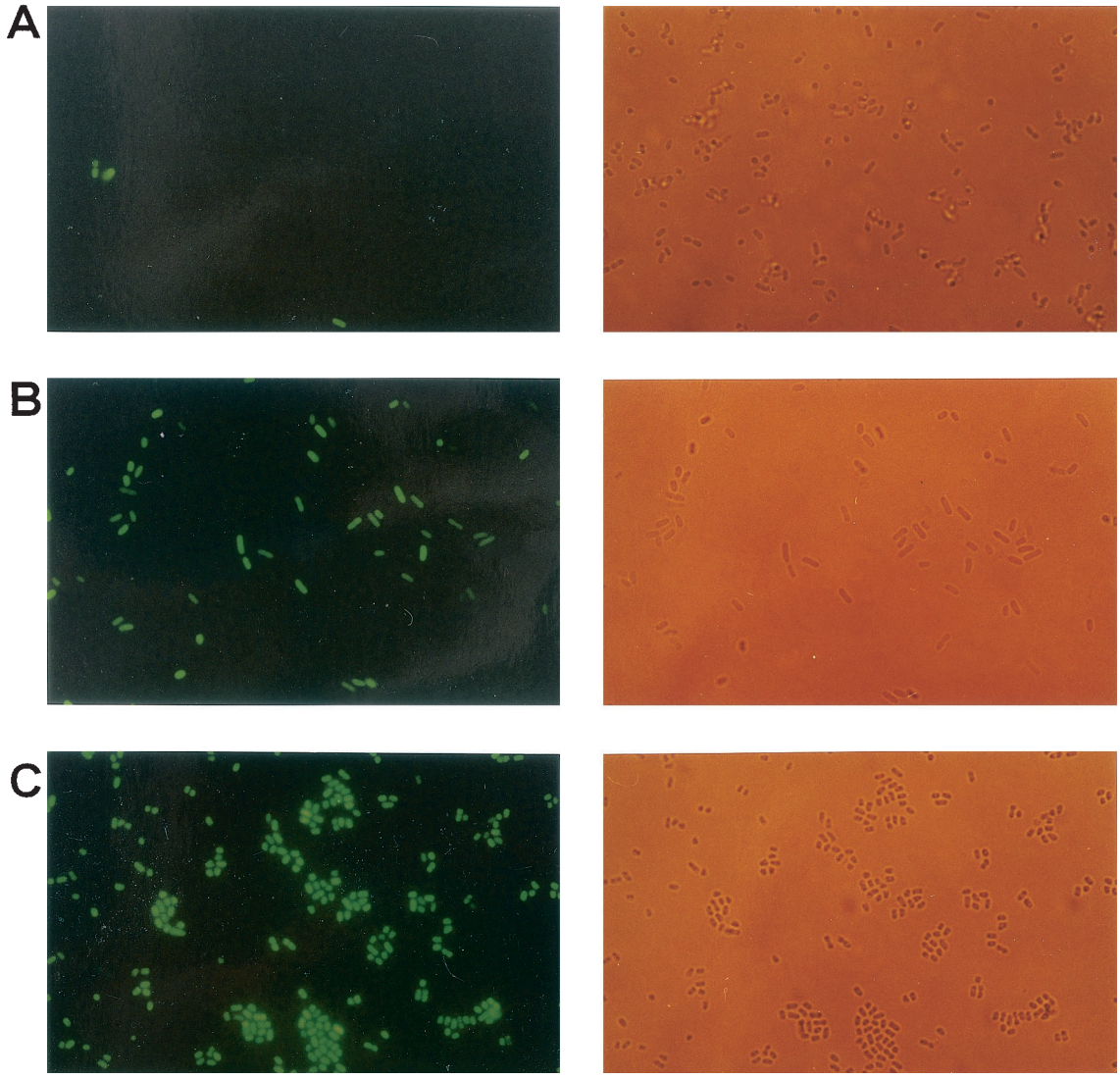

FIG. 2. Differential expression of the colicin K *cka* and *cki* genes through the growth cycle. Shown are images of MC4100 with the *cka-gfp* fusion in stationary phase (A) and of MC4100 with the *cki-gfp* fusion in the exponential (B) and stationary (C) phases, taken under fluorescence microscopy (left panels) and bright-field microscopy (right panels). The experiments were repeated four times, and representative results are shown.

TABLE 2. Promoter activity of the colicin K activity (*cka*) and immunity (*cki*) genes through the growth cycle at the single-cell level

Growth phase	Fluorescence (geometric mean)[a]					
	pKCT3[b]	RW542[c]	RO98[d]	pKCT4[e]	+[f]	−[g]
Exponential	12	71.8	10	91	208	6.1
Stationary	23.3	136.3	22	137	234	6.2

[a] Determined from 10,000 individual particles per sample. Experiments were carried out in duplicate, and representative results are shown.
[b] *cka-gfp* fusion.
[c] *cka-gfp* fusion in RW542 *lexA51*.
[d] *cka-gfp* fusion in a *relA spoT* mutant.
[e] *cki-gfp* fusion.
[f] +, random insertion of promoterless *gfp* expressed throughout the growth cycle.
[g] −, plasmid pColK-K235 without *gfp*.

Colicin synthesis is characteristically regulated by the SOS response with LexA binding sites in the promoter regions of colicin-encoding operons. To try to resolve how differential expression of *cka* is accomplished, expression of the *cka-gfp* fusion was studied in the *lexA51* strain RW542, encoding a defective LexA that cannot bind to LexA binding sites, and in the isogenic strain RW118, encoding the wild-type LexA. Fluorescence microscopy (data not shown) and flow cytometry revealed expression of the *cka* gene, from the *cka-gfp* fusion, in almost all (99% [Table 2]) of the cells analyzed throughout the growth cycle in the *lexA51*-defective strain RW542, compared with 3% in the wild-type strain RW118 (data not shown). The levels of GFP synthesized from the *cka-gfp* fusion were also measured in the *lexA51* mutant and in the wild-type strain. GFP levels from washed cells lysed by 0.1% Triton X-100 (Sigma) and chloroform were determined fluorimetrically using the Spectrofluorometer JASCO FP 750. In the *lexA51*

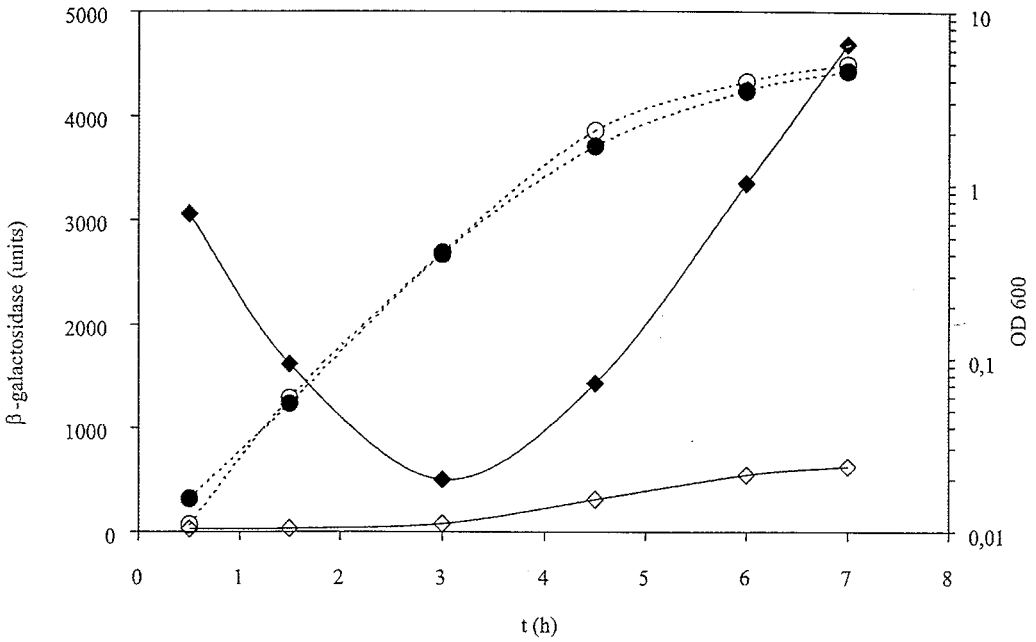

FIG. 3. Expression of the *cka-lacZ* fusion in the *lexA51* mutant (solid symbols) and in RW118 (open symbols). β-Galactosidase activity in Miller units (diamonds) and growth expressed as the optical density at 600 nm (circles) are presented (8). The experiment was carried out three times, and representative results are shown.

strain, GFP levels were 5- and at least 10-fold higher than in the wild-type strain in the exponential and late-exponential phases, respectively. These results demonstrate that the LexA protein represses *cka* promoter activity in the majority of colicinogenic cells.

Expression of a *cka-lacZ* fusion is increased in LexA-defective strain. Previous studies based on mitomycin C induction of β-galactosidase activity of a *cka-lacZ* fusion showed an approximately threefold increase in β-galactosidase activity (8). These results seemed to indicate that the SOS response is not a strong regulatory signal for *cka* expression. The finding that the *cka-gfp* fusion is expressed in the large majority of cells in the *lexA*-defective strain, as well as following mitomycin C induction (data not shown), was unexpected. To resolve the discrepancy between results obtained using the *cka-lacZ* and *cka-gfp* fusions, and to more directly study the effect of LexA on *cka* expression, the β-galactosidase activity of the former fusion from plasmid pIK471 (8) was also examined in strain RW542, with the defective LexA protein, and in the isogenic strain RW118, encoding the wild type LexA protein. In the *lexA51* strain, instability of the *cka-lacZ* fusion in particular was evident, as loss of ampicillin resistance encoded by plasmid pIK471 was observed during progression through the growth cycle in spite of antibiotic selection. *cka* expression was therefore assayed in transformants isolated immediately prior to each experiment. The results of our study showed approximately 5- and 10-fold-higher *cka* expression in the *lexA51* strain in the exponential and late-exponential phases, respectively (Fig. 3). Further, direct assays of β-galactosidase activity of newly isolated transformants scraped from plates and of a sample taken half an hour after growth medium inoculation were performed. On the basis of the β-galactosidase activity of the *cka-lacZ* fusion, more than 100-fold-greater *cka* expression was shown in the *lexA51* strain than in the wild type. Our results demonstrate, on the basis of *cka* expression from both *cka-lacZ* and *cka-gfp* fusions, that LexA is indeed a decisive regulatory element of *cka* expression.

Differential expression of *cka* is independent of ppGpp. Previously, ppGpp had been shown to be the main positive effector of *cka* expression (8, 9). Therefore, expression of the *cka-gfp* fusion was analyzed in strain RO98, with mutations in *relA* and *spoT*. Strains with *relA spoT* double mutations produce no ppGpp. Expression of fluorescence in cells carrying *cka-gfp* occurred in a slightly lower percentage of cells, 2%, than in the wild-type strain (Table 2). Since ppGpp regulates the translation efficiency of colicin K mRNA, these data confirm that differential expression of *cka* is accomplished primarily at the level of transcription.

***cki* is expressed throughout the growth cycle.** The Cki immunity protein protects the cell from extracellular colicin K. It has been accepted that the immunity protein is synthesized constitutively at a low level to protect the colicinogenic population from its native colicin. About 500 molecules of the immunity protein are inserted in the cytoplasmic membrane, where they inhibit channel formation. To determine whether all cells in fact express the immunity gene, strain MC4100-1, resistant to colicin K, carrying pCKT4 with a *cki-gfp* transcriptional fusion, was grown with aeration at 37°C. Again, samples were periodically removed and prepared for microscopy as described above. In contrast to that of the *cka-gfp* fusion, expression of the *cki-gfp* fusion, as determined by fluorescence and flow cytometry, was detected throughout the growth cycle in almost all (98.9%) of the cells analyzed (Fig. 2 and Table 2).

Colicin synthesis is characteristically regulated by the SOS response with LexA binding sites in promoter regions of colicin-encoding operons. In this study we present evidence, on the

basis of fluorescence of a *cka-gfp* transcriptional fusion, that upon entry into stationary phase, only 3% of a colicin K-producing population of cells express the colicin K activity gene *cka*. We show that the LexA protein exerts a strong negative effect, repressing colicin K expression almost completely in the exponential phase and in about 97% of the population in the stationary phase. We conclude that the LexA protein is a decisive regulatory element in establishing differential expression of colicin synthesis at the level of transcription.

Thus, in the stationary phase, transcription from the *cka* promoter is derepressed in only approximately 3% of the colicinogenic population. At the posttranscriptional level, the *cka* mRNA is translated more efficiently due to increased levels of ppGpp (9). Possibly, some other regulatory protein whose concentrations vary in response to environmental signals could displace LexA from the *cka* binding boxes or, alternatively, activate transcription without displacing LexA.

Bacteria live in complex associations that in many ways resemble multicellular organisms. Even though bacterial populations consist of identical cells, examples of differential gene expression are known in which parts of a population perform specialized functions. Some of these functions have been extensively studied: bacterial development, exemplified by sporulation in *Bacillus subtilis* (reviewed in reference 15) and fruiting-body formation in *Myxococcus xanthus* (reviewed in reference 7), as well as genetic exchange, for example, the development of competence in *B. subtilis* (6). Colicin production is without doubt another specialized function, and there could be other examples not yet identified.

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