NOTES

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

Janez Mulec,1 Zdravko Podlesek,2 Peter Mrak,2 Andreja Kopitar,3 Alojz Ihan,3 and Darja Žgur-Bertok2*

Karst Research Institute, Scientific Centre of the Slovenian Academy of Sciences and Arts, Postojna,1 and Department of Biology, Biotechnical Faculty,2 and Institute for Microbiology and Immunology, Medical Faculty,3 University of Ljubljana, Ljubljana, Slovenia

Received 21 August 2002/Accepted 16 October 2002

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 interspecies 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 fusion gene. 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' gene from plasmid pUC19 was introduced into pColK-K235. For this purpose, pUC19 was digested with TaqI, and the two TaqI fragments encoding the Ap' gene were cloned into the ClaI site of plasmid pColK-K235, producing pKCT1. Subsequently, pKCT1 was cut with EcoRI, and the longer,
and the Ap r gene was isolated. The 2.1-kb cation. Finally, plasmid pKCT3 occurred during PCR amplification with the Eco/h11032 fragment to the 7-kb Eco/h11032 without the 5’ Eco/h11032 end, was used as a template for a PCR with two primers, K1P (5’/H11032-TCGAATTC-3’), designed on the basis of the Kpn I site and ligating with the Kpn I site (boldfaced). The KPCR-amplified 2.1-kb EcoRI fragment with the Kpn I site was subsequently ligated to the 7-kb EcoRI 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 fusion gene. 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’-GAATTGGGACAATCCAG 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.

cki is fully expressed in the stationary phase in 3% of the bacterial population. On the basis of the β-galactosidase activity of a cki-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 cki-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 Axiosvert 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 cki-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, cki, encoding colicin K, is induced in only a fraction of the population.

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

---

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139 Δ(argF-lac)U169 rpsL50 relA1 fhlB3501 ptsF25 deoC1</td>
<td>10</td>
</tr>
<tr>
<td>MC4100-1</td>
<td>MC4100 resistant to colicin K</td>
<td>This work</td>
</tr>
<tr>
<td>DH5α</td>
<td>thi-1 relA hsdR17 lac</td>
<td>A. Francy</td>
</tr>
<tr>
<td>AB1133</td>
<td>Sensitive to all colicins</td>
<td>B. Bachman</td>
</tr>
<tr>
<td>RO98</td>
<td>MC4100 relA251:kan spoT:cat</td>
<td>10</td>
</tr>
<tr>
<td>RW118</td>
<td>thr-1 araD139 Δ(gpt-proA)62 lacY1 tss-33 supE44 galk2 hisG4</td>
<td>R. Woodgate</td>
</tr>
<tr>
<td>RW542</td>
<td>RW118 lecA51 (Del)</td>
<td>R. Woodgate</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pColK-K235</td>
<td>Encoding colicin K</td>
<td>14</td>
</tr>
<tr>
<td>pAG408</td>
<td>Promoter-probe gfp minitransposon suicide delivery system</td>
<td>16</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap' cloning vector</td>
<td>17</td>
</tr>
<tr>
<td>pKCT1</td>
<td>TaqI fragments with pUC19 Ap' gene cloned into pColK-K235</td>
<td>This work</td>
</tr>
<tr>
<td>pKCT2</td>
<td>cka cki ckl Ap' and KpnI restriction site</td>
<td>This work</td>
</tr>
<tr>
<td>pKCT3</td>
<td>pKCT2 with cka-gfp</td>
<td>This work</td>
</tr>
<tr>
<td>pKCT4</td>
<td>pColK-K235 cki-gfp</td>
<td>This work</td>
</tr>
<tr>
<td>pIK471</td>
<td>cka-lacZ</td>
<td>8</td>
</tr>
</tbody>
</table>
FIG. 1. Strategy for construction of plasmid pKCT4 with the cka-gfp fusion. Arrows indicate transcription polarity. Cleavage sites used for cloning are designated.
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. 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.](image)

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Fluorescence (geometric mean)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pkCT3</td>
<td>RW542</td>
</tr>
<tr>
<td>Exponential</td>
<td>12</td>
<td>71.8</td>
</tr>
<tr>
<td>Stationary</td>
<td>23.3</td>
<td>136.3</td>
</tr>
</tbody>
</table>

* Determined from 10,000 individual particles per sample. Experiments were carried out in duplicate, and representative results are shown.

* \( cka-gfp \) fusion.

* \( cki-gfp \) fusion.

* \( cka-gfp \) fusion in a \( relA spoT \) mutant.

* \( cki-gfp \) fusion.

* +, random insertion of promoterless \( gfp \) expressed throughout the growth cycle.

* −, plasmid pColK-K235 without \( gfp \).
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 /H9252-galactosidase activity of a cka-lacZ fusion showed an approximately threefold increase in /H9252-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 /H9252-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 transpositional fusion, that
upon entry into stationary phase, only 3% of a colicin-
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 different-
ential 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 col-
icinogenic population. At the posttranscriptional level, the cka 
RNA is translated more efficiently due to increased levels of 
ppGpp (9). Possibly, some other regulatory protein whose con-
centrations 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 re-
semble multicellular organisms. Even though bacterial popu-
lations 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 ex-
tensively studied: bacterial development, exemplified by spor-
ulation in Bacillus subtilis (reviewed in reference 15) and fruit-
body morphogenesis in Myxococcus xanthus (reviewed in 
reference 7), as well as genetic exchange, for example, the 
development of competence in B. subtilis (6). Colicin produc-
tion is without doubt another specialized function, and there 
could be other examples not yet identified.

This work was supported by grant P0-0508-0487 from the Slovene 
Ministry of Science and Technology.

We thank R. Hengge-Aronis, A. P. Pugsley, and R. Woodgate for 
providing bacterial strains and C. A. Guzmán for the generous gift of 
the promoter-probe gfp-based minitransposon suicide delivery system.

REFERENCES
Green fluorescent protein as a marker for gene expression. Science 263:802– 
803.
Baquero, and C. Torres. 2001. Bacteriocin production in vancomycin-resis-
frequency of colicinogeny in Escherichia coli from house mice. Microbiology 
144:2233–2240.
5. Gordon, D. M., and M. A. Riley. 1999. A theoretical and empirical investi-
dependent checkpoint limits growth during the escape from competence. 
542.
K cka gene reveals induction of colicin synthesis by differential responses to 
2001. Codon-usage based regulation of colicin K synthesis by the stress 
scriptional start sites and the role of ppGpp in the expression of 
iproS, the structural gene for the σ3 subunit of RNA polymerase in Escherichia coli. J. 
11. Pilsl, H., and V. Braun. 1995. Strong function-related homology between the 
711.
isolates of Escherichia coli and an investigation into the stability of Col-
Curr. Opin. Microbiol. 3:561–566.
cloning vectors and host strains: nucleotide sequences of the M13mp18 and 