Mol Genet Genomics (2007) 277:391–401
DOI 10.1007/s00438-006-0185-x

ORIGINAL PAPER

Heterogeneity in expression of the *Escherichia coli* colicin K activity gene *cka* is controlled by the SOS system and stochastic factors

Peter Mrak · Zdravko Podlesek · Jos P. M. van Putten · Darja Žgur-Bertok

Received: 30 August 2006 / Accepted: 17 October 2006 / Published online: 11 January 2007

Abstract Phenotypic diversity provides populations of prokaryotic and eukaryotic organisms with the flexibility required to adapt to and/or survive environmental perturbations. Consequently, there is much interest in unraveling the molecular mechanisms of heterogeneity. A classical example of heterogeneity in *Escherichia coli* is the subset (3%) of the population that expresses the colicin K activity gene (*cka*) upon nutrient starvation. Here, we report on the mechanism underlying this variable response. As colicin synthesis is regulated by the LexA protein, the central regulator of the SOS response, we focused on the role of LexA and the SOS system in the variable *cka* expression. Real-time RT-PCR showed that the SOS system, without exogenous DNA damage, induces moderate levels of *cka* expression. The use of *cka–gfp* fusions demonstrated that modification of the conserved LexA boxes in the *cka* promoter region affected LexA binding affinity and the percentage of *cka–gfp* expressing cells in the population. A *lexA–gfp* fusion showed that the *lexA* gene is highly expressed in a subset of bacteria. Furthermore, c*ka–gfp* fusions cloned into higher copy plasmid vectors increased the percentage of *cka–gfp* positive bacteria. Together, these results indicate that the bistability in *cka* expression in the bacterial population is determined by (1) basal SOS activity, (2) stochastic factors and possibly (3) the interplay of LexA dimers at *cka* operator. Other LexA regulated processes could exhibit similar regulation.

Keywords Heterogeneity · Gene expression · LexA · Stochastic · Colicin

Communicated by D. Andersson.

P. Mrak · Z. Podlesek · D. Žgur-Bertok (✉)
Department of Biology, Biotechnical Faculty,
University of Ljubljana, 1000 Ljubljana, Slovenia
e-mail: darja.zgur.bertok@bf.uni-lj.si

Present Address:
P. Mrak
Lek Pharmaceuticals d.d., Mengeš, Slovenia

J. P. M. van Putten
Department of Infectious Diseases and Immunology,
Utrecht University, Utrecht, 3584 CL, The Netherlands

Introduction

Phenotypic heterogeneity among isogenic cells has been investigated in natural systems (Novick and Weiner 1957; Ferrell and Machleder 1998; Hernday et al. 2003; Maamer and Dubnau 2005) and by theoretical studies (Ferrell 2002). Simulations have shown that in fluctuating conditions dynamic heterogeneity can enhance fitness (Thattai and van Oudenaarden 2004) and is subject of natural selection (Fraser et al. 2004). Elucidation of the molecular basis of heterogeneity has become a major focus of research. Here, we investigate the heterogeneity in expression of the *Escherichia coli* colicin activity gene *cka*.

Colicins are plasmid-encoded bacteriocins, synthesized by and active against cells of *E. coli* and sometimes against related species such as *Salmonella*. Among natural isolates colicin producing strains are found with high frequency and more than 20 colicin types have been characterized (Riley and Wertz 2002). In natural populations, a dynamic equilibrium appears to exist between colicin producing strains, colicin

sensitive strains and colicin resistant strains. This has been shown to promote microbial diversity within *E. coli* populations in the mammalian colon (Kirkup and Riley 2004).

Colicin synthesis is characteristically regulated by the LexA protein, the key regulator of the SOS response (Friedberg et al. 1995). In *E. coli*, LexA regulates at least 28 genes belonging to the LexA regulon (Fernández de Henestrosa et al. 2001). The protein represses gene expression by binding as a dimer to a 16-mer consensus sequence CTG-N_{10}-CAG designated as SOS boxes (Erill et al. 2003). The 5′ CTG and CAG were shown to be most critical for LexA binding (Wertman and Mount 1985). One factor that determines the cellular level of LexA is DNA damage. This causes stalling of replication forks and exposure of single-stranded DNA. The ssDNA is bound by RecA and the formed nucleoprotein filament activates the autoproteolytic cleavage of LexA. A number of other bacterial species, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *K. oxytoca*, *Serratia marcescens*, *Hafnia alvei* and others, synthesize bacteriocins that are also regulated by the LexA protein (Riley et al. 2001; Michel-Briand and Baysee 2002; Ferrer et al. 1996; Wertz and Riley 2004).

One of the colicin types is colicin K. Colicin K production is encoded by three genes: *cka* encoding the colicin activity protein, *cki* encoding the immunity protein which protects the producing strain, and *ckl* encoding the lysis protein (Pilsl and Braun 1995). Colicin K belongs to the group of pore-forming colicins which destroy the electrochemical potential of the cytoplasmic membrane. We have previously shown that synthesis of colicin K is primarily induced by an increase in the alarmone ppGpp due to nutrient depletion (Kuhar and Žgur-Bertok 1999). ppGpp indirectly regulates translation of colicin K mRNA (Kuhar et al. 2001). As a number of other colicins, colicin K is released semi-specifically by cell lysis. Therefore, only a part of the population should express the colicin activity and lysis genes. It was shown that in the stationary phase, transcription from the *cka* promoter is derepressed in only approximately 3% of the colicinogenic population and that the LexA protein is a decisive regulatory element repressing expression at the level of transcription in the large majority of the population (Mulec et al. 2003).

Although the diversity between *E. coli* populations appears to be based on a dynamic equilibrium between colicin producing, sensitive, and resistant strains (Kirkup and Riley 2004), the molecular basis for the dynamic equilibrium between cells derived from the same parental strain is still unknown. In the present study, we searched for the regulatory mechanism used to sustain *cka* expression in only a small fraction of a colicinogenic population. Several mechanisms can be hypothesized (1) *cka* expression may follow intracellular events that activate the SOS response without exogenous DNA damaging treatment e.g., inactivated replication forks and generation of long-lived single-stranded DNA (ssDNA), (2) variable gene expression can involve stochastic mechanisms (McAdams and Arkin 1999; Ozbudak et al. 2002). In this scenario expression of the *cka* gene could be a random outcome possibly depending on the intracellular concentration of the LexA protein, and (3) *cka* expression may involve another regulatory protein that, dependent upon environmental signals, could displace LexA from the *cka* SOS boxes or activate transcription without displacing LexA. To test these possibilities real-time RT-PCR was used to quantify *cka* mRNA levels in a wild type and isogenic SOS defective *recA* mutant strain. Additionally, modifications were introduced into the *cka* SOS boxes and assessed for their effect on *cka* expression using *cka–lacZ* and *cka–gfp* fusions. To observe the effect of titration of the LexA protein, *cka–gfp* expression from higher copy number plasmids was measured, at the single cell level. Taken together our results indicate that both the SOS response induced in the absence of exogenous DNA damage, stochastic factors as well as possibly the interplay of LexA dimers binding to the *cka* SOS boxes are decisive in establishing differential expression of colicin synthesis among the bacterial population.

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used in this study are presented in Table 1. Bacteria were grown in Luria-Bertani medium (LB) with aeration at 37°C with the appropriate antibiotics.

General DNA manipulation techniques

Plasmid DNA isolation, ligation, and transformation experiments were performed using standard methods (Sambrook et al. 1989). Restriction endonuclease digestion was carried out as specified by the manufacturer (Promega). DNA fragments were isolated from agarose gels by using a QIAquick gel extraction kit (Qiagen). DNA sequencing was performed using cycle sequencing with BigDye terminator cycle sequencing ready reaction kit and an ABI PRISM 310.

Table 1 Bacterial strains and plasmids

Strain/plasmid	Relevant properties	Source/reference
E. coli strains		
RW118	*thr-1 araD139Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 sulA211*	R. Woodgate
RW464	RW118 *recA*	R. Woodgate
AB1157L	AB1157 with *lexA-gfp*	Ronen et al. 2002
Plasmids		
pCB267	*lacZ* promoter–probe vector Ap^R	Schneider and Beck 1986
pIK471	*cka* fragment (−397 to + 74) in pCB267	Kuhar and Žgur-Bertok 1999
pIK471–606	pIK471 with distal SOS box HI 3.7	This study
pIK471–656	pIK471 with proximal SOS box HI 15.9	This study
pColK-JA533	*cka* wild-type plasmid	Kuhar and Žgur-Bertok 1999
pKCT1	pColK-JA533 with Ap^R	Mulec et al. 2003
pKCT2	pKCT1 with *Kpn*I site	Mulec et al. 2003
pKCT3	pKCT1 with *cka–gfp*	Mulec et al. 2003
pERO19	*cka–gfp* fusion cloned into pUC19	This study
pERO22	*cka–gfp* fusion cloned into pBR322	This study
pKCT5	pKCT2 with deletion of *IS2* sequences	This study
pKCT5-Up1	pKCT5 with mutations in 5′ upstream LexA binding sequence	This study
pKCT3–606	pKCT5 with distal SOS box HI 3.7	This study
pKCT3–656	pKCT5 with proximal SOS box HI 15.9	This study
pKCT3-Up1	pKCT5 with mutations in 5′ upstream LexA binding sequence	This study
pKCT3-Up2	pKCT5 with mutations in 5′ and 3′ LexA binding sequences	This study
pKCT3-Up3	pKCT5 with mutations in all three LexA binding sequences	This study

β-galactosidase assays

To measure β-galactosidase activity strains were grown overnight at 37°C in LB medium and diluted 1:500 into fresh LB medium. Samples were periodically removed and assayed for β-galactosidase activity. β-galactosidase assays were performed as described previously (Miller 1972) with bacteria treated with sodium dodecyl sulfate-chloroform and washed with Z buffer and *o*-nitrophenyl-β-D-galactopyranoside as a substrate. Enzyme activity was expressed in Miller units (Miller 1972).

RNA isolation

RNA was isolated from approximately 10^9 cells from 7 h cultures of strains RW118, RW434, and RW464, harbouring plasmid pColK-JA533. RNA was purified with RNAzolB (Campro Scientific). Following isopropanol precipitation, the RNA was dissolved in 40 µl of H_2O. Samples were treated with RNase-free DNase I (Invitrogen) and stored at −70°C.

Quantitation of mRNA levels

Real-time RT PCR was performed using the Quanti-Tect SYBR Green RT-PCR Kit from Qiagen. The reactions were performed on 0.2 µg DNase I-treated RNA with 100 pmol of both primers. Amplification was performed with an ABI PRISM 7000 sequence detection system as follows: reverse transcription 30 min at 50°C; activation 15 min at 95°C followed by 35 cycles, with 1 cycle consisting of 15 s at 94°C, 30 s at 51°C, and 30 s at 72°C. Each was analysed from three independent RNA samples and performed in triplicate. Controls were performed by omission of reverse transcriptase to determine extent of residual genomic DNA contamination and a no-template control to measure interference from primer dimer formation. Additionally, no product was detected using strain RW118, without plasmid pColK-JA533, as a negative control. To further verify that only a single band was produced all samples were analysed on a 2% agarose gel. For detection of *cka* and *lpp* mRNA, primers cka1 (5′-CAGAGGTCGCTGAACATGAA-3′) and cka2 (5′-CTAGCTAAACGCTTCTCTGCTT-3′) and lpp1 (5′-GCAATGCGTTCCGACGTT-3′) and lpp2 (5′-TGTCCAGACGCT-3′) were used. Data were analysed by use of the $2^{-\Delta\Delta CT}$ method described previously (Livak and Schmittgen 2001).

Site-specific mutagenesis of SOS boxes

Site-directed mutagenesis was performed by PCR using primers based on the *cka* LexA binding sites with

point mutations (indicated in bold). The primers used to introduce mutations into the *cka* LexA binding sites were SOS1 (5′-GTAGGTTTTTACTGTA**T**ATA**T**A **T**CCAGTGG-3′), for the distal site, and SOSp (5′-AA CCAGTGGTTATA**AT**TGTAC**G**GTATA-3′), for the proximal site. PCR was performed using primers SOS1 or SOSp and primer K2 (5′-TC**TCTAGA**TGATTCA GATTCGCCCCTG-3′) corresponding to colicin K nucleotides 74–55 (Kuhar and Žgur-Bertok 1999) and with an added *Xba*I restriction sequence (boldface) with plasmid pIK471 as the template. The isolated (158 or 138 bp, respectively) amplified fragment was subsequently used together with primer K3 (5′-TC**GGAT CC**TTCACTGACCACATGACGC-3′) corresponding to sequences 395–376 upstream of the *cka* ATG codon and with an added *Bam*HI restriction sequence (boldface). The generated PCR products were cut with *Bam*HI and *Xba*I and ligated into plasmid pCB267 digested with the same enzymes, generating plasmids pIK471–606 and pIK471–656. Sequencing reactions were performed to verify the mutations.

Construction of plasmids with *cka–gfp* fusions

Plasmids pKCT3-606 and pKCT3-656 were constructed by replacing the approximately 2,000 bp long *Hind*III *Kpn*I fragment of plasmid pKCT3 with fragments amplified by PCR from plasmids pIK471-606 and pIK471-656 using primers ColKH (5′-TC**AAGCTT**AGTCAG CCGTTGTCTCCGGGT-3′) with an added *Hind*III restriction sequence (boldface) and ColKK (5′-TC**GGT ACC**TCTTCTGTCATAACAATACCA-3′) with an added *Kpn*I restriction sequence (boldface). Construction of the *cka–gfp* fusions Up1, Up2, and Up3 were unsuccessful, most likely due to transposase activity, until an *IS*2 sequence directly upstream of the *cka* regulatory region was deleted. For this purpose the approximately 2,000 bp long *Hind*III *Kpn*I fragment of plasmid pKCT3, which harbours a large part of the *IS*2, was replaced by a shorter fragment without the *IS*2. The latter was prepared by PCR using pKCT1 as the template and primer ColZP (5′-CACT**AAGCTT** AACGGCAGCAAAG-3′) on the basis of sequences approximately 500 bp upstream of the *cka* gene (nucleotide sequence accession number AY929248) and with an added *Hind*III restriction site (boldface) as well as primer K2A (5′-TTGAATT**GGTACC**GTCATAAC AATA-3′), corresponding to colicin K nucleotides 338–331 and with a *Kpn*I site (boldface). The amplified product was digested with both enzymes and ligated to the 6.3 kb *Hind*III *Kpn*I fragment of plasmid pKCT4. Thus plasmid pKCT3-S was prepared into which subsequently modifications of the SOS boxes were introduced by PCR using the method described by Michael 1994. For this purpose three primers were used: two external, one ColZP, the second K2A, and the third with the modified LexA binding sequence. Modified primers were either UP1d (5′-GTAGTAGGTTTTTA C**CA**TACATA-3′), UP2 (5′-TACT**A**TACATAAAA CCAGTGGTTATATGTA**G**AGTA-3′) or UP3 (5′-T AC**CA**TACATAAAACC**T**GTGGTTATATGTA**GC** GTA-3′). The obtained PCR product was digested with *Hind*III and *Kpn*I and ligated into plasmid pKCT5, digested with the same enzymes. Subsequently, a *Kpn*I digested cassette from plasmid pAG408 (Suarez et al. 1997) bearing the promoterless *gfp* and Km^R genes was inserted into the *Kpn*I site within the *cka* gene. All modifications were verified with sequencing reactions. For preparation of plasmid pKCT5-Up1, the *gfp* encoding casette was not inserted into the *Kpn*I site.

The pUC19 derived plasmid pERO19 was constructed by cloning plasmid's pKCT3 3,894 bp long *Hind*III and *Xba*I restriction fragment carrying the *cka–gfp* fusion into plasmid pUC19 digested by *Hind*III and *Hind*II. Subsequently, the pBR322 derivative plasmid pERO22 was prepared by cloning plasmid's pERO19 *Hind*III and *Sma*I fragment with the *cka–gfp* fusion, into plasmid pBR322 digested by *Hind*III and *Eco*RV.

Mobility shift assays using agarose gels

The mobility shift assays were performed essentially as previously described by Lewis et al. (1994). Protein/DNA complexes were run on 2% agarose gels following incubation at 30°C for 30 min. A 100 bp DNA ladder (MBI Fermentas) was used as the competitor DNA. The 230 bp fragment with the two *cka* LexA boxes employed in the mobility shift assays was amplified using primers GS1 (5′-GCCTCCCCATAGTGTG ATCCTTATTATTGC-3′) and primer K2 (5′-TCTCT AGATGATTCAGATTCGCCCCTG-3′).

Fluorescence microscopy

The bacterial cells were prepared for fluorescence microscopy as described previously (Mulec et al. 2003). Fluorescence in single cells was detected using a Carl Zeiss Axiovert 135 microscope, equipped with a GFP filter (Chroma). For each experiment approximately 10,000 cells were examined. The fluorescence intensity of individual cells was estimated using image analysis software Scion Image (http://www.scioncorp.com) in the following manner. The fluorescent micrographs were converted to greyscale images. The density window was

determined by using density slice matching the shape of the cells with the highest fluorescence intensity and that of the cells with the lowest intensity, gaining the top and the bottom boundaries (respectively) of the density window. For greater clearness the density index scale is determined from 0 (black) to 256 (white). All micrographs were taken at exactly the same conditions; hence the density window gives good correlation to the fluorescence intensity of the analysed population.

Colicin production assay

Colicin production was determined employing an in vivo biological assay as described previously (Jerman et al. 2005). Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. AY929248

Results

The role of spontaneous SOS activity in *cka* expression

Previous studies on the transcriptional regulation of the colicin E1 activity gene *cea* indicated that colicin expression in a population of colicinogenic bacteria is due to a spontaneous SOS response (Salles et al. 1987). To investigate the role of SOS activity in *cka* expression, *cka* mRNA levels were quantified for the wild type RW118 and the isogenic *recA* defective strain RW464, both harbouring the colicin K encoding plasmid pKCT1, using real-time RT-PCR. Samples were taken from stationary phase cells when colicin K levels are highest (Kuhar et al. 2001). mRNA levels of the constitutively expressed *lpp* gene, encoding the *E. coli* major outer membrane lipoprotein, served as a control. Our results revealed that transcription of the *cka* gene was decreased by 3.9-fold in the *recA* defective strain compared to the wild type, indicating that maximal expression of the *cka* gene requires SOS induction.

To investigate how the observed decrease in *cka* mRNA levels in a *recA* defective background are related to the expression in individual cells, the expression of a *cka–gfp* fusion from plasmid pKCT3-S was analysed with fluorescence microscopy in the *recA* defective and wild type strains. This revealed an approximately 20-fold reduction in the number of *cka–gfp* expressing cells as only 0.15% expressed the *cka* gene in the *recA* defective strain RW464 (Table 2). These results indicate that *cka* expression is also regulated by stochastic factors.

Effect of modification of the SOS boxes on *cka* expression

All ColE operons including the *cka* gene have two overlapping SOS boxes in their regulatory regions, each with a characteristic affinity of LexA binding. The SOS boxes are designated as distal and proximal with regard to the ATG codon. The term heterology index (HI) was defined to determine the degree of divergence of any 20 nucleotide sequences from the consensus LexA box (Lewis et al. 1994). Sequences with a low HI value are closer to the consensus sequences and bind LexA with greater affinity than sites with a higher HI. It was shown that LexA binds to sequences with an HI value of 12.6 or lower but not to sequences with an HI value >15 (Fernández et al. 2001). On the basis of the nucleotide sequence of the operator sites, the distal SOS box of the *cka* gene has an HI value of 8.6 and the proximal 11.6.

To investigate the potential role of the *cka* operator in differential expression of the colicin K activity gene, site-specific mutagenesis of the SOS boxes was

Table 2 Single cell fluorescence microscopy analysis

Strain/plasmid	Percentage of fluorescence	Density window	Background[a]	Cells counted
RW118				
pKCT1	0	0	0	9,753
pKCT3	2.98	34–78	0	10,303
pKCT3-UP1d	5	61–169.75	16.25–3	11,404
pKCT3-UP2	95	78.5–161.75	0	9,517
pKCT3-UP3	96	134.25–191	0	9,239
pERO19	60	61.5–146	0	9,625
pERO22	6.9	35.75–80	0	10,336
pKCT3–606	1.42	30.75–69.75	0	9,664
pKCT3–656	1.73	24–57.25	0	10,308
RW464				
pKCT3	0.15	16–34	0	10,123
AB1157L	0.3	70.5–112	19.5–33	9,577
pUC19	0.9	72–115.8	20–34.5	9,125

[a] The large majority of cells exhibiting lower fluorescence

performed. Two *cka–lacZ* fusions were constructed. One plasmid, pIK471-606, harboured a distal SOS box with a higher affinity of LexA binding (HI 3.7) and the other, pIK471-656, a proximal SOS box with a lower affinity of binding (HI 15.9) due to mutation of one of the conserved SOS boxes and nucleotide insertion (Fig. 1). Analysis of *cka* expression on the basis of β-galactosidase activity of the *cka–lacZ* fusion from plasmid pIK471-606 revealed an approximately fourfold reduction of enzyme activity throughout the growth cycle. Unexpectedly, the mutant *cka–lacZ* pIK471-656 fusion, with a proximal SOS box with a lower affinity of binding, also exhibited reduced β-galactosidase activity (Fig. 2).

Electrophoretic mobility shift assays to assess the affinity of LexA binding to the altered SOS boxes

Two SOS boxes are present in the *cka* regulatory region and presumably to each a LexA dimer binds. To assess LexA binding, electrophoretic mobility shift assays were performed with a 230 bp PCR-amplified and gel-purified fragment containing the two *cka* SOS binding sites and various concentrations of purified LexA. As shown in Fig. 3b, mutation of the distal SOS box to a higher affinity of binding site resulted in an approximately 50% shift at 0.15 nM of LexA, due to binding to one LexA box, while with the wild type *cka* operator region only binding to both SOS boxes was observed, suggesting that binding of the dimers is cooperative. Full binding to both boxes was detected at 0.5 nM LexA (Fig. 3a). Similar experiments with plasmid pIK471-656 that has a lower affinity of LexA binding (Fig. 3c) due to alterations in the proximal SOS box, also resulted in clearly evident binding of one dimer at 0.05 nM LexA. An approximately 50% shift was evident at 0.25 nM LexA, whereas at 0.5 nM LexA practically all DNA bound to one or two dimers. Full binding to both boxes was detected at 1 nM LexA. These last results indicate some interplay between the binding of the two LexA dimers. Cooperative binding of LexA dimers to both operators has previously been shown for the colicin A (Lloubes et al. 1991) and colicin E1 operons (Ebina et al. 1983). The specificity of LexA binding was confirmed by competitive agarose shift assay using a 100 bp ladder (MBI) as the competitor DNA. Much higher concentrations were needed to alter the migration of random DNA as a shift in competitor DNA was observed at 10 nM LexA (data not shown).

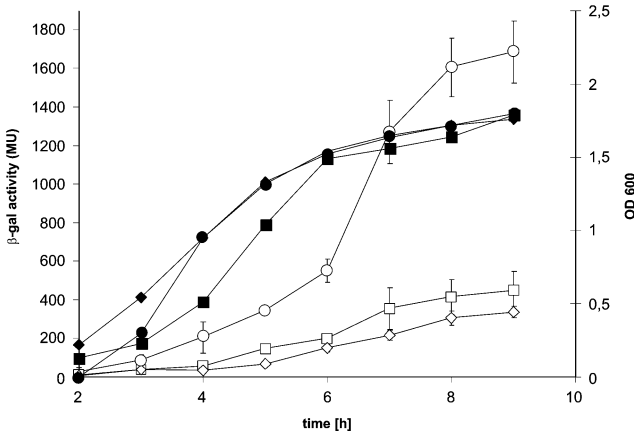

Fig. 2 Expression of the *cka–lacZ* fusion in plasmids pIK471, pIK471–606 and pIK471–656. β-galactosidase activity in Miller units and growth expressed as optical density at 600 nm are presented. *Circles* wild type plasmid; *squares* pIK471–606 and *diamonds* pIK471–656 with mutations in *cka* SOS boxes. This figure shows that *cka* expression, on the basis of β-galactosidase activity of the *cka–lacZ* fusion, is lower in both mutants. Levels of β-galactosidase activity in Miller units (*open symbols*) and growth OD_{600} (*solid symbols*) are depicted. The experiment was performed three times and the means ± standard errors of the mean (*error bars*) are shown

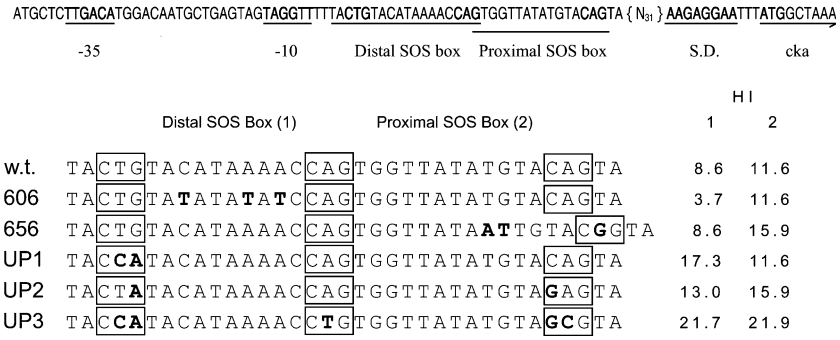

Fig. 1 Regulatory region of the *cka* gene and wild type as well as mutant *cka* SOS boxes. *Top* regulatory region of the *cka* gene including the −10 region, the −35 region, the two SOS boxes and the ATG are indicated. *Bottom* comparison of the DNA sequence of the wild type *cka* SOS boxes and the investigated mutant *cka* SOS boxes. Nucleotide substitutions introduced by oligonucleotide mutagenesis are shown in *bold*. The most highly conserved sequences are indicated

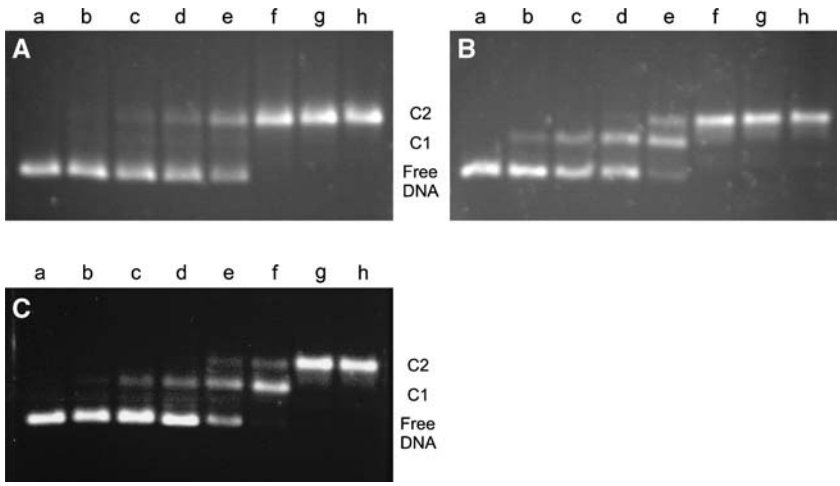

Fig. 3 Electrophoretic mobility shift assay with the wild type and mutant SOS boxes. **a** wild type and **b** mutant *cka* SOS boxes of plasmid pIK471–606 as well as **c** mutant *cka* SOS boxes of plasmid pIK471–656. The following LexA concentrations: *lanes a–h*, 0, 0.05, 0.1, 0.15, 0.25, 0.5, 1, and 1.5 nM. *C1* complex 1, consisting of DNA and one bound LexA dimer; *C2* complex 2, consisting of DNA and two bound LexA dimers

Expression of *cka–gfp* fusions with altered SOS boxes at the single cell level

To observe whether modifications introduced into the *cka* SOS boxes affected expression of the colicin K activity gene at the single cell level, the two above-described modified SOS sequences were introduced into plasmid pKCT5, carrying a *cka–gfp* fusion. Thus pKCT3-606, harbouring a distal SOS box with a lower HI index than the wild type and pKCT3-656 with a proximal SOS box with a higher HI than the wild type were generated. Single cell analysis using fluorescence microscopy of the strains carrying these plasmids showed a small but reproducible decrease in the percentage of *cka* expressing cells compared to the strain with the original SOS boxes with percentages dropping from approximately 3% fluorescent bacteria for the wild type to 1.4 and 1.7% for the strains carrying pKCT3-606 and pKCT3-656, respectively (Table 2).

Three additional *cka–gfp* fusions (Up1, Up2, and Up3) with nucleotide modifications in one, two, and all three of the most conserved sequences of the SOS boxes were subsequently constructed and analysed (Fig. 1). Fluorescence microscopy revealed that when bacteria carried modifications in only the 5′ most conserved LexA binding sequence the percentage of cells expressing the *cka* gene increased slightly from 3 to 5%, and fluorescence was more intense than in the parent strain, indicative of increased *cka* expression. Furthermore, it was noted that the remaining cells in the population exhibited a low level of *cka* expression. This is in contrast to the wild type where only highly expressing cells and fluorescence-negative bacteria were observed. In strains with modifications in two or all three of the conserved LexA binding sites, the large majority of the population exhibited (high) expression of the *cka* gene (Fig. 4). Bacteria carrying modifications in all three most conserved LexA binding sequences showed highest intensity i.e., highest expression of the *cka* gene fusion (Table 2). To further corroborate the *cka–gfp* results, colicin production was measured using in vivo bioassays. This demonstrated that strains carrying the plasmid pKCT3-SUp1 with the Up1d mutation exhibited approximately 20-fold higher colicin production than the strain with the wild type *cka* promoter on plasmid pKCT3 (data not shown).

Effect of titration of LexA on *cka* expression at the single cell level

The finding that the HI index of the SOS boxes influenced LexA dependent *cka* expression led us to investigate whether intracellular levels of the LexA protein could also play a decisive role in establishing differential expression of the *cka* gene in the population. To test this hypothesis we increased the intracellular number of LexA binding sites which should result in titration of the LexA protein. For this purpose plasmids pERO22 and pERO19 were constructed, harbouring the wild type c*ka–gfp* fusion (Mulec et al. 2003) in pBR322 and pUC19 that are present in approximately 50 and 500 copies per cell, respectively. Expression of the *cka–gfp* fusion was subsequently analysed at the single cell level by fluorescence microscopy. These experiments demonstrated that among the population harbouring pERO22::*cka–gfp*, approximately 7% of the cells was fluorescent compared with approximately 3% for the wild type carrying plasmid pKCT3. For the population carrying the high copy number plasmid pERO19::*cka–gfp* a 20-fold increase in the percentage of fluorescent cells (60%) was observed (Fig. 5). For control strain RW542 *lexA542* (Def) with a defective LexA protein that cannot bind

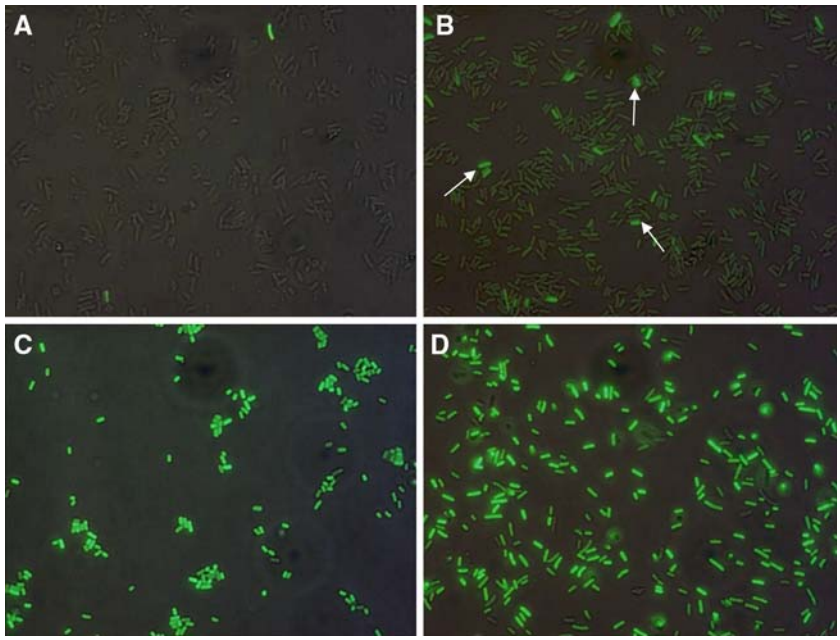

Fig. 4 Mutations in the *cka* SOS boxes affect *cka–gfp* expression at the single cell level. A merged image of the phase contrast and fluorescence images of strain RW118 with the wild type and mutant *cka–gfp* fusions. **a** Wild type *cka–gfp* fusion on plasmid pKCT3; **b** mutant plasmid pKCT3-Up1; **c** mutant plasmid pKCT3-Up3 and **d** mutant plasmid pKCT3-Up2. The experiment was repeated four times and a representative experiment is shown

to DNA, virtually all cells showed intense fluorescence (Mulec et al. 2003). To ensure that the effects were due to LexA activity rather than the general SOS response, a high copy number plasmid without LexA binding sequences (pUC19) was tested for its effect on *lexA–gfp* expression in strain AB1157L. While an increase in the percentage of fluorescent cells was observed with this plasmid, it was not comparable to the 20-fold increase observed with plasmid pERO19 (Table 2). These results strongly suggest that the ratio between the quantity of LexA relative to its DNA binding sites is a major determinant of *cka* expression.

The *lexA* gene is not equally expressed in all cells

To determine whether fluctuations in expression of the *lexA* gene within the bacterial population could be responsible for differential expression of the SOS response and the *cka* gene, single cell fluorescence of a *lexA–gfp* fusion (Ronen et al. 2002) was analysed. Fluorescence microscopy revealed that *lexA* is expressed in all cells. However, on the basis of the intensity of fluorescence, expression was higher in approximately 0.3% of the cells. The bright cells also exhibited pronounced elongation, suggesting that they were highly induced for SOS expression in the absence of external DNA damage (Fig. 6; Table 2). It should be noted that the morphology of cells expressing the *cka* gene from the *cka–gfp* fusion did not differ from non-expressing cells (Mulec et al. 2003).

Together, our data indicate that while the LexA protein efficiently represses expression of the *cka* gene in the large majority of the cells, in a minor fraction, maximal expression is due to the combined effect of SOS induction as a result of intracellular events, the interplay of LexA dimers binding to operator sequences and possibly a limited intracellular concentration of LexA.

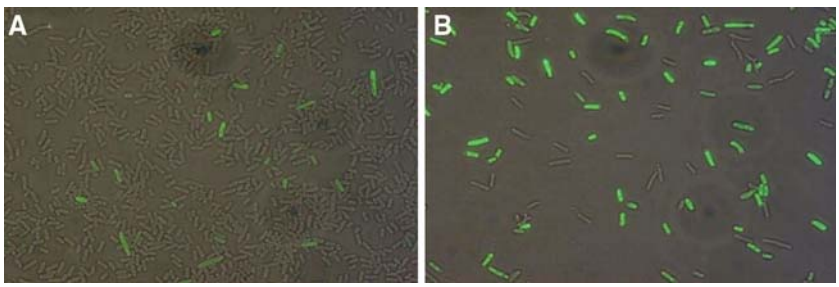

Fig. 5 A merged image of the phase contrast and fluorescence images of RW118 with **a** plasmid pERO22 and **b** plasmid pERO19. This figure shows the effect of titration of LexA on *cka* expression in individual cells. The experiment was repeated four times and a representative experiment is shown

Fig. 6 A merged image of the phase contrast and fluorescence images of strain AB1157L with *lexA-gfp*. The *arrow* denotes cell with highly induced SOS expression. The experiment was repeated four times and a representative experiment is shown

Discussion

We previously demonstrated that the LexA protein establishes repression of the *cka* gene in the large majority of the colicin K encoding population (Mulec et al. 2003) and that disruption of the *lexA* gene results in strong *cka* expression in the entire population. The molecular basis for the differential expression of *cka* expression under normal conditions, however, remained obscure. In the present study we provide evidence that variable expression of colicin K in a population of *E. coli* is regulated by the 'spontaneous' SOS response, i.e., without exogenous induction of DNA damage, the affinity of LexA binding to the *cka* SOS boxes and stochastic factors.

The first indication that the 'spontaneous' SOS response contributed to *cka* expression was obtained by comparing the level of the *cka* transcript in the wild type RW118 and isogenic *recA* defective strain RW464, using real-time RT-PCR. The observed lower transcript level for the *recA* strain compared to the wild type clearly showed that SOS system does induce *cka* expression without exogenous induction of DNA damage. Consistent with this finding, fluorescence microscopy of the *recA* and isogenic wild type strains both harbouring the wild type *cka–gfp* fusion, showed a nearly 20-fold drop in the number of *cka–gfp* expressing cells for the *recA* mutant. However, clearly a small but detectable fraction did express the *cka* gene in the *recA* defective strain, indicating that additional, stochastic factors also affect the expression of *cka*.

As SOS induction and *cka* expression could be due to fluctuations in *lexA* expression within a population, we hypothesized that variation in the level of LexA within the population also contributed to the heterogeneity in *cka* expression.

Fluorescence microscopy of a strain harbouring a *lexA–gfp* fusion revealed that from the *lexA* promoter, *gfp* was expressed in all cells. However, a small fraction of the population exhibited more pronounced *lexA* expression and inhibition of cell division consistent with high SOS expression in the absence of exogenous DNA damage. Cells expressing the *cka–gfp* fusion were not inhibited in cell division, demonstrating that they did not undergo high SOS induction.

To further decipher the mechanism via which LexA may cause heterogenous *cka* expression in the population, we focused on the binding of the protein to the LexA binding sites in the DNA. Most of the *E. coli* SOS genes have a single operator site to which a LexA dimer binds. However, several operons including those of the colE colicins, *lexA* and the *recN* gene, have two or more operator sites also designated as SOS boxes. Among the LexA regulated operons the position of the operator relative to the promoter, as well as the ability to bind LexA varies, indicating a very complex regulatory scheme which ensures an optimal cellular response to DNA damage. All of the colE colicins have two overlapping SOS boxes and to each the LexA protein binds as a dimer to repress expression. Colicins are released by lysis of the producing cell, and the two SOS boxes have been shown to effectively repress expression protecting the producer from overt lysis (Lu and Chak 1996). Comparison of a number of operator sites of colicin encoding operons revealed that even though three have identical sequences, namely colicin E2, E6, and E7, others exhibit divergence and variability in their heterology index value and consequently in the affinity of LexA binding (Lewis et al. 1994; Riley et al. 2001). We speculated that the specific affinity of the two *cka* SOS boxes for LexA binding could be significant in establishing differential expression and tested this hypothesis using directed mutagenesis of the SOS boxes. Surprisingly, this showed that mutations both in the distal SOS box to provoke a higher affinity of binding and in the proximal SOS box for lower affinity of LexA binding inhibited expression of a *cka–lacZ* fusion. Furthermore, electrophoretic mobility shift experiments showed that both boxes exhibited a higher affinity of LexA binding as judged from the binding of one dimer at lower LexA concentrations. Thus both mutations increased the affinity of LexA binding to the distal site. At the single cell level in strains with the above-described modifications of the two SOS boxes in the natural colicin K encoding plasmid harbouring a *cka–gfp* fusion, both modifications resulted in a lower

percentage of expressing cells, consistent with increased gene repression. These results also revealed that the distal LexA binding site has a more pronounced role in repression of *cka* transcription. This is in agreement with the higher affinity of LexA binding to the distal site. A comparison of several colicin two-operator LexA binding sites and their HI (Lewis et al. 1994) showed that for most, the distal site has a higher affinity of binding LexA. Previous studies investigating LexA binding to colicin operators employed DNA with deletions of one of the LexA sites. Our studies using modifications introduced into one of the *cka* LexA binding sites indicate that cooperative binding of LexA dimers depends on the affinities (HI) of LexA for the two binding sites. A shift in the binding affinity of LexA for one of the two sites (for the w.t. *cka* distal site an HI of 8.6 and the proximal site with an HI of 11.6), results in binding of only one dimer at lower protein concentrations.

Evidence that intracellular concentrations of the LexA protein could be decisive in enabling expression in a small portion of the population was obtained by introducing the *cka–gfp* fusion into cloning vectors pBR322 and pUC19. As observed by fluorescence microscopy, the number of expressing cells increased to approximately 7 and to 60%, respectively. Colicin K is encoded on plasmids belonging to the ColIb group of colicins which are present in 15–30 copies per cell. By introducing the *cka–gfp* fusion into pBR322 and pUC19, an approximately 1.6- to 3-fold and 17- to 30-fold increase in *cka* LexA binding sites was achieved resulting in titration of LexA. Thus, by increasing the number of *cka* LexA binding sites from 1.6- to 3-fold, a small but reproducible increase in *cka* expression was achieved at the single cell level. A 17- to 30-fold increase in *cka* LexA binding sites resulted in a 20-fold increase in the number of expressing cells. These results strengthen the hypothesis that the intracellular level of LexA could influence colicin expression and that variation in the quantity of LexA among the population may contribute to the heterogeneity in colicin expression.

The molecular basis for variation of the quantity of LexA in the population of cells remains to be defined. Bacterial populations are composed of identical cells. However, within isogenic populations considerable variation exists with regard to development, surface structures important in virulence as well as in the molecular components of cells. Well-documented examples of differential gene expression include lactose utilization (Novick and Weiner 1957), bacterial development, exemplified by sporulation in *Bacillus subtilis* (Sonenshein 2000), and genetic exchange, such as the development of competence in *B. subtilis* (Haijema et al. 2001). Heterogeneity in the *E. coli lac* operon was found to involve a positive feedback loop, whereas positive autoregulation at the *comK* promoter was shown to be required for bistability of *B. subtilis* competence (Maamar and Dubnau 2005). Further, theoretical studies also described population heterogeneity in systems with two mutually repressing negative feedback loops (Ferell 2002). Whether similar systems or, perhaps, variation in random partitioning of the LexA protein during cell division, contribute to LexA heterogeneity awaits further study. Our approach of using *cka–gfp* fusions as well as the *lexA–gfp* fusion described in Ronen et al. 2002, which enable investigation of heterogeneity in gene expression at the single cell level, may aid to resolve this issue. On the basis of our results we believe that regulation of expression of colicin K synthesis within a population of colicinogenic cells is a multifactorial process that involves SOS induction without exogenous DNA damaging agents, the affinity of LexA binding to operator sequences, as well as stochastically, possibly due to the intracellular concentrations of the LexA protein. Significant is also the possibility that other LexA regulated processes, including mutagenesis, could exhibit similar regulation.

Acknowledgments We thank Roger Woodgate for generously providing strains RW118 and RW464 as well as the LexA protein and Uri Alon for strain AB1157 carrying the *lexA–gfp* fusion. Irena Kuhar and John Little are acknowledged for insightful comments. This work was supported by grant P0-0508-0487 from the Slovene Ministry of Higher Education and Science.

References

Ebina Y, Takahara Y, Shirabe K, Yamada M, Nakazawa T, Nakazawa A (1983) Plasmid-encoded regulation of colicin E1 gene expression. J Bacteriol 156:487–492

Erill I, Escribano M, Campoy S, Barbé J (2003) In silico analysis reveals substantial variability in the gene contents of the gamma proteobacteria LexA-regulon. Bioinformatics 19:2225–2236

Fernández De Henestrosa AR, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H, Woodgate R (2001) Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. Mol Microbiol 35:1560–1572

Ferrel JE Jr (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr Opin Cell Biol 14:140–148

Ferrel JE Jr, Machleder EM (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus oocytes*. Science 280:895–898

Ferrer S, Viejo M B, Guasch J F, Enfedaque J, Regue M (1996) Genetic evidence for an activator required for induction of colicin like bacteriocin 28b production in *Serratia marcescens* by DNA-damaging agents. J Bacteriol 178:951–960

Fraser HB, Hirsh AE, Giaver G, Kumm J, Eisen MB (2004) Noise minimization in eukaryotic gene expression. PLoS Biol 2:0834–0838

Friedberg EC, Walker GC, Siede W (1995) DNA repair and mutagenesis. American society for microbiology, Washington DC

Haijema BJ, Hahn J, Haynes J, Dubnau D (2001) A ComGA-dependent checkpoint limits growth during the escape from competence. Mol Microbiol 40:52–64

Hernday A, Braaten BA, Low D (2003) The mechanism by which DNA adenine methylase and PapI activate the *pap* epigenetic switch. Mol Cell 12:947–957

Jerman B, Butala M, Žgur-Bertok D (2005) Sublethal concentrations of ciprofloxacin induce bacteriocin synthesis in *Escherichia coli*. Antimicrob Agents Chemother 49:3087–3090

Kirkup BC, Riley MA (2004) Antibiotic mediated antagonism leads to a bacterial game of rock-paper-scissors in vivo. Nature 428:412–414

Kuhar I, Žgur-Bertok D (1999) Transcription regulation of the colicin K *cka* gene reveals induction of colicin synthesis by differential responses to environmental signals. J Bacteriol 181:7373–7380

Kuhar I, van Putten JP, Žgur-Bertok D, Gaastra W, Jordi BJ (2001) Codon-usage based regulation of colicin K synthesis by the stress alarmone ppGpp. Mol Microbiol 41:207–216

Lewis KL, Harlow GR, Gregg-Jolly LA, Mount DW (1994) Identification of high affinity binding sites for LexA which define new DNA damage-inducible genes in *Escherichia coli*. J Mol Biol 241:507–523

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{\mathbf{T}}$ method. Methods 25:402–408

Lloubes R, Granger-Schnarr M, Lazdunski C, Schnarr M (1991) Interaction of a regulatory protein with a DNA target containing two overlapping binding sites. J Biol Chem 266:2303–2312

Lu FM, Chak KF (1996) Two overlapping SOS-boxes in ColE operons are responsible for the viability of cells harboring the Col plasmid. Mol Gen Genet 251:407–411

Maamer H, Dubnau D (2005) Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. Mol Microbiol 56:615–624

McAdams HH, Arkin A (1999) It's a noisy business. Genetic regulation at the nanomolar scale. Trends Genet 15:65–69

Michael SF (1994) Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. BioTechniques 16:409–412

Michel-Briand Y, Baysee C (2002) The pyocins of *Pseudomonas aeruginosa*. Biochimie 84:499–510

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor

Mulec J, Podlesek Z, Mrak P, Kopitar A, Ihan A, Žgur-Bertok D (2003) A *cka–gfp* transcriptional fusion reveals that the colicin K activity gene is induced in only 3 percent of the population. J Bacteriol 185:654–659

Novick A, Weiner M (1957) Enzyme induction as an all-or-none phenomenon. Proc Natl Acad Sci USA 43:553–567

Ozbudak EM, Thattai M, Kurster I, Grossman AD, van Oudenaarden A (2002) Regulation of noise in the expression of a single cell. Nat Genet 31:69–73

Pilsl H, Braun V (1995) Strong function-related homology between the pore-forming colicins K and 5. J Bacteriol 177:6973–6977

Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology and application. Annu Rev Microbiol 56:117–137

Riley MA, Pinou T, Wertz JE, Tan Y, Valletta CM (2001) Molecular characterization of the klebicin b plasmid of *Klebsiella pneumoniae*. Plasmid 45:209–221

Ronen M, Rosenberg R, Shraiman BI, Alon U (2002) Assigning numbers to the arrows: parametrizing a gene regulation network by accurate expression kinetics. Proc Natl Acad Sci USA 99:10555–10560

Salles B, Weisemann JM, Weinstock GM (1987) Temporal control of colicin E1 induction. J Bacteriol 169:5028–5034

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor

Schneider K, Beck CF (1986) Promoter-probe vectors for the analysis of divergently arranged promoters. Gene 42:37–48

Sonenshein AL (2000) Control of sporulation initiation in *Bacillus subtilis*. Curr Opin Microbiol 3:561–566

Suarez A, Güttller A, Strätz M, Staendner LH, Timmis KN, Guzmán CA (1997) Green fluorescent protein-based reporter system for genetic analysis of bacteria including monocopy applications. Gene 196:69–74

Thattai M, van Oudenaarden A (2004) Stochastic gene expression in fluctuating environments. Genetics 167:523–530

Wertman KF, Mount DW (1985) Nucleotide sequence binding specificity of the LexA repressor of *Escherichia coli* K-12. J Bacteriol 163:376–384

Wertz JE, Riley MA (2004) Chimeric nature of two plasmids of *Hafnia alvei* encoding the bacteriocins alveicins A and B. J Bacteriol 186:1598–1605