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Double locking of an *Escherichia coli* promoter by two repressors prevents premature colicin expression and cell lysis

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Summary

The synthesis of *Eschericha coli* colicins is lethal to the producing cell and is repressed during normal growth by the LexA transcription factor, which is the master repressor of the SOS system for repair of DNA damage. Following DNA damage, LexA is inactivated and SOS repair genes are induced immediately, but colicin production is delayed and induced only in terminally damaged cells. The cause of this delay is unknown. Here we identify the global transcription repressor, IscR, as being directly responsible for the delay in colicin K expression during the SOS response, and identify the DNA target for IscR at the colicin K operon promoter. Our results suggest that, IscR stabilizes LexA at the *cka* promoter after DNA damage thus, preventing its cleavage and inactivation, and this cooperation ensures that suicidal colicin K production is switched on only as a last resort. A similar mechanism operates at the regulatory region of other colicins and, hence, we suggest that many promoters that control the expression of 'lethal' genes are double locked.

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Introduction

The bacterial SOS response enables cells to deal with DNA damage and associated stresses. The response is controlled by the LexA global transcription factor that represses transcription of dozens of SOS genes that are involved in coping with and repairing DNA damage (Courcelle *et al.*, 2001; Wade *et al.*, 2005). DNA damage results in exposed single-stranded DNA that is recognized by RecA which forms a helical nucleoprotein filament. It is this filament that triggers cleavage of LexA (Little, 1991). Only unbound LexA is proteolytically inactivated, thus the repressor's dissociation from its DNA targets instigates the induction of the LexA regulon (Butala *et al.*, 2011).

In *Escherichia coli* and related bacteria, where the SOS response has been most studied, it has been found that the LexA regulon includes many genes encoding colicins (Ebina *et al.*, 1982; Lloubes *et al.*, 1986). Colicins are toxic suicide proteins that kill other bacteria by a single-hit mode of action, targeting either cell membranes, DNA or RNA (Cascales *et al.*, 2007; Kleanthous, 2010). In *E. coli*, most colicins are encoded by plasmids and transcribed from strong promoters whose activity is firmly repressed by LexA, and hence colicin expression is triggered by agents that induce the SOS response (Ebina *et al.*, 1982; Cascales *et al.*, 2007). Most LexA-repressed promoters are induced immediately upon DNA damage (Courcelle *et al.*, 2001), but induction of the majority of colicin genes is delayed and triggered only upon severe and persistent DNA damage (Herschman and Helinski, 1967; Salles *et al.*, 1987). This makes sense as colicins play no role in DNA repair, but rather, the producer cell dies as they are released, and their role appears to be to assist surviving cells by killing potential competitors (Majeed *et al.*, 2011). It has been postulated that the lag period in colicin production after SOS induction provides cells with time for damage repair before induction of the lethal colicin (Salles *et al.*, 1987), but the cause of the delay is unknown.

In previous work, we established how LexA represses the promoter of the *E. coli cka* gene (p*cka*) that encodes colicin K, a pore-forming toxin that kills susceptible cells by collapsing the membrane potential (Kuhar and Zgur-Bertok, 1999; Mulec *et al.*, 2003; Jerman *et al.*,

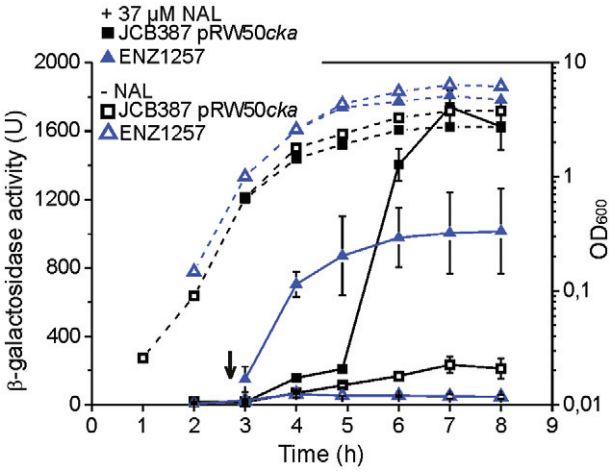

Fig. 1. Induction of the *cka* gene promoter is delayed, after triggering the SOS response. Measured β-galactosidase activities of JCB387 carrying pRW50*cka*, with a *cka–lacZ* transcriptional fusion, and of strain ENZ1257 harbouring a *sulA–lacZ* fusion are indicated. Each value represents the mean ± SEM of at least three independent measurements, the arrow indicates the time of addition of nalidixic acid (NAL) where relevant and the dashed lines represent optical density measured at 600 nm.

2005). Here, we have studied the timing of *cka* transcription after SOS induction and we report that the IscR global transcription repressor is directly responsible for delaying *cka* expression. We show that IscR and LexA co-dependently ensure tight and timed regulation of colicin K expression. Our results argue that, in SOS-induced cells, IscR stabilizes LexA dimers at the *cka* promoter until nutrients are depleted, which leads to reduction of IscR levels. Thus, in the DNA-damaged cells, IscR links *cka* expression to the cellular nutritional status. This double locking mechanism is rare at bacterial promoters but appears to be conserved at pore-forming colicin gene regulatory regions. We conclude that it has evolved to control the expression of 'dangerous' gene products whose expression needs to be tightly repressed.

Results

Delayed induction of the colicin K gene during the SOS response

To study the kinetics of induction of p*cka* after DNA damage we exploited a p*cka*::*lacZ* fusion carried by the pRW50 *lac* expression vector plasmid (Lodge *et al*., 1992). After triggering the SOS response with a sub-inhibitory concentration of nalidixic acid, we observed a pronounced delay in the induction of the p*cka*, compared with the expression of the *sulA* LexA regulon gene (Fig. 1; Table S1).

We previously showed that LexA represses p*cka* by binding to tandem DNA sites for LexA located downstream from the –10 promoter element (Mrak *et al*., 2007). Results in Fig. S1 show that LexA can both block RNA polymerase binding at p*cka* and displace pre-bound polymerase, but this cannot explain the delay in induction of p*cka*. Thus, we searched for another regulator by using affinity chromatography methods, using a DNA fragment containing p*cka* in complex with LexA as bait, and cleared SOS-induced cell extracts (see *Experimental procedures*). After elution of bound proteins and analysis by SDS-polyacrylamide gel electrophoresis (Fig. 2A) and mass spectroscopy, we identified the nucleoid associated factor H-NS and the known transcription factors Lrp, GlcC, DeoR, IscR, LexA as regulatory factors that had associated with the bait (Table S2). Since LexA was expected, and H-NS and Lrp were previously shown not to be involved in the *cka* regulation (Kuhar and Zgur-Bertok, 1999), we focussed on GlcC, DeoR and IscR and assayed *cka* promoter activity following SOS induction from a p*cka–lacZ* fusion in the corresponding deletion mutant strains from the Keio collection (Baba *et al*., 2006). Results illustrated in Fig. 2B show that the *glcC* and *deoR* deletions have minimal effects, but disruption of *iscR* resulted in induced p*cka* activity immediately after addition of nalidixic acid, indicating that IscR represses expression from p*cka*.

IscR regulates cka *expression*

The IscR (iron–sulphur cluster regulator) protein was originally identified as a transcription repressor that regulates genes involved in the formation and the repair of iron–sulphur clusters in proteins (Schwartz *et al*., 2001). IscR exists in two forms, holo-IscR that contains a Fe-S cluster, and apo-IscR, which is formed upon destruction of the Fe-S cluster, for example, in response to oxidative stress. It is now known that certain targets require holo-IscR for repression, while the majority of targets are repressed by both forms (Nesbit *et al*., 2009).

To investigate whether IscR can bind to the *cka* regulatory region and restore repression of p*cka* in the Δ*iscR* strain, we exploited plasmids encoding arabinose-inducible IscR or an IscR mutant locked in the apo- form due to alanine substitutions of the cysteine Fe-S cluster ligands (IscR-CTM) (Wu and Outten, 2009). The experiment illustrated in Fig. 2C shows that, both wild-type IscR and IscR-CTM complemented the *iscR* deletion, and strongly repressed p*cka* in spite of DNA damage. From this, we conclude that both holo- and apo-IscR can repress p*cka*, and inspection of the base sequence identified a perfect palindrome, overlapping the promoter –35 element (Fig. 3A), that corresponds well to the established consensus sequence (Nesbit *et al*., 2009). To show the importance of this site, we modified the two most critical nucleotides in the predicted site (Fig. 3A): the base at position 44 upstream of the p*cka* transcript start (p-44C to

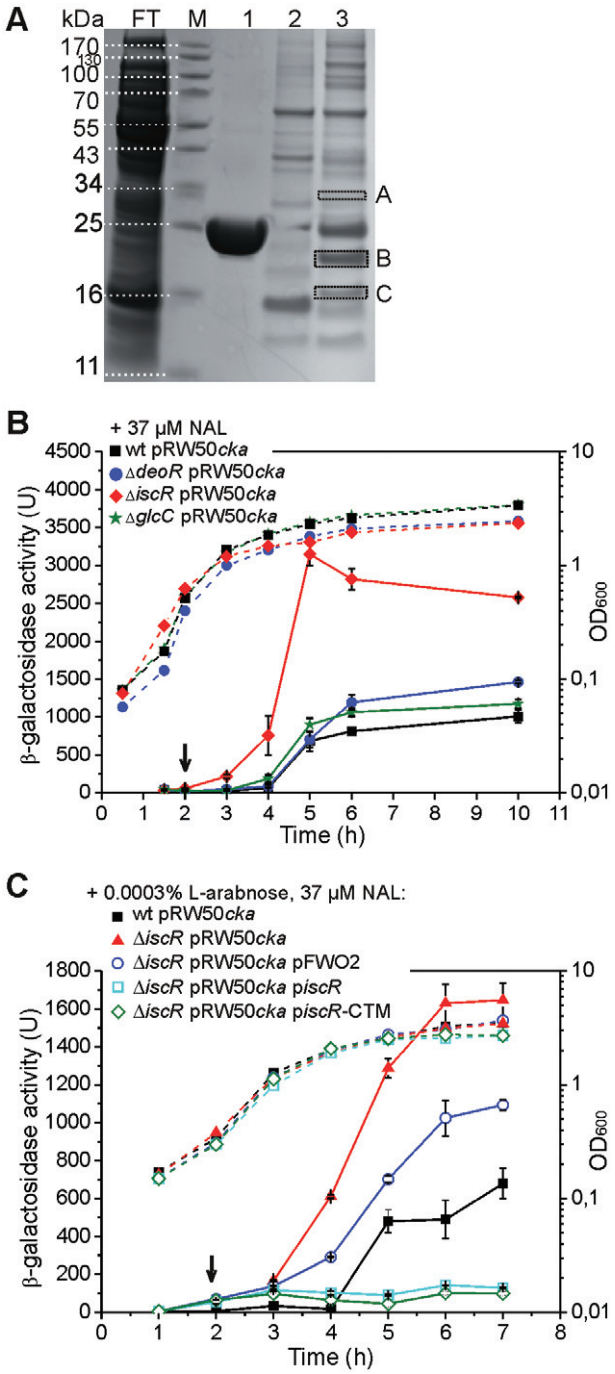

Fig. 2. LexA and IscR regulate induction of the *cka* gene promoter.
A. Coomassie stained protein profile of flow through (FT), protein standards (M), denatured beads and LexA (lane 1) and eluates from the control (lane 2) or p*cka* affinity chromatography (lane 3). Proteins in three gel slices (denoted by boxes) were trypsin digested and analysed by mass spectrometry. Proteins in the bands marked A, B and C were identified as DeoR, GlcC; Lrp, IscR; H-NS respectively.
B. Expression of the *cka–lacZ* fusion either in starting strain BW25113 (wt), or in the Δ*deoR*, Δ*glcC* or Δ*iscR* mutants. For (B) and (C) each value represents the mean ± SEM of at least three independent measurements, the arrow indicates the time of addition of nalidixic acid (NAL) and the dashed lines represent optical density measured at 600 nm.
C. Expression of the *cka* promoter in strain BW25113 (wt) or the Δ*iscR* mutant derivative complemented with holo-IscR (p*iscR*) or apo-IscR (p*iscR*-CTM). Empty parent vector pFWO2 was used as a control plasmid. The highest concentration of L-arabinose that had a minimal effect on cell growth was added at the time of inoculation.

G) and the symmetric modification at position 28 (p-28G to C). Results illustrated in Fig. 3B show that the mutations have similar effects on the expression of p*cka* as the *iscR* deletion, strongly suggesting that the palindrome is the target for IscR binding.

Next we purified the IscR protein and performed surface plasmon resonance (SPR) analysis directly to measure IscR binding at p*cka* using the DNA fragments illustrated in Fig. 3C. Our results show that IscR interacts with the chip-immobilized DNA fragment in a concentration dependent manner (Fig. 3D). Association of IscR with the DNA fragment harbouring mutation p-44G was decreased by ~ 10-fold in comparison to the wild-type *cka* fragment, and the affinity of IscR for the DNA fragment harbouring both the p-44G and the p-28C mutations was negligible (Fig. 3E).

To support the observation that IscR can, in conjunction with LexA, repress p*cka* activity, we constructed an artificial SOS promoter where the IscR repressor binding site was added to the promoter of *sulA* in pRW50, creating *sulA*1::*lacZ* fusion reporter (Fig. 4A). Results illustrated in Fig. 4B show that this results in a promoter where induction is suppressed even after the SOS response is triggered and that IscR and LexA function together to repress an otherwise IscR-independent promoter (Fig. 4B).

IscR activity reflects the nutritional status of the cell

Since our data indicate that p*cka* is repressed by both holo- and apo-IscR, we considered that relief of IscR-dependent repression could be due to changes in IscR levels, rather than oxidative stress. Thus, we used Western blotting to monitor intracellular concentrations of IscR during normal growth or during the SOS response in an *E. coli* MG1655 derivative expressing FLAG-tagged IscR from its native promoter. A threefold decrease of the IscR level was observed when either normal growing or SOS-induced cells entered into the late exponential phase and early stationary phase after early exponential growth (Fig. 5A). This suggests that *cka* transcription in SOS-induced cells is induced when IscR levels fall below a threshold.

Our data imply that IscR derepression occurs upon nutrient depletion. To test this hypothesis we maintained

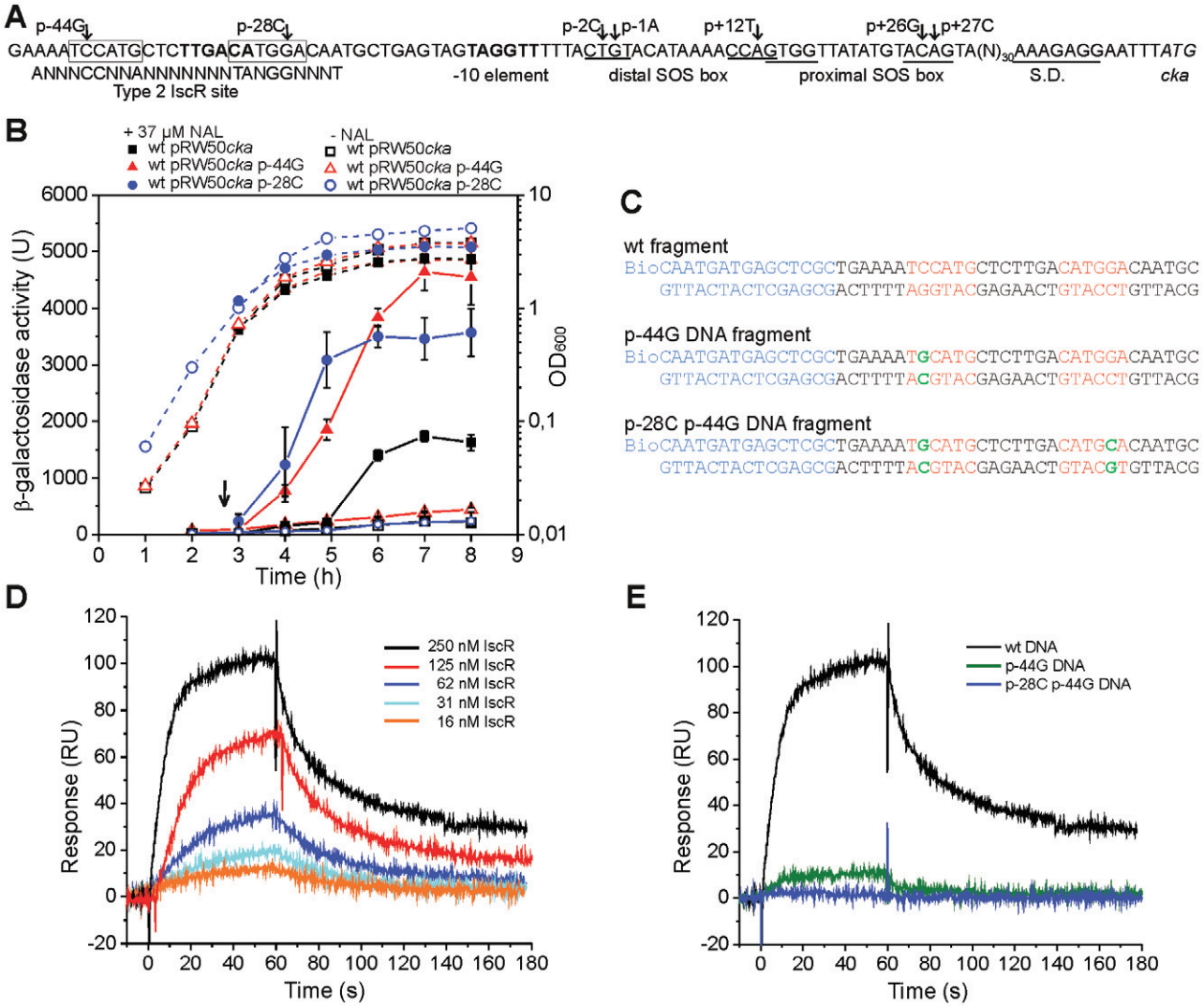

Fig. 3. IscR interacts with the *cka* regulatory region.
A. Regulatory elements of the *cka* promoter region. The boxes indicate the predicted palindromic target for IscR binding which corresponds to the consensus (Nesbit *et al*., 2009). The promoter –10 and –35 elements are in bold type, and the SOS box targets for LexA, the Shine Dalgarno sequence (S.D.) and the translation start site (*cka*) are also indicated. Positions of the site-directed mutations described in the paper are indicated above the sequence.
B. Measured β-galactosidase activities in BW25113 (wt) carrying either the p*cka*, p*cka* p-44G or p*cka* p-28C fragments subcloned into pRW50. The arrow indicates the time of addition of NAL where relevant. Each value is the average ± SEM of at least triplicate experiments and the optical density measured at 600 nm is shown as dashed lines.
C. Biotinylated DNA fragments used in the SPR analysis. The DNA linker by which fragments were attached to the chip surface is indicated in blue font, the palindromic sequence in red and the point mutations in green.
D. SPR sensorgrams of interactions of IscR (16–250 nM) with chip-immobilized wt DNA fragment.
E. Sensorgram of 250 nM IscR interacting with either wt DNA fragments or derivatives with mutations in the predicted IscR site.

the SOS-induced cells in a nutritionally rich environment by periodic subculturing to ensure that the optical density measured at 600 nm remains under 0.4. Under these conditions, expression from p*cka* remains repressed, and hence we can conclude that nutritional status is the signal for IscR deactivation (Fig. 5B), and that *cka* induction requires both DNA damage and nutritional starvation (Fig. 5C).

*Co-dependence of the p*cka *on IscR and LexA after DNA damage*

To investigate the effect of IscR alone on p*cka* activity, we used a *cka* promoter variant with mutated LexA operators (pRW50UP3, Fig. 6A; Table S1), unable to bind LexA specifically (Mrak *et al*., 2007). In the SOS-induced or the uninduced strain harbouring pRW50UP3, measured levels

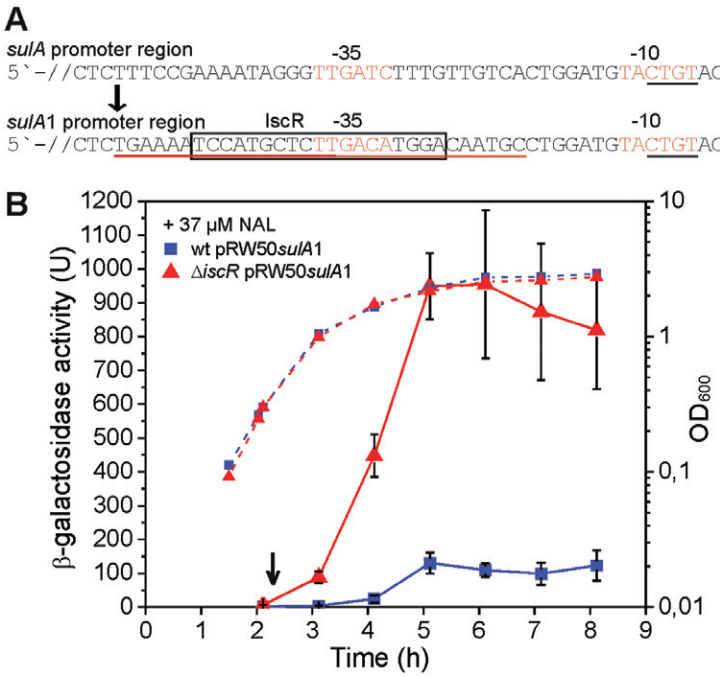

Fig. 4. IscR represses *sulA*1 promoter variant.
A. The wild-type *sulA* and the altered promoter region, *sulA*1. Marked are the –35 and –10 promoter elements, the LexA binding site (SOS boxes are underlined), inserted IscR target site of the *cka* (underlined with red line) and the palindromic sequence denoted by box.
B. Measured β-galactosidase activities in BW25113 (wt) or the Δ*iscR* mutant derivative carrying 330-bp-long *sulA*1 promoter region subcloned into pRW50. The arrow indicates the time of addition of NAL. Each value is the average ± SEM of at least triplicate experiments and the optical density measured at 600 nm is shown as dashed lines.

of p*cka* activity were high and were unaffected by SOS induction (Fig. 6A and B). Hence, IscR alone is not sufficient to repress p*cka*. To explain our results we supposed that, because only unbound LexA can be proteolytically inactivated (Butala *et al*., 2011), IscR assists LexA binding here and prolongs the time needed for LexA to dissociate from the operators. To test this, we measured promoter activities in the wild-type or the Δ*iscR* strain harbouring p*cka*::*lacZ* variants with weakened LexA-tandem operator interactions. We separated the tandem operators at p*cka*::*lacZ* by one base pair (Fig. 6A, pRW50*cka*1), in order to prevent LexA from interacting cooperatively at *cka* (Ebina *et al*., 1983), and constructed a p*cka*1::*lacZ* variant (pRW50*cka*2) with consensus SOS boxes (Butala *et al*., 2009b), to increase the LexA affinity for the separated tandem targets. LexA was able to repress all p*cka* derivatives during normal bacterial growth (Fig. 6C and D, non-treated cells). As anticipated, in the DNA damaged cells, promoter activities were p*cka*1 > p*cka*2 > p*cka* respectively (Fig. 6C and D). For both promoter variants, IscR caused delayed induction in the wild-type, after DNA damage (Fig. 6C and D). Hence, our data argue that IscR stabilizes LexA at the *cka* gene implying, that the co-dependence between the transcriptional factors governs the temporal induction of the p*cka* after DNA damage.

IscR controls the expression of different colicins

To investigate the effects of IscR on the expression of other colicins, we introduced the Δ*iscR* allele into strains that produce the pore-forming colicins K, E1, A and N. Following SOS induction of the colicinogenic cultures, cell growth and colicin production was compared in the starting strains and the Δ*iscR* mutants. We observe that IscR confers viability to most of the tested strains (Fig. 7A). Crude cell extracts were prepared from cultures before and after SOS induction and colicin levels were compared by bioassays (Fig. 7B) or by SDS-PAGE (Fig. 7C). The results show that nalidixic acid induces an immediate increase in colicin K, E1 and N levels in the Δ*iscR* strains in comparison to the delayed colicin production in the wild-type strains. In contrast, only a small difference in colicin A production was detected, which could be due to additional posttranscriptional factors (Yang *et al*., 2010). Colicin promoter regions were sequenced and alignment of these sequences (Fig. 7D) revealed potential SOS boxes and IscR binding sites present in the same organization and location as at p*cka*.

Discussion

Modulation of transcription initiation is widely deployed in bacteria as a strategy to regulate gene expression

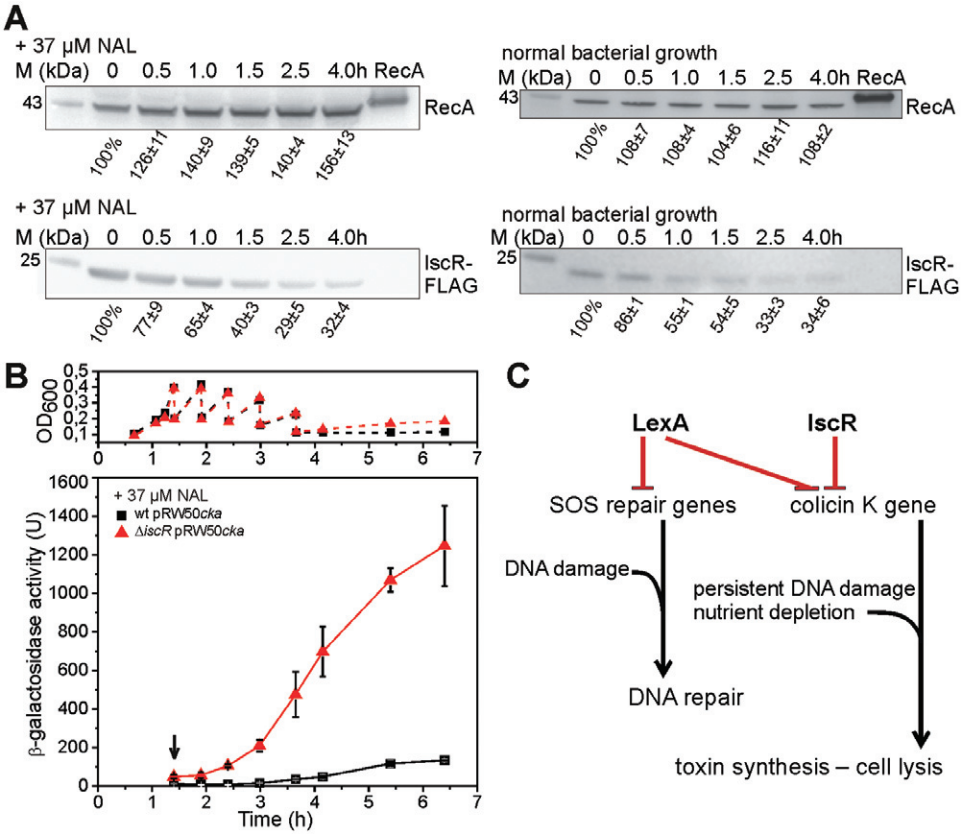

Fig. 5. Deactivation of IscR control is driven by a decline of cellular nutritional status.
A. Western blot analysis of the growth phase-dependent variation in the levels of RecA and FLAG-tagged IscR at various growth phases in MG1655. Samples were taken at time intervals before or after induction of the SOS reponse with nalidixic acid (NAL) at OD_{600} 0.3 (0 h), as indicated, or during normal growth. Cells entered the stationary phase of growth after 2.5 h. Purified $RecA(His)_6$ (0.18 μg) loaded in the last lane was used as a control. Quantification of proteins is presented below the gels as the ratio (%) of the protein density value of the initial sample (0 h) relative to the density value obtained from the samples harvested through the bacterial growth, shown with the standard deviation.
B. Cells were maintained in a nutritionally rich environment by periodic subculturing the cells in fresh LB media supplemented with NAL thus, not allowing the cell density to exceed 0.4 OD_{600} (dashed lines). Measured β-galactosidase activities in BW25113 (wt) or Δ*iscR* cells carrying pRW50*cka* (solid lines). The arrow indicates the time of addition of NAL. Values are the average ± SEM of at least triplicate experiments.
C. Model for the delayed expression of p*cka*. During normal growth, LexA and IscR bind and repress transcription from p*cka*. Upon DNA damage, e.g. caused by antibiotics, SOS DNA repair commences due to the decrease in intracellular LexA concentrations, but IscR levels are not affected. p*cka* becomes de-repressed after long-lasting DNA damage due to decreased IscR levels driven by a decline of cellular nutritional status.

(Browning and Busby, 2004). Thus, the genomes of bacteria such as *E. coli* encode hundreds of transcription activators and repressors, each of which modulates the expression of specific genes in response to specific environmental and metabolic signals. A large number of bacterial promoters are regulated by more than one transcription factor and this ensures that inputs from different signals are integrated. Interestingly, although there are scores of cases where bacterial promoters are regulated by combinations of activators and repressors, or by activators alone (Lee *et al.*, 2012), to date, there appear to be very few cases where regulation is solely due to two or more repressors acting in combination. Our observation that the potentially suicidal expression of different *E. coli* colicins is controlled by the LexA and IscR repressors, acting in concert, suggests that regulation by repression may be prevalent in controlling 'lethal' genes.

Colicin production by a bacterial cell is suicidal and it is thought that this is an example of bacterial altruism (Majeed *et al.*, 2011). Although it was well known that the transcription of most *E. coli* genes encoding colicins is repressed by the LexA global repressor, and thus induced in response to DNA damage (Cascales *et al.*, 2007), it is not known how induction of colicin expression is uncoupled from temporal induction of the SOS response to deal with repairable DNA damage. The work with p*cka* presented here shows that the key is a second repressor, IscR. Hence, IscR and LexA bind to adjacent sites in the *cka* regulatory region and repress transcription in a co-dependent manner. Our results argue that bound IscR

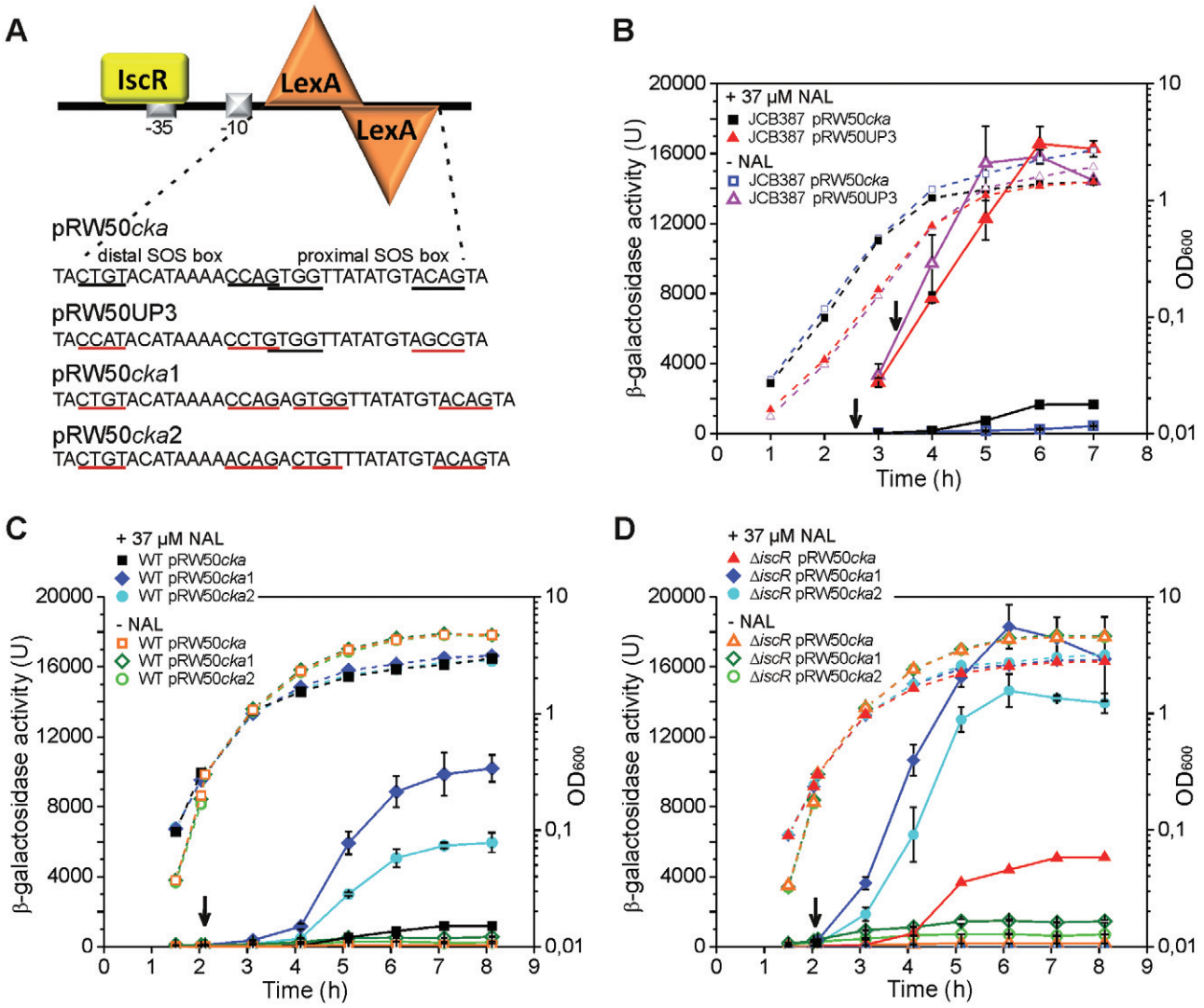

Fig. 6. IscR and LexA co-dependently repress p*cka* after DNA damage.
A. A schematic representation of the important elements involved in regulation of the *cka* promoter. Marked are the −35 and the −10 promoter elements, IscR binding site and the overlapping tandem DNA sites for LexA. The LexA binding sites of the *cka* promoter region inserted in pRW50 and the derivates are indicated. The SOS boxes and the modified targets are underlined with black and red line respectively.
B. Expression of the p*cka* in strain JCB387 carrying pRW50*cka* or pRW50UP3.
C and D. (C) Measured β-galactosidase activities of BW25113 (wt) or the (D) Δ*iscR* mutant strain carrying either pRW50*cka*, pRW50*cka*1 or the pRW50*cka*2 variant. For (B), (C) and (D) each value is the average ± SEM of at least triplicate experiments and the optical density measured at 600 nm is shown as dashed lines. The arrow indicates the time of addition of nalidixic acid (NAL) where relevant.

must interact with LexA to stabilize its binding thereby protecting it against inactivation triggered by RecA fibre formation. Hence, as well as being an example of regulation of bacterial transcription by two repressors, it is also a rare example of interactions between transcription factors. Recall that, in contrast to the situation in eukaryotes, most bacterial transcription factors exert their effects by binding independently at their target promoters (Lee *et al.*, 2012). Note that cooperative binding of two LexA dimers to an operator just downstream of the p*cka* transcript start also plays a role in keeping the *cka* promoter silent after SOS induction. Hence, even in the absence of IscR, there is a short lag in expression of the *cka* promoter, but this lag is lost if one base pair is inserted between the two DNA sites for LexA (Fig. 6).

Previous studies identified IscR as a regulator of the expression of gene products involved in the synthesis or repair of Fe-S proteins (Schwartz *et al.*, 2001; Tokumoto and Takahashi, 2001). It has homologues in eukaryotes, which sustain fundamental life processes (Lill and Muhlenhoff, 2005). IscR exists in two states apo-IscR and holo-IscR, which contains a Fe-S cluster (Schwartz *et al.*, 2001). For some targets, the ability of IscR to repress is dependent on the Fe-S cluster. This is the case for the

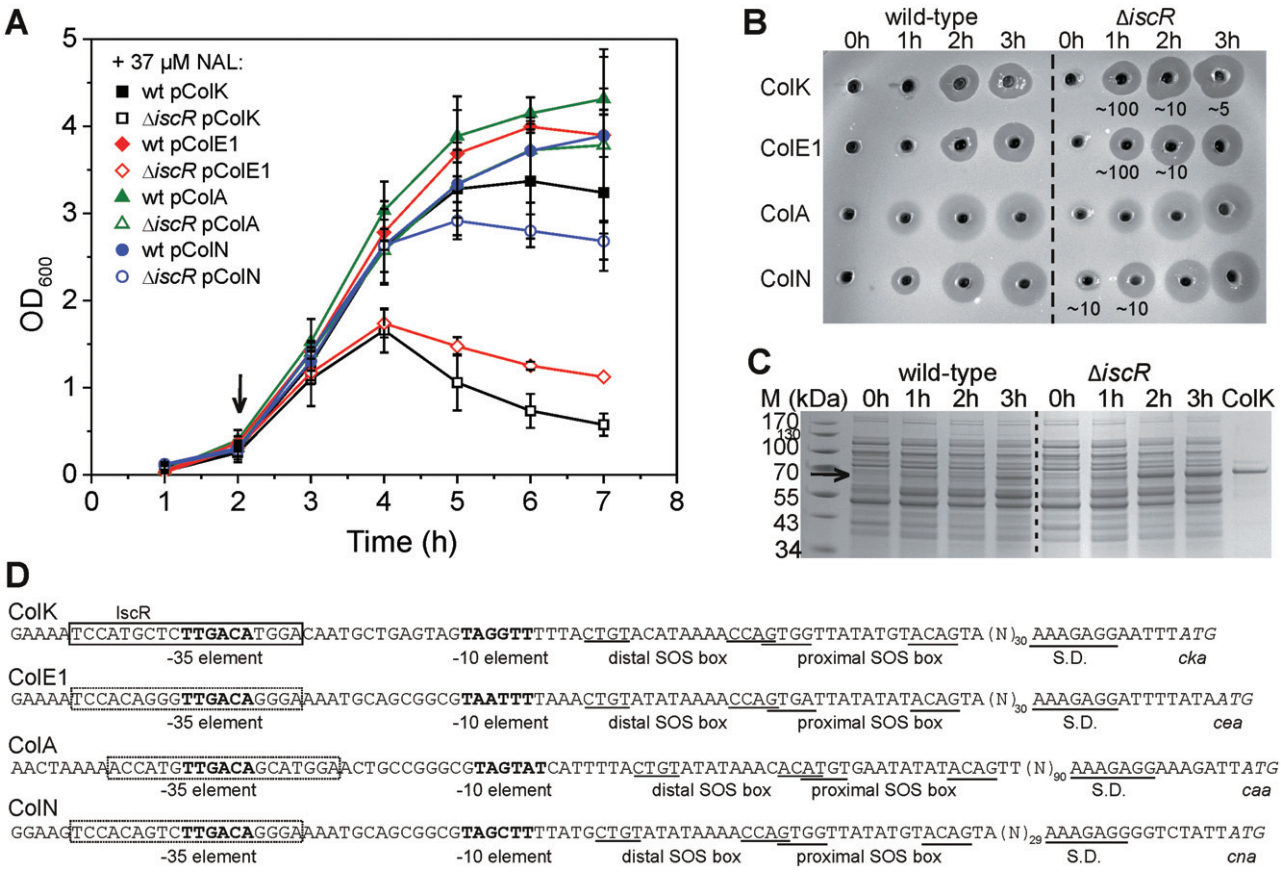

Fig. 7. IscR protein manages temporal induction of different colicins.
A. Growth curve of BW25113 (wt) or Δ*iscR* cells harbouring naturally occurring plasmids encoding pore forming colicins either K (pColK), E1 (pColE1), A (pColA) or N (pColN). The arrow indicates the time of addition of nalidixic acid (NAL), each value is the average ± SEM of duplicate experiments.
B. Assays of colicin production in cells harbouring colicin-encoding plasmids. Equal amounts of cells were collected at hourly time points from the time of addition of nalidixic acid (0 h) and cell extracts were placed into wells in an LB plate overlaid with soft agar harbouring an indicator strain. Numbers below the lysis zones indicate the fold increase of colicin production in the Δ*iscR* strain compared with the wild-type strain at the same time point, as determined from the dilution of crude colicin extracts (Fig. S2). Experiments were performed in duplicate.
C. SDS-PAGE analysis of total cell extracts of BW25113 or Δ*iscR* cells carrying pColK. The arrow indicates the position of colicin K as determined in comparison to the size of the purified $(His)_6$-tagged colicin K.
D. The sequence alignments highlight regulatory elements in colicin gene promoter regions, annotated as in Fig. 3A, and the predicted IscR sites are marked with dashed boxes.

iscR promoter itself and hence IscR levels vary greatly depending on the oxidation status of the cell (Nesbit *et al*., 2009). For most targets, both apo- and holo-IscR bind and repress transcription, and regulation appears to be due to changes in the cellular concentration of IscR. Our data suggest that this is the case for IscR binding at the *cka* promoter. It was previously shown that the *cka* and colicin E1 genes are induced due to lack of nutrients and not by an inducer released from the surrounding cells (Eraso *et al*., 1996; Kuhar and Zgur-Bertok, 1999). Thus, in metabolically active cells in the absence of DNA damage, colicin K synthesis is carefully locked off by IscR and LexA repressors. However, following a prolonged SOS response, when nutrients are depleted and metabolism slows, colicin synthesis is turned on and defective cells are eradicated. This may be in order to donate nutrients to related neighbours or to maintain a low mutation rate in a microbial community.

To conclude, here we have shown that IscR has a role in programmed bacterial cell death, which is part of the developmental process in a number of bacterial species (Lewis, 2000) and, in cooperation with LexA, it affects the expression of many colicin operons by carefully orchestrating colicin gene induction following the SOS response.

Experimental procedures

The following materials and methods are described in the *Supplementary Experimental Procedures*: plasmids and promoter constructs, computer modelling and electromobility shift assays.

Proteins

Escherichia coli RNA polymerase holoenzyme containing σ^{70} was purchased from Epicentre Technologies (Madison). The LexA protein was overexpressed and purified as described (Butala *et al*., 2011). The MH1 strain and the pQ-ORF2-95 plasmid to overexpress the IscR protein were donated by Yonesaki T. The IscR protein was expressed as described (Otsuka *et al*., 2010) and isolated to > 95% purity by the Ni-NTA affinity chromatography and stored at –20°C in 20 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, 0.5 mM EDTA, 40% glycerol, 0.2% Triton-X. Concentration of the LexA repressor was determined using NanoDrop1000 (Thermo SCIENTIFIC) and the extinction coefficients at 280 nm of 6990 M^{-1} cm^{-1}. The concentration of IscR was determined by a biuret procedure and standardized against the BSA protein.

DNA affinity purification

Escherichia coli JCB387 harbouring the pRW50*cka* plasmid (0.5 l) were induced with 8.5 μg ml^{-1} nalidixic acid when the OD_{600} reached 0.5, and after 45 min, cells were harvested and cell extracts prepared as described (Butala *et al*., 2009a). Biotinylated ~ 180 bp *cka* promoter fragments were generated by PCR using primers Pull_F, Pull_R and pRW50*cka* as a template and were purified by GeneJET PCR purification kit (Fermentas), which was attached to 2.5 mg of M-280 streptavidin Dynabeads (Invitrogen) according to the manufacturer's instructions. In binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA], 60 μg LexA repressor was bound to 50 μg of DNA immobilized to the magnetic beads and excess LexA was washed off in wash buffer [20 mM Hepes-Na (pH 7.4), 100 mM NaCl, 0.1% (v/v) Tween 20]. Binding reactions were performed in binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA] containing: beads either with or without the immobilized *cka* promoter-LexA complex. Dynabeads were collected with a magnet and washed four times with wash buffer. Proteins were eluted from the DNA with buffer containing 800 mM NaCl, and concentrated by TCA precipitation. Proteins were resolved on a 12% SDS-PAGE gel (Invitrogen); 1 mm gel slices were excised and analysed by the Birmingham Functional Genomics and Proteomics Unit (http://www.genomics.bham.ac.uk/) using a Thermo-Finnigan LTQ Orbitrap mass spectrometer. Three protein bands specific for the *cka* promoter–LexA complex were recovered from the high stringency 0.8 M NaCl eluate. These bands, which corresponded to molecular weights of approximately 15, 19 and 35 kDa (Fig. 2A), were recovered and analysed. We ignored candidate proteins with less than 20% identity and selected those that exhibited DNA binding properties but ignored the ones that were previously shown not to regulate p*cka* (Table S2).

β-Galactosidase assays

To assay the promoter activity of the *cka* promoter and its promoter variants or the *sulA*1 promoter variant, pRW50 harbouring the promoter fragments (listed in Table S1) were transformed into the wild-type *E. coli* strains BW25113 or the JCB387 or into the BW25113 Keio collection Δ*iscR* derivative JW2515 (Baba *et al*., 2006). For the Δ*iscR* complementation experiments the above-described strains were co-transformed with the arabinose-inducible plasmids pFWO2, p*iscR* or p*iscR*-CTM (Table S1) (Wu and Outten, 2009). To measure the promoter activity of the chromosomally encoded SOS gene, *sulA*, the ENZ1257 strain was used (Moreau, 2004). Cells were grown aerobically (180–200 r.p.m.) at 37°C in Luria–Bertani (LB) broth medium supplemented with tetracycline (12.5 μg ml^{-1}, Tc) or without antibiotic for the ENZ1257 strains. To induce the SOS response, sub-inhibitory concentration, 8.5 μg ml^{-1} of nalidixic acid was added to the culture when the OD_{600} reached 0.3 (Butala *et al*., 2009a). For complementation experiments 0.0003% of L-arabinose was added to the LB media at the time of strain inoculation. To test the effect of the cellular nutritional status on the deactivation of IscR control, we maintained cells in a nutritionally rich environment by periodically subculturing in fresh LB media supplemented with Tc and a sub-inhibitory concentration of nalidixic acid thus, not allowing cell density to exceed 0.4 OD_{600}. Culture samples were assayed for β-galactosidase activity according to the Miller (1972) procedure. Data presented on the same graph are obtained from the cultures inoculated at approximately the same time point. Each value is the average of at least three independent experiments; representative growth curves are shown.

Surface plasmon resonance assays

The SPR measurements were performed on a Biacore X (GE Healthcare) at 25°C. The streptavidin (SA) sensor chip (GE Healthcare) was equilibrated with buffer containing SPR_1 buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.005% surfactant P20 (GE Healthcare). Approximately 100 response units (RU) of 3′-biotinylated S1 primer was immobilized on the flow cells of the SA chip. To prepare double stranded DNA with the predicted IscR operator at the *cka* or its two mutant derivates p-44G and p-28C p-44G, complementary primers IscR_F and IscR_R or IscRm_F and IscRm_R or IscR2m_F and IscR2m_R (Table S1) in 20 mM Tris-HCl (pH 7.5), 0.1 mM NaCl were mixed in 1:1.5 (mol : mol) ratio respectively. Primers were annealed in temperature gradient from 94°C to room temperature (~ 1.5 h) in PCR machine (Eppendorf). So prepared 31 bp duplex DNA with a 15-nucleotide overhang complementary to the streptavidin chip-immobilized S1 primer was passed for 1–5 min at 2 μl min^{-1} across the flow cell 1 to immobilize ~ 90 RU of either IscR operator *cka* fragment or its derivates. The interaction between the IscR repressor and the chip-immobilized DNAs was studied by injecting solutions of the desired concentration of the IscR in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 0.005% surfactant P20 at 100 μl min^{-1} for 1 min. Dissociation was followed for 2 min. The DNA-sensor chip surface was regenerated by injecting buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl and the hybridized DNA fragments separated by 50 mM NaOH. SPR experiments were performed at the Infrastructural Centre for Surface Plasmon Resonance, University of Ljubljana.

Colicin production assays

Colicin synthesis was monitored in the wild-type or the Δ*iscR* strain harbouring naturally occurring colicinogenic plasmids

by a colicin production assay as described previously (Jerman *et al.*, 2005). Cultures of colicin-producing strains were grown in LB broth supplemented with ampicillin (100 μg ml^{-1}) with aeration at 37°C. Samples were collected just before nalidixic acid treatment at an OD_{600} of 0.3 and 1, 2 and 3 h after induction. Cells were diluted in LB broth to the OD_{600} of 0.3 to make a millilitre of the sample and crude colicin extracts were prepared by sonication (Sonics VCX750) at 40% power for 30 s on ice. Subsequently, 100 μl of the crude extracts were injected into wells in an LBTc plate overlayed with 4 ml of soft agar with 40 μl of the indicator strain DH5α harbouring pBR322 (laboratory stock). For an estimation of colicin production ratio among the strains, a 10-fold dilution series of crude colicin extracts were prepared and 5 μl samples were spotted on the LBAp plate overlayed with the indicator strain. To determine the ratio of colicin production in wild-type or Δ*iscR* strain, the sizes of the colicin produced lysis zones were compared and dilution of the cell lysates was taken into account. The remaining crude colicin K extracts were TCA precipitated and protein bands resolved on the 12% SDS-PAGE gel (Invitrogen) and visualized as described above. Experiments were performed in duplicate. Colicin promoter regions were sequenced with primers used previously (Kamensek *et al.*, 2010).

Western blot analysis

The PK10016 strain (*iscR*-FLAG) harbouring the pRW50*cka* was grown in LB broth supplemented with Tc (12.5 μg ml^{-1}) with aeration at 37°C. Samples were collected at an OD_{600} of 0.3 and after 0.5, 1.0, 1.5, 2.5 and 4.0 h of growth in normal or SOS-induced conditions. DNA damage was elicited with 8.5 μg ml^{-1} nalidixic acid at an OD_{600} of 0.3, where relevant. Samples were equilibrated to an OD_{600} of 0.6 to detect protein levels in equal number of cells during bacterial growth. Cell pellets were resuspended in 10 μl NuPAGE LDS sample buffer, 10 μl of DTT and 20 μl of dH_2O and heated (95°C, 5 min) before loading equal amount of the samples on a 12% SDS-PAGE gel (Invitrogen). For blotting, proteins were transferred to polyvinylidene difluoride membranes (Millipore), blocked in 4% bovine serum albumin at room temperature. Primarily the proteins were labelled with monoclonal mouse anti-flag M2 antibody (Sigma-Aldrich) and secondary antibodies conjugated by horseradish peroxidase. The same membrane was re-labelled by primary anti-RecA antibody (Anti-RAD51 polyclonal antibody, Thermo Scientific). Antibodies were used at a concentration of 0.5 μg ml^{-1}. Bands were stained using 4-chloro-1-naphtol/H_2O_2. The resolved bands were quantified using a G:Box (Syngene). The integrated optical densities of the IscR-FLAG or the RecA protein were determined. The IscR levels throughout the growth were compared and are presented as the ratio of the density value for the sample harvested at time indicated as 0 h relative to the density value obtained from the samples harvested later in the bacterial growth. Experiments were performed in duplicate.

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