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SOS response promotes horizontal dissemination of antibiotic resistance genes

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Mobile genetic elements have a crucial role in spreading antibiotic resistance genes among bacterial populations. Environmental and genetic factors that regulate conjugative transfer of antibiotic resistance genes in bacterial populations are largely unknown[1]. Integrating conjugative elements (ICEs) are a diverse group of mobile elements that are transferred by means of cell–cell contact and integrate into the chromosome of the new host[2]. SXT is a ~100-kilobase ICE derived from *Vibrio cholerae* that encodes genes that confer resistance to chloramphenicol, sulphamethoxazole, trimethoprim and streptomycin[3]. SXT-related elements were not detected in *V. cholerae* before 1993 but are now present in almost all clinical *V. cholerae* isolates from Asia[4]. ICEs related to SXT are also present in several other bacterial species and encode a variety of antibiotic and heavy metal resistance genes[4–7]. Here we show that SetR, an SXT encoded repressor, represses the expression of activators of SXT transfer. The 'SOS response' to DNA damage alleviates this repression, increasing the expression of genes necessary for SXT transfer and hence the frequency of transfer. SOS is induced by a variety of environmental factors and antibiotics, for example ciprofloxacin, and we show that ciprofloxacin induces SXT transfer as well. Thus, we present a mechanism by which therapeutic agents can promote the spread of antibiotic resistance genes.

SXT transfer requires *recA* in donor cells, but the molecular basis for this requirement was unclear[3]. We identified genes at the 3′ end of the integrated SXT that regulate SXT transfer[8]. Two loci, *setC* and *setD*, encode transcriptional activators required for SXT excision and transfer[8]. Overexpression of these activators was toxic to cells that harboured SXT but not in cells lacking SXT. *setR*, the 3′-most gene in integrated SXT, is similar to the λ bacteriophage CI repressor and, like this repressor, is predicted to contain both a helix–turn–helix DNA-binding motif and a protease motif (see Supplementary Information). *setR* cannot be deleted from SXT unless the mutation is complemented *in trans* from a plasmid[8], indicating that the removal of SetR repression of some SXT-encoded factor(s) might be deleterious to cell growth. Because the overexpression of *setC* and *setD* is toxic, we hypothesized that SetR represses these SXT transcriptional activators; we found that *setR* could be deleted in a *setCD* deletion strain.

To measure *setC* and *setD* gene expression, we replaced *setC* and *setD* with a promoterless *lacZ* reporter gene (*setCD*::*lacZ*). β-Galactosidase activity from this reporter was relatively low (15 Miller units) in the wild-type background; in contrast, the activity was 1,870 Miller units in the *setR* deletion background (Table 1, rows 1 and 2). Introduction of a plasmid carrying *setR* restored the repression of *setCD*::*lacZ* in the *setR* deletion background (data not shown), confirming that SetR represses *setC* and *setD* expression.

The similarity of SetR to the λ-phage repressor CI suggested that the regulation of SXT transfer might be similar to the regulation of λ lysogeny. In λ lysogens, CI represses prophage gene expression. After DNA damage and the induction of the SOS response, the co-protease activity of RecA is stimulated and promotes the autoproteolysis of CI, alleviating CI-mediated repression and beginning the phage lytic cycle[9]. Because SXT transfer requires *recA* in donor cells[3] and SetR is similar to CI, triggering the SOS response might result in a RecA-dependent cleavage and inactivation of SetR, increasing *setC* and *setD* expression and enhancing

Table 1 **Mitomycin C activates expression of SXT conjugation-associated loci**

Site of fusion (background)	β-Galactosidase activity	
	Without mitomycin C	With mitomycin C
Δ*setCD*::*lacZ*	15	171
Δ*setCD*::*lacZ*(Δ*setR*)	1,870	1,710
Δ*s003*::*lacZ*	11	73
Δ*traG*::*lacZ*	17	70
Δ*floR*::*lacZ*	34	33
Δ*setCD*::*lacZ* (*setR*^{G94E})	112	124

All strains were derivatives of *E. coli* MG1655 harbouring SXT. Mutations in *setR* are indicated in parentheses. The values presented are the means of at least three experiments; standard deviations were less than 10%.

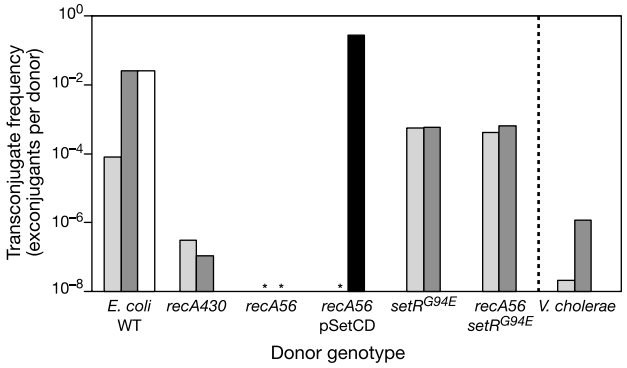

Figure 1 SOS-inducing agents activate SXT transfer. Transconjugate frequency was calculated as transconjugants observed per donor cell as described[3]. The wild type (WT) and *setR*^{G94E} donor strains are derivatives of *E. coli* strain BW25113 (MG1655 *lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$)[20] containing SXT. The *recA56* and *recA430* donor strains are derivatives of MG1655. *V. cholerae* transfer experiments used MO10 as a donor[3]. In all cases the recipient was CAG18439 (ref. 21). pSetDC contains *setDC* under the control of an arabinose-inducible promoter[8]. The different growth conditions used for the donor cells before their use in the conjugation assay are represented by light grey bars for growth in LB, dark grey bars for growth in LB containing mitomycin C, white bars for growth in LB containing ciprofloxacin, and black bars for growth in LB containing arabinose. Asterisks represent data that were below the detection limits of the assay (~10^{-8}). Values presented are the averages of three independent assays; standard deviations were less than 30%.

SXT transfer. We used conjugation and gene expression assays to explore whether the SOS response enhances SXT transfer and gene expression.

Induction of the SOS response markedly enhanced SXT transfer. Growth of SXT-containing *Escherichia coli* donor cells in sublethal concentrations of mitomycin C, a DNA-damaging agent that stimulates the SOS response[10], augmented the transfer frequency more than 300-fold (Fig. 1). Similar results were observed with the related element R391 (data not shown). Growing donor cells in ciprofloxacin, a widely used fluoroquinolone antibiotic that activates the SOS response[11], also increased SXT transfer (Fig. 1). *V. cholerae* donor cells had a similar response to mitomycin C (Fig. 1) and—in matings of *V. cholerae* to *V. cholerae*—ciprofloxacin (data not shown). To confirm that the stimulation of SXT transfer by mitomycin C was a consequence of SOS induction, we treated *recA E. coli* SXT donors with mitomycin C. Both a point mutation that renders RecA co-protease-deficient (*recA430*)[12] and a *recA*-null allele (*recA56*) prevented the induction of SXT transfer by mitomycin C (Fig. 1). Thus, activation of the SOS response in both *E. coli* and *V. cholerae* greatly stimulates the transfer of SXT and SXT-related elements.

recA could have a regulatory or direct role in enabling mitomycin C-stimulated SXT transfer. We found that the requirement for *recA* in an SXT donor was bypassed by overexpressing *setDC in trans* (Fig. 1), indicating that *recA* is a regulator and that SetC and SetD act downstream of RecA in the activation of SXT transfer by SOS.

Mitomycin C and the SOS response induced expression of genes required for SXT transfer. *lacZ* reporters for *s003* (a gene in the same operon as the SXT integrase gene), *traG* and *setDC* were all induced 4–11-fold by mitomycin C (Table 1), whereas *floR*, the gene mediating chloramphenicol resistance, was not induced, indicating that induction is specific to transfer-associated loci. Mitomycin C did not augment expression from these reporters in *recA*-null cells, indicating that induction of SXT gene expression depends on the SOS response (data not shown). These findings suggest that the SOS response stimulates SXT transfer by increasing the transcription of SXT genes required for the element's transfer.

Mitomycin C might act by relieving SetR-mediated repression. The *setCD*::*lacZ* reporter showed an 11-fold induction when wild-type cells were grown in mitomycin C (Table 1). However, in a strain lacking *setR*, the basal activity of the reporter was 125-fold higher and the reporter was not affected by mitomycin C. In the *setR* deletion strain β-galactosidase activity was 10-fold higher than in the wild-type strain after mitomycin C treatment, suggesting that only a portion of the total cellular SetR is inactivated during the SOS response.

To assess whether SetR cleavage and inactivation were required for SXT's response to mitomycin C, we generated a SetR variant expected to be resistant to cleavage by RecA. The substitution of glutamate for glycine at the Ala-Gly RecA cleavage site in the CI phage repressor resulted in a non-cleavable repressor and a prophage that was no longer inducible by SOS (ref. 13). Because SetR contains the same putative Ala-Gly cleavage site as CI^{434} (see Supplementary Information), we introduced a G94E substitution into SetR. We hypothesized that a non-cleavable SetR would prevent the activation of SXT transfer by mitomycin C, because the repression of *setD* and *setC* by SetR would not be relieved after treatment with mitomycin C. In fact, mitomcyin C did not increase SXT transfer in the strain harbouring $setR^{G94E}$ (Fig. 1), suggesting that SetR inactivation mediates the SOS enhancement of SXT transfer.

Surprisingly, the basal rate of SXT transfer in the strain producing $SetR^{G94E}$ was slightly increased (Fig. 1); in contrast, basal phage production by strains harbouring prophages with non-cleavable repressors is typically decreased[13]. This difference probably reflects a decrease in the repressor activity of $SetR^{G94E}$. In fact, in the $setR^{G94E}$ background, the basal expression of *setCD*::*lacZ* was 7.5-fold greater than in the wild-type *setR* background (Table 1). However, the activity of this reporter (Table 1) and those of the other reporters (data not shown) were not augmented by mitomycin C, indicating that $SetR^{G94E}$ might not be inactivated by mitomycin C.

The frequency of transfer of the $setR^{G94E}$ SXT in a *recA*-null background was nearly identical to that in a wild-type ($recA^+$) background (Fig. 1). This is in marked contrast to the transfer frequency of the wild-type *setR* SXT in a *recA* background (Fig. 1), indicating that the $setR^{G94E}$ mutation is epistatic to the *recA56* mutation. This result places SetR downstream of RecA in the regulatory circuit controlling SXT transfer.

The intergenic region between *setR* and *s086* (see Fig. 2a) was

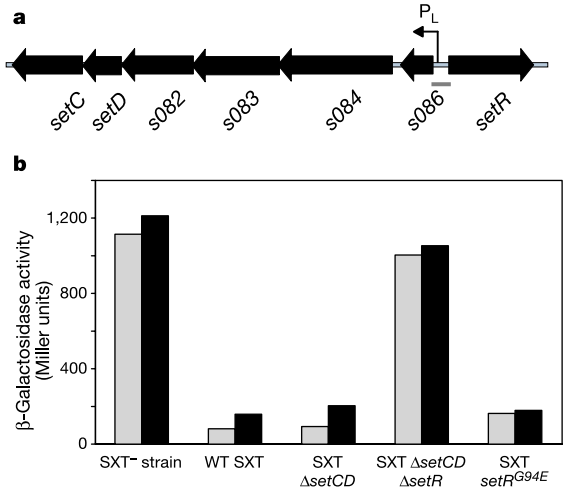

Figure 2 SetR represses expression from P_L. **a**, Schematic representation of the 3′ end of SXT. P_L is denoted by the arrow but its precise position is not known. The region cloned into the P_L reporter is indicated by the grey line. **b**, β-Galactosidase activity from the P_L reporter plasmid was determined in cells containing no SXT, wild-type (WT) SXT or mutant SXT derivatives grown in LB in the absence (grey bars) or presence (black bars) of mitomycin C. All strains are derivatives of BW25113. The values presented are the means of at least three independent experiments; standard deviations were less than 10%.

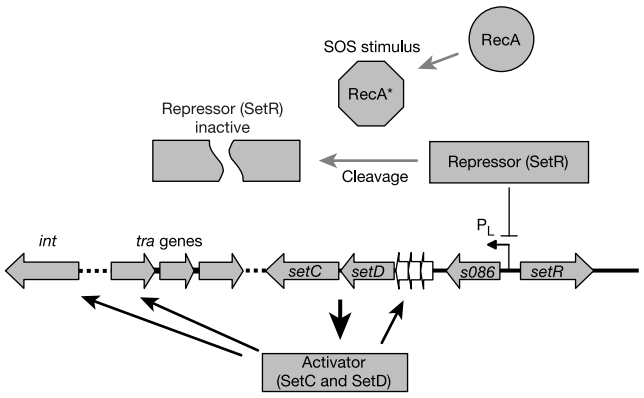

Figure 3 Model of the regulatory pathway by which the SOS response augments SXT transfer. The co-protease activity of RecA protein becomes activated during the SOS response. Activated RecA (RecA*) facilitates the autocleavage of SetR, alleviating the repression of *s086*, *setD* and *setC* expression. Increased levels of SetC and SetD lead to the autoactivation of *setD* and *setC* (ref. 8) at a promoter that has not been identified, and also the activation of the *tra* and *int* loci. Thin black arrows indicate transcriptional activation. The inverted-T-shaped object indicates repression. The thick black arrow indicates translation. Grey arrows indicate changes in protein activities.

investigated as a potential target of SetR repression because it is upstream of a putative operon that extends from *s086* to *setD* and *setC* and because prokaryotic repressors often act at divergently transcribed promoters. Expression from the promoter upstream of *s086* (designated P_L) was high in a strain lacking SXT (Fig. 2b), but was decreased 13-fold in a strain containing SXT, indicating that P_L is repressed by an SXT-encoded factor (Fig. 2b). *setDC* did not influence expression from P_L in either the presence or the absence of mitomycin C. In contrast, SetR accounted for this repression at P_L because the β-galactosidase activity of the P_L reporter was virtually the same in the Δ*setR* and SXT^- backgrounds (Fig. 2b). As with the *setCD*::*lacZ* fusion, treatment with mitomycin C alleviated the SetR repression of P_L only partly, indicating that only a fraction of the total cellular SetR might be inactivated during the SOS response. In the $setR^{G94E}$ background P_L was not induced by mitomycin C. SetR repression at P_L seems to be direct because purified epitope-tagged SetR bound to a probe encompassing the region between *s086* and *setR*, which includes P_L (see Supplementary Information).

SXT has co-opted a global cellular response to DNA damage, the SOS response, to control the activity of the SXT repressor, SetR (Fig. 3). SOS might promote the inactivation of SetR by stimulating its autocleavage. Inactivation of SetR relieves the repression of *setC* and *setD*, the transcriptional activators of both the SXT conjugative transfer and integrase genes. Because SetR and the other genes in the 3′ regulatory region of SXT seem to be conserved in related elements[7,14,15], this regulatory network probably governs the conjugative transfer of all SXT-related elements. Environmental stimuli such as ultraviolet radiation trigger the SOS response and might induce the transfer of SXT. In addition, the SOS response is activated by at least two classes of antibiotics, fluoroquinolones (such as ciprofloxacin) and dihydrofolate reductase inhibitors (such as trimethoprim). Because SOS enhances the conjugative transfer of SXT, the use of certain antimicrobial agents, either clinically or in agricultural settings, might potentiate the horizontal dissemination of antibiotic resistance genes to a broad range of bacterial species. All of these stimuli could account for the rapid manner in which SXT and related elements have spread. □

Methods

Bacterial strains and culture conditions

All bacteria were cultured in Luria–Bertani broth (LB) at 37 °C. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; ciprofloxacin, 10 ng ml^{-1} (*E. coli*) or 1 ng ml^{-1} (*V. cholerae*); mitomycin C, 200 ng ml^{-1} (*E. coli*) or 20 ng ml^{-1} (*V. cholerae*); sulphamethoxazole, 160 μg ml^{-1}; trimethoprim, 32 μg ml^{-1}; tetracycline, 10 μg ml^{-1} (*E. coli*) or 1 μg ml^{-1} (*V. cholerae*). Arabinose was added to a final concentration of 0.02%. HW220 $setR^{G94E}$ was constructed by allelic exchange by using pOrfRGE as described[8].

Molecular biology procedures and plasmid construction

Plasmid DNA was prepared by using the Qiaprep Spin Miniprep Kit and Qiaprep Miniprep Kit (Qiagen). Recombinant DNA manipulations were performed by standard procedures[16]. The TA Cloning Kit and pBAD TOPO TA Cloning Kit (Invitrogen) were used to clone PCR products. pRep6, which encodes a carboxy-terminally His_6-tagged SetR (SetR-H_6), was constructed by cloning *setR* without a stop codon into pBAD-TOPO. pPs086 was constructed by cloning a PCR fragment of the 250-base-pair region between *s086* and *setR* into pCRII. The fragment was then subcloned into pCB182 (ref. 17). pOrfRGE was made by first amplifying *setR* with primers RepA1 (5′-AAAACTTTATCC GAACGACT-3′) and RepA2 (5′-CCAGAAATCGATGATAGCTTG-3′). The resulting fragment was cloned into pBAD TOPO and the G94E mutation was introduced by using the Quick Change kit and primers RepGE1 (5′-CTGGGTTCAGGCCGAGGATTGGACT GAAATAGCGG-3′) and RepGE2 (5′-CCGCTATTTCAGTCCAATCCTCGGCCTGAA CCCAG-3′). The $setR^{G94E}$ was then subcloned into pWM91 (ref. 18), yielding pOrfRGE.

β-Galactosidase assays

Overnight cultures of cells containing either chromosomal or plasmid-borne β-galactosidase fusions were diluted 1:100 into LB containing antibiotics to select for plasmid maintenance and grown for 2 h. Mitomycin C, ciprofloxacin or arabinose was then added to half of the culture and the cells were grown for a further 2 h. β-Galactosidase activity, reported in Miller units, was measured as described[19].

Bacterial matings

Conjugation experiments were conducted as described previously[3]. In brief, overnight cultures of differentially marked donor and recipient cells were diluted 1:100 into fresh medium and grown separately for 2 h at 37 °C. Mitomycin C or ciprofloxacin was added as appropriate and cells were grown for a further 1 h at 37 °C. Equal volumes of donor cells and recipient cells were then mixed and spread on a filter on a LB plate. *E. coli* matings were incubated for 1 h at 37 °C; matings of *V. cholerae* to *V. cholerae* were incubated for 16 h at 37 °C. Afterwards, cells were resuspended in LB and dilutions were plated on selective media to enumerate donors, recipients and transconjugants. SXT transfer frequency was calculated as transconjugants per donor cell.

Gel-shift assays

SetR-H_6 was affinity-purified on Ni^{2+}-nitrilotriacetate resin (Qiagen) from lysates of *E. coli* LMG194 (Invitrogen) pRep6 grown in the presence of 0.02% arabinose in accordance with the manufacturer's protocol. Probes for gel-shift experiments were made by labelling restriction fragments with ^{32}P by using the Klenow subunit of DNA polymerase and then gel-purifying them from a 6% retardation gel (Invitrogen). Gel-shift reactions (20 μl volume) were performed by incubating 2,000 c.p.m. of each probe with decreasing amounts (2.5, 1.7, 0.63, 0.25 and 0 ng) of purified SetR-H_6 in a reaction buffer consisting of 80 mM NaCl, 25 mM Tris-HCl pH 8.0, 0.12 mM EDTA, 2.0 mM dithiothreitol, 50 μg ml^{-1} BSA and 25 μg ml^{-1} sonicated salmon-sperm DNA at 4 °C for 1 h. Reactions were analysed on a 6% DNA retardation gel.

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