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Induction of the SOS response by bacteriophage lytic development in *Salmonella enterica*

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Abstract

Infection of *Salmonella enterica* with lytic mutants of either P22 or SE1 bacteriophages triggers the expression of its DNA damage-inducible SOS response through a *lexA*-dependent pathway. This induction of the SOS system strictly requires the presence of the bacteriophage *kil* gene. Accordingly, plasmid overexpression of the *kil* gene also promotes the *S. enterica* SOS network induction. Furthermore, *S. enterica* Gifsy prophages are induced following the infection with SE1 and P22 lytic derivatives. The observed data reveal a hitherto unknown SOS system-mediated fail-safe mechanism of resident prophages against infection with heteroimmune lytic bacteriophages and suggest a novel role for the *kil* family of proteins.
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Keywords: Bacteriophage lytic cycle; SOS system; *Salmonella enterica*; Resident prophages; *Kil* gene

Introduction

The bacterial SOS system comprises a battery of genes aimed at guaranteeing cell survival in the presence of extensive DNA damage (Walker, 1984). The SOS response is induced by the activation of the RecA protein after binding to single-stranded DNA fragments (ssDNA) (Sassanfar and Roberts, 1990). Activated RecA (RecA*) promotes the autocatalytic cleavage of the Ala^{84}-Gly^{85} bond of the *Escherichia coli* LexA repressor (Little, 1991). This cleavage, mediated by LexA residues Ser^{119} and Lys^{156}, is similar in mechanism to that observed for serine proteases (Luo et al., 2001) and prevents LexA from binding to its specific recognition motif in the promoter region of SOS genes. The cleavage of the LexA protein in many other bacterial species belonging to different Phyla follows the same mechanism that in *E. coli* (Campoy et al., 2003; Fernández de Henestrosa et al., 1998; Winterling et al., 1998). The set of genes induced during the SOS response of both *E. coli* (Kenyon and Walker, 1980) and *Salmonella enterica* (Smith et al., 1991) includes, among others, error-prone polymerases (UmuDC and DinB), excision repair proteins (UvrAB), and a protein determining the reversible inhibition of cell division (SulA).

In addition to the genes directly regulated by LexA, the induction of the SOS response via ssDNA activation of RecA promotes cleavage of other repressors. Among these, several lytic cycle repressors of temperate bacteriophages have been shown to undergo RecA*-mediated autocatalytic cleavage through their serine protease domain (Roberts and Devoret, 1983; Sauer et al., 1982), which is very similar to that of LexA. Another class of temperate bacteriophages carry a gene (*tum*) encoding an anti-repressor protein that is under direct negative control of LexA (Shearwin et al., 1998). Whether it is due to the direct RecA*-mediated cleavage of the phage repressor or to the binding of SOS-induced Tum protein to the repressor, the net result of both mechanisms is the inactivation of the phage repressor as a result of DNA damage.

Infection of bacterial cells with either non-temperate bacteriophages or lytic mutants of temperate bacteriophages interferes with several cellular processes (Nechaev and Severinov, 2003), including the normal replication of the

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bacterial chromosome (Smith and Levine, 1965). Furthermore, and depending on the bacteriophage, either a significant amount of ssDNA (Inciarte et al., 1980) or a massive degradation of the bacterial chromosome (Schmiger and Buch, 1975; Woodworth-Gutai et al., 1972) may be generated during the development of the bacteriophage lytic cycle. Nevertheless, the putative impact of bacteriophage lytic cycle development on the bacterial SOS response has not been analyzed to date.

In the present work, the behavior of the *S. enterica* SOS system after cell infection with either wild type or lytic mutants of P22 and SE1 bacteriophages has been studied. In addition, and taking advantage of the fact that *S. enterica* contains several prophages (Bunny et al., 2002, Figueroa-Bossi et al., 1997), the effect of the infection with either P22 or SE1 derivatives on these resident prophages has also been analyzed.

Results and discussion

Infection of S. enterica serovar Typhimurium with P22 and SE1 lytic c2 mutants induces the SOS system

To analyze the behavior of the *S. enterica* SOS regulon during phage infection, the expression of its *recA* gene was measured by quantitative RT-PCR assays. Total RNA of *S. enterica* cells was extracted after infection with either wild-type P22 bacteriophage or a lytic derivative carrying a mutation inactivating the *c2* gene which encodes the lytic cycle repressor (Table 1).

Fig. 1 shows how *recA* expression in P22*c2*-infected cells remained invariable up to 10 min post-infection. By 20 and 30 min following phage infection, however, the level of *recA* transcription has clearly increased (Fig. 1A). In contrast, no significant change in *recA* transcription levels was detected when the wild-type P22 bacteriophage was used (Fig. 1A).

Table 1
Spontaneous lytic mutants of P22, SE1 and their *kil* derivatives used in this work

Phage	*kil* Genotype	Characteristics of *c2* mutation [a]	Position [b]
*P22*c2	Wild type	Frameshift (A insertion)	+127
P22 kil c2	129-bp internal deletion [c]	Nonsense (A → T; Lys^{16} → Amber codon)	+46
*SE1*c2	Wild type	Transition (C → T; Arg^{9} → Cys^{9})	+25
SE1 kil c2	129-bp internal deletion [c]	Transition (T → C; Ser^{113} → Pro^{113})	+337

[a] For each lytic mutant bacteriophage, the *c2* gene was sequenced by dideoxy method (Sanger et al., 1977) on an ALF Sequencer (Pharmacia Biotech) using the suitable oligonucleotide and the Thermo Sequenase Cy5 Dye Terminator cycle sequencing kit (Amersham Biosciences).

[b] Position of each mutation with respect to the translational starting point of *c2* gene of either P22 or SE1 bacteriophages or their derivatives.

[c] The 129-bp deletion introduced into the *kil* gene is described in Materials and methods section.

To confirm that the *recA* gene induction is not P22 exclusive, the effect of infection with another *S. enterica* specific bacteriophage (SE1) (Llagostera et al., 1986) was also studied. In accordance with the abovementioned results, a similar increase in *recA* expression is detected after infection with a *c2* mutant (Table 1) of SE1 (Fig. 1A), while no change in the *recA* transcription level can be appreciated when the SE1 wild-type temperate bacteriophage is used for *S. enterica* infection (Fig. 1A). To further determine whether this induction was LexA-dependent, a *lexA*(Ind) *S. enterica* derivative was constructed (Table 2), and its *recA* expression after bacteriophage infection was also analyzed. As expected, the *lexA*(Ind) mutant strain did not show any increase in *recA* transcription levels after infection with either P22 or SE1 *c2* mutant bacteriophages (Fig. 1B). Likewise, a *S. enterica recA*-defective mutant (Table 2) did not show bacteriophage-mediated SOS response activation (Fig. 1B).

Phage infection-mediated induction of the SOS system requires the bacteriophage kil gene

As many other bacteriophages (Casjens et al., 2004; Juhala et al., 2000; Perna et al., 2001; Sato et al., 2003), λ and P22 phages (Greer, 1975; Vander Byl and Kropinski, 2000) contain a gene annotated as *kil* that has been hypothetically linked to the bacterial cell-cycle regulation, since its expression has been shown to transiently block cell division (Semerjian et al., 1989; Sergueev et al., 2002). Similarly, sequencing of the SE1 genome has revealed the presence of a *kil* gene with a 74% amino acid sequence identity to that of P22. (Busquets et al., 2005). Because of the relationship between the induction of the SOS response and the inhibition of cell division (Walker, 1984), we decided to analyze here the effect of infection with a *kil* knockout mutant of both P22 and SE1 lytic derivatives on the behavior of *S. enterica recA* expression.

An internal 129-bp deletion was introduced in the P22 bacteriophage *kil* gene as described in Materials and methods and, through RT-PCR studies, it was confirmed that the transcription of genes located downstream of *kil* is not affected (data not shown). Afterwards, a spontaneous clear mutant of the P22 *kil* derivative was isolated (Table 1), and the effect upon the SOS response was analyzed. Fig. 2A shows that the *c2* lytic derivative of P22 *kil* mutant is unable to trigger the expression of the *S. enterica recA* gene. The same results were obtained when *S. enterica* was infected with an SE1 *kil* lytic mutant, suggesting that the *kil* gene product might be responsible for the observed SOS induction. To further confirm this hypothesis, the *kil* gene was cloned into the pUA1085 plasmid (Table 2), downstream the Ptac promoter that is negatively regulated by the LacI protein. As expected, real-time RT-PCR assays clearly pointed out that the IPTG-mediated overexpression of the *kil* gene induces the *recA* expression (Fig. 2B).

The function of the *kil* gene has been traditionally assumed to be related to cell division inhibition, although the mechanism by which the *kil* gene would block cell division

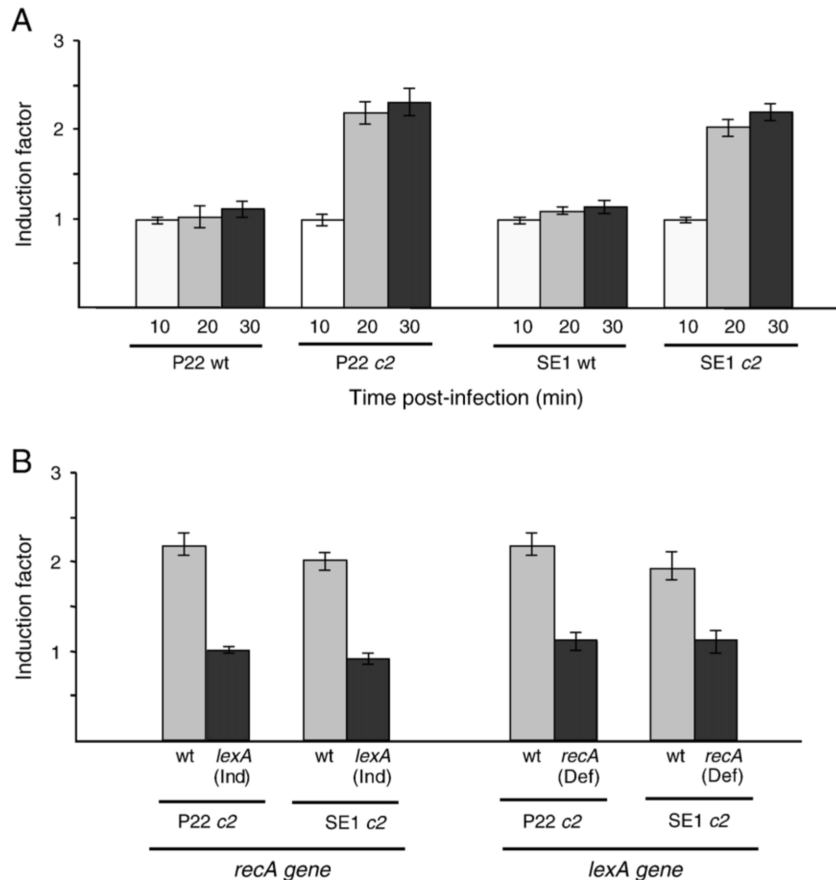

Fig. 1. (A) Induction factor of *recA* gene at 10, 20, and 30 min after infection of the *S. enterica* ATCC14028 wild-type strain with either P22 or SE1 wild-type or *c2* mutant derivatives at MOI of 5. (B) Induction factor of the *recA* and *lexA* genes, respectively, in *S. enterica* ATCC14028 *lexA*(Ind) and *recA*(Def) mutants. Samples were taken at 20 min post-infection with either P22*c2* or SE1*c2* at MOI of 5. As a control, the induction factor of both genes in the wild-type strain is also shown. The induction factor measured by quantitative RT-PCR is the ratio of the relative mRNA concentration for the studied gene (*recA* or *lexA*) in infected cells to that in non-infected cells. The relative mRNA concentration for each gene is normalized to that of the *trpA* gene. In each case, error bars indicate the standard error of the mean from three independent experiments (each in triplicate).

has yet to be elucidated. However, and since SOS induction leads to cell filamentation due to expression of the cell division inhibitor SulA (Schoemaker et al., 1984), the above data suggest that the *kil* cell division inhibitory effect (Semerjian et al., 1989; Sergueev et al., 2002) might be actually mediated by SulA after bacteriophage-triggered expression of the host SOS response.

Gifsy prophages are induced by infection with heteroimmune lytic bacteriophages

The inactivation of the *lexA* gene and the consequent deregulation of the SOS response in *S. enterica* stimulates the expression of several genes (e.g., *tum*, STM2605 or STM1048) encoded in Gifsy-1 and Gifsy-2 prophages (Bunny et al., 2002). Therefore, we analyzed here the effect of P22*c2* infection on the lysogenic cycle stability of Gifsy prophages using several approaches.

Firstly, the expression of genes STM2605 and STM1048 of the Gifsy-1 and Gifsy-2 prophages, respectively, was studied. Induction of these two genes can be taken as an indicator of lytic gene expression, since it has been shown that neither is a moron, and each is located in the morphogenesis module of their respective prophages (Bunny et al., 2002; Frye et al., 2005). The behavior of these genes was analyzed at 20 min post-infection with a P22*c2* mutant. Levels of expression of STM2605 and STM1048 increased about 3- to 4-fold in these conditions (Fig. 3A). Likewise, the mRNA levels of Gifsy-1 and Gifsy-2 *tum* genes were also enhanced following P22*c2* infection (Fig. 3A), even though the induction levels for all the monitored Gifsy-1 and Gifsy-2 genes were lower than those observed following mytomicin-C induction of the SOS response (Fig. 3B). The same results were obtained when the expression levels of these Gifsy genes were analyzed after SE1*c2* mutant infection (data not shown). On the other hand, *tum*, STM1048 and STM2605 expression was not stimulated in a *S. enterica lexA*(Ind) genetic background (data not shown).

Secondly, and since this method has been recently employed to monitor Gifsy prophage induction (Alonso et al., 2005; Frye et al., 2005), the amount of Gifsy phage produced DNA was monitored by real-time PCR experiments. In agreement with the abovementioned results for prophage gene expression (Fig. 3), a significant increase of intracellular

Table 2
Bacterial strains and plasmids used in this work

	Relevant features	Source
Bacterial strains		
ATCC14028	*S. enterica* serovar Typhimurium ATCC14028 wild type strain.	ATCC
GE13	*S. enterica* strain *lex*A3(Ind) *malE*::Tn10. Cm^R.	(Salles et al., 1987)
MA6684	As ATCC14028 but *bio*-106::Tn10, *galE*496 Gifsy-1^{-} Gifsy-2 [-]	Dr. Nara Figueroa-Bossi
UA1770	As ATCC14028 but Rif^R	(Campoy et al., 2002)
UA1820	As UA1770 but *recA*ΩKm. Rif^R and Km^R	This work
UA1821	As ATCC14028 but *recA*ΩKm. Km^R	This work
UA1822	As ATCC14028 but *lexA3*(Ind). Obtained by transduction using GE13 strain as donor. Cm^R.	This work
UA1823	As ATCC14028 but carrying the pUA1092 plasmid. Ap^R.	This work
Plasmids		
pGE108	ColE1 derivative carrying the *cea::lacZ* fusion. Km^R.	(Salles et al., 1987)
pGP704	Suicide vector. Ap^R.	(de Lorenzo et al., 1990)
pGEX 4T-1	Glutathione S-transferase gene fusion vector.Ap^R.	Amersham Biosciences
pUA1085	pGEMT vector containing the *E. coli* P*tac* promoter and the *lacI*^{q} gene of the pGEX 4T-1 vector. Ap^R.	This laboratory
pUA1092	As pUA1085 but with the P22 *kil* gene placed downstream of the P*tac* promoter. Ap^R.	This work

Gifsy DNA concentration was observed after P22*c2* infection (Fig. 4A), and accordingly, the same rise in intracellular Gifsy DNA concentration was obtained when SE1*c2* bacteriophage was used (data not shown).

Finally, an increase in the production of Gifsy infective particles was also detected 80 min after P22 (Fig. 4B) or SE1 bacteriophage infection (data not shown).

The P22 genome encodes the *ant* gene whose product has been shown to block the c2 repressor of this bacteriophage, as well as that of those of other related phages (Levine et al., 1975; Prell, 1978, 1979). It must be noted that this *ant* gene is negatively regulated by the product of the P22 *arc* gene (Levine et al., 1975; Susskind and Botstein, 1978), and that P22 mutants presenting a constitutive expression of the *ant* gene exhibit a virulent phenotype (Levine et al., 1975). In this context, it could be argued that the stimulatory effect upon the lytic cycle of Gifsy phages described in this work was due to the effect of an overproduction of the *ant* product, which could then inhibit the action of the Gifsy c2 repressor. However, it must be stressed that the SE1 phage genome lacks the *ant* gene (Busquets et al., 2005), thus providing a convenient negative control for this effect.

Several mechanisms of defensive strategy against heteroimmune infecting phages, as *rexAB* or *sieAB* (Hofer et al., 1995; Ranade and Poteete, 1993; Shinedling et al., 1987), have been described for prophages. These mechanisms aim at guaranteeing prophage persistence and include evasive strategies, like super-infection exclusion or lysogenic conversion of the host cell wall, as well as active mechanisms, such as targeted DNA restriction systems. Nonetheless, our data demonstrate that, at least in *S. enterica*, infection of a lysogenic cell by a heteroimmune lytic bacteriophage triggers the SOS system, and that this response, in turn, induces lytic cycle of the resident prophages. Thus, SOS-mediated induction of prophage lytic cycle in lysogenic cells may not have evolved only as an early warning mechanism of relatively infrequent DNA damage, but also, as a hitherto unknown fail-safe mechanism to prevent loss of these prophages in an evolutionary arms race with competing lytic phages. Finally, and given that bacterial virulence factors are frequently encoded in the genome of temperate bacteriophages (Boyd and Brussow, 2002), it must be noted that the

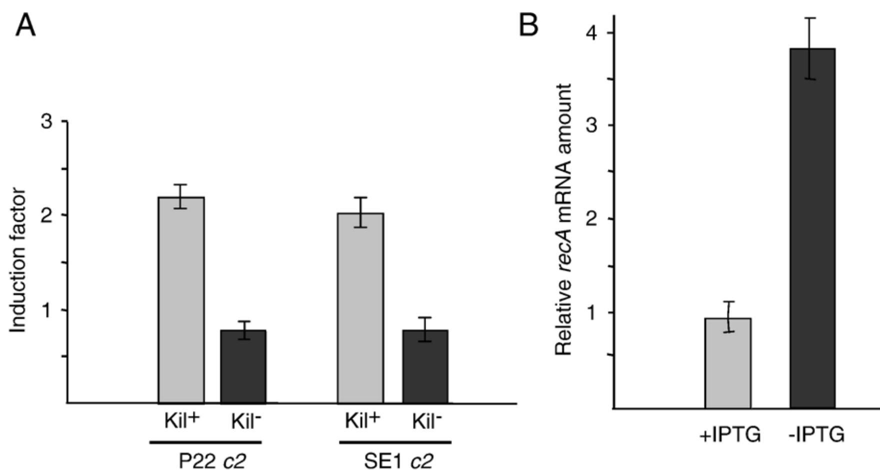

Fig. 2. (A) Induction factor of the *S. enterica* ATCC14028 *recA* gene at 20 min post-infection with *c2* virulent derivatives of either P22 or SE1 *kil* mutants. As a control, the induction factor of this gene after infection with either P22*c2* or SE1*c2* is also shown. The induction factor is the ratio of the relative mRNA concentration for *recA* gene measured by quantitative RT-PCR in infected and non-infected wild-type cells. (B) RT-PCR analysis of *recA* gene induction after *kil* gene overexpression. The relative *recA* mRNA amount in the absence (-IPTG) or presence (+IPTG) of IPTG (1 mM) is shown. Always, the relative mRNA concentration for *recA* gene is normalized to the *trpA* gene. In each case, error bars indicate the standard error of the mean from three independent experiments (each in triplicate).

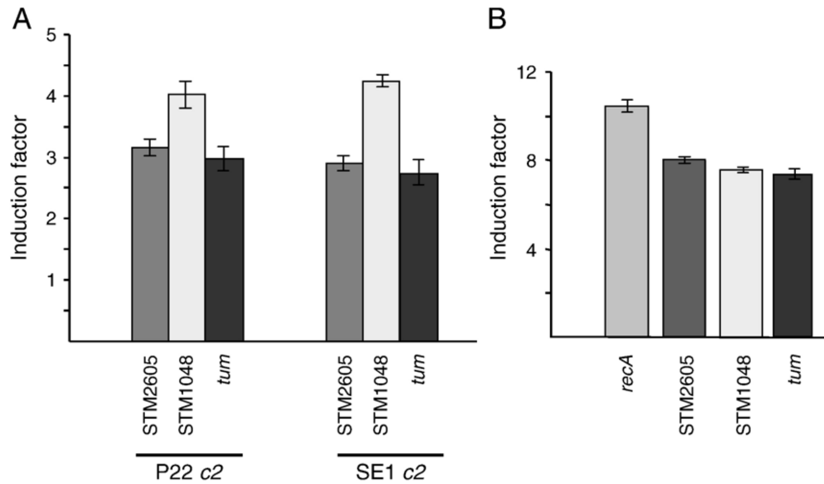

Fig. 3. (A) Induction factor of STM2605 (Gifsy-1), STM1048 (Gifsy-2) and *tum* (from Gifsy-1 and Gifsy-2) genes at 20 min post-infection with a P22*c2* mutant. (B) Induction factor of *recA*, STM2605, STM1048 and *tum* after 2 h of mitomycin-C treatment. In all cases, the induction factors were measured by quantitative RT-PCR, and they are the ratio of the relative mRNA amount of each gene in infected cells with respect to that of non-infected cells. The relative mRNA concentration for each gene is normalized to *trpA* gene. In each case, error bars indicate the standard error of the mean from three independent experiments (each in triplicate).

prophage induction mechanism described in this work may be a relevant dissemination pathway of these factors among bacterial populations.

Materials and methods

Bacteria and plasmids

S. enterica strains and plasmids used in this work are listed in Table 2. *S. enterica* was grown either at 37° or 42° in LB. When necessary, ampicillin (100 μg/ml), kanamycin (100 μg/ml) or chloramphenicol (34 μg/ml) were added to the bacterial culture. DNA extractions, cloning, transformation and other molecular techniques used in this work were performed as described elsewhere (Sambrook and Russell, 2001).

Construction of P22 and SE1 kil mutants and isolation of their c2 bacteriophage derivatives

P22 and SE1 *kil* defective mutants were obtained from their respective lysogenic strains via a PCR-based gene replacement method (Datsenko and Wanner, 2000). The antibiotic resistance cassette from the pKD3 plasmid was amplified using KilP1 and KilP2 70-nt long oligonucleotides. These primers contain 20-

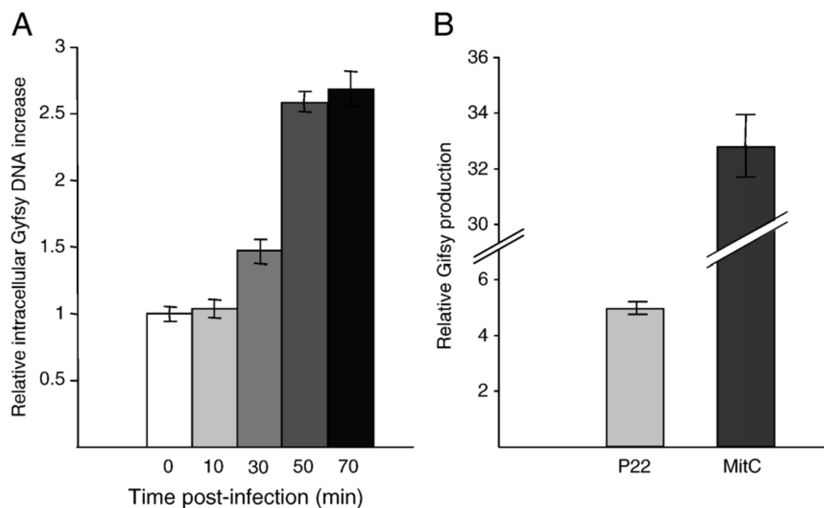

Fig. 4. (A) Increase of relative intracellular Gifsy bacteriophage DNA amount following P22*c2* mutant infection. Samples were taken 10, 30, 50, and 70 min after P22*c2* adsorption to *S. enterica* cells. It is worth noting that the lysis of P22*c2*-infected bacteria occurred 80 min post-infection. The DNA increase was measured by quantitative PCR and it is the ratio of the STM2633 (from Gifsy-1) and STM1008 (from Gyfsy-2) relative gene amount in infected cells with respect to that of non-infected cells. The behavior of both Gifsy genes was simultaneously analyzed. The intracellular Gifsy DNA quantity was normalized to that of the *trpA* gene as non-bacteriophage gene indicator. (B) Increase of relative Gifsy infective particles production after either 80 min post P22*c2* infection or 2 h treatment with mitomycin-C (20 μg/ml). The relative increase is the concentration of the infective particles detected after each treatment with respect to that of non-treated cultures. In all cases, error bars indicate the standard error of the mean of three independent experiments (each in triplicate).

mers that prime with pKD3 vector (Datsenko and Wanner, 2000) and 50-mers presenting *kil* homology (from position −21 to +33 and from +150 to +200 with respect to the *kil* translation start point, in KilP1 and KilP2 primers, respectively). After PCR product transformation into *S. enterica* ATCC14028 strain lysogenic for P22 phage, and containing the pKD46 expressing the λ Red-recombinase vector (Datsenko and Wanner, 2000), *kil* defective chloramphenicol resistant derivatives were selected. Afterwards, and using the pCP20 FLP-recombinase expression vector (Cherepanov and Wackernagel, 1995), the chloramphenicol resistance cassette was eliminated. Spontaneous clear mutants of either P22 or SE1 wild type, as well as of their *kil* defective derivatives, were isolated as described (Davis et al., 1980). Afterwards, their *c2* gene was amplified from bacteriophages DNA, with the appropriate oligonucleotides, and sequenced twice by the dideoxy method (Sanger et al., 1977) on an ALF Sequencer (Amersham-Pharmacia) using the Thermo Sequenase Cy5 Dye Terminator Sequencing Kit (Amersham Biosciences). Mutations in the *c2* gene of each virulent bacteriophage derivative used in this work are listed in Table 1.

Construction of S. enterica mutants

S. enterica serovar *typhimurium* ATCC14028 *lexA*(Ind) strain, was obtained by transduction using GE13 strain (Salles et al., 1987) as a donor (Table 2). Among kanamycin-resistant tranductants, the presence of the *lexA*(Ind) mutation was confirmed through β-galactosidase assay after introduction of the pGE108 plasmid (Table 2) which contains a LexA-regulated *cea*::*lacZ* fusion (Salles et al., 1987).

The *S. enterica* serovar Typhimurium *recA* knockout mutant was constructed as described before (Campoy et al., 2002). Briefly, the *S. enterica* ATCC14028 *recA* gene was amplified, cloned in the pGEMT vector and a 2.2 kb cassette encoding kanamycin resistance (ΩKm) was inserted into its *Cla*I internal site. After cloning the *recA*ΩKm construction into the pGP704 suicide vector (de Lorenzo et al., 1990), the obtained plasmid was introduced by triparental mating into a *S. enterica* ATCC14028 Rif^R derivative. Kanamycin-resistant transconjugants were screened for the loss of vector-mediated ampicillin resistance and the presence of the *recA* mutation was confirmed by PCR. Afterwards, the *recA*ΩKm construction was transferred by transduction to a *S. enterica* ATCC14028 wild-type strain as described before (Campoy et al., 2002). The absence of P22*int7*(HT) prophage in kanamycin-resistant tranductants was determined by streaking the mutants onto green plates in which *S. enterica* P22 lysogenic colonies were dark green whereas non-lysogenic cells formed light-colored colonies (Davis et al., 1980). Afterwards, the presence of *recA*ΩKm construction was confirmed by PCR.

Cloning and overexpression of kil gene

The *kil* gene was amplified by PCR using Ndekil (G*GAATTCC*ATATGACCATTACGCCTG) and Bamkil (CG*GGATCC*GTCATGAACATGACGCCTC) oligonucleotides carrying *Nde*I and *Bam*HI restriction sites at their 5′-end, respectively. The PCR product was digested using *Nde*I and *Bam*HI endonucleases and cloned into a pUA1085 plasmid (Table 2), placing the *kil* gene downstream the Ptac promoter to obtain plasmid pUA1092. Afterwards, the construction was confirmed by sequencing, and the plasmid pUA1092 was electro-transformed into *S. enterica* ATCC14028, giving rise to the UA1823 strain. For *kil* overexpression, IPTG (1 mM) was added to an exponential growing culture of the UA1823 strain. After 2-h incubation at 37 °C, total RNA was extracted using RNeasy Mini kit (Quiagen).

Quantitative RT-PCR and PCR experiments

Quantitation of the expression and the DNA amount for several genes in *S. enterica* cells following bacteriophage infection was analyzed through quantitative RT-PCR and PCR analysis, respectively, according the following procedure. P22 or SE1 bacteriophages were added to a multiplicity of infection of 5 pfu/cfu into fresh-growing *S. enterica* cultures previously treated with KCN (5 mM) during 10 min, allowing absorption for 25 min without agitation. Afterwards, the mixture was centrifuged, and the pellet was resuspended in LB media and incubated with agitation at 37 °C. Samples were periodically taken and either total RNA or DNA extractions were performed using RNeasy Mini kit (Quiagen) or Easy-DNA (Invitrogen), respectively. For RT-PCR assays, absence of DNA in RNA extractions was confirmed by PCR analysis.

The level of *recA*, STM2605, STM1048 or *tum* genes transcription was in all cases, and in concordance with standard methodology (Brooks et al., 2001; Campoy et al., 2003), normalized with respect to that of *trpA* gene, since this gene has been shown to be non-inducible by DNA damage in the Gamma Proteobacteria (Courcelle et al., 2001).

The increase in intracellular DNA concentration for Gifsy-1 and Gifsy-2 bacteriophages was measured, as described previously, by analyzing the behavior of STM2633 and STM1008 genes, respectively (Frye et al., 2005). Both genes were tested simultaneously using the same primer pair in infected and non-infected cells. In all cases, the amount of Gifsy DNA was normalized with respect to that of *S. enterica trpA* gene as a non-bacteriophage gene indicator.

When required, *S. enterica* ATCC14028 cultures were treated with mitomycin-C as described before (Campoy et al., 2003).

Measurement of in vivo Gifsy phage induction

Samples of *S. enterica* ATCC14028 cultures either infected with P22*c2* or SE1*c2* bacteriophages or treated during 2 h with mitomycin-C were taken out, centrifuged and filtered. Afterwards, the presence of Gifsy infective particles in these samples was determined as described before (Figueroa-Bossi and Bossi, 1999) by using *S. enterica* MA6684 as an indicator strain. The MA6684 strain is resistant to both P22

and SE1 bacteriophages because it is a *galE* defective mutant (Table 2).

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