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Overexpression of the *recA* Gene Decreases Oral but Not Intraperitoneal Fitness of *Salmonella enterica*▽

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Transcription of the *Salmonella enterica recA* gene is negatively controlled by the LexA protein, the repressor of the SOS response. The introduction of a mutation (*recAo6869*) in the LexA binding site, in the promoter region of the *S. enterica* ATCC 14028 *recA* gene, allowed the analysis of the effect that RecA protein overproduction has on the fitness of this virulent strain. The fitness of orally but not intraperitoneally inoculated *recAo6869* cells decreased dramatically. However, the SOS response of this mutant was induced normally, and there was no increase in the sensitivity of the strain toward DNA-damaging agents, bile salts, or alterations in pH. Nevertheless, *S. enterica recAo6869* cells were unable to swarm and their capacity to cross the intestinal epithelium was significantly reduced. The swarming deficiency in *recAo6869* cells is independent of the flagellar phase. Moreover, swimming activity of the *recAo6869* strain was not diminished with respect to the wild type, indicating that the flagellar synthesis is not affected by RecA protein overproduction. In contrast, swarming was recovered in a *recAo6869* derivative that overproduced CheW, a protein known to be essential for this function. These data demonstrate that an equilibrium between the intracellular concentrations of RecA and CheW is necessary for swarming in *S. enterica*. Our results are the first to point out that the SOS response plays a critical role in the prevention of DNA damage by abolishing bacterial swarming in the presence of a genotoxic compound.

In bacteria, RecA is a key protein in homologous recombination, enabling the alignment of DNA molecules prior to strand exchange (17). RecA is also the positive regulator of the SOS response, one of the most-well-studied DNA repair systems in bacteria (54). In *Escherichia coli* and other *Enterobacteriaceae*, the cellular SOS network consists of more than 40 genes whose products act together to ensure cell survival after DNA damage (25). The negative regulator of the SOS system, LexA, binds to a specific sequence, the SOS box, which is located in the promoter region of the controlled genes (54). In *Gammaproteobacteria*, the SOS box is an imperfect palindrome, comprising the sequence $CTGTN_8ACAG$ (54) and varying among the different bacterial phyla (22).

The induction pathway of the SOS response seems to be conserved in all bacteria in which this DNA repair system is found. The RecA protein is activated after binding to single-stranded DNA resulting from inhibition of chromosomal replication (48). In turn, the activated RecA triggers autocatalytic cleavage of the LexA repressor. In *E. coli*, this cleavage occurs in the Ala^{84}-Gly^{85} bond of the regulator (36) in a process mediated by the residues Ser^{119} and Lys^{156}. Autocleavage resembles that described for serine proteases (38), and it prevents the binding of LexA to its specific recognition motif in the promoter region of SOS genes.

In addition to DNA repair genes, the SOS response involves lytic-cycle repressors of temperate bacteriophages (54). In some cases, such as in λ or P22 phages, RecA promotes autocleavage of the repressor (45), whereas for another class of temperate bacteriophages, an anti-lytic-cycle repressor protein, encoded by the *tum* gene, is under the direct negative control of LexA (49). For instance, *Salmonella enterica* strains contain at least three prophages (Fels-2, Gifsy-1, and Gifsy-2) whose lytic cycle is regulated by a *tum*-encoded protein (7). In fact, constitutive expression of the LexA regulon is lethal in this bacterial species, as it results in spontaneous induction of the lytic cycle of these three resident phages (7). The loss of these prophages suppresses the lethality of *S. enterica lexA*(Def) (*lexA* defective) cells (7), but it is worth noting that some of these *S. enterica* resident phages harbor genes that are required for bacterial virulence (24), so they are useless for virulence studies.

It is known that high RecA protein levels increase the frequency of DNA recombination (16). Moreover, the virulence of *recA*-defective mutants is significantly decreased in *S. enterica* as well as in other bacterial pathogens (5, 6, 11, 26, 34). However, due to the dual role of RecA (i.e., in recombination and as a positive regulator of the SOS response), the decrease in virulence of *recA*-defective mutants may be attributed to a reduction of the recombination frequency, to the inhibition of the SOS system induction, or both. To further elucidate the relative importance of each of the roles of RecA in the virulence process, two *recA* mutants were constructed: an *S. enterica* ATCC 14028 *recAo6869* mutant that constitutively expressed *recA* and a *recAo6869 lexA3*(Ind^-) (*lexA* noninducible)

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TABLE 1. Bacterial strains and plasmids used in this work

S. enterica strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
ATCC 14028	Wild type	ATCC
UA1876	As ATCC 14028, but *recAo6869* Km^r	This study
UA1822	As ATCC 14028, but *lexA3*(Ind^-) Cm^r	9
UA1877	As UA1876, but *lexA3*(Ind^-) Km^r Cm^r	This study
UA1878	As ATCC 14028, but Δ*recX*::*cat* Cm^r	This study
UA1879	As UA1876, but Δ*recX*::*cat* Km^r Cm^r	This study
UA1885	As ATCC 14028, but Km^r	This study
UA1886	As ATCC 14028, but Δ*ruvC*::*cat* Cm^r	This study
UA1887	As UA1876, but Δ*ruvC*::*cat* Km^r Cm^r	This study
Plasmids		
pKOBEGA	Amp^r, temperature sensitive	14
pKD3	Amp^r Cm^r	20
pKD4	Amp^r Km^r	20
pGEM-T	Cloning vector; Amp^r	Promega
pGEX 4T-1	Glutathione *S*-transferase gene fusion vector carrying the P*tac* promoter and the *lacI*^{q} gene; Amp^r	Amersham Biosciences
pUA1108	pGEX 4T-1 derivative plasmid carrying the P*tac* promoter and the *lacI*^{q} gene; Amp^r	This study
pUA1109	pUA1108 derivative plasmid containing the *recA* gene under the control of the P*tac* promoter	This study
pUA1110	pUA1108 derivative plasmid containing the *cheW* gene under the control of the P*tac* promoter	This study

strain derivative harboring a mutated LexA repressor that was unable to induce the SOS response. The fitness of these mutants in intraperitoneal (i.p.) and oral infection was analyzed compared with that of the wild-type strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *S. enterica* was grown at 37°C in Luria-Bertani (LB) broth or agar plates. When necessary, ampicillin (100 μg ml^{-1}), kanamycin (150 μg ml^{-1}), or chloramphenicol (34 μg ml^{-1}) was added to the bacterial cultures. DNA techniques were performed as described elsewhere (46). For anoxic conditions, the cultures were grown in the appropriate atmosphere by using the GasPak EZ anaerobe container system from Becton Dickinson.

Construction of *S. enterica* ATCC 14028 mutant derivatives. The *S. enterica recAo6869*, *ruvC*, and *recX* mutants were constructed using the one-step PCR-based gene replacement method (20). The kanamycin resistance (Km^r) and chloramphenicol resistance (Cm^r) cassettes from plasmids pKD4 and pKD3, respectively, were amplified using suitable 100-nucleotide (nt)-long oligonucleotides containing 80-nt stretches homologous to the insertion sites and the point mutation in the SOS box when needed (*S. enterica recAo6869* mutant). The PCR product was transformed into *S. enterica* ATCC 14028 (Table 1) containing plasmid pKOBEGA (14). After the transformant clones were selected, pKOBEGA was eliminated by taking advantage of its temperature sensitivity at 42°C. The presence of the deletions or the point mutation in the SOS box was confirmed by PCR and sequencing.

For the construction of the *S. enterica recAo6869 recX* double mutant, the same strategy was used, but the recipient strain of the Cm^r cassette was the previously constructed *recAo6869* strain harboring pKOBEGA (Table 1).

The *S. enterica* wild-type strain harboring the Km^r cassette insertion was obtained using the same method. In this case, the 100-nt-long oligonucleotides harboring the 80-nt stretches homologous to the insertion sites contained no point mutations in the SOS box.

In all cases, the construct resulting from this procedure was moved into a clean wild-type strain background by transduction using the P22*int7*(HT) bacteriophage (10). The absence of the prophage in the transductants was determined by streaking them onto green plates as previously described (21). The obtained strains were verified both by PCR using suitable oligonucleotides and by sequencing.

S. enterica recAo6869 lexA3(Ind^-) and *recAo6869 ruvC* strains were obtained by transduction. The *recAo6869* construct from *S. enterica* UA1876 strain was transduced to the previously described (9) *lexA3*(Ind^-) strain UA1822, and the *ruvC* deletion from UA1886 was transduced to the *S. enterica* UA1876 strain. The presence of the mutations was confirmed by PCR and sequencing.

For the flagellar phase-reversal process, the Sven Gard method was used (15). Briefly, *Salmonella enterica* was inoculated into the center of swimming plates where sterile antiserum against the identified flagellar phase had been added. The antiserum immobilizes the bacterial cells with the homologous flagella, whereas the cells with the heterologous flagella swim from the center of the plate. After overnight (ON) incubation at 37°C, cells from the edge of the growing area were selected for further studies.

Real-time quantitative RT-PCR assays. Reverse transcription (RT)-PCR analyses of gene expression were carried out for all bacterial strains as previously reported (8) and using the primer pairs suitable for each gene. The results were normalized with respect to those obtained for the *hisG* housekeeping gene, since the latter does not belong to the SOS response (23). The induction factor (IF) of each gene for a given strain was defined as the ratio between the studied gene expression level in the presence and in the absence of mitomycin C (80 ng ml^{-1}) in the respective culture.

Quantification of RecA protein. *S. enterica* wild-type or *recAo6869*, *lexA3*(Ind^-), or *recAo6869 lexA3*(Ind^-) mutant cells were grown in LB broth at 37°C to an optical density at 550 nm (OD_{550}) of 0.4. Then, 4 ml of the culture was removed and treated as the preinduction control. An equal volume of cells was treated with mitomycin C (0.8 mg ml^{-1}) for 2 h. Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM EDTA, 10% glycerol), and the samples were stored in buffer B (50 mM Tris-HCl, pH 6.8, 3% β-2-mercaptoethanol, 3% SDS, 30% glycerol, 0.1% bromophenol blue). Samples from each induction experiment were electrophoresed alongside purified RecA protein standard (10 to 500 ng).

Polyclonal rabbit antiserum was raised against purified RecA protein according to standard protocols. The IgG fraction of the sera was purified by affinity chromatography with protein A-Sepharose 4B (Pharmacia Biotech) as described by the suppliers. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were transferred and probed with anti-RecA antibodies as described previously (13). RecA protein bands on developed immunoblots were quantified with a scanning densitometer. Purified RecA protein standards yielded a linear relationship between antibody signal and the RecA protein concentration. The amount of RecA protein in each induced sample was interpolated from the purified RecA protein standard curve.

***S. enterica in vivo* and *in vitro* competition assays.** The competitive assays were performed as reported previously (4, 10). Briefly, wild-type and mutant strains were grown separately and then mixed together in a ratio of approximately 1:1. The initial concentration for each strain was checked by plating serial dilutions of the bacterial suspensions onto LB media. For *in vitro* competition assays, the bacterial mixture was inoculated onto fresh LB medium at a final concentration

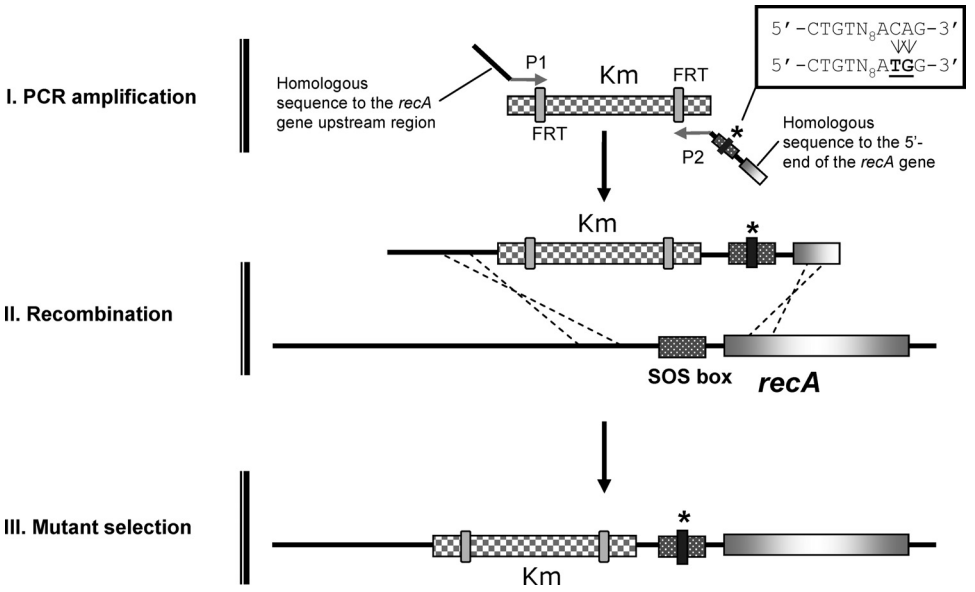

FIG. 1. Construction of the *S. enterica recAo6869* mutant using a one-step PCR-based gene replacement method. The kanamycin resistance gene (Km) was amplified from plasmid pKD4 using oligonucleotides containing sequences homologous to the chromosomal DNA of *S. enterica* (step I). This construct was used to transform the *S. enterica* ATCC 14028 wild-type strain (step II), and Km^r colonies were selected (step III). The presence of the mutation in the SOS box was verified by both PCR and sequencing. FRT, FLP recombination target. The asterisk indicates the point mutation in the LexA binding sequence that is boxed in the figure.

of 2×10^7 CFU ml^{-1} and the culture was grown overnight (ON) at 37°C under aerobic or anaerobic conditions. Afterwards, serial dilutions of the culture were plated onto LB broth. To determine the proportion of each strain, replica platings were performed on appropriate antibiotic-supplemented LB plates. All of the *in vitro* experiments were performed at least in triplicate. For *in vivo* studies, in each experiment 6 or 12 female (4 to 6 weeks old) BALB/c mice were inoculated either orally or intraperitoneally (i.p.) with 2×10^8 CFU or 2×10^3 CFU, respectively, of the bacterial mixture per animal. Blood samples taken from the heart immediately after the mice died due to septicemia (generally 4 to 5 days after inoculation) were cultured in LB medium. The proportion of each strain appearing in the cultures was determined by replica plating onto appropriate antibiotic-supplemented LB plates. In all cases, the competitive index (CI) was calculated for each mutant as the ratio between the mutant and the wild-type strain in the output (bacteria recovered from the ON cultures in *in vitro* assays or from the host after infection in *in vivo* studies) divided by their ratio in the input (initial inoculum) (4). Statistical analyses consisted of the two-tailed *t* test and the Wilcoxon signed-rank test, with a P of ≤ 0.05 considered significant.

pH, bile, H_2O_2, and UV survival assays. *S. enterica recAo6869*, *lexA3*(Ind^-), and *recAo6869 lexA3*(Ind^-) survival assays were performed as previously described (50, 52, 53), but with some modifications. All bacterial strains were grown ON in LB medium, diluted to a final concentration of 3×10^7 CFU ml^{-1} in the same medium, and then incubated with the appropriate compound. For pH resistance assays, ON cultures were diluted and incubated in pH 3.3 LB broth for 300 min without previous adaptation (52). Survival was assessed in samples withdrawn at 0, 50, 100, 150, 200, 250, and 300 min. For bile salts survival assays, diluted ON cultures were incubated for 300 min in LB broth supplemented with sodium choleate (Sigma) at a final concentration of 30%; survival was assessed in samples withdrawn at 0, 200, 250, and 300 min (53). For H_2O_2 survival assays, diluted ON cultures were incubated for 120 min in LB medium containing 10 mM H_2O_2. Samples were removed at 0, 15, 30, 60, 90, and 120 min after H_2O_2 addition and diluted in LB broth containing catalase (10 μg ml^{-1}) to neutralize the H_2O_2 (50). The UV resistance assays were performed as described previously (3). Briefly, bacterial cells in the exponential growth phase were washed by centrifugation, suspended in AB medium (3) to a concentration of about 2×10^8 cells ml^{-1}, and then irradiated at several doses of UV (0, 6, 12, 18, 24, and 30 J m^{-2}) in a glass petri dish in thin layers, with a General Electric GY1578 germicidal lamp, at a rate of 1 J m^{-2} s^{-1} (determined with a Latarjet dosimeter). Afterwards, for each UV dose, samples were taken, diluted and plated in LB media, and grown at 37°C. All experiments with UV-irradiated cells were performed under yellow light or in the darkness to prevent photoreactivation. For all treatments, cell survival was calculated as the survival ratio of the treated versus the untreated cells.

***S. enterica* invasion assays.** The protocol used to test the invasiveness of several *S. enterica* strains into Caco-2 cells was similar to that used by Kim and Wei (33), with some modifications. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX I medium (Difco) supplemented with 20% fetal bovine serum (Difco) at 37°C in 95% air and 5% CO_2. Once the monolayer was formed, cells were trypsinized and the suspension was seeded at 10^6 cells ml^{-1} onto tissue culture-treated 6-well plates (Corning Incorporated, Corning, NY). The plates were incubated at 37°C in 95% air and 5% CO_2 until confluence was reached in every well.

S. enterica wild-type and *recAo6869* and *recAo6869 lexA3*(Ind^-) mutant strains were inoculated into LB broth and incubated overnight at 37°C. The bacterial cultures were centrifuged at 5,000 × *g* for 10 min and washed twice with phosphate-buffered saline (PBS), and the OD_{600} value was adjusted to 0.4. Bacterial suspensions were inoculated to a final concentration of 10^6 CFU ml^{-1} into the confluent monolayers of Caco-2 cells in the 6-well plates. Plates were incubated for 1 h at 37°C in 5% CO_2. The cells were then rinsed once with 1 ml of DMEM (Gibco) and incubated for 2 h at 37°C in 5% CO_2 with 1 ml of cell culture medium containing 100 μg of gentamicin ml^{-1} to remove extracellular bacteria. The cells were finally rinsed 3 times with PBS and lysed at room temperature for 10 min with 0.1% Triton X-100 in PBS. Viable bacterial counts were determined in duplicate by plating serial dilutions on LB agar. The plates were incubated at 37°C for 24 h. The invasion assay was carried out in triplicate.

***S. enterica* coinfection analysis in murine ileal loops.** To examine the *in vivo* interaction of *S. enterica* wild-type and *recAo6869* strains with murine intestinal epithelial cells, the ligated-ileal loop coinfection model was used as described previously (30), with some modifications. Six-week-old BALB/c female mice were starved for 24 h and then anesthetized by i.p. injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) before surgery. A small incision was made to expose the small bowel, and an ileal loop was then created by ligating a section of intestine containing a grossly identifiable Peyer's patch to a site proximal to the cecum. The blood supply to the loop was kept intact. Equal numbers of cells of the parental and *recAo6869* mutant strains (~10^7 CFU ml^{-1}) in PBS (200 μl) were then injected through a 25-gauge needle, after which the bowel was returned to the abdomen and the incision stapled. The mice were kept alive for 60 min and then killed. The ileal loop was excised intact, opened longitudinally, and placed into a tube containing PBS. Extracellular bacteria were eliminated by three washes with PBS and a further incubation for 90 min in PBS containing gentamicin (100 μg ml^{-1}). The samples were then rinsed with PBS to remove

residual gentamicin, and the entire ileal segment was processed in a stomacher (Seward Medical) using 1 ml of PBS. To determine intracellular parental and mutant bacteria, appropriate dilutions of the homogenized samples were spread onto LB and LB-kanamycin plates.

Overexpression of *S. enterica recA* and *cheW*. The overexpression plasmid was constructed by inverted PCR using the pGEX up (5′-CGGGATCCCGCATATGTACTGTTTCCTGTGTGAA-3′) and pGEX dw (5′-CGGGATCCCCGGAATTCCCG-3′) primers, containing the NdeI and BamHI restriction sites, together with the pGEX 4T-1 vector as a template. The constructed plasmid (pUA1108), containing the *tac* promoter and the *lacI* gene in pGEX 4T-1, was checked by sequencing. Both the *recA* and the *cheW* coding regions were amplified using suitable oligonucleotides containing the NdeI and BamHI restriction sites. The PCR products were cloned into the pGEM-T vector and confirmed by sequencing. Afterwards, the NdeI-BamHI *recA* and *cheW* fragments were obtained by digestion and cloned into pUA1108, yielding plasmids pUA1109 and pUA1110, respectively. In both cases, *recA* and *cheW* were under the control of the *tac* promoter. Plasmids pUA1109 and pUA1110 were transformed in the *S. enterica* wild-type strain and *recAo6869* mutant derivative, respectively. IPTG (isopropyl-β-D-thiogalactopyranoside)-mediated overexpression of either RecA or CheW was confirmed in crude extracts by SDS-PAGE (data not shown). Briefly, the wild-type strain, harboring pUA1109, and the *recAo6869* mutant, containing pUA1110, were grown on LB broth to an OD_{550} of 0.5, after which IPTG (1 mM) was added to the cultures followed by 3 h of incubation at 37°C. The crude protein extracts were loaded onto 15% acrylamide gels, and SDS-PAGE was conducted for 2 h. The protein profiles of the wild-type and mutant strains were visualized by Coomassie blue staining of the gels, which in each case confirmed the overexpression of either RecA or CheW.

Swimming and swarming assays. Swimming and swarming assays were carried out as described previously (28). In short, the bacterial strains were grown in LB broth at 37°C. Then, 10 μl of the stationary cultures was inoculated in the middle of either swimming or swarming plates, supplemented when necessary with IPTG or mitomycin C. The plates were incubated ON at 37°C and then photographed.

RESULTS AND DISCUSSION

Construction of an *S. enterica* mutant constitutively expressing the *recA* gene. The *S. enterica recAo6869* mutant (UA1876), harboring a mutation in the LexA binding site of the *recA* gene promoter, was obtained using the one-step mutagenesis method (Fig. 1). As expected, this mutant presents a higher amount of RecA protein (~25,000 protomers per cell) than that measured in the wild-type strain (~5,000 protomers per cell) (Fig. 2A). The basal level of the RecA protein was essentially the same in the wild-type and *lexA3*(Ind$^-$) strains (Fig. 2A). Likewise, the basal level of RecA protein in the *recAo6869 lexA3*(Ind$^-$) mutant was the same as that in the *recAo6869* strain (Fig. 2A). It is worth noting that the basal number of molecules of *S. enterica* RecA protein per cell was similar to that obtained for *E. coli* (~5,000) and *Bacillus subtilis* cells (~4,500) (37).

Furthermore, in mitomycin C-treated wild-type cells, the rate of RecA protein accumulation increased about 5-fold, whereas no induction was observed in the *lexA3*(Ind$^-$) strain (Fig. 2A). Besides, the levels of RecA protein were essentially the same in *recAo6869* and *recAo6869 lexA3*(Ind$^-$) strains and both wild-type and mutant mitomycin C-treated cells (Fig. 2A). This rate probably represents maximum induction of the RecA protein by DNA damage because it was essentially the same as that from the *recAo6869* mutant.

Nonetheless, the behavior of the *S. enterica recAo6869* mutant was the same as that of the wild-type strain with respect to the DNA-damage-mediated induction of other SOS genes, such as *sulA* and *uvrA* (Fig. 2B). In contrast, in the *S. enterica recAo6869 lexA3*(Ind$^-$) strain and the *lexA3*(Ind$^-$) strain, these SOS genes were not induced after cellular DNA damage (Fig. 2B). In concordance with these results, and as previously reported in an *E. coli recAo98* mutant (27), the survival of UV-irradiated *S. enterica recAo6869* cells was the same as that of wild-type cells (data not shown). Altogether, these data indicated that constitutive expression of the *recA* gene in the *recAo6869* strain affects neither the kinetics of the SOS response nor the resistance of *S. enterica* to DNA-damaging agents. Similar results were reported for *E. coli recAo6869* cells (27).

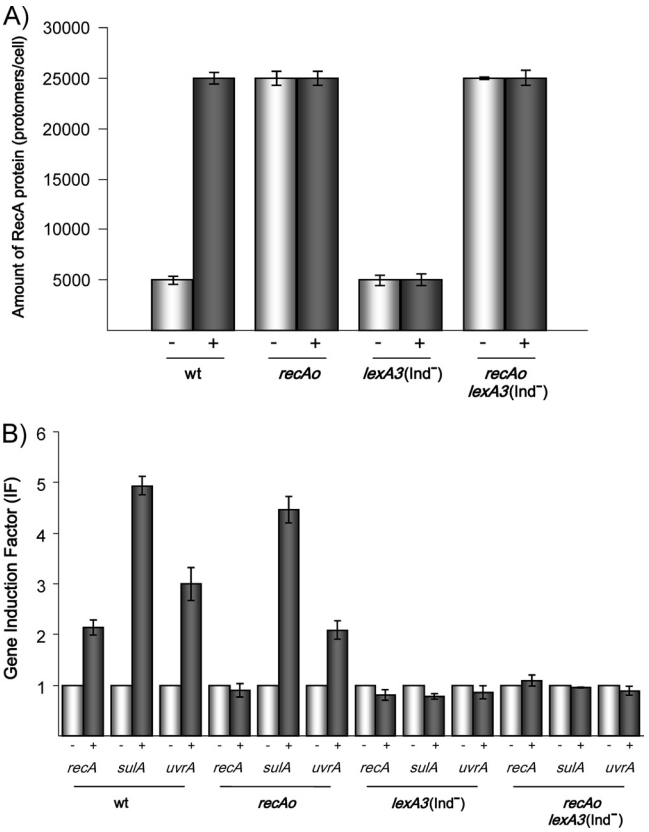

FIG. 2. Mitomycin C-mediated induction of the RecA protein levels and of the expression of several SOS genes in *S. enterica recAo6869* (*recAo*), *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutants. (A) Levels of RecA protein in the *S. enterica* ATCC 14028 wild type (wt) strain and in each mutant strain treated (+) or untreated (−) with mitomycin C. All determinations were performed at least three times. (B) Expression levels of the *recA*, *uvrA*, and *sulA* SOS genes in the *S. enterica* wild type or in each mutant derivative in the presence (+) or absence (−) of mitomycin C. In this case, the induction factor (IF) was defined as the ratio between the relative mRNA concentration of each gene in cells treated with mitomycin C and in untreated cells. The data are the means of two independent quantitative real-time RT-PCRs (each performed in triplicate), and the standard deviation of any value was never greater than 10%.

To further characterize the *S. enterica recAo98*, *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutants and to determine whether their growth was affected, *in vitro* competitive assays were carried out and growth kinetics were measured in minimal medium and in rich medium. The results showed that neither the overproduction of RecA protein nor the inability to induce the SOS system seems to have an effect on *S. enterica* growth *in vitro* (data not shown). It must be noted that similar results have been reported for both *E. coli recAo98* and *lexA*(Ind$^-$) mutants (27, 42).

TABLE 2. Competitive indexes of several *S. enterica* mutants in murine model

Strain comparison (mixed infection)	Challenge route	CI[a]
recAo6869 mutant vs wild type	i.p.	0.42
lexA3(Ind$^-$) mutant vs wild type	i.p.	0.42
recAo6869 lexA3(Ind$^-$) mutant vs wild type	i.p.	0.48
recAo6869 ruvC mutant vs wild type	i.p.	0.00019*
recAo6869 mutant vs wild type	Oral	0.00027*
lexA3(Ind$^-$) mutant vs wild type	Oral	2.73
recAo6869 lexA3(Ind$^-$) mutant vs wild type	Oral	0.0054*

[a] CI is calculated as the ratio between the mutant and the wild-type strain in the output (bacteria recovered from the host after infection) divided by their ratio in the input. *, virulence of the mutants was significantly lower than that of the wild-type strain (in all cases with a P value of ≤ 0.05).

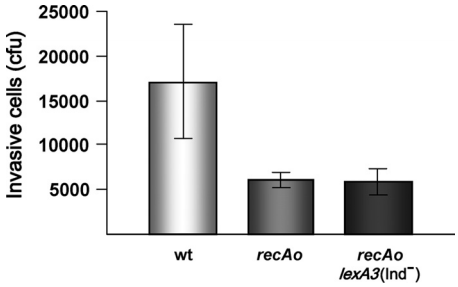

FIG. 3. Invasiveness of *S. enterica* strains in Caco-2 cells. In all cases, a final concentration of 10^6 CFU ml^{-1} of either the wild type (wt) or the *recAo6869* (*recAo*) mutant derivatives was inoculated into confluent monolayers of Caco-2 cells. After 1 h of incubation and a gentamicin treatment, Caco-2 cells were lysed; the number of invasive cells is shown. In all cases, the invasion assay was carried out in triplicate.

***In vivo* fitness of the *S. enterica recAo6869* strain.** To elucidate whether RecA-mediated recombination or SOS activation is more important for the infection process, the *S. enterica recAo6869*, *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutants were examined in competition assays with the wild-type strain. Neither deregulation of the *S. enterica recA* gene nor the inability to induce the SOS response because of the presence of a *lexA3*(Ind$^-$) mutation had any effect upon the virulence of intraperitoneally (i.p.) inoculated *S. enterica* (Table 2). Proteins encoded by *ruvABC* genes play an essential role in the late steps of homologous recombination (55), whereas RecA is involved in the early stages of this process (35). For this reason, an *S. enterica recAo6869 ruvC* mutant was constructed and its competitive index (CI) following i.p. inoculation, in comparison with that of the wild-type strain, was analyzed. Table 2 demonstrates that *S. enterica recAo6869 ruvC* cells present a 10^4-fold decrease in their CI. These results, together with those reported for *recA* mutants of *S. enterica recA* and other bacterial species (5, 6, 11, 26, 34), indicate that all stages of the DNA recombination process are important for a full bacterial infective capacity. Furthermore, and following oral inoculation, the *lexA3*(Ind$^-$) strain exhibited the same behavior as the wild-type strain (Table 2), in concordance with results recently published (19) in which no defect in *lexA3*(Ind$^-$) mutant is observed when it is i.p. inoculated. On the other hand, the fitness of the *S. enterica recAo6869* and *recAo6869 lexA3*(Ind$^-$) mutants was dramatically reduced when orally inoculated (Table 2). These results demonstrated that overexpression of *recA* has a negative effect on the oral fitness of *S. enterica*.

Overproduction of the RecA protein decreases invasiveness of *S. enterica*. It should be noted that, following infection via the oral route, the bacterial cells cross the intestinal epithelium, and then the infection process proceeds as if they had been i.p. inoculated (40). Since no differences between the wild type and *recAo6869* and *recAo6869 lexA3*(Ind$^-$) mutants were observed in i.p. competition assays, our results suggested that the negative effect of RecA overexpression upon the oral fitness of the *recAo6869* mutant derivatives must have occurred before *S. enterica* entered the bloodstream.

In this context, several factors that could explain the reduced fitness of the *S. enterica recAo6869* mutant derivatives when orally inoculated were analyzed. First, *in vitro* competition assays carried out under anoxic conditions did not reveal any difference between the *recAo6869* mutant and the wild-type strain (data not shown). Similar results were reported in *E. coli recAo6869* cells (27). Second, the results of survival assays in which the bacteria were exposed to bile salts, acidic pH, and H_2O_2 likewise suggested that the sensitivity of the *S. enterica recAo6869* cells to the above DNA-damaging agents was similar to that of the wild-type strain (data not shown). Third, as mentioned above, some *S. enterica* prophages have an important role in virulence (24). Accordingly, the maintenance of some resident prophages was examined in the *S. enterica*

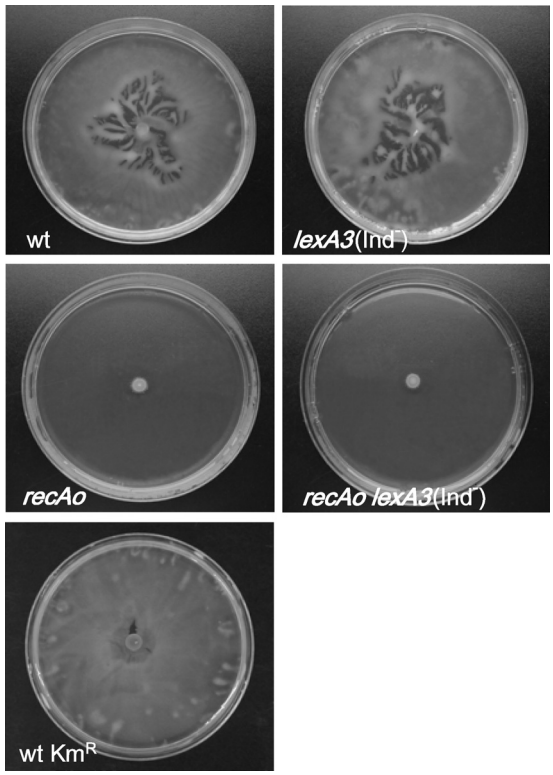

FIG. 4. Swarming ability of the *S. enterica* ATCC 14028 wild-type strain (wt) and the wild-type Km^r (wt Km^r), *recAo6869* (*recAo*), *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutant derivatives. Bacterial colony swarming patterns as they appeared on a semisolid (Difco) agar surface following incubation of the cultures for 24 h at 37°C are shown.

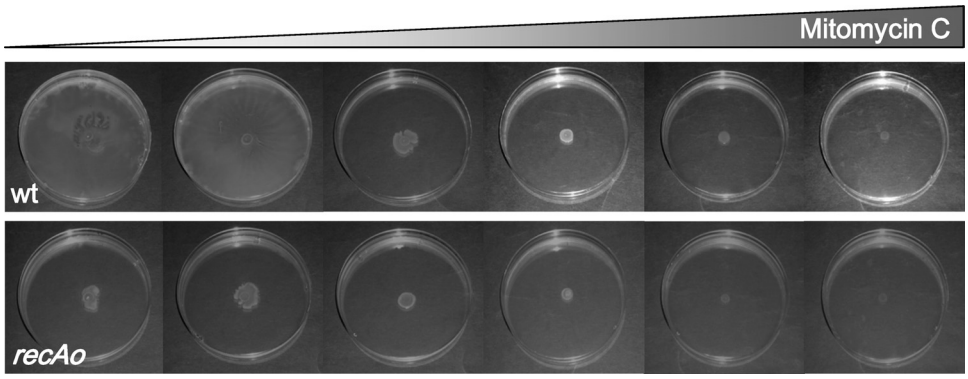

FIG. 5. Swarming ability of the *S. enterica* wild-type (wt) and *recAo6869* (*recAo*) mutant strains in the presence of an SOS inductor. Colony swarming patterns developed on a semisolid agar surface (Difco) in the presence of increasing concentrations of mitomycin C (0, 8, 80, 200, 800, and 1,600 ng ml^{-1}) are shown.

recAo6869 mutants. The results of PCRs showed that the reduced fitness of the *recAo6869* cells could not be attributed to a loss of the prophages Gifsy-1 and Gifsy-2, since both were present in the *recAo6869* mutant strains (data not shown).

Another critical point during infection is the passing of the bacteria through the intestinal epithelium (40). Thus, invasiveness of *S. enterica recAo6869* cells was tested using the human intestinal epithelial cell line Caco-2. Figure 3 indicates that both *S. enterica recAo6869* and *recAo6869 lexA3*(Ind$^-$) mutants have a decreased ability to invade Caco-2 cells (statistically significant at $P = 0.0286$). In concordance with this, coinfection studies in murine ileal loops using the *S. enterica* wild-type and *recAo6869* and *recAo6869 lexA3*(Ind$^-$) mutant strains show that the competitive indexes for both mutants in the ileal loops were 0.25 ± 0.04 and 0.376 ± 0.045, respectively. These data clearly indicate that the *S. enterica recAo6869* and *recAo6869 lexA3*(Ind$^-$) mutants were less able to reach the systemic compartment. It can therefore be concluded that the reduced fitness of *S. enterica recAo6869* cells was associated with its deficiency in crossing the intestinal epithelium.

***S. enterica recAo6869* mutants do not swarm.** It has been demonstrated that *S. enterica* mutants defective in swarming as a consequence of mutations in either the *cheW* or the *cheB* gene show a lower invasiveness than the wild-type strain under anaerobic conditions (31). Furthermore, through comparative analyses among several *S. enterica* serovars, it has been suggested that a relationship between swarming and intestinal colonization must exist in this bacterial species (32). Likewise, swarming is known to be important for the virulence of other bacterial pathogens, such as *Proteus mirabilis*, *Helicobacter pylori*, *Vibrio parahaemolyticus*, and *Campylobacter jejuni* (43, 44, 51, 57). In addition, it has been reported that the RecA protein is necessary for the swarming motility of *E. coli*, since *recA* mutants of this species do not swarm (28). For all these reasons, the swarming behavior of the *S. enterica recAo6869*, *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutants was studied (Fig. 4). The data indicated that neither the *S. enterica recAo6869* strain nor the *recAo6869 lexA3*(Ind$^-$) strain swarmed, while the swarming capacity of *lexA3*(Ind$^-$) cells was similar to that of the wild-type strain (Fig. 4). Thus, and in

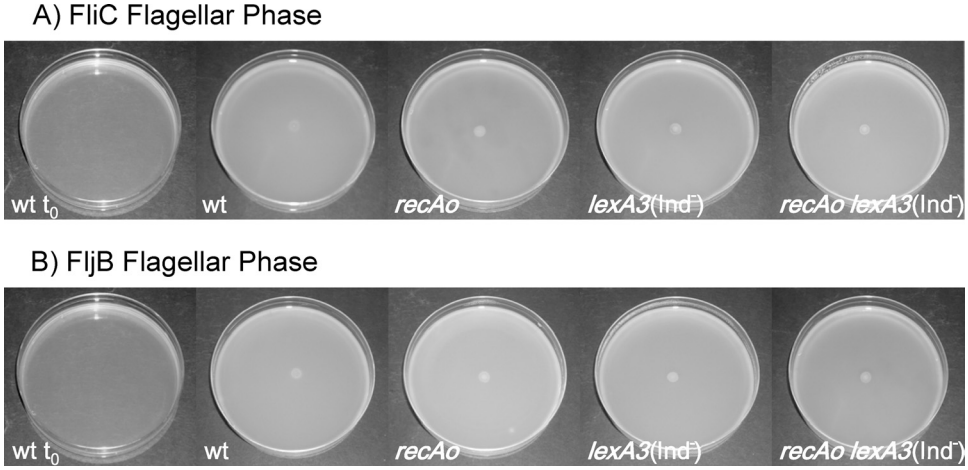

FIG. 6. Swimming ability of *S. enterica* wild-type strain (wt) and its *recAo6869* (*recAo*), *lexA3*(Ind$^-$), and *recAo6869 lexA3*(Ind$^-$) mutant derivatives. Colony swimming patterns developed by strains presenting either the FliC (A) or the FljB (B) flagellar phase are shown. As a control, the wild-type plate immediately after inoculation (wt t_0) is shown.

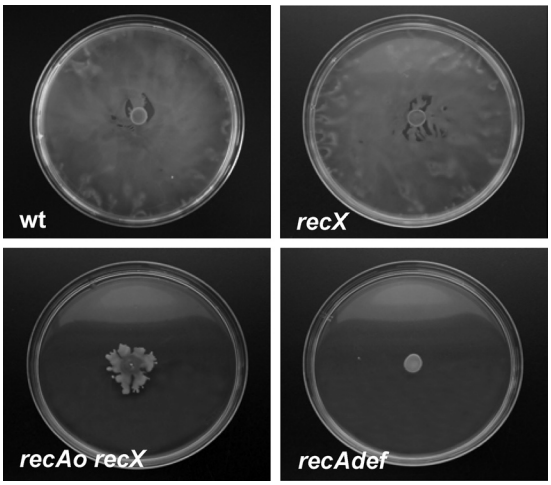

FIG. 7. Swarming ability of *S. enterica recX* and *recAo6869* (*recAo*) *recX* mutant derivatives. Colony swarming patterns developed on a semisolid (Difco) agar surface following incubation of the cultures for 24 h at 37°C are shown. As a control, the negative swarming phenotype of the *S. enterica recA*-defective mutant (*recAdef*) is also presented.

concordance with the results obtained in the competition assays, the inhibition of swarming was only associated with constitutive levels of *recA* and not with the presence of the *lexA3*(Ind$^-$) mutation.

To ensure that the *recAo6869* mutation was the only mutation responsible for this swarming-defective phenotype, a wild-type strain harboring the Km^r cassette in the same position as the *recAo6869* mutants but without any mutation in the LexA binding site was constructed. This strain presents a swarming pattern identical to that of the *S. enterica* ATCC 14028 wild-type strain with no insertion (Fig. 4). Furthermore, it must be noted that in the wild-type strain, the increase in SOS-mediated *recA* expression following the addition of sublethal concentrations of mitomycin C abolished swarming (Fig. 5).

It is worth noting that the swarming deficiency shown by the *S. enterica recAo6869* cells is not dependent upon the flagellar phase, since strains expressing either *fliC* or *fljB* genes do not swarm (data not shown). Moreover, swimming motility of *S. enterica recAo6869* cells is not affected regardless of the flagellar phase which they present (Fig. 6), indicating that this mutant does not display any difficulties in synthesizing flagella.

The *S. enterica recA* and *recX* genes form a single transcriptional unit (18, 41). To rule out the possibility that *recX* overexpression was responsible for the swarming defect, knockout mutant derivatives of this gene were constructed and the organism's ability to swarm was tested. The *S. enterica recX* mutant was able to swarm, while the *recAo6869 recX* double mutant was not (Fig. 7). These results again implicated *recA* constitutive expression in the swarming defect. To further confirm that the overexpression of RecA fully accounts for the inability of the mutant strain to swarm, the *recA* gene was cloned into plasmid pUA1108 under the control of an IPTG-inducible promoter (the *tac* promoter), giving rise to pUA1109. The pUA1109 plasmid was used to transform the *S. enterica* wild-type strain. The swarming phenotype of *S. enterica* carrying pUA1109 was then analyzed in the presence of increasing concentrations of IPTG. As expected, the swarming ability decreased in an IPTG-dependent manner (Fig. 8), unequivocally confirming that the overexpression of *recA* suppresses swarming.

CheW overexpression reestablishes swarming in the *S. enterica recAo6869* mutant. The swarming phenotype has been widely studied in several bacterial species (29, 44). In *Salmonella* species, swarming is under the control of the CheA-CheY two-component signal transduction system (39), and a critical role in this process has been also demonstrated for the CheW protein (2). Specifically, CheW is bound to CheA (a histidine protein kinase) and participates in its autophosphorylation, which leads to swarming activation (2). However, it is also known that the overexpression of CheW inhibits swarming (47) and that a precise quantitative association between the CheW and CheA proteins is required for this kind of cellular motility (12, 56). Furthermore, CheW and RecA have been shown to interact *in vivo* in *E. coli* (1), and, as mentioned above, *E. coli* mutants defective in *recA* do not swarm (28). Since both the

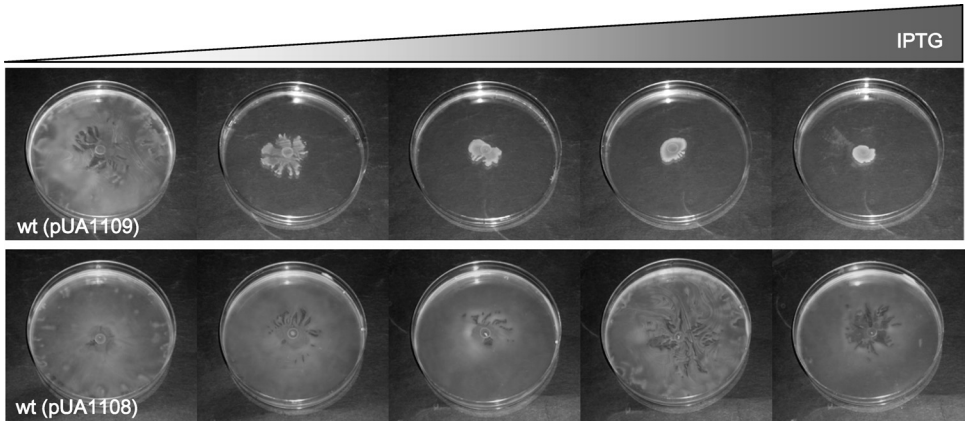

FIG. 8. Swarming ability of the *S. enterica* ATCC 14028 wild-type (wt) strain harboring the *recA*-overexpressing plasmid pUA1109. Colony swarming patterns developed on a semisolid (Difco) agar surface in the presence of increasing concentrations of IPTG (0, 5, 10, 20, and 30 μM) after incubation of the cultures for 24 h at 37°C. As a control, the swarming phenotype of the wild-type strain harboring the pUA1108 overexpression vector without the *recA* gene in the presence of IPTG is also presented.

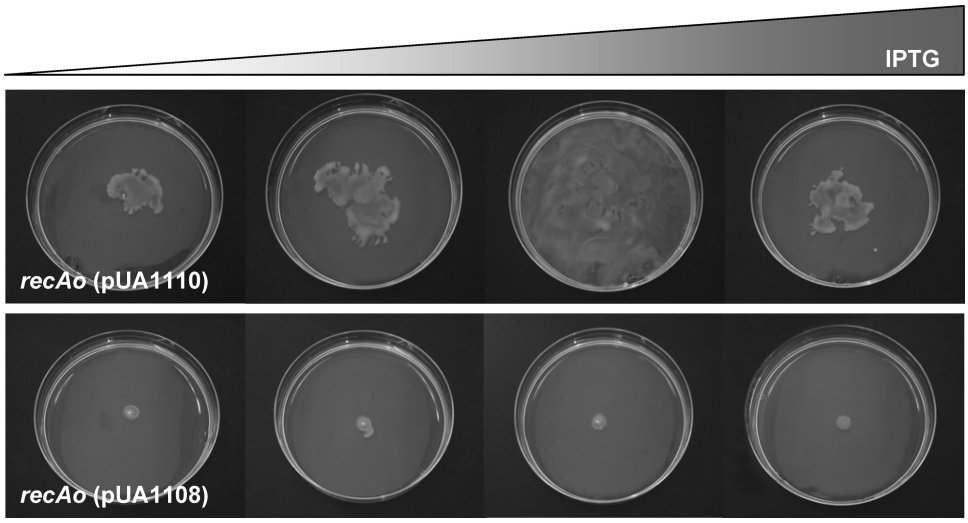

FIG. 9. Swarming ability of the *S. enterica recAo6869* (*recAo*) mutant harboring the *cheW*-overexpressing plasmid pUA1110. Colony swarming patterns developed on a semisolid (Difco) agar surface in the presence of increasing concentrations of IPTG (0, 10, 20, and 30 μM) in cultures incubated for 24 h at 37°C. As a control, the swarming phenotype of the *recAo6869* mutant harboring the pUA1108 overexpression vector without the *cheW* gene in the presence of IPTG is also presented.

absence of RecA and the overexpression of RecA result in an inability to swarm, we postulated that an equilibrium between the cellular amounts of RecA and CheW proteins must be critical for bacterial swarming.

To test this hypothesis, the *S. enterica cheW* gene was cloned under the control of the *tac* promoter to obtain plasmid pUA1110, which was then transformed in the *S. enterica recAo6869* mutant. The swarming phenotype of this strain was analyzed in the presence of increasing IPTG concentrations. As shown in Fig. 9, the *S. enterica recAo6869* (pUA1110) strain recovered the swarming ability in an IPTG-dependent manner. However, when the amounts of CheW were large enough, and in accordance with previously published works (47), swarming was abolished (Fig. 9). These data confirmed that the *in vivo* equilibrium between CheW and RecA is important for bacterial swarming.

In this context, it is tempting to speculate that bacterial colonies use the amplification of intracellular RecA as a tool to sense the presence of DNA-damaging compounds (antibiotics and bacteriocins, among other natural molecules) around them and, consequently, to cease swarming. In this scenario, the presence of a DNA-damaging agent would induce the SOS response, initiating an increase in the amount of RecA protein and thus an alteration of the equilibrium between RecA and CheW, which in turn would abolish the coordinated multicellular surface migration. Accordingly, exposure of the bacterial genetic material to even higher concentrations of the DNA-injuring agent is avoided. Thus, from a bacterial population point of view, the LexA regulon not only would play a fundamental role in DNA damage repair but also could serve to avoid DNA injuries by preventing bacterial colonies from being exposed to high concentrations of genotoxic agents.

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