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Characterization of the SOS Regulon of *Caulobacter crescentus*▽‡

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The SOS regulon is a paradigm of bacterial responses to DNA damage. A wide variety of bacterial species possess homologs of *lexA* and *recA*, the central players in the regulation of the SOS circuit. Nevertheless, the genes actually regulated by the SOS have been determined only experimentally in a few bacterial species. In this work, we describe 37 genes regulated in a LexA-dependent manner in the alphaproteobacterium *Caulobacter crescentus*. In agreement with previous results, we have found that the direct repeat GTTCN$_7$GTTC is the SOS operator of *C. crescentus*, which was confirmed by site-directed mutagenesis studies of the *imuA* promoter. Several potential promoter regions containing the SOS operator were identified in the genome, and the expression of the corresponding genes was analyzed for both the wild type and the *lexA* strain, demonstrating that the vast majority of these genes are indeed SOS regulated. Interestingly, many of these genes encode proteins with unknown functions, revealing the potential of this approach for the discovery of novel genes involved in cellular responses to DNA damage in prokaryotes, and illustrating the diversity of SOS-regulated genes among different bacterial species.

The prototypical cellular response to DNA damage in prokaryotes is the SOS response. Best characterized in *Escherichia coli*, it can be viewed as a global stress response system, which controls the expression of several genes in response to a wide variety of environmental challenges (reviewed in reference 24). In a noninduced state, the genes belonging to the SOS network are repressed by the LexA protein, which binds in a dimeric conformation to an operator located in the regulatory region of target genes, termed the SOS box. The SOS regulon is activated in response to single-stranded DNA regions, which can be a result of DNA replication inhibition or the processing of broken ends in this molecule (53). The RecA protein is able to associate with such regions, acquiring an activated conformation (RecA*). This active form can act as a coprotease in the cleavage of the Ala^{84}-Gly^{85} bond of the LexA repressor (reviewed in reference 63). This cleavage, similar to that mediated by serine proteases, prevents LexA from binding to the SOS operator sequences. Once freed from the repression imposed by LexA, the genes belonging to the SOS regulon can be transcribed, helping the cell to manage the DNA damage. After the DNA lesions have been repaired, the RecA protein activation ceases, and LexA can regain the transcriptional control of the SOS genes. Besides contributing to cell survival after DNA injury, the SOS response has also been proposed to play an important role in bacterial evolution, by up-regulating mutation rates in growth-inhibiting environments (11, 36, 40).

The SOS response is extremely important for prokaryotic cells, and therefore, it is widespread among the *Bacteria*. However, two interesting features have been demonstrated to be highly variable among different bacterial species: the sequence recognized by LexA as the SOS operator and the actual set of genes subject to LexA control (2, 11, 18). The structure of the regulon can vary depending on the organism, but still, a canonical set of genes has been proposed to be LexA regulated in all *Proteobacteria* studied to date. It is comprised of *lexA*, *recA*, *ssb*, *uvrA*, and *ruvCAB* (19).

Several different strategies have been used to identify the genes directly regulated by RecA-LexA. Microarray technology has extensively been used for *E. coli* (13, 31, 48) to compare the level of induction in noninduced and induced states (UV-mediated DNA damage, *lexA* Ind$^-$ versus *lexA* Def strains, and mitomycin C-mediated DNA damage). Another successful strategy has been the in silico identification of LexA-regulated genes based on algorithms devoted to the identification of the SOS operator and/or the search of the genome sequence for promoters containing the conserved motif. This strategy helped to expand the array of *E. coli* genes known to be SOS regulated (21, 33). The use of this type of approach has prompted the in silico characterization of the SOS regulatory circuit in several bacterial species (16, 18, 19) and is constantly expanding our knowledge about the SOS response in models other than *E. coli*. Nevertheless, most of the in silico analyses of SOS regulons done to date lack extensive biological validation, making the assumptions of gene content less robust and missing important information about differential levels of SOS induction for different genes.

The characterization of the SOS response in other bacteria led to the conclusion that the *E. coli* SOS genes cannot be assumed to be universally LexA regulated in all bacterial species. In the deltaproteobacteria *Bdellovibrio bacteriovorus*, for example, some genes considered to be part of the core of the proteobacterial SOS genes (*recA*, *uvrA*, *ruvCAB*, and *ssb*) are not repressed by the LexA protein (9). Likewise, dissection of the SOS regulon in other species may show different genes involved in the DNA damage response which are not present

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
NA1000	Parental strain, *C. crescentus* CB15 derivative	20
lexA	NA1000 (Δ*lexA*)	This study
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pNPTS138	pNPTS129 derivative, *oriT sacB* Kan^r	61
pMR20	Broad-host-range, low copy vector, Tet^r	52
pRKlacZ290	pRK2-derived vector with a promoterless *lacZ* gene, Tet^r	26
pP3213	*imuA* promoter cloned in the pLacZ290 vector	25
pP3213Oc	*imuA* promoter fragment containing the O^c mutation in the SOS operator, cloned in the pLacZ290 vector	This study
pPLEXA	*lexA* promoter cloned in the pRKlacZ290	This study
pLEXDEL	In-frame deletion of the *lexA* gene and flanking regions cloned in pNPTS138	This study
pMRLEXA	*lexA* gene plus promoter region cloned in the pMR20 vector	This study

in the *E. coli* genome. Recently, we have demonstrated that a three-gene operon encoding a hypothetical protein (ImuA), a protein similar to members of the Y family of DNA polymerases (ImuB), and a second copy of *dnaE* (DnaE2) is responsible for the DNA damage-inducible mutagenesis in *Caulobacter crescentus*, a function performed by the UmuDC proteins in *E. coli*. (25). This SOS-regulated operon is widespread in *Bacteria* (1) and was recently shown to be part of the SOS-mediated response to the antibiotic ciprofloxacin in *Pseudomonas aeruginosa* (11), demonstrating the relevance of expanding the knowledge about the SOS regulatory circuit in other models.

In the present work, we report the characterization of the SOS regulon of *Caulobacter crescentus.* We have performed a computational screening for the LexA binding motif in the whole genome of this organism and found that the SOS operator is in good agreement with the direct repeat $GTTCN_7G$ TTC, the same repeat suggested previously to be universal among alphaproteobacteria (19). We have confirmed the functionality of this operator with site-directed mutagenesis experiments of the LexA-regulated *imuA* promoter and by mapping its position relative to the actual transcriptional start site of two SOS genes. The expression of several genes identified in silico as potentially SOS regulated, by means of the presence of the operator in their putative regulatory regions, was investigated. The assays were performed by comparing the levels of expression of the selected genes in a *C. crescentus lexA* strain and its parental counterpart. We have been able to identify some unreported genes that are part of the SOS regulon, as well as confirm the inducibility of previously described LexA-regulated genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used in this study are shown in Table 1; for a list of all primers cited in this article, see Table S1 in the supplemental material. The *C. crescentus* strains were grown in PYE medium (17) at 30°C with constant shaking. Plasmids were introduced into *C. crescentus* by conjugation with *E. coli* strain S17-1. When appropriate, the culture medium was supplemented with kanamycin (50 μg/ml), nalidixic acid (25 μg/ml), spectinomycin (50 μg/ml), or tetracycline (2 μg/ml). *E. coli* strain DH10B (Invitrogen, CA) was used for cloning purposes. The *E. coli* strain was grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml), when necessary.

The construction of gene-targeting plasmids was performed with PCR products amplified with the appropriate primers. These products were cloned in the pGEM-T Easy vector (Promega) and sequenced to ensure sequence integrity. For the disruption of *lexA*, fragments were amplified with primers lexA5ext2 and lexA5int and primers lexA3int and lexA3ext and fused in the pNPTS138 vector by use of the restriction sites introduced in the oligonucleotides. This strategy generated a 1,824-bp fragment containing the first 96 bp fused to the last 42 bp of *lexA* plus flanking regions, leading to a 600-bp in-frame deletion of *lexA.* The resulting plasmid, pLEXDEL, was introduced into *C. crescentus* NA1000 by conjugation with *E. coli* S17-1. Genetic disruption was achieved by two consecutive recombination events. The vector contains the *nptI* gene, conferring kanamycin resistance, and the *sacB* gene, conferring sucrose sensitivity. First, Kan^r conjugants of *C. crescentus* were selected in the screening for plasmid integration. The loss of the plasmid after the second recombination was selected in PYE media containing 3% sucrose. Strains generated in this way were analyzed by diagnostic PCR to confirm gene disruptions.

In order to promote complementation of the phenotypes of the *lexA* strain, a full-length gene, including the promoter region, was amplified with the lexA5ext2 and lexAre oligonucleotides and cloned in the low-copy-number pMR20 vector, using the restriction sites introduced in the primers. The resulting fragment contains 1,481 bp and includes the whole *lexA* gene plus 597 bp before the transcription initiation codon and 146 bp after the gene stop codon.

Growth determination experiments. Overnight cultures of the *C. crescentus* strains were diluted to an initial optical density at 600 nm (OD_{600}) of 0.1 in PYE medium and incubated with constant shaking at 30°C. At each indicated time interval, two aliquots of the cultures were removed. The first one was utilized for the optical density determination, and the other one was subjected to serial dilutions and plated on solid PYE medium for CFU counting, which was carried out 2 days after the plating.

In silico analysis. Ab initio searches for overrepresented DNA motifs were done with the Gibbs Motif Sampler program (60) in regions from −250 bp to +50 bp of the predicted start codons of the current set of known LexA-regulated genes in *C. crescentus.* A motif width of 19 bp was used as a parameter in these searches to account for the previously reported 15-bp alphaproteobacterial LexA binding motif (19) and to allow for additional conserved positions in its vicinity. A position weight matrix derived from the most abundant motif found in each search was used to score candidate LexA binding sites. This matrix is given by $m_{bi} = -ln\ (f_{bi}/p_b)$, where f_{bi} is the frequency of the base b in position i of the motif and p_b is the frequency of base b in the whole genome (55). Genome sequence and gene annotations were obtained from the TIGR database (http://www.tigr.org/cmr/).

Real-time analysis of gene expression. The relative expression levels of the genes belonging to the SOS regulon in *C. crescentus* were determined by quantitative reverse transcription (RT)-PCR experiments comparing the level of expression in the wild-type strain with that of the strain containing the *lexA* gene disruption. RNA from exponentially growing cells was extracted with Trizol reagent (Invitrogen) and treated with DNase I to eliminate contaminant DNA. An aliquot of 2 μg of RNA pretreated with DNase I was used as a template for total cDNA synthesis in 20-μl reaction mixtures with random hexamers by use of the Superscript First-Strand synthesis system for RT-PCR (Invitrogen). For

quantitative PCR, an amount of cDNA corresponding to 25 ng of input RNA was used in each reaction. Reactions were performed with the Sybr green PCR master mix (Applied Biosystems) and analyzed in the ABI 7500 real-time system. Relative expression levels were calculated as described previously (47), using the *rho* gene as an endogenous control.

Site-directed mutagenesis of the *imuA* promoter and β-galactosidase assays. PCR-mediated mutagenesis of the *imuA* promoter was accomplished using primers with the desired mutation. The introduced mutations led to two base substitutions in P*imuA*. Two complementary mutagenic primers (see Table S1 in the supplemental material) were used with external primers to amplify fragments of P*imuA* containing the desired mutation. Both fragments were used in a further reaction with only the external primers to generate full-length fragments containing the mutation. All the PCRs were carried out with the high-fidelity Pfx enzyme (Invitrogen), using the cloned P*imuA* (pP3213) (25) as a template. After the full-length mutant promoter fragment was produced, it was subcloned in the pGEM-T Easy vector (Promega) and sequenced to ensure that the correct mutation was introduced and that no additional mutations were generated during amplification. The fragment was then subcloned in the pRKLacZ290 plasmid to create a transcriptional fusion with *lacZ*. Measurements of promoter activity with *lacZ* transcriptional fusions were performed as described previously (25), both before and 60 min after UV irradiation in exponentially growing cells in PYE medium.

UV irradiation. All the cell irradiations were carried out in rich medium (PYE). A germicidal lamp that preferentially emits UVC (254 nm, dose rate of 3.26 J/m^2/s) was used. The UV dose was monitored by a VLX 3W radiometer with a CX-254 monochromatic sensor (Vilber Lourmat, Marne-la-Vallée, France). A UVC dose of 45 J/m^2 was used in the β-galactosidase assays described above. This dose was shown previously to yield about 6,000 β-galactosidase units in a *lacZ* gene fusion with the *imuA* gene promoter in a wild-type background (25).

Determination of transcriptional start sites by 5′ RACE experiments. RNA was extracted from exponentially growing *lexA* cells with the Trizol reagent. 5′ rapid amplification of cDNA ends (RACE) experiments using the 5′ RACE system (Invitrogen) were conducted as follows: three primers (GSP1 to GSP3) were designed for each gene. GSP1 was used for gene-specific cDNA synthesis. A poly(T) tail was added to the 5′ end of the cDNA with terminal deoxynucleotidyl transferase (Tdt). The tailing reactions were used as templates for PCR with the primers GSP2 and 3′ RACE. A nested PCR was performed to increase specificity, using primers GSP3 and AUAP, and the amplification products were cloned in the pGEM-T Easy vector (Promega). Nine clones were sequenced for the CC_2272 5′ RACE reactions, and 10 for the *imuA* 5′ RACE reactions. The transcriptional start site was then identified as the base adjacent to the poly(T) tail represented more among the sequenced clones, which meant 70% of the clones for *imuA* and 66.7% of the clones for CC_2272.

RESULTS

Construction of a *lexA*-deficient strain of *Caulobacter crescentus*. The open reading frame (ORF) CC_1902 was annotated in the *C. crescentus* genome as *lexA*. BLAST analysis using the *E. coli* LexA protein as a probe showed the gene product of this ORF as the single significant hit, confirming its annotation (E value of 8.9e-25). A strain containing an internal deletion of 600 base pairs in the *lexA* gene was constructed by double recombination (61). The deletion of this gene disruption caused a severe growth defect in the *lexA* strain in rich medium at 30°C. Its growth rate was approximately half of that observed for the wild-type NA1000 strain (generation time of about 240 min, contrasted with the 120 min observed for NA1000) (Fig. 1A). A decrease in cell viability in the *lexA* strain was observed compared to the wild-type strain (Fig. 1B); although both cultures started from the same OD_{600}, the *lexA* strain always exhibited fewer CFU per ml. When observed under light microscopy, the cells exhibited a filamentous aspect, as expected for *lexA* null strains (Fig. 1C and D). In *E. coli*, an SOS-induced filamentation is mediated by the *sulA* gene product, which inhibits cell division by blocking FtsZ ring formation (4, 54). *lexA* knockouts are not viable in *E. coli*, unless a *sulA* mutation is present. As mentioned above, although viable, the *C. crescentus lexA* mutant presents a severe filamentous phenotype. Interestingly, *sulA* homologs are not present in *C. crescentus*, as noted previously (25), indicating that a distinct mechanism of a divisional checkpoint might be responsible for the SOS-mediated cell division suppression in this organism. Although this mutant strain could have accumulated suppressor mutations which might have allowed its survival, we were able to consistently obtain independent *lexA* knockouts, suggesting that this gene is not essential in *C. crescentus.* The filamentous phenotype of the *lexA* strain is complemented by a low copy vector expressing the wild-type *lexA* gene (Fig. 1E), indicating that no additional mutations are responsible for this phenotype of the *lexA* strain.

In silico determination of the LexA operator and identification of the SOS regulon of *C. crescentus*. We adopted an iterative approach, combining the in silico prediction of LexA binding sites and gene expression measurements to successively augment the set of known LexA-regulated genes and improve the current LexA binding site model. Each iteration consisted of the following steps. (i) The promoter regions of the current set of known LexA-regulated genes are used in an ab initio search for overrepresented DNA motifs with the Gibbs Motif Sampler program (60). The set of genes used in the first iteration consisted of *lexA*, *recA*, and *recN*, whose orthologs are part of the SOS regulon in several different bacterial species, and also *imuA* and *uvrA*, which are known to be LexA regulated in *C. crescentus* (25). (ii) The position-specific nucleotide frequency matrix corresponding to the most abundant motif found in step 1 is taken as the current LexA box model and is used to scan the *C. crescentus* genome sequence in search of candidate binding sites. (iii) The expression levels of genes immediately downstream of high-score sites are measured in the *lexA* and wild-type strains by real-time PCR experiments. Genes whose expression levels changed at least twofold in the *lexA* strain are added to the set of LexA-regulated genes and are used in step 1 of the next iteration.

After a number of iterations of the procedure described above, we obtained the set of genes regulated by LexA shown in Table 2. The promoter regions of the 32 genes that have a twofold expression change in the *lexA* strain and that are in the first position of the respective putative operon were used in the construction of the final LexA binding site model, whose logo (14) is shown in Fig. 2. See Fig. S1 in the supplemental material for the genomic regions comprising the highest scoring putative LexA sites, obtained in a whole-genome scan of the final box model.

As shown in Fig. 2, the LexA binding site model is in good agreement with previously described alphaproteobacterial SOS box models, namely the pattern $GTTCN_7GTTC$ and the matrix model obtained previously (19). However, due to the larger number of sequences used in model construction, it was possible to detect new positions in the box with a partial degree of conservation, as in the vicinity of each GTTC block shown in Fig. 2. Such positions may also contribute to the binding of LexA.

Gene expression analysis of the SOS regulon. To ascertain which of the genes identified in silico as potentially belonging to the SOS regulon were actually regulated by LexA in vivo, we

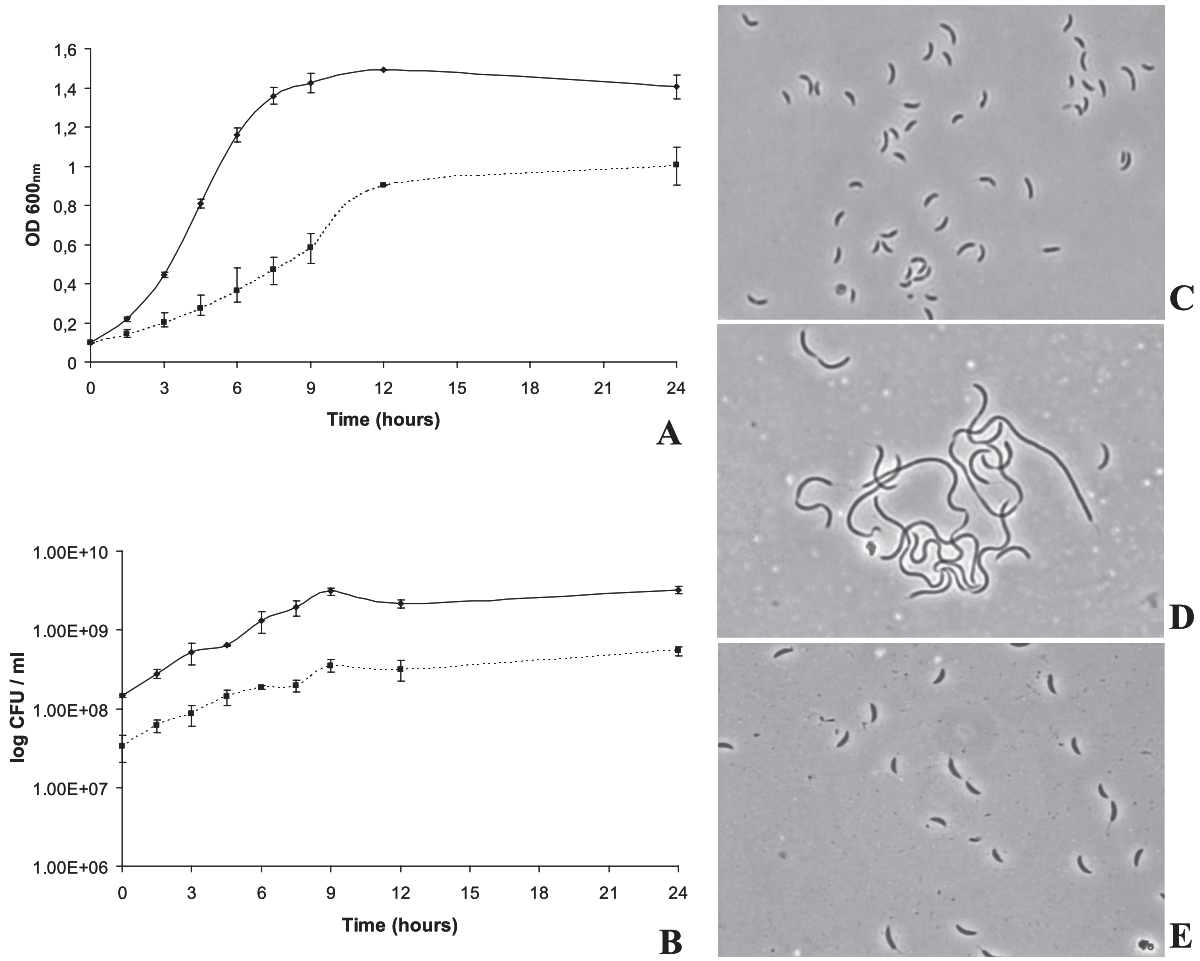

FIG. 1. Phenotypic characterization of the *C. crescentus lexA* strain. (A and B) Growth curve of the wild-type NA1000 and *lexA* strains. Cells were grown in PYE medium at 30°C and monitored for up to 9 h (CFU counting) and 24 h (OD_{600}). OD_{600} determination (A) and CFU determination (B). Error bars indicate standard errors. Solid lines, wild-type NA1000 strain; dotted lines, the *lexA* strain. (C to E) Light microscopy of *C. crescentus* strains using a 100× objective. Wild-type NA1000 strain (C), the *lexA* strain (D), and the *lexA* strain after genotypic complementation with a low-copy-number vector containing the wild-type allele of *lexA* (E).

conducted quantitative RT-PCR assays during each of the iteration steps. The assays were performed by measuring the relative expression levels of the selected genes in the *lexA* null strain versus the wild-type strain. We used as the endogenous control the *rho* gene, which encodes the transcription terminator Rho and is not induced in a LexA-dependent fashion, as determined by β-galactosidase assays (data not shown).

We performed the quantitative RT-PCR assays for 44 genes (Table 2). Among these, there were four putative operons, for which we tested all the genes possibly cotranscribed (CC_1926-CC_1927, CC_2332-CC_2333, CC_2879-CC_2880-CC_2881, and CC_3238-CC_3237-CC_3236, the *ruvCAB* operon). On the other hand, when an SOS operator was found in the noncoding region between two divergently transcribed genes, we performed the assay for both of them in order to establish if the same box could repress both promoters. This was the case for genes CC_1086 and CC_1087, CC_1531 and CC_1532, CC_1927 and CC_1928, CC_2589 and CC_2590, CC_2878.1 and CC_2879, CC_3038 and CC_3039, and CC_0382 and CC_0383 (Table 2; see Fig. S1 in the supplemental material). Among the divergently transcribed genes, we found that CC_3039 and the putative operon CC_3036-CC_3037-CC_3038, CC_1531 and CC_1532, and CC_0382 and CC_0383 were induced in a *lexA* null background. Some of the putative operons (CC_2332-CC_2333, CC_2879-CC_2880, CC_3036-CC_3037-CC_3038, and the *ruvCAB* operon) had similar variations of transcriptional levels, supporting the idea that they are arranged in operons that are part of the SOS regulon. In other cases, some of the putative operons presented clearly different levels of transcript (CC_1926 and CC_1927; CC_2879 and CC_2881), suggesting that they are not transcribed as operons.

In total, of the 44 genes tested, 35 showed increased expression in the *lexA* strain (Table 2), exhibiting at least twofold increased expression in the *lexA* strain compared to the wild type. Among them, there were some genes known to be part of the canonical SOS response in other proteobacteria, such as *recA* (CC_1087), *lexA* (CC_1902), *uvrA* (CC_2590), *ssb* (CC_1468), and *recN* (CC_1983). The *ruvCAB* operon was proposed to be part of the SOS regulon in all alphaproteobac-

TABLE 2. Genes identified by in silico analyses as belonging to the SOS regulon of *Caulobacter crescentus* and comparison of in vivo expression of these genes in the *lexA* and wild-type strains

ORF[c]	Gene name/annotation	Box or description	Strand[a]	Box score	Box position[b]	Relative expression level (*lexA* mutant/wild-type)
CC_1902*	*lexA*	AATGTTCTCCTGGTGTTCC	−	14.3	−51	43.2 ± 9.3
CC_0627*	Hypothetical protein	AAAGTTCGCGTTATGTTCT	−	18.8	−9	40.3 ± 11.8
CC_3467*	Conserved hypothetical protein	CATGTTCCAGCTTTGTTCG	−	13.6	−20	37.5 ± 10.5
CC_3518*	Conserved hypothetical protein	GATGTTCATGTATTGTTCT	+	18.8	−3	27.4 ± 4.8
CC_2332*	Conserved hypothetical protein	ATCGTTCTTGATTTGTTCT	−	18.7	−13	18.8 ± 6.8
CC_2333	Uracil DNA glycosylase-related protein	Putative operon with CC_2332				8.3 ± 1.2
CC_1926	*dnaE*/DNA polymerase III, alpha subunit	CATATTCCGGTTTTGTTCT	−	16.4	−165	1.6 ± 0.6
		AGATTTCTTGTTTTGTTCC	−	11.4	−181	
		TCTGTTCACAAGATGTTCC	−	11.1	−147	
CC_1927*	Hypothetical protein	Putative operon with CC_1926				17.7 ± 6.2
CC_3213*	*imuA*/inducible mutagenesis protein A; in operon with ImuB, a Y family polymerase, and DnaE2, a C family DNA polymerase	CATGTTCCACTTTTGTTCT	−	17.9	−73	16.2 ± 4.7
CC_2272*	Endonuclease III family protein	AATGTTCTTGTTATGTTCT	+	23.2	−26	14.7 ± 3.1
CC_3424*	Conserved hypothetical protein	AATGTTCCTGAATTGTTCT	−	20.7	−26	13.62 ± 5.5
CC_1330*	Radical SAM domain protein	TATGTTCTTGTTATGTTCG	+	20.6	−33	11.7 ± 3
CC_1054*	Hypothetical protein	TTTGTTCTCGGCTTGTTCT	+	16.3	−3	11.3 ± 5.5
CC_2040*	ATP-dependent RNA helicase, DEAD/DEAH family	CATGTTCCCTTTCTGTTTC	−	14.0	−24	9.1 ± 3.4
CC_1087*	*recA*/DNA recombination protein A	CATGTTCGCAAGATGTTCC	−	15.5	−114	9.0 ± 1.5
CC_2879*	Hypothetical protein	CATGTTCTGACTATGTTCC	−	14.3	56	8.0 ± 1.6
CC_2880*	Hypothetical protein	Putative operon with CC_2879				9.74 ± 3.7
CC_2881	*uvrC*/exinuclease ABC, subunit C	Putative operon with CC_2879				1.7 ± 0.3
CC_3038*	Conserved hypothetical protein	AATGTTCCTATAATGTTCT	+	21.5	−160	5.3 ± 0.8
CC_3037	Conserved hypothetical protein	Putative operon with CC_3038				7.7 ± 1.9
CC_3036	Hypothetical protein	Putative operon with CC_3038				7.3 ± 1.2
CC_3039*	Hypothetical protein	AATGTTCCTATAATGTTCT	−	21.5	−159	4.4 ± 0.2
CC_3356*	Hypothetical protein	CATGTTCTCGTATTGTTCG	−	18.1	−52	6.3 ± 2.5
CC_1531*	Hypothetical protein	ATTGTTCTTGATATGTTCC	+	20.2	−31	5.9 ± 1.1
		TATGTTCCAACTTCGTTTG		11.3	−20	
CC_1983*	*recN*	GATGATCCCGTTTCGTTCC	+	11.9	−56	5.6 ± 1.6
CC_0140*	*comM*/competence protein ComM	AACGTTCGTTTTTCGTTCT	+	15.7	−72	4.7 ± 0.1
CC_0383*	Hypothetical protein	TATGTTCCTGAAAAGTTCT	−	18.5	−14	5.0 ± 2.7
CC_3238*	*ruvC*	CGCGTTCATCATGTGTTCT	−	10.4	2	5.1 ± 1.9
CC_3237	*ruvA*	Putative operon with CC_3238				5.0 ± 0.8
CC_3236	*ruvB*	Putative operon with CC_3238				3.4 ± 0.4
CC_3225*	Sensory box sensor histidine kinase/response regulator	TTTGTTCGCCAGATTTTTT	+	12.9	8	4.8 ± 1.9
CC_0382*	*tag*/DNA-methyladenine glycosylase I	TATGTTCCTGAAAAGTTCT	+	18.5	−44	3.4 ± 1.2
CC_2590*	*uvrA*/exinuclease ABC, subunit A	TTTGTTCGCATCTTGTTCT	+	17.7	−87	3.5 ± 1.0
		CTTGTTCTCGCGACGTTCG	−	10.6	−268	
CC_1532*	Conserved hypothetical protein	ATTGTTCTTGATATGTTCC	−	20.2	+32	3.1 ± 0.7
		TATGTTCCAACTTCGTTTG		11.3	+21	

Continued on following page

TABLE 2—*Continued*

ORF[c]	Gene name/annotation	Box or description	Strand[a]	Box score	Box position[b]	Relative expression level (*lexA* mutant/wild-type)
CC_3515*	Conserved hypothetical protein	AGAGTTCGCATTATGTTCT	+	15.7	−79	3.1 ± 0.6
CC_1468*	*ssb*/single-stranded binding protein	TTTGTTCTCATAACGTTCT	−	18.6	−93	2.1 ± 1.0
CC_3130*	Glutamine synthetase family protein	TTTGTTCTCGAAAGGTTTC	−	14.4	−52	2.1 ± 0.9
		GTTTTTCCGGATTTGTTCT	−	11.7	−41	
CC_2878.1	Conserved hypothetical protein	CATGTTCTGACTATGTTCC	+	14.3	−55	1.4 ± 0.7
CC_2589	Hypothetical protein	TTTGTTCGCATCTTGTTCT	−	17.7	−154	0.9 ± 0.3
		CTTGTTCTCGCGACGTTCG	+	10.6	+27	
CC_1928	Inosine-uridine-preferring nucleoside hydrolase	CATATTCCGGTTTTGTTCT	+	16.4	−126	0.8 ± 0.2
		AGATTTCTTGTTTTGTTCC	+	11.4	−110	
		TCTGTTCACAAGATGTTCC	+	11.1	−144	
CC_1086	Sensory box protein	CATGTTCGCAAGATGTTCC	+	15.5	−114	0.8 ± 0.2
CC_3214	Carbamoyl-phosphate synthase/carboxyl transferase	CATGTTCCACTTTTGTTCT	+	17.9	−103	0.6 ± 0.5
CC_1665*	*dnaB*/replicative DNA helicase	GATGTTCTGTGTATGTTTT	−	14.5	−73	0.3 ± 0.1
CC_2433*	Conserved hypothetical protein	ATTATTTTCATTATGTTTT	+	16.5	−105	0.1 ± 0.0

[a] + indicates the template strand, and − indicates the complementary strand.
[b] The box position is relative to the annotated start codon.
[c] An asterisk indicates a gene that was used in the construction of the model shown in Fig. 2.

teria (19), and all three genes (CC_3238, CC_3237, and CC_3236) are in fact induced in the *lexA* null background. We also observed that *imuA*, the first gene in the operon devoted to mutagenic DNA repair in *C. crescentus*, is among the most highly expressed in the *lexA* strain, confirming our previous results (25). Some of the genes identified in the approach have putative functions related to DNA metabolism, such as CC_2272 (encoding an endonuclease III family protein), CC_0382 (*tag* gene, DNA methyladenine glycosylase I), CC_2332 and CC_1330 (which exhibit a photolyase domain), and CC_3518 (UvrC-like domain). Most of the genes encode proteins of previously uncharacterized function, here determined for the first time to be involved in the proteobacterial DNA damage response. One of the surprises of this study was the finding that *dnaB* (a replicative helicase) and the ORF CC_2433 (encoding a conserved hypothetical protein) were downregulated in the strain containing the *lexA* deletion, in spite of containing SOS boxes with significant scores. This is indicative of a positive direct or indirect regulation of these genes by the LexA protein, a feature that is not commonly found in the SOS regulon of other bacteria, and highlights the importance of gene expression data in the validation of in silico predictions.

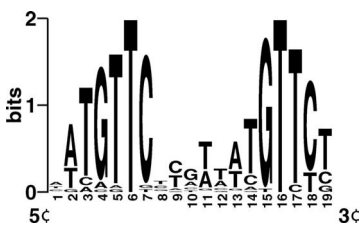

FIG. 2. *Caulobacter crescentus* LexA binding site model. The sequence logo was generated using the WebLogo program (14).

In our analysis, we did not observe any significant correlation between the box score and the relative expression obtained from the quantitative RT-PCR experiments (Table 2). This is an indication that, at least in *C. crescentus*, the level of repression of the LexA-regulated genes does not directly reflect the similarity of their SOS operator to the consensus pattern. Other factors, like intrinsic promoter strength and the positioning of the operator relative to sigma factor binding sites, are likely to contribute to the final level of induction.

In vivo functionality of the SOS operator. The in silico analysis identified the model shown in Fig. 2 as the potential LexA binding site in *C. crescentus*, in good agreement with previous studies in several alphaproteobacteria (19). A high-score candidate binding site (Table 2; see Fig. S1 in the supplemental material) is present in the promoter region of the *imuA* gene, the first gene of an operon involved in mutagenic DNA repair in *C. crescentus* (25). In order to determine the role of the identified operator in the regulation of P*imuA*, we have conducted site-directed mutagenesis in the promoter region previously shown to drive high levels of expression after UV irradiation. We have introduced two base substitutions in the potential LexA binding site, as shown in Fig. 3, creating an operator-constitutive (O^c) mutant for the *imuA* promoter. The activities of both the wild- type and the mutagenized promoters (P*imuA* and P*imuA*O^c) were measured in transcriptional fusions with the *lacZ* gene, both in the wild-type and *lexA* strains. As shown before, P*imuA* is highly UV inducible. Remarkably, the basal levels of transcription of P*imuA*O^c are much higher than those observed for the wild-type promoter (~10-fold), showing that the operator identified in silico contributes significantly to the repression of *imuA* expression under physiological conditions (Fig. 3). It is also interesting to note that the levels of β-galactosidase expression achieved with P*imuA*O^c in nonirradiated cells are even higher than those observed when

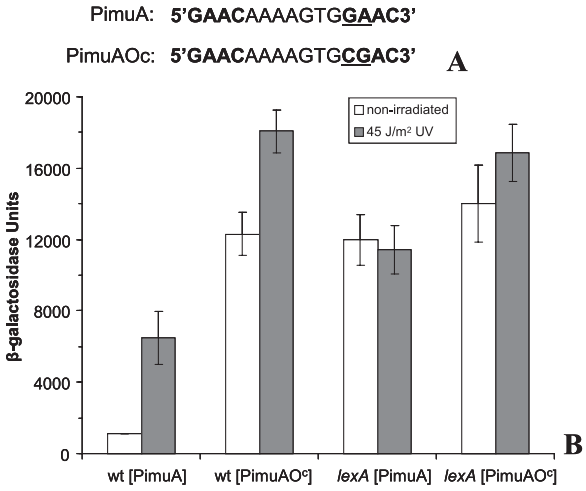

FIG. 3. Site-directed mutagenesis of the *imuA* promoter and analysis of promoter activity in β-galactosidase assays. (A) The SOS box in the promoter of the *imuA* gene is shown, with the conserved, directed repeats shown in bold. The underlined bases were altered in P*imuA*O^c, eliminating the directed repeat. (B) The chart shows the average of three β-galactosidase assays performed with the wild-type (wt) and *lexA* strains with plasmids pP3213 and pP3213Oc, containing P*imuA* and P*imuA*O^c, respectively. Induction of the SOS response was achieved by the irradiation of cells with 45 J/m^2 of UVC.

cells carrying the wild-type promoter fusion are exposed to 45 J/m^2 of UV light. Nonetheless, transcription from P*imuA*O^c is still slightly stimulated by UV light, suggesting that residual binding of the repressor may still occur. These results confirm that the SOS operator identified in silico in this study and previously by others (19) is indeed responsible for the repression of the SOS genes in *C. crescentus*. In the *lexA* strain, both P*imuA* and P*imuA*O^c show similar levels of basal transcriptional activity, which is not significantly modified after UV irradiation of cells.

After the demonstration of the function of the SOS operator in vivo, we determined the positioning of this sequence relative to the RNA polymerase binding sites in the promoters of *imuA* and the endonuclease III glycosylase-related gene CC_2272, another tightly SOS-regulated gene (Table 2). For that purpose, 5′ RACE experiments were performed to determine the transcriptional start site of these two SOS genes. As shown in Fig. 4, the transcriptional start site for the *imuA* gene was determined to be located 71 bases upstream from the annotated start codon of this ORF and 23 to 25 bases upstream from the ATG in the case of the CC_2272 gene. In the latter, it was impossible to precisely determine the start site, given that the primer used in the 5′ RACE experiments contains a run of T's (see Material and Methods) and that the 5′ RACE products start with the sequence $T_{(13\text{-}20)}$ATGTTCTCG. Thus, the starting site for the CC_2272 gene can be any of the three bases marked in Fig. 4. Considering these transcriptional start sites, we identified the regions with similarity to the −10 and −35 consensus sequences for the vegetative sigma factor of *C. crescentus* (34). Remarkably, the SOS operator overlaps the transcriptional start site and the end of the −10 region in both promoters, even with the three-base imprecision in mapping the transcriptional start site of the gene CC_2272. The positioning of the operators could clearly impose a block to RNA polymerase access and/or promoter unwinding upon LexA binding, showing that the position of the identified operators is consistent with the proposed function.

5' Race CC2272:

AGCCTGTGGACAAACCTCGACGCTGAGCATAATGTTCTTGTTATGTTCTCGCGCGGAGA
GGTTCCATGCAGCTGTCCCTAGCCCTGGCCCGGTCACCGCTGGAAAGCGTGCGGGACGC

5' Race *imuA*:

CAAGCCTTGACTCTCCAGTCCGGGAAGATCAGAACAAAAGTGGAACATGGAGTTGGGAA
TGGCCGGATCGCGCGAGGCGCGTCTTGCGGGCCCTGAGAGGCCGGATCGCCGCGATGGAA
GCGGGGACTCGGACTCCGACTCCGGTGCTGTCGTTCGGAGAGCCGTCGATCGACGGGTG

Consensus sigma 70: TTGaCgS $N_{(16\text{-}14)}$ GCtANAWC $N_{(5\text{-}7)}$ +1

FIG. 4. Determination of the transcriptional start sites of *imuA* and CC_2272. The sequence around the predicted transcriptional start site is shown, with the coding sequence highlighted in bold. The transcriptional start sites are shown inside the black boxes and in italics. The SOS box is shown in gray shading, and the conserved −35 and −10 sequences are underlined. The consensus promoter for the vegetative sigma factor (34) is shown at the bottom.

DISCUSSION

In the present work, the SOS regulon of *Caulobacter crescentus* was elucidated by the combined use of in silico analysis and quantitative RT-PCR assays. We were able to identify 35 genes that are up-regulated in the *lexA* strain and that are thus part of the SOS regulon in this organism. A previous survey of DNA repair-related genes in *C. crescentus* identified many interesting features of this bacterium concerning the maintenance of genome integrity (35), and this work expanded these studies, revealing genes that are involved in the DNA damage response.

We have confirmed the motif GTTCN$_7$GTTC as the SOS operator in this organism, in good agreement with previous reports (19). In the *imuA* promoter, this sequence was shown to be responsible for a high level of repression, as determined by the site-directed mutagenesis experiments. Furthermore, this repression was shown to be mediated by LexA, since the operator is not functional in the *lexA* strain.

C. crescentus displays the core of SOS-regulated genes in proteobacteria, which is comprised of *recA*, *lexA*, *ssb*, *uvrA*, and *ruvCAB* (19). It is important to highlight that the most induced gene in this analysis was *lexA*, the negative regulator of the regulon. Among the genes shown to be part of the SOS regulon in this bacterium, some are interesting and reveal potentially new pathways that may be induced by DNA damage.

The CC_2272 gene encodes an endonuclease III family DNA glycosylase, responsible for the removal of pyrimidine adducts other than dimers and (6-4) photoproducts, the most representative of pyrimidine lesions created by UV irradiation (24). Another important gene is CC_0382, which encodes the DNA-methyladenine glycosylase I gene (*tag*). This protein catalyzes the removal of the cytotoxic lesion 3-methyladenine (3meA), induced by alkylation damage (41). To our knowledge, the induction of these two genes represents the first evidence of base excision repair genes regulated by the SOS response. The case of the *tag* gene is interesting if we consider that bacteria usually possess two DNA-methyladenine glycosylases, Tag and AlkA. The latter is described as being induced

by sublethal doses of alkylating agents, while *tag* is constitutively expressed in *E. coli* (5). The regulation of *tag* by the SOS response adds a new layer of complexity in its regulation and in the removal of 3meA from the genome. Further examination of the SOS regulon in other bacterial species will reveal if this is a specific feature of *C. crescentus* physiology or a more widespread phenomenon.

Some other genes of unknown function, but which are potentially involved in DNA repair activities, were shown to be SOS regulated in *C. crescentus*. The CC_3518 gene encodes a protein with high similarity to the N-terminal end of UvrC endonucleases. In *E. coli*, the gene *cho* (*ydjQ*) encodes a 295-amino-acid protein with similarity to the N terminus of UvrC (33, 43, 62). Interestingly, the protein encoded by CC_3518, one of the most strongly SOS-upregulated genes in *C. crescentus*, also bears similarity to UvrC, although it is even smaller than Cho, consisting of only 123 amino acids. A BLAST analysis reveals that CC_3518 orthologs, with similar size, are widespread among bacteria. Another interesting family of genes includes CC_2332 and CC_1330 (35). They are both similar to the radical *S*-adenosylmethionine superfamily of proteins, which includes the *splB* gene of *Bacillus subtilis* that encodes the spore photoproduct photolyase. Possibly, these genes represent a different class of SOS-regulated bacterial photolyase genes. Interestingly, the *Mycobacterium tuberculosis* gene Rv2578c encodes a homolog of CC_2332 and CC_1330 and has been shown to be part of the SOS regulon in that organism (16), further supporting a role for these genes in the DNA damage response. CC_2332 is in a putative operon with another SOS-regulated gene, CC_2333, which is related to another base excision repair-related protein, uracil-DNA glycosylase (35).

On the other hand, *dnaB* and CC_2433 were consistently down-regulated in the *lexA* strain, suggesting that they are actually repressed upon SOS induction. For *Rhodobacter sphaeroides*, LexA has been proposed to both repress and activate transcription of the *recA* promoter (59). It is possible that in these two genes, LexA may act as a transcriptional activator. Since the *lexA* strain is severely affected in cell division and growth, the down-regulation of these genes might just reflect a pleiotropic effect of this gene disruption. However, it is interesting to note that the operators identified in both promoters are corrupted in the direct repeat, lacking one or both of the GTTC units (Table 2).

The case of *dnaB* is especially interesting, given that in previous reports it was shown to be damage inducible independently of RecA in *E. coli* (32); in *C. crescentus*, however, this gene is down-regulated in a LexA-dependent fashion. The implications of this fact are not completely clear. Considering the complex and coordinated cell cycle of *C. crescentus*, it would not be surprising to find a gene involved in replication initiation being repressed in the presence of DNA damage (simulated by the constitutive expression of the SOS-regulated genes in this *lexA* background), since it could act as an additional mechanism ensuring that replication initiation would be allowed only after the lesions are removed.

The gene *comM* (CC_0140), which had already been proposed to be part of the LexA regulon of alphaproteobacteria (19), was also confirmed by this analysis. It was annotated as a Mg^{2+} chelatase in *Brucella suis* and *Brucella melitensis*; thus, this gene could be involved in the regulation of polymerase fidelity synthesis during SOS activation, as has been proposed for members of this class of proteins in *E. coli* (64). In *Haemophilus influenzae*, the ComM protein was discovered on the basis of a transformation-deficient mutant that exhibited normal DNA uptake but possessed low transformation and phage recombination efficiencies (27).

Our analysis identified also some genes with known function that have never been associated with the SOS regulon in other organisms. One of these is CC_3225, which encodes a sensory box histidine kinase/response regulator. Two-component signal transduction systems are known to be the major cellular signaling molecules in prokaryotes. They are usually comprised of a sensor histidine kinase and a response regulator that mediates an appropriate cellular response to an endogenous or exogenous signal (46). The CC_2040 gene encodes an ATP-dependent RNA helicase, a member of the DEAD/DEAH family. *C. crescentus* possesses four members of this family of proteins, but only one seems to be LexA-regulated. These proteins are generally energy motors involved in the conformational change of RNAs, and as such, they are involved in many aspects of RNA metabolism, like transcription, ribosome biogenesis, translation, and RNA degradation (28); the term RNA helicase, however, has to be considered carefully, since for the vast majority of proteins, the exact biochemical activity has not been defined yet (57). We also identified the gene CC_3130, responsible for a glutamine synthetase family protein, as being regulated by LexA in *C. crescentus*. This enzyme is involved in nitrogen assimilation through the incorporation of ammonia in bacteria (49). It is still not clear how these genes would be involved in the celullar response to DNA damage, and thus further analyses are needed.

The results also indicated SOS regulation of a large number of hypothetical proteins, some of which did not display significant similarity to any other known protein in the microbial genomes sequenced so far. Representatives of this class of genes are some with highly increased expression in the *lexA* mutant, like CC_0627 (the second-most induced gene in the *lexA* strain [see Table 2]), CC_1927, CC_3356, CC_1531, CC_1532, CC_0383, and the first gene (CC_2879) of a putative operon of two genes. The CC_3467 gene, which encodes a conserved hypothetical protein related to proteins of unknown function in other bacteria, can also be included in this group. Another class of genes controlled by LexA in *C. crescentus* is that of the putative transcriptional regulators; this analysis identified CC_1054, CC_3036, and CC_3037 belonging to this group. CC_1054 is a member of the CopG transcriptional repressor family, CC_3036 belongs to the AlgR/AgrA/LytR family of transcriptional regulators, and CC_3037 is a helix-turn-helix-containing protein. Therefore, the indirect effects of SOS induction on global gene expression patterns are likely to occur.

Another interesting feature revealed by this work is the potential existence of an SOS-mediated divisional checkpoint in *C. crescentus*. In *E. coli*, the SulA protein inhibits cell division after SOS induction by blocking FtsZ polymerization (4, 54). As a result of this blockage, *lexA* knockouts are not viable, unless *sulA* is also disrupted. In contrast, in *Bacillus subtilis*, *lexA* knockouts are viable, although cells do show a filamentous aspect and poor growth; this filamentation is dependent on the

yneA gene, which is unrelated to *sulA* (30). The *sulA* and *yneA* orthologs are not present in the *C. crescentus* genome (45) or in many other bacterial genomes (data not shown), and it will be of special interest to determine the genes responsible for this *lexA*-dependent divisional checkpoint in *C. crescentus*.

In total, 37 genes were identified as part of the SOS regulon in *C. crescentus*, including 35 genes that were induced and 2 genes that were repressed. Although the regulons in *E. coli* (13) and *B. subtilis* (2) are substantially bigger, the *C. crescentus* regulon contains more genes than the *Pseudomonas aeruginosa* (11) and *Staphylococcus aureus* (12) regulons, which are comprised of 15 and 16 genes, respectively. A table comparing the known SOS regulons of several bacterial species to that of *C. crescentus* is provided (see Table S2 in the supplemental material). Almost all SOS regulons described so far control the *recA* and *lexA* genes, as well as one or more recombination/repair functions and one or more Y family polymerases. We found in this study that *C. crescentus* is not an exception to this rule. However, some exceptions to this fact are known. In the bacteria "*Dehalococcoides ethenogenes*" (22), *Bdellovibrio bacteriovorus* (9), a "*Magnetococcus*" sp. (23), *Petrotoga miotherma* (38), *Acidobacterium capsulatum* (39), *Deinococcus radiodurans* (44), and *Geobacter sulfurreducens* (29), *recA* is not regulated by LexA. There are also *lexA* genes that are not autoregulated, as in *Leptospira interrogans* (15). The most dramatic situation has been found in *Thermotoga maritima*, where neither *lexA* nor *recA* is part of the SOS regulon (38); this is not commonly found in bacteria that have not suffered major genome reduction and ended up losing the *lexA* gene (*Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Campylobacter jejuni*, for example).

In conclusion, this work has further expanded the current knowledge of the prokaryotic responses to DNA damage, revealing a number of new genes which are part of the SOS regulon. These findings are especially relevant in the context of recent efforts focusing on unraveling the SOS response in several different bacteria, since it might play relevant roles in bacterial evolution and the development of antimicrobial resistance (3, 10, 11, 12, 42).

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