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An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*

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ABSTRACT

DNA polymerases of the Y-family, such as *Escherichia coli* UmuC and DinB, are specialized enzymes induced by the SOS response, which bypass lesions allowing the continuation of DNA replication. *umuDC* orthologs are absent in *Caulobacter crescentus* and other bacteria, raising the question about the existence of SOS mutagenesis in these organisms. Here, we report that the *C.crescentus dinB* ortholog is not involved in damage-induced mutagenesis. However, an operon composed of two hypothetical genes and *dnaE2*, encoding a second copy of the catalytic subunit of Pol III, is damage inducible in a *recA*-dependent manner, and is responsible for most ultraviolet (UV) and mitomycin C-induced mutations in *C.crescentus*. The results demonstrate that the three genes are required for the error-prone processing of DNA lesions. The two hypothetical genes were named *imuA* and *imuB*, after *i*nducible *mut*agenesis. ImuB is similar to proteins of the Y-family of polymerases, and possibly cooperates with DnaE2 in lesion bypass. The mutations arising as a consequence of the activity of the *imuAB dnaE2* operon are rather unusual for UV irradiation, including G:C to C:G transversions.

INTRODUCTION

DNA damage-induced mutagenesis is, in a major extent, an active process that requires specialized DNA polymerases able to perform translesion synthesis (TLS). In *Escherichia coli*, three SOS-regulated DNA polymerases are involved in damage tolerance: Pol II, encoded by the *polB* gene, Pol IV and Pol V. The major role of Pol II is the rescue of stalled replication forks (1), although it can bypass some types of DNA damage (2). *dinB* (also called *dinP*) encodes a member of the Y-family of DNA polymerases, DNA Pol IV (3,4). This enzyme is responsible for untargeted SOS mutagenesis of phage λ, and induces frameshift mutations when overexpressed (5). More recently, *dinB* has been associated with the phenomenon of mutation under stress conditions, or adaptive mutagenesis [reviewed in (6)].

In *E.coli*, ultraviolet (UV) and chemical mutagenesis is heavily dependent on the SOS-regulated *umuDC* operon (7,8). The UmuC protein has been identified as a DNA polymerase (Pol V), a distributive enzyme able to perform TLS and highly inaccurate even when using undamaged templates (9,10), which interacts with the processed form of the co-expressed protein UmuD. Both *in vivo* and *in vitro* experiments have shown that efficient TLS requires the $UmuD'_2$ C complex, RecA, Ssb, the catalytic subunit of DNA Pol III encoded by the *dnaE* gene, and the β clamp [reviewed in (11)].

DNA polymerase III is a multiprotein complex responsible for the replication of the bacterial chromosome. Although the Y-family DNA polymerases, specialized in TLS, seem to be ubiquitous throughout nature, it has been shown that the α subunit of Pol III, which bears the catalytic polymerase activity, is also able to perform TLS. In *Bacillus subtilis*, the *dnaE* gene is SOS regulated and the purified enzyme can perform TLS *in vitro*, an activity also shown for the *Streptococcus pyogenes* protein (12,13). Depletion of *B.subtilis* DnaE inhibits UV-induced mutagenesis, although it is difficult to ascertain whether this requirement reflects the need for this enzyme to extend the bypass reaction performed by Y-family DNA polymerases, or results from actual lack of lesion bypass (13).

It has recently been shown that a second copy of the *dnaE* gene mediates SOS mutagenesis in *Mycobacterium tuberculosis* (14). The expression of this gene is regulated by the SOS response in this organism, being induced by

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DNA damaging agents in a *recA*-dependent manner (15,16). The *dnaE* gene is presented in duplicate in several bacterial genomes, most frequently in a potential operon with two hypothetical genes, one of which is a *dinB*-related one. This widespread operon has been shown to be regulated by LexA in *Pseudomonas putida*, indicating that it is involved in DNA damage response (17). This operon is present in several α-proteobacteria genomes completely sequenced to date, including *C.crescentus* (18).

In *C.crescentus*, a *recA*-dependent inducible repair system was demonstrated through the existence of Weigle reactivation of irradiated phage (19). This organism is also mutable by UV radiation, suggesting the existence of error-prone pathways, although *umuDC* orthologs are absent from its genome (18,19). In this work, we investigated the involvement of the *dnaE2*-containing gene cassette and the *dinB* ortholog in damage-induced mutagenesis, detected in rifampicin resistance assays. The results show that the *dnaE2* operon is upregulated in response to DNA damage in a *recA*-dependent manner, and demonstrate that all three genes that compose this expression unit are involved in UV and mitomycin C-induced mutagenesis. On the other hand, *dinB* is not necessary for mutagenic repair. We propose to name the genes co-expressed with *dnaE2*, after *i*nducible *mu*tagenesis, as *imuA* and *imuB*, and show that the *imuAB*/*dnaE2*-dependent mutational spectra of UV-induced lesions is different from the prototypical *E.coli* one, including a high proportion of G:C → C:G transversions.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table 1. *C.crescentus* strains were grown in PYE medium (20) at 30°C with constant shaking. Plasmids were introduced into *C.crescentus* by conjugation with *E.coli* S17-1 strain. When appropriated, the culture medium was supplemented with kanamycin (50 μg/ml), nalidixic acid (25 μg/ml), spectinomycin (50 μg/ml) or tetracycline (1 μg/ml). *E.coli* strain DH10B (Invitrogen, CA) was used for cloning purposes. *E.coli* was grown at 37°C in Luria–Bertani medium supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml) or tetracycline (15 μg/ml) when necessary.

Genetic disruptions were achieved using the pNPTS138 vector, and selecting for two consecutive recombination events. First, Kan^r conjugants of *C.crescentus* were selected in the screening for the first recombination event. Loss of the plasmid after the second recombination was selected in PYE media containing 3% sucrose. When appropriated, a concomitant Spc^r selection was also applied. Strains generated in this way were analyzed by Southern blot to confirm gene disruptions. The construction of gene targeting plasmids was performed with PCR products amplified with the primers shown in Supplementary Table 1S. These products were cloned in the pGEM-T easy vector and sequenced to assure sequence integrity. For disruption of *recA*, fragments amplified with primers recaA × recaB, and recaC × recaD were fused in the pNPTS138 vector using the restriction sites introduced, generating a 1.8 kb fragment containing the first 81 bp fused to the last 75 bp of *recA* plus flanking regions, leading to an in-frame deletion of *recA*. In-frame deletions were constructed in the same way for *imuA* and *imuB* using, respectively, primers CC3213A to D and CC3212A to D. *imuA* disruption resulted in a deletion containing only the first 36 and the last 54 bp of the gene, and *imuB* disruption resulted in a deletion containing only the first 63 and the last 81 bp of the gene. *dnaE2* disruption was performed by the insertion of the Ω spec^r cassette (21) in the natural EcoRI site of a 1629 bp fragment of this

Table 1. Bacterial strains and plasmids used in this study

	Description	Source
Strain		
NA1000	Parental strain, *C.crescentus* CB15 derivative	(49)
GM10	NA1000, (Δ*recA*)	This study
GM20	NA1000, (Δ*imuA*)	This study
GM30	NA1000, (Δ*imuB*)	This study
GM40	NA1000, (*dnaE2*:: Ω)	This study
GM50	NA1000, (*dinB*:: Ω)	This study
GM34	NA1000, (Δ*imuB*, *dnaE2*:: Ω)	This study
GM35	NA1000, (Δ*imuB*, *dinB*:: Ω)	This study
S17.1	*E.coli* strain for plasmid mobilization	(50)
DH10B	*E.coli* strain for cloning purposes	Invitrogen
Plasmid		
pGEM-T easy	Cloning vector	Promega
pNPTS138	pNPTS129 derivative, *oriT sacB* Kan^r	(51)
pMR20	Broad-host-range low copy vector, Tet^r	(52)
pHP45Ω	Ω cassette (Spc^r) containing vector	(21)
pRKlacZ290	pRK2 derived vector with a promoterless *lacZ* gene, Tet^r	(53)
pPUVRA	*uvrA* promoter cloned in the pLACZ290 vector	This study
pP3213	CC3213 promoter cloned in the pLACZ290 vector	This study
pRECDEL	In-frame deletion of *recA* gene and flanking regions cloned in pNPTS138	This study
pDINDEL	*dinB* interrupted with the Ω cassette in the internal EcoRV site, cloned in pNTPS138	This study
pDNAE2DEL	*dnaE2* interrupted with the Ω cassette in the internal EcoRI site, cloned in pNTPS138	This study
p3212DEL	In-frame deletion of CC3212 gene and flanking regions cloned in pNPTS138	This study
p3213DEL	In-frame deletion of CC3213 gene and flanking regions cloned in pNPTS138	This study
pMR3213	CC3213 gene cloned in the pMR20 vector	This study
Cosmid3A4	pLAFR5 containing nt 3 469 882–3 497 288 of the *C.crescentus* genome	(54)

gene, amplified with the dnaE2re and dnaE2fo primers. The insertion in the EcoRI site resulted in a predicted truncation at position 598 of the protein, leading to loss of the last 485 amino acids. *dinB* knock-out was constructed by insertion of the Ω spec^r cassette in the EcoRV site of a 1.2 kb fragment, amplified with primers dinbfo and dinbre. This insertion resulted in a predicted truncation at position 167 of the 419 amino acid DinB protein.

PCRs were also carried out to amplify promoter regions of *uvrA* and *imuA* genes. The primers puvrafo and puvrare were used to obtain a fragment ranging from –492 to +54 of the start codon of *uvrA* [open reading frame (ORF) CC2590]. Primers p3213fo and p3213re amplified a fragment ranging from −513 to +74 of the *imuA* start codon. Both fragments were cloned in the pRKlacZ290 vector using the restriction sites introduced in the primers.

In order to promote complementation of the phenotypes of the *imuA* strain, a full-length gene including the promoter region was amplified with the 3213A and 3213D oligonucleotides, and cloned in the low copy number pMR20 vector, using the restriction sites introduced in the primers.

Survival and mutagenesis experiments

Caulobacter crescentus strains were grown until mid-log phase (5×10^8 cells/ml), and then submitted to the different treatments. A 5 ml batch of culture was UV-irradiated with a Philips TUV 15W/G15T8 germicidal lamp (mainly 254 nm) in Petri dishes. Aliquots were removed before and after irradiation for serial dilutions and plating in PYE medium, for the determination of viable cell counts after 48 h incubation at 30°C. For mutagenesis experiments, a 200 µl aliquot of irradiated cells was inoculated in 1 ml of PYE liquid medium and cultivated overnight, to allow mutation fixation. Then, the cultures were submitted to serial dilutions and plating on PYE medium for viable cell counts. The cultures were also plated in PYE medium containing 100 µg/ml rifampicin to score Rif^r cells. Mutation frequencies were calculated by dividing the numbers of mutants by the estimated total number of cells analyzed in the experiments. Mitomycin C (mit C) survival and mutagenesis experiments were conducted in a similar manner, with drug treatments performed in 2 ml of culture for 1 h at 30°C. For mit C mutagenesis, cells were centrifuged and resuspended in PYE before inoculating for overnight incubation, to remove the drug from the medium.

Determination of the mutation spectra in the *rpoB* gene

Rifampicin-resistant clones, obtained after UV irradiation in the experiments described above, were cultivated overnight for the extraction of chromosomal DNA. The primers rpoB2fo and rpoB2re (Supplementary Table 1S) were used in PCRs designed for the amplification of the region II of rifampicin resistance. PCR products were purified and sequenced in both strands using rpoB2fo and rpoB2re primers with the Big Dye Terminator Cycle Sequencing kit and analyzed in the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA). Only independent mutations from each experiment were scored.

RT–PCR experiments and β-galactosidase assays

The operon organization of genes CC3211, CC3212 and CC3213 was assayed through RT–PCR experiments. The primers 1112A and 1112B were used to check *imuB*/*dnaE2* co-expression, and primers 1213A and 1213B were used to check *imuA*/*imuB* co-expression. RNA from exponentially growing cells was extracted with Trizol® reagent (Invitrogen), and treated with DNAseI to eliminate contaminant DNA. RT–PCR experiments were carried out with 300 ng of RNA using the Superscript One Step kit (Invitrogen), in the following cycling conditions: 50°C for 20 min; 94°C for 2 min; 40 cycles of 95°C for 1 min; 61°C for 30 s; 72°C for 50 s; followed by incubation at 72°C for 7 min and cooling to 4°C. A control for DNA contamination of samples was performed with PCR lacking reverse transcriptase.

Measurements of promoter activity with *lacZ* transcriptional fusions were performed after UV irradiation, in the same conditions used for survival experiments (see above), allowing cells to recover in PYE medium for 90 min after treatment. After recovery, cells were assayed for β-galactosidase activity as described previously (22).

Real-time analysis of gene expression

Relative expression of *imuA*, *imuB*, *dnaE2* and *dinB* after UV irradiation was determined with quantitative PCR experiments. An aliquot of 2 µg of RNA pre-treated with DNAse I were used as template for total cDNA synthesis in 20 µl reactions with random hexamers using the Superscript First-Strand Synthesis System for RT–PCR (Invitrogen). For real-time 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 (23), using the *rpoD* gene as the endogenous control. Primers used in this analysis are listed in Supplementary Table 1S.

Phylogenetic analyses

Protein sequences of genes CC3211, CC3212 and CC3213 were obtained from the National Center of Biotechnology Information GenBank database (www.ncbi.nlm.nih.gov/Entrez/). The accession numbers are respectively NP_422005, NP_422006 and NP_422007. Orthologous protein sequences from complete genomes were obtained by BLAST analyses (24). The lack of orthologs in several genomes was ascertained by exhaustive BLAST analysis.

For phylogenetic analysis, the protein sequences were aligned using the ClustalX multiple sequence alignment program (25) with manual adjustment using Genedoc v2.6.001 (26) (www.psc.edu/biomed/genedoc). Only unambiguously aligned positions (excluding poorly conserved and gap regions) were used. Phylogenetic trees were generated for each group of protein homologs from sequence alignments using the PHYLIP program version 3.5 (27). Distance analyses were performed using the neighbor-joining method in PHYLIP, with the distance PAM matrix model (28). Bootstrap support (resampled 1000 times) was calculated, and strict consensus trees constructed. Only bootstrap values >50% are shown. The consensus trees obtained were viewed through TreeView software (29). The same set of prokaryotic species was used in all analyses, and includes CC3211 (*dnaE2*) and CC3212 (*imuB*) orthologs from fully sequenced genomes and *E.coli* proteins.

RESULTS

Genomic context and phylogenetic analyses

The CC3211 gene of *C.crescentus* encodes a second copy of the *dnaE* gene, hereafter referred to as *dnaE2*. *dnaE2* is in a conserved organization with two genes encoding hypothetical proteins, CC3212 (*imuB*) and CC3213 (*imuA*), similar to other proteobacteria (Figure 1). This operon organization is eventually associated with a duplication of *lexA*, such as in *P.putida*. However, this is not the case for *C.crescentus* and related bacteria, which have only one copy the *lexA* gene (30). It was previously shown that the operon organization of *imuAB* and *dnaE2* is conserved in all genomes of proteobacteria containing these genes (17). However, other bacterial species, such as several Actinomycetales and the planctomycete *Rhodopirellula baltica*, also possess a *dnaE2* gene, although in different genomic contexts (Figure 1). A total of six different gene arrangements were found in bacterial genomes containing *dnaE2*. Remarkably, the large majority of organisms presenting *dnaE2* also possess an *imuB* ortholog, although not necessarily in an operon organization. In *R.baltica*, *imuA* and *imuB* form a potential operon, which does not include *dnaE2*, located apart in the genome. The *M.tuberculosis imuB* gene, encoded by the Rv3394c ORF, is in an operon with Rv3395c, a putative RecA-like protein. Most interestingly, both Rv3395c and Rv3394c are damage inducible in *M.tuberculosis*, and the promoter of Rv3395c has been shown to contain a Mycobacterial SOS box (14,16). A similar picture can be observed

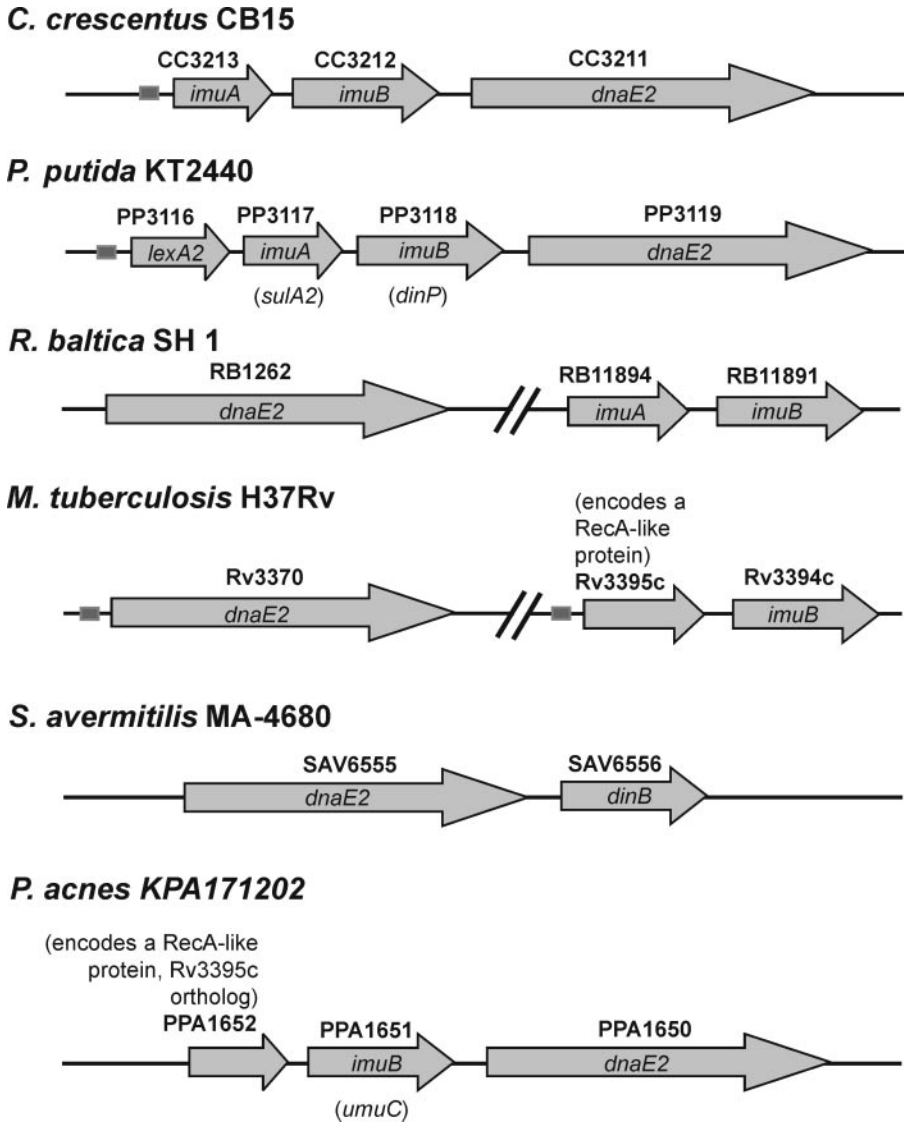

Figure 1. Prototypical genomic organizations of *dnaE2* and accompanying *imuA* and *imuB* genes. The same organization found in *C.crescentus* is also present in α-proteobacterial genomes, *Pseudomonas aeruginosa*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Ralstonia solanacearum*, *Bordetella bronchiseptica* and *Bordetella parapertussis*. A duplication of *lexA* preceding the operon, as found in *P.putida*, is also present in *Methylococcus capsulatus*, *Pseudomonas syringae*, *Xanthomonas campestris* and *Xanthomonas axonopodis*. The *M.tuberculosis* gene arrangement is conserved in all species of the genera *Mycobacterium* and *Corynebacterium*, and in *Nocardia farcinica*. Small boxes represent known SOS operators, and interrupted lines indicate that the genes are not contiguous in the genome. Loci numbers as provided by the genome annotation are indicated above, the arrows representing genes, and our annotation is given inside the arrows. Different original gene annotations are indicated in parentheses.

in the genomes of other Actinomycetales, except *Propionibacterium acnes*, where the Rv3395c ortholog forms a putative operon with both *imuB* and *dnaE2*. Although proteins with significant similarity to ImuA cannot be found in Actinomycetales, the genomic context analysis suggests that the RecA-like protein encoded by *M.tuberculosis* Rv3395 may represent a functional counterpart of ImuA in these organisms. It is evident that several rearrangements of the genomic organization of *dnaE2* and *imuB* have taken place during evolution, but notably these genes always co-exist in bacterial genomes. These data indicate that the *imuB* and *dnaE2* genes compose a highly conserved co-evolving genetic system, which is likely to be involved in DNA damage response.

Phylogenetic analyses of the DnaE proteins of the microorganisms presenting such duplication indicate that this is most probably a very ancestral duplication event, present in the vast majority of fully sequenced genomes from α-proteobacteria, but also found in other proteobacteria and in Actinomycetales (Figure 2). The branch of DnaE includes the *E.coli* protein and the product of the CC1926 gene, which seems to be involved in DNA replication in *C.crescentus* (31). DnaE2 orthologs clearly form a distinct branch, including the DnaE2 from *C.crescentus*. The prototypical member of this branch is the *M.tuberculosis* protein Rv3370c. This protein has been found to be damage inducible, and implicated in cell survival and mutagenesis after UV treatment (14).

The CC3213 ortholog from *P.putida*, PP3117, was annotated as *sulA2* (32). However, PSI-BLAST analyses of the protein encoded by CC3213 shows only very low levels of similarity to SulA (24), and also some similarity to RecA/RadA recombinases (data not shown). A phylogenetic tree of this protein and its orthologs has been presented

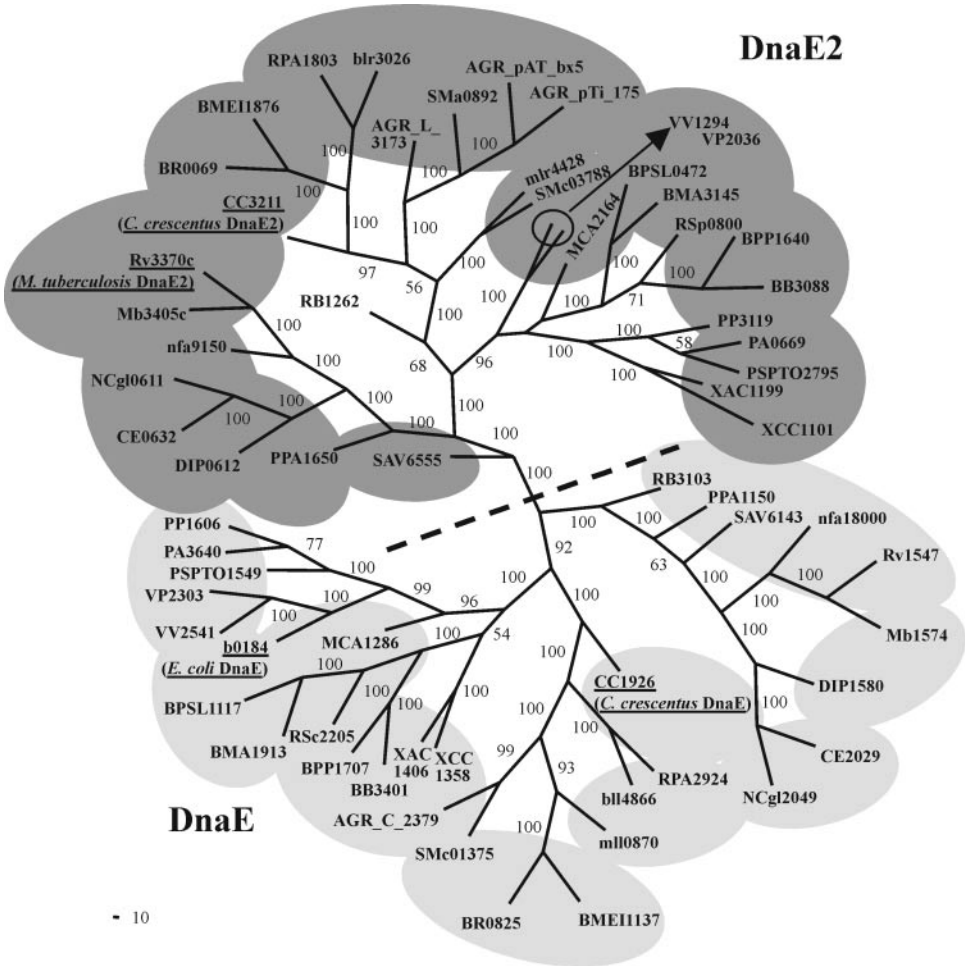

Figure 2. Phylogenetic relationships between DnaE and DnaE2. Phylogenetic analyses included DnaE and DnaE2 proteins of all fully sequenced genomes where such duplication could be found. Only *M.tuberculosis* and *Mycobacterium bovis* were included for simplification, although the duplication can be found in several other Mycobacterial genomes. The names of the ORFs and species name abbreviations shown are the same adopted in the genome annotation of each organism. Abbreviations of organism names are as follows: AGR, *A.tumefaciens* str. C58; b, *E.coli* K12; BB, *B.bronchiseptica* RB50; bll, *Bradyrhizobium japonicum* USDA 110; BMA, *Burkholderia mallei* ATCC 23344; BMEI, *Brucella melitensis* 16M; BPP, *B.parapertussis* 12822; BPSL, *Burkholderia pseudomallei* K96243; BR, *Brucella suis* 1330; CC, *C.crescentus* CB15; CE, *Corynebacterium efficiens* YS-314; DIP, *Corynebacterium diphtheriae* NCTC 13129; Mb, *M.bovis* AF2122/97; MCA, *M.capsulatus* str. Bath; mll and mlr, *Mesorhizobium loti* MAFF303099; NCgl, *Corynebacterium glutamicum* ATCC 13032; Nfa, *N.farcinica* IFM 10152; PA, *Pseudomonas aeruginosa* PAO1; PP, *P.putida* KT2440; PPA, *P.acnes* KPA171202; PSPTO, *P.syringae pv. tomato* str. DC3000; RB, *R.baltica* SH 1; RPA, *Rhodopseudomonas palustris* CGA009; Rsc and Rsp, *R.solanacearum GMI1000*; Rv, *M.tuberculosis* H37Rv; SAV, *S.avermitilis* MA-4680; Smc and Sma, *S.meliloti* 1021; VP, *V.parahaemolyticus* RIMD 2210633; VV, *V.vulnificus* YJ016; XAC, *X.axonopodis* pv. *citri* str. 306; and XCC, *Xanthomonas campestris* pv. *campestris* str. ATCC 33913. The numbers indicate the bootstrap values >50%.

previously (17), but the levels of protein similarities between CC3213 orthologs and known proteins, such as SulA and RecA, are too low to precisely ascribe any protein function. We tested the filamentaion in both the wild type and the derivative CC3213 deletion strain in several conditions by microscopic examination. *C.crescentus* shows a filamentous morphology after prolonged exposure to mit C, and the deletion of CC3213 did not affect this cellular response, indicating that this gene is not involved in cell division suppression (Supplementary Figure 1S). Therefore, we propose to name the CC3213 gene *imuA*, after *i*nducible *mu*tagenesis, given the functional characterization conducted (see below).

Similarly, CC3212 orthologs identified by BLAST analysis present diverse annotations: the *P.putida* gene PP3118 was annotated as *dinP* (32), and the PPA1651 gene of *P.acnes* was annotated as *umuC* (33), while most other orthologs (including CC3212 itself) were annotated as genes with unknown function. In this case, phylogenetic analysis was conducted including all fully sequenced genomes where CC3212 orthologs were found, for comparison between these proteins and DinB, the closest related protein of known function. All organisms with a CC3212 ortholog also have one or more typical *dinB* genes. The product of CC3212 and its orthologs form a branch that is distinct from the DinB proteins of the same organisms, indicating that this is a divergent DinB/UmuC homolog (Figure 3). The only possible exception is the SAV6556 protein of *Streptomyces avermitilis*, which cannot be surely assigned as ImuB through phylogenetic analyses, although it is present in a putative operon with *dnaE2* (Figure 1). Protein similarity comparison among these proteins reinforces the difference between ImuB and DinB, e.g. *E.coli* DinB and UmuC proteins share 45% identity, while *C.crescentus* DinB and the ImuB proteins share only 35% identity. Therefore, we concluded that the CC3212 gene product is a member of a distinct branch in the UmuC superfamily of proteins, and not a DinB ortholog. We named this gene *imuB*, in accordance with the nomenclature adopted for CC3213.

Characterization of the *imuAB*/*dnaE2* expression

The genomic organization of the CC3213 (*imuA*), CC3212 (*imuB*) and CC3211 (*dnaE2*) genes suggests that they may constitute an operon (Figure 1). In fact, in *P.putida*, *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*, the orthologs of these genes are co-expressed in a single RNA (17). In order to evaluate the co-expression of these genes in *C.crescentus*, we designed RT–PCR experiments with the

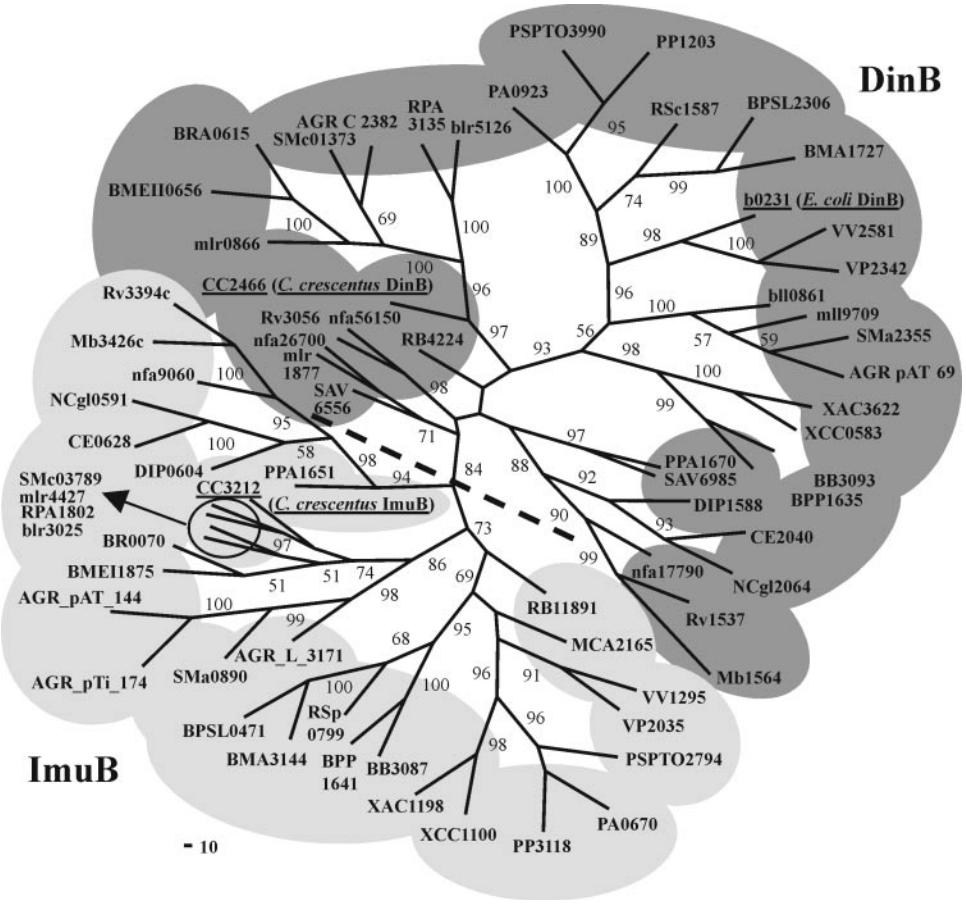

Figure 3. Phylogenetic relationships between ImuB and DinB. Phylogenetic analyses included ImuB and DinB proteins of all fully sequenced genomes where the *imuB* gene could be found. Only *M.tuberculosis* and *M.bovis* were included for simplification, although *imuB* can be found in several other Mycobacterial genomes. The names of the ORFs and species name abbreviations shown are the same adopted in the genome annotation of each organism. Abbreviations of organism names are indicated in the legend of Figure 2. The numbers indicate the bootstrap values >50%.

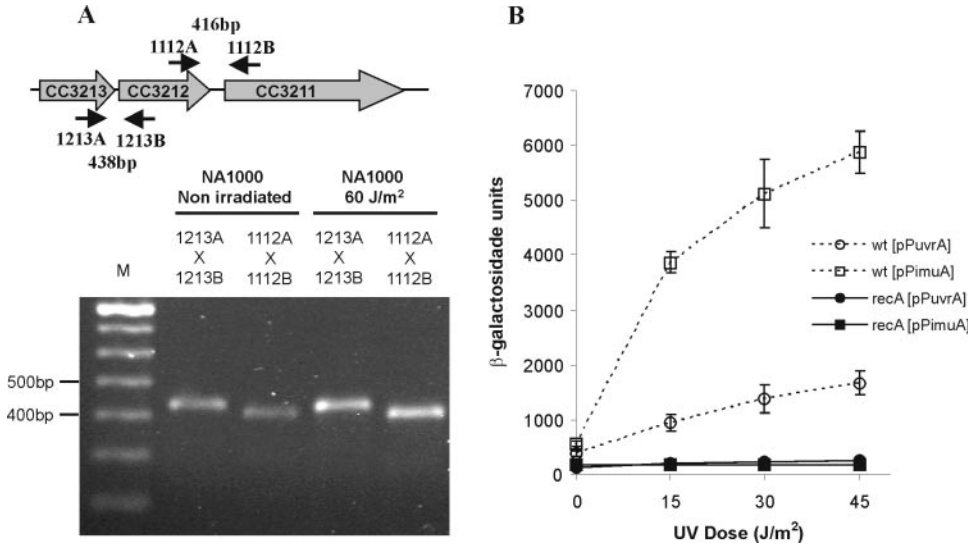

Figure 4. Expression of the *imuAB dnaE2* operon. (**A**) Schematic diagram for this operon indicating the position of the oligonucleotides (small arrows) used in the RT–PCR strategy to detect the co-expression of the three genes. Template RNA was extracted from exponentially growing cells, both from non-irradiated cultures and from cultures irradiated with 60 J/m^2 of UV and recovered after 45 min. The RT–PCR products were visualized after electrophoresis in 1% agarose gel stained with ethidium bromide. (**B**) Expression of *uvrA* and *imuA* promoters in transcriptional fusions with the *lacZ* gene. β-Galactosidase activity was measured 90 min after irradiation with UV light, both in the wild-type and *recA* strains.

oligonucleotides represented in Figure 4A. The results confirmed that the three genes are expressed as a single operon, and the polycistronic RNA can be detected both in UV-irradiated and non-irradiated exponentially growing cells.

In α-proteobacteria, the LexA-binding site has been characterized for many species. The typical sequence GTTCN$_7$GTTC is apparently well conserved in all species examined to date (34,35). The operon containing *imuA*, *imuB* and *dnaE2* presents this typical LexA-binding sequence 66 bp upstream from the *imuA* start codon, indicating that this operon is SOS regulated. In order to examine whether the expression of this operon is regulated in response to DNA damage, we cloned the promoter of *imuA* upstream the reporter gene *lacZ* in the pRK*lacZ*290 vector. For comparison, the promoter of the *uvrA* gene, which also bears a putative α-proteobacteria type SOS box 78 bp upstream the start codon, was cloned in the same vector. Determination of β-galactosidase activity was performed both for the wild-type NA1000 strain, and for a derivative *recA* deletion mutant, constructed in this work. Both promoters were strongly induced 90 min after UV irradiation in a dose-responsive fashion in the wild-type strain (Figure 4B), although the expression of *lacZ* under control of the *imuAB*/*dnaE2* promoter is much higher than the one observed for the *uvrA* promoter.

In our conditions, optimal promoter expression was achieved 90–120 min after irradiation (data not shown), which is in good agreement with the previous reports of Weigle reactivation time course experiments in this organism (19). On the other hand, damage-induced activity of both promoters is completely abolished in a *recA*-deleted strain, indicating that both are regulated in a RecA-dependent manner. These results confirm the prediction that both *uvrA* and the *imuAB*/*dnaE2* operon are controlled by the SOS regulon in *C.crescentus*.

The increased activity of the *imuA* promoter resulting in the induction of all three genes in the operon was further investigated by quantitative RT–PCR experiments. In addition, the expression of the *dinB* gene (encoded by the CC2466 ORF), which does not present an SOS box in its putative promoter region, was also tested. The results shown in Figure 5 demonstrate that *imuA*, *imuB* and *dnaE2* expression levels are increased similarly in response to UV damage. Furthermore, the damage induction of the three genes is abolished in the *recA* strain, indicating that they are coordinately upregulated by the SOS response. The SOS box-containing promoter upstream of *imuA* presumably mediates this coordinated expression. In contrast, the expression of *dinB* remains unaffected by DNA damage, suggesting that this gene is not involved in damage tolerance in *C.crescentus*.

Genetic characterization of damage-inducible mutagenesis in *C.crescentus*

Several strains disrupted in the *imuA*, *imuB* and *dnaE2* genes, as well as in the *dinB* gene, were constructed (Table 1). These gene disruptions had no effect upon cell growth (data not shown). Compared with the wild type, all the single mutant strains deficient in the genes of the operon were slightly sensitive to UV (Figure 6A). In contrast, the *dinB* strain showed wild-type levels of UV resistance, indicating that DinB is not involved in UV damage tolerance. The double mutant *imuB dnaE2* showed no increased sensitivity to UV, indicating that the products of these genes act in the same tolerance pathway. A *dinB imuB* double mutant was also constructed, to evaluate if DinB could be acting in DNA damage tolerance in an *imuAB*-deficient background. The *dinB* mutation had no effect on the resistance to UV, further indicating that this gene is not involved in UV damage resistance.

The mutagenesis of these strains was determined by the rifampicin resistance assay. This assay detects point mutations in the *rpoB* gene, which encodes the β-subunit of bacterial

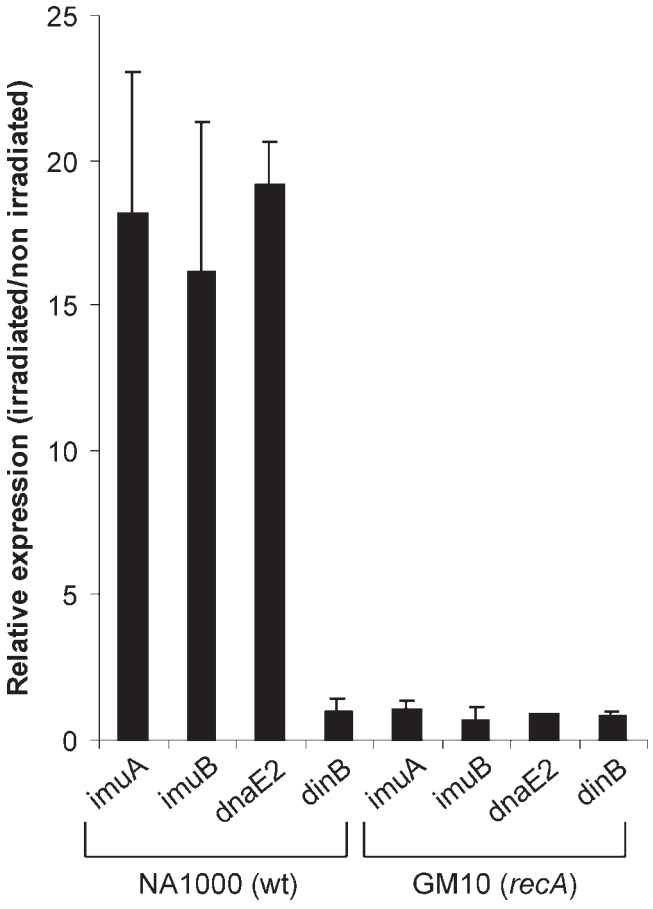

Figure 5. Quantitative RT–PCR analysis of UV-induced gene expression. RNAs were extracted from exponentially growing *recA* and wild-type cells before and 45 min after irradiation with 60 J/m^2 UVC. Relative levels of expression shown represent the average of two independent experiments done in triplicate.

RNA polymerase, and is very useful due to the high conservation of the target gene, allowing its utilization in a wide variety of bacterial species (36,37). Figure 6B shows the mutagenesis induced by UV light in all the strains. It is clear that *dinB* depletion did not affect UV-induced mutagenesis, in contrast to the disruption of *imuA*, *imuB* and *dnaE2*. The UV-induced mutations are almost equally diminished in these three strains, and reduced to approximately the same extent in the *imuB dnaE2* double mutant. Altogether, the results shown in Figure 6 demonstrate that the *imuAB dnaE2* operon is involved in mutagenic repair of UV lesions, and suggest that the proteins encoded by this operon cooperate in the same pathway, as evidenced by the similar phenotypes of the single mutants, and by the epistatic effect of *imuB* and *dnaE2* mutations. The *dinB imuB* strain presents the same levels of UV-induced mutagenesis as the *imuB* single mutant, confirming that DinB is not implicated in error-prone repair in the conditions tested.

Similar experiments were performed to investigate the effects of the UV mimetic drug mit C in *C.crescentus*. The effects of mit C on the survival of the different strains are much more pronounced than those of UV light (Figure 7A). The single mutants *imuA*, *imuB* and *dnaE2* mutants are extremely sensitive to this condition, revealing that this operon plays a major role in the tolerance to the mit C-induced DNA lesions. The *imuB dnaE2* double mutant does not exhibit an exacerbated phenotype, indicating again that these genes cooperate in the same pathway of damage tolerance. On the other hand, the *dinB* mutant is as resistant as the wild type, and the *dinB imuB* double mutant shows no further sensitivity enhancement, demonstrating that DinB is not involved in the tolerance to mit C-induced DNA lesions. Both the UV and the mit C sensitivity phenotypes of *imuA* could be complemented by vector pMR3213, containing the wild-type *imuA* allele. Similarly, the *imuB* strain could be complemented by cosmid 3A4, which contains integral *imuA* and *imuB* genes, but lacking

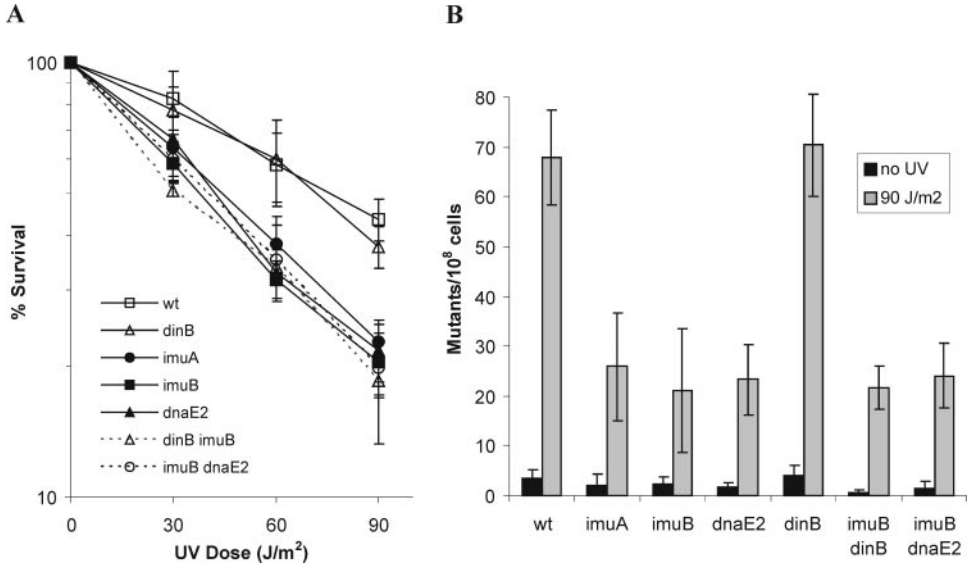

Figure 6. Effects of *imuAB*, *dnaE2* and *dinB* mutations on UV resistance and mutagenesis in *C.crescentus*. (**A**) Survival curves. (**B**) Frequency of Rif^r mutations induced by UV in the *rpoB* gene. The results shown are the mean of at least five independent experiments done in triplicate. Error bars indicate the SD.

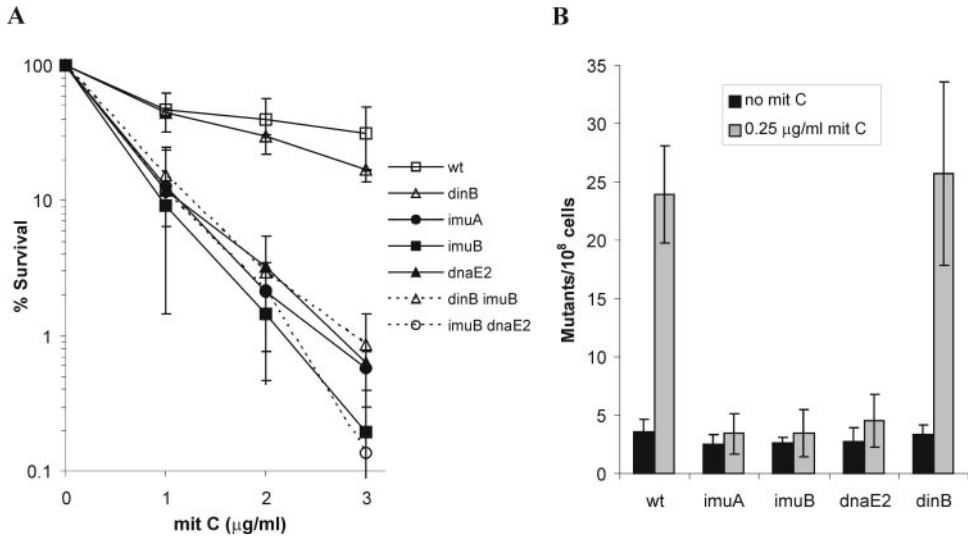

Figure 7. Effects of *imuAB*, *dnaE2* and *dinB* mutations on mit C resistance and mutagenesis in *C.crescentus*. (**A**) Survival curves. (**B**) Frequency of Rif^r mutations induced by mit C in the *rpoB* gene. The results shown are the mean of at least four independent experiments done in triplicate. Error bars indicate the SD.

dnaE2 (data not shown). These results confirm that the phenotypes of *imuA* and *imuB* strains are not due to negative effects on the expression of downstream genes in the operon.

The mutagenesis induced by mit C was also investigated using the rifampicin assay (Figure 7B). The results show a dramatic decrease in mit C mutagenesis in all strains devoid of genes belonging to the damage-inducible operon of *C.crescentus*. On the other hand, *dinB* is clearly not required at all for mit C mutagenesis. These data confirm that the *imuAB*/*dnaE2* operon accounts for most of the mutagenic DNA repair in *C.crescentus* cells exposed to UV light and mit C.

Mutational specificity of UV light in *C.crescentus*

The nature of *imuAB*/*dnaE2*-dependent and independent mutations caused by UV light was analyzed in the *rpoB* gene. Rifampicin-resistant mutants obtained after UV radiation of the wild-type, *dnaE2* and *imuB* strains were randomly selected for sequencing of the *rpoB* cluster II of rifampicin resistance (37), which contained the Rif^r mutations in all mutants analyzed. The mutation spectra observed are shown in Figure 8. The mutation signature of UV light is remarkably similar in *dnaE2* and *imuB* strains. G:C → A:T transitions are by far the most common type of substitution, constituting ~90% of the base changes in both strains (Table 2). On the other hand, UV mutagenesis in the parental strain is significantly modified to a high proportion of G:C → C:G transversions and tandem substitutions concentrated in a hotspot consisting of a run of three Cs, which gives rise to only G:C → A:T mutations in *dnaE2* and *imuB* strains (Figure 8). This increase in transversions and tandem substitutions in the parental strain is accompanied by concomitant reduction in the fraction of G:C → A:T transitions, as shown in Table 2. These other types of mutations are probably an outcome of the mutagenic activity of the *imuAB*/*dnaE2* operon of *C.crescentus*, since they are not observed in the *dnaE2* and *imuB* mutants. G:C → C:G transversions are not a significant part of the mutagenic spectrum of UV light in *E.coli* (37,38). These results indicate that *imuB* and *dnaE2* cooperate in a lesion bypass pathway, which has a different specificity than that involving *umuDC* in *E.coli*.

DISCUSSION

In the present work, we demonstrate that a highly conserved gene expression unit consisting of the genes *imuAB* and *dnaE2*, controlled by the SOS regulon, is responsible for most of the damage-inducible mutagenesis in *C.crescentus*. The genes *imuA* and *imuB* encode previously uncharacterized proteins, which are shown here for the first time to be involved in DNA damage tolerance. These genes are present in several genomes where *umuDC* orthologs cannot be found, such as the α-proteobacteria branch, and it is intuitive to argue that they may functionally replace these proteins in damage-inducible mutagenesis in a wide variety of bacterial species. It is very interesting to note that, independently of being organized as single transcriptional units or as independent genes, *imuB* and *dnaE2* always co-exist in bacterial genomes, strongly suggesting that their activities are interconnected.

The data obtained here clearly indicate that this operon plays a major role in damage-induced mutagenesis in *C.crescentus*, especially after mit C exposure. The lack of mit C mutagenesis in *imuAB*/*dnaE2*-deficient strains correlates well with the extreme sensitivity they show to this agent. Although mit C mutagenesis is mostly dependent on these genes, at least one-third of the UV-induced mutations are still generated in the *imu*- or *dnaE2*-deficient genetic backgrounds. These mutations are not caused by the action of the *dinB* gene, as evidenced by the phenotypes of *dinB* and *imuB dinB* mutant strains. The nature of this mutagenic process remains to be elucidated. In *E.coli*, it has been shown that *umu*-independent UV mutagenesis may occur, depending on the experimental system used (39). Also, in *B.subtilis*, residual UV mutagenesis can be detected in varying levels, even in

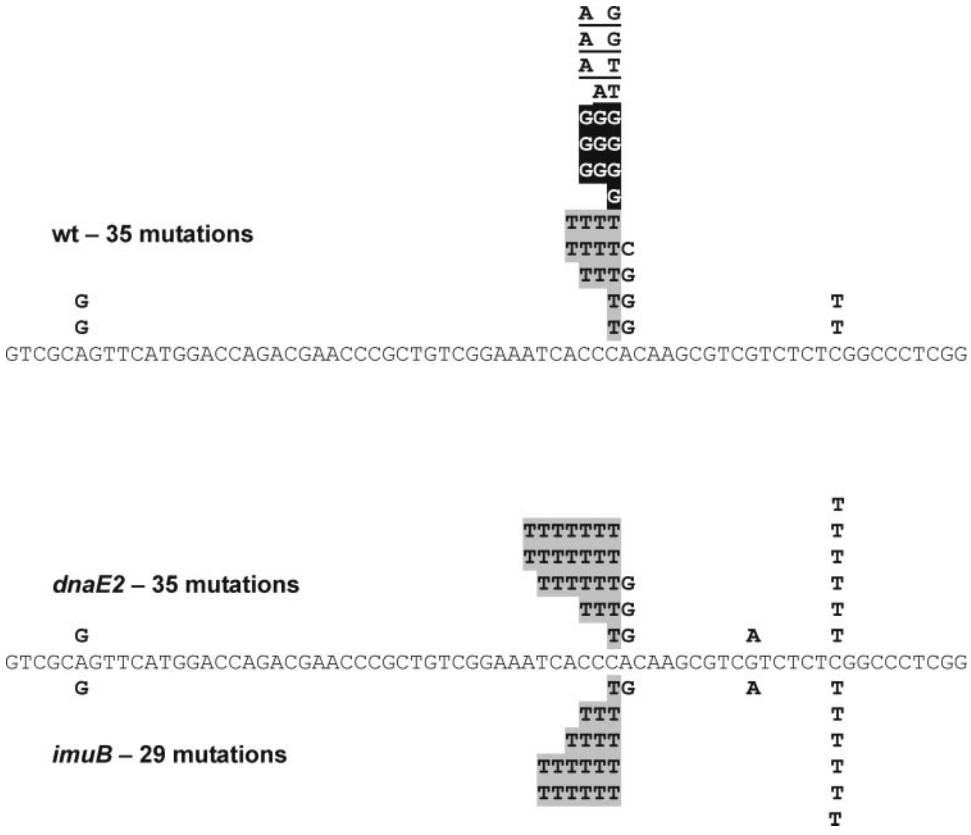

Figure 8. UV mutation spectra in the *rpoB* gene. Nucleotides 1557–1625, comprising the cluster II of rifampicin resistance, are shown in this figure. All mutants analyzed had mutations in this region of the *rpoB* gene. Mutations in shaded background correspond to repetitions of the same base substitution, and tandem mutations are underlined. The total number of independent mutations sequenced is indicated for each strain.

Table 2. Frequencies of base substitutions in the *rpoB* gene induced by UV light

Type of mutation	Wt (%)	*dnaE2* (%)	*imuB* (%)
G:C → A:T	42.9	88.6	93.1
G:C → C:G	28.5	—	—
A:T → G:C	14.3	11.4	6.9
A:T → C:G	2.9	—	—
Tandem substitutions	10.4	—	—

cells devoid of both Y-family DNA polymerases (40,41). It is interesting to note that no other protein belonging to the UmuC superfamily can be found in the *C.crescentus* genome, and neither there are *polB* orthologs. Thus, *imuAB*/*dnaE2*-independent UV mutagenesis might occur via a still uncharacterized pathway. Recently, it has been suggested that the *dnaE* gene of *C.crescentus* may also be a part of the SOS regulon (42). Overexpression of the replicative polymerase encoded by *dnaE* may account for the *imuAB*/*dnaE2*-independent UV mutagenesis. On the other hand, mit C mutagenesis shows an absolute dependence on the activity of the *imuAB dnaE2* operon, indicating that DnaE and the ImuAB DnaE2 machinery may have different lesion bypass capacities.

The mutation spectra of UV-induced lesions in *C.crescentus* show a dramatic qualitative difference between wild-type and *imuB* or *dnaE2* strains. G:C → A:T transitions, a hallmark of UV mutagenesis in *E.coli*, are observed in the mutant strains, while wild-type cells also have a high proportion of tandem substitutions and G:C → C:G transversions. This novel mutational signature, dependent on the activity of the *imuAB*/*dnaE2* operon, probably reflects a difference in polymerase preference for nucleotide incorporation during TLS. On the other hand, the mutations induced by UV light in *M.tuberculosis* in a *dnaE2*-dependent manner are different from the observed in *C.crescentus*, resembling the UV mutagenic spectrum of *E.coli* (14). More detailed studies of both systems are still needed to clarify this discrepancy, although it is clear that there are fundamental differences between these organisms, since *imuA* orthologs are not present in *M.tuberculosis* and related bacteria. In addition, the use of the *rpoB* gene as target for mutational analysis has intrinsic limitations, such as the underestimation of frameshifts and the limited number of mutations detectable as gain of function alleles for Rif^r. The development of more sophisticated genetic tools for the study of mutagenesis is mandatory for a deeper understanding of the basic mechanisms of this aspect of DNA metabolism in *C.crescentus*.

The DNA polymerase III holoenzyme is responsible for chromosomal replication. The α-subunit of DNA polymerase III, encoded by the *dnaE* gene, is essential in *E.coli* (43). In *C.crescentus*, the involvement of the *dnaE* gene (ORF CC1926) in DNA replication has also been demonstrated previously (31). However, in *M.tuberculosis*, the deletion of *dnaE2* causes no growth defect (14), similar to what was observed for *C.crescentus*. It was demonstrated years ago

that *dnaE* is required for UV mutagenesis in *E.coli* (44,45). Currently, it is thought that successful TLS takes place after a blockage of Pol III and formation of a RecA filament on single-stranded DNA. Then, Y-family polymerases displace the Pol III catalytic subunit and mediate lesion bypass, synthesizing patches of DNA sufficiently sized to allow the hand-off of replication back to Pol III. These polymerase switches are mediated by interactions with the β-clamp, the processivity factor of Pol III (46–48). The observation that several bacterial species bear a second copy of the *dnaE* gene, which seems to be solely devoted to DNA damage response, is very intriguing. The presence of this second, damage inducible, Pol III catalytic subunit may be an alternative manner some bacteria have found to control polymerase switch during lesion bypass. Additionally, DnaE2 may itself act as a TLS polymerase, as shown in *B.subtilis* and *S.pyogenes* (12,13).

In spite of the functional and phylogenetic characterization provided in this work, the actual biochemical functions of *imuA* and *imuB* genes remain to be elucidated. However, the genetic data shown here indicate that these genes cooperate with *dnaE2* in an error-prone repair pathway. ImuB is a member of the UmuC superfamily, and it is tempting to speculate about a model in which ImuB and DnaE2 would act in concert, as shown for Y-family polymerases and Pol III in TLS in *E.coli*. This hypothesis is consistent with the genetic requirement for both *imuB* and *dnaE2* in damage-inducible mutagenesis shown in this work, and is further supported by the epistatic effect of these gene disruptions. This model would present a functional conservation of the overall TLS scenario in bacteria, although natural selection has chosen different role-players within different domains of the bacterial kingdom. Shedding light on the reasons why different bacteria should have distinct mutagenic repair machinery is a challenging question, which deserves further examination.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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