Determination of the *Paracoccus denitrificans* SOS box

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**INTRODUCTION**

DNA damage induces in *Escherichia coli* cells the expression of a set of genes whose encoded proteins are directly involved in DNA repair or in enabling the cell to tolerate the lesion until repair occurs (Little & Mount, 1982; Walker, 1984). This set of genes, which constitutes the SOS system, is under the control of both *recA* and *lexA* proteins. The *LexA* protein is the common repressor of the SOS network, which also includes both *recA* and *lexA* genes. *RecA* promotes proteolytic inactivation of *LexA* in DNA-damaged cells (Little, 1991). Several studies indicate that the *RecA* protein binds to single-stranded DNA regions, originated by DNA-damage-mediated replication inhibition or enzymic processing of broken DNA ends, and is converted into an active conformation (Sassanfar & Roberts, 1990). Once activated, *RecA* facilitates autocatalytic cleavage of *LexA*, giving rise to the expression of the SOS genes. When DNA has been repaired, the *RecA* protein is no longer activated and the level of the *LexA* repressor increases, again blocking expression of the SOS genes.

Similar DNA repair systems have been reported in other bacteria (Miller & Kokjohn, 1990). In this way, the regulatory genes of the SOS system, *lexA* and *recA*, of different Gram-positive and Gram-negative bacteria have been characterized (Raymond-Denise & Guillen, 1991; Garriga *et al.*, 1992; Roca & Cox, 1997). *E. coli* and *Bacillus subtilis* *LexA* proteins bind to a specific site located at the 5′ end of their respective SOS genes which is known as the SOS box. So far, three different SOS boxes have been identified. The first one, which was initially described in *E. coli*, displays the dyad symmetrical sequence 5′ CTGT-(AT),-ACAG 3′ (Wertman & Mount, 1985; Lewis *et al.*, 1994). This *E. coli*-like SOS box is present in a great number of Gram-negative bacterial species belonging to several taxonomic families (*Enterobacteriaceae*, *Pseudomonadaceae* and *Pasteurellaceae*, among others). The second SOS box known, whose consensus sequence is 5′ CGAACRNYGTYC 3′ (Winterling *et al.*, 1998), was first reported in *B. subtilis* (Cheo *et al.*, 1991), although it is also present in many other Gram-positive bacteria (Movahedzadeh *et al.*, 1997; Durbach *et al.*, 1997; Johnston *et al.*, 1997). Finally, a third SOS box belonging to the phototrophic bacterium *Rhodobacter sphaeroides* has recently been identified (Fernández de Henestrosa *et al.*, 1998). This displays the GTTCN,GTTC motif, and is the first SOS box known whose sequence is a direct repeat.

**By gel retardation experiments with crude cell extracts of *Paracoccus denitrificans* it was demonstrated that a protein specifically binds to the promoter of the *P. denitrificans recA* gene. PCR mutagenesis of the *recA* promoter showed that the GAACN,GAAC motif is required for the formation of the DNA–protein complex. This protein also binds to the GTTCN,GTTC motif, which is present in the promoter of the *P. denitrificans uvrA* gene. Mutational analysis of the promoter regions of both *P. denitrificans recA* and *uvrA* genes indicated that the GAACN,GAAC and GTTCN,GTTC sequences are required for DNA-damage-mediated induction of these two genes in vivo. Furthermore, the *P. denitrificans recA* gene was DNA-damage-inducible when introduced into cells of the phylogenetically related phototrophic bacterium *Rhodobacter sphaeroides*, although this inducibility was lost in mutants in the GAACN,GAAC motif. These results indicate that *P. denitrificans* possesses the same SOS box as *R. sphaeroides*, which, in agreement with previous work, is proposed as being the GTTCN,GTTC motif.

**Keywords:** *recA* gene, *uvrA* gene, SOS box, *Paracoccus denitrificans*, DNA damage
**Paracoccus denitrificans** is an aerobic soil bacterium which, taxonomically, has been grouped in the α subclass of the Proteobacteria together with *R. sphaeroides* (Woese et al., 1984). The *recA* gene of *P. denitrificans* has been isolated and sequenced, and it has been shown to be DNA damage-inducible despite its promoter lacking an *E. coli*-like SOS box (Fernandez de Henestrosa et al., 1997). In this work, to determine whether the *R. sphaeroides* SOS box is uniquely present in this organism or is also found in other members of the Proteobacteria, the promoters of the *P. denitrificans recA* and *uvrA* genes were mutagenized and their behaviour in *vitro* and *in vivo* was analysed.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium (Miller, 1991), and *P. denitrificans* and *R. sphaeroides* strains were normally grown at 30 °C in Brain-Heart Infusion (BHI) (Miller, 1991) or 290A (Calero et al., 1994) media, respectively. Antibiotics were added to the culture media at the appropriate concentrations for each bacterial species (Miller, 1991).

**General genetic techniques.** Plasmid DNA was transformed into competent *E. coli* cells as described by Silhavy et al. (1984). Bacterial matings were carried out as reported previously (Fernández de Henestrosa & Barbé, 1994). Mitomycin C treatment was performed as described previously (Barbé et al., 1985).

**Biochemical, and RNA and DNA methods.** β-Galactosidase activity assays, RNA extraction, DNA techniques and gel mobility experiments were as reported previously (Fernández de Henestrosa et al., 1998). In all shift mobility experiments, the protein concentration of the crude cellular extract, when used, was 2.5 pg ml⁻¹. In the competition experiments with a specific DNA, a 100-fold molar excess of unlabelled DNA was always added to the reaction mixture.

To determine the transcriptional starting point of the *P. denitrificans recA* gene, the oligonucleotide 5' GAACTGCC-
GTTCGATCTGGGCC 3', which hybridizes to nucleotides +75 to +96 relative to the translational starting site, was used. This primer was synthesized and 5'end-labelled with digoxigenin-N-hydroxysuccinimide ester (MWG-Biotech). Primer extension was carried out as previously reported (Tapia et al., 1997).

The nucleotide sequence of the *P. denitrificans* *uwr*A gene was determined from both DNA strands by the dideoxy method (Sanger et al., 1977) on an ALF Sequencer (Pharmacia Biotech). Primer extension was carried out as previously reported (Tapia et al., 1997).

Primers used in the construction of the different *P. denitrificans recA* and *uwr*A probes, supplied by Boehringer Mannheim, are presented in Table 2. To facilitate subcloning of PCR-DNA fragments and construction of the *lacZ* fusions, specific restriction sites (shown in Table 2) were incorporated into the oligonucleotide primers. The DNA sequence of all PCR-mutagenized fragments was obtained in all cases from both DNA strands.

### Table 2. Oligonucleotide primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Position†</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>5'TCGAAAAGTGTTGCCCC 3'</td>
<td>+20</td>
<td>Lower primer, 5' digoxigenin end-labelled; used in the construction of probes for mobility shift assays (Fig. 2b)</td>
</tr>
<tr>
<td>LF</td>
<td>5'GGATCCTGCAAAAGTGTTGCCCC 3'</td>
<td>+20</td>
<td>Lower primer used in <em>recA</em> fusion constructions</td>
</tr>
<tr>
<td>LPE</td>
<td>5'GAATCGCGGTTCGATCTGGGCC 3'</td>
<td>+96</td>
<td>Lower primer used for primer extension experiments</td>
</tr>
<tr>
<td>UR1</td>
<td>5'GAGGCGCAAATAGGAACA 3'</td>
<td>-90</td>
<td>Upper primer used to obtain P1 probe (Fig. 2b)</td>
</tr>
<tr>
<td>UR2</td>
<td>5'GATGAAAGATGGCGG 3'</td>
<td>-69</td>
<td>Upper primer used to obtain P2 probe (Fig. 2b)</td>
</tr>
<tr>
<td>UF1</td>
<td>5'GATTCAGGACTTGGATCGGCCG 3'</td>
<td>-112</td>
<td>Upper primer used to obtain fusion constructions</td>
</tr>
<tr>
<td>UF2</td>
<td>5'GATTCAGGACTTGGATCGGCCG 3'</td>
<td>-112</td>
<td>Upper primer used to obtain the mutagenized <em>recA</em> OR probe; also used in mutagenized fusion constructions</td>
</tr>
<tr>
<td>UF3</td>
<td>5'GATTCAGGACTTGGATCGGCCG 3'</td>
<td>-112</td>
<td>Upper primer used to obtain the mutagenized <em>recA</em> OR probe; also used in mutagenized fusion constructions</td>
</tr>
<tr>
<td>LUF1</td>
<td>5'AGACTTGGCCAGAC 3'</td>
<td>+122</td>
<td>Upper primer used to obtain wild-type <em>uwrA</em> fusion</td>
</tr>
<tr>
<td>LUF2</td>
<td>5'GGATCCTGGCCGACAGGCGCAGGCG 3'</td>
<td>+111</td>
<td>Upper primer used to obtain <em>uwrA</em> probes; also used in mutagenized fusion</td>
</tr>
<tr>
<td>UV1</td>
<td>5'GCCGATTTGCGACCCGC 3'</td>
<td>-189</td>
<td>Upper primer used to obtain wild-type <em>uwrA</em> fusion</td>
</tr>
<tr>
<td>UV2</td>
<td>5'CCTGCACTTGGAGGCGACAGGCG 3'</td>
<td>-126</td>
<td>Upper primer used to obtain <em>uwrA</em> probe (Fig. 2b)</td>
</tr>
<tr>
<td>UV3</td>
<td>5'GATTCAGGACTTGGATCGGCCG 3'</td>
<td>-126</td>
<td>Upper primer used to obtain the mutagenized <em>uwrA</em> OR probe; also used in mutagenized fusion constructions (Fig. 2b)</td>
</tr>
<tr>
<td>UV4</td>
<td>5'GATTCAGGACTTGGATCGGCCG 3'</td>
<td>-126</td>
<td>Upper primer used to obtain the mutagenized <em>uwrA</em> OR probe; also used in mutagenized fusion constructions (Fig. 2b)</td>
</tr>
</tbody>
</table>

* Distance from the 5' end of the oligonucleotide to either the transcriptional or the translational starting point of *P. denitrificans recA* and *uwrA* genes, respectively.

#### RESULTS

Characterization of the *Paracoccus denitrificans recA* transcriptional starting point

It has been reported that some *recA* genes are expressed from a polycistronic transcript (Martin et al., 1995). To determine whether this was the case in the *P. denitrificans recA* gene, and also to locate its putative regulatory sequences, the transcriptional starting point (Table 2) containing 114 bp of the *uwrA* coding region. For each fusion, desired restriction fragments were recovered from the appropriate pGEM-T derivative and subcloned into the pU108 plasmid upstream of the promoterless *trp-lacZ* region. Afterwards, the *NotI* fragment harbouring the created fusion was recovered from the agarose gel, filled with T4 DNA polymerase to obtain blunt ends and inserted into the single *SacI* cloning site of the pLV106 plasmid. The presence of the desired mutations was again tested by DNA sequencing of each fusion using the 5' fluorescein CGAACGGCCAGTGA-3' primer, which extended from nucleotides +32 to +14, with respect to the translational starting point of the *E. coli lacZ* gene. Finally, plasmids containing the constructed fusions were introduced by mating into *P. denitrificans* cells.
A. DEL REY and OTHERS

comparing the relationship existing between them analyzed in vivo and in vitro. To isolate the \textit{P. denitrificans uvrA}, the same strategy used to clone its \textit{recA} gene was employed, taking advantage of the phylogenetic relationship existing between \textit{P. denitrificans} and \textit{R. sphaeroides} (Fernández de Henestrosa \textit{et al.}, 1997). Thus, a gene bank of \textit{P. denitrificans} constructed in the \textit{\textsc{Agem}12} vector was probed with a 0.3 kb \textit{SacII} internal fragment of the \textit{R. sphaeroides uvrA} gene (Mackenzie \textit{et al.}, 1995). Among 1200 plaques screened, four reacted with the probe. One of these positive phage was analyzed by restriction enzyme digestion and Southern blot hybridization. A 5 kb \textit{SmaI} fragment of this phage, which hybridizes with the \textit{R. sphaeroides} probe, was isolated and ligated into the single \textit{SmaI} site of the \textit{pBluescript SK(+) plasmid}. Further subcloning and sequencing experiments have enabled us to obtain the nucleotide sequence of the promoter and the 5' end coding region of the \textit{P. denitrificans uvrA} gene.

It is known that not all \textit{uvrA} genes are DNA-damage-inducible. Thus, it has been demonstrated that expression of the \textit{Pseudomonas aeruginosa} and \textit{Neisseria gonorrhoeae uvrA} genes is not triggered following DNA damage (Rivera \textit{et al.}, 1997; Black \textit{et al.}, 1998). For this reason, it was decided to analyze the behavior of the \textit{P. denitrificans uvrA} promoter towards DNA injury. To do this, the \textit{pUA694} plasmid containing a \textit{uvrA–lacZ} fusion (constructed as reported in Methods) was introduced into a Rif\textsuperscript{R} mutant of \textit{P. denitrificans}. Addition of mitomycin C to \textit{P. denitrificans} (\textit{pUA694}) cells induced the expression of the \textit{uvrA–lacZ} fusion in RecA\textsuperscript{−} but not in RecA\textsuperscript{+} cells, indicating that this inducibility is \textit{recA}-dependent (data not shown).

**Electrophoretic mobility of the \textit{P. denitrificans recA} and \textit{uvrA} promoters**

The above-established +1 position of the \textit{P. denitrificans recA} gene overlaps with the GAAC\textsubscript{N}{\textsubscript{GAAC}} direct repeat (Fig. 1b). This motif, GAAC\textsubscript{N}{\textsubscript{GAAC}}, is also present upstream of the \textit{R. sphaeroides recA} gene and controls its DNA-damage-mediated expression (Fernández de Henestrosa \textit{et al.}, 1998). Gel retardation experiments were therefore carried out with different derivatives of the \textit{P. denitrificans recA} promoter to determine whether a protein binds to its GAAC\textsubscript{N}{\textsubscript{GAAC}} motif. Fig. 2(b) shows that the mobility of a DNA fragment containing the GAAC\textsubscript{N}{\textsubscript{GAAC}} sequence shifts in the presence of \textit{P. denitrificans} cell extract. On the other hand, no changes were detected in the mobility of a fragment lacking the GAAC\textsubscript{N}{\textsubscript{GAAC}} motif (Fig. 2b, lane 4). This DNA–protein complex is formed by the binding of a sequence-specific protein to the probe since the addition of unlabelled \textit{pBSK-DNA} did not affect the formation of the DNA–protein complex (Fig. 2b, lane 6), whereas the presence of unlabelled wild-type \textit{recA} promoter inhibited it (Fig. 2b, lane 7). Furthermore, the addition of competitor fragments carrying a mutation in any of the two GAAC

![Fig. 1. (a) Determination of the 5' end of the \textit{P. denitrificans recA} transcript by primer extension. Lanes T, C, G and A contain products of sequencing reactions of plasmid \textit{pUA}617 carried out with the same primer as used for primer extension. The transcriptional starting point is indicated (+1). (b) Nucleotide sequence of the \textit{P. denitrificans recA} upstream region. The +1 position, the predicted -10, -35 and ribosome-binding site (RBS) sequences, and the translational starting point are indicated. The GAAC\textsubscript{N}{\textsubscript{GAAC}} direct repeat present in the upstream region of the \textit{recA} gene is underlined.](image-url)
Paracoccus denitrificans SOS box

Fig. 2. (a) Sequence of the operator regions of the P. denitrificans recA and uvrA genes. P1 and P2 indicate the starting point of the upper primers used to obtain fragments containing the recA gene employed in gel mobility experiments. The changes introduced by PCR mutagenesis in the GAACN,GAAC and GTTCN,GTTC direct repeats present in the operators of the recA and uvrA genes, respectively, are also indicated. (b) Gel mobility of the recA promoter of P. denitrificans under several experimental conditions. Lanes 1–4 show the behaviour of P1 and P2 fragments in the absence (−) or in the presence (+) of P. denitrificans crude cell extract. Lanes 5–9 show the mobility of the P1 fragment (lane 5) in the presence of P. denitrificans crude extract as well as the effect on this mobility of unlabelled 100-fold molar excess of pBSK-DNA (lane 6), P1 fragment (lane 7), or mutants RL and RR of the P1 fragment (lanes 8 and 9, respectively). The effect of an excess of unlabelled-fragment containing either the wild-type or mutant derivatives of the P. denitrificans uvrA promoter on the mobility of the P1 fragment in the presence of crude extract is also shown (lanes 12–14). All binding assays were done simultaneously, although competitive experiments with uvrA fragments (lanes 12–14) were run in a different gel. For this reason, and as an experimental control, the mobility of the P1 fragment is also included in lanes 10–11.

Submotifs of the GAACN,GAAC sequence does not affect the band shift (Fig. 2b, lanes 8 and 9). All these data indicate that the binding of the protein requires the presence of the GAACN,GAAC sequence. It is worth noting that the same protein binds to the P. denitrificans uvrA promoter since its presence prevents P. denitrificans recA–protein complex formation (Fig. 2b, lane 12).

The gel retardation experiments indicate that a protein binds the promoters of both recA and uvrA of P. denitrificans. Between the −66 and −91 positions, with
Table 3. Effects of mutations on the expression of the P. denitrificans recA and uvrA genes measured as the β-galactosidase specific activity of recA-lacZ and uvrA-lacZ fusions

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>β-Galactosidase specific activity (units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal level</td>
</tr>
<tr>
<td>pUA699 (recA wild-type)</td>
<td>1200</td>
</tr>
<tr>
<td>pUA700 (recA OL−)</td>
<td>6500</td>
</tr>
<tr>
<td>pUA701 (recA OR−)</td>
<td>6400</td>
</tr>
<tr>
<td>pUA694 (uvrA wild-type)</td>
<td>1600</td>
</tr>
<tr>
<td>pUA702 (uvrA OL−)</td>
<td>4800</td>
</tr>
<tr>
<td>pUA703 (uvrA OR−)</td>
<td>6600</td>
</tr>
</tbody>
</table>

*β-Galactosidase specific activities were measured in the absence of mitomycin C (basal level) or 180 min after the addition of this compound at 10 µg ml⁻¹ (induced level). All determinations are the means of at least three experiments (each in triplicate), and a single standard error of any value was never greater than 10%.
† The induction factor is the ratio between the specific activity of β-galactosidase of mitomycin C-treated cells and the specific activity in non-treated cells for each strain.

### DISCUSSION

We have previously shown that GTTCN,GATC and GAACN,GAAC motifs control the DNA-damage-mediated induction of the R. sphaeroides recA gene. Likewise, we have also demonstrated that the GTTCN,GTTC motif regulates the induction of the R. sphaeroides uvrA gene. Since the GAACN,GAAC motif is the reverse and complementary sequence of GTTCN,GTTC, together with the fact that the GTTCN,GATC and GTTCN,GTTC motifs are also present in both DNA-damage-inducible genes, we have proposed the GTTCN,GTTC motif as the SOS box of R. sphaeroides (Fernández de Henestrosa et al., 1998). In the present study, it has been established that the GAACN,GAAC and GTTCN,GTTC motifs control the expression of the P. denitrificans recA and uvrA genes, respectively. Thus it is clear that the R. sphaeroides and P. denitrificans uvrA genes have the same regulatory sequence (GTTCN,GTTC), whereas the recA genes of these two organisms differ in the presence of a second regulatory sequence in one of them: in both recA genes the GAACN,GAAC sequence is found, but the R. sphaeroides recA gene also has the GTTCN,GTAC motif. Furthermore, expression of the P. denitrificans recA gene is triggered by DNA damage in R. sphaeroides cells unless the GAACN,GAAC motif is mutagenized. These data are in agreement with the fact that P. denitrificans and R. sphaeroides have both...
been phylogenetically classified within the α-subclass of the Proteobacteria.

In this context, and in agreement with our previous work (Fernández de Henestrosa et al., 1998), we propose the GTTCN-GTTC motif as the P. denitrificans SOS box. From a biological point of view, and despite the sequences being different with respect to the transcriptional sense, the mechanism by which the LexA repressor of P. denitrificans binds to either recA (GAACN-GAAC) or wuA (GTTCN-GTTC) operators must be the same since, in fact, the sequences of both motifs (which are a direct repeat) are complementary and reverse. In this respect, it is also worth noting that the sequence of the R. sphaeroides and P. denitrificans SOS box shows several specific and different properties from either of the other SOS boxes or protein-binding sequences described. The first is that this sequence is a direct repeat instead of a palindrome. The second is that, to our knowledge, this is the only case shown where the controlling sequences of two genes of the same regulatory network are found in the opposite orientation in each of them. Because of these characteristics, the model LexA repressor-SOS box of P. denitrificans may be very useful for progressing knowledge of DNA–protein interactions in bacteria.

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REFERENCES


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