The *recA* gene from *Clostridium perfringens* is induced by methyl methanesulphonate and contains an upstream Cheo box

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The *recA* gene from *Clostridium perfringens* was cloned using degenerate oligonucleotide primers designed from conserved regions of RecA proteins from other bacteria. The 1089 bp gene encoded a putative RecA protein with 69% amino acid sequence similarity to the RecA protein from *Bacillus subtilis*. The *C. perfringens* *recA* gene was induced by exposure to methyl methanesulphonate and complemented a *recA* mutant of *Escherichia coli*. A Cheo box was identified in the region upstream of the gene. Since this SOS-like operator site is conserved in many DNA-damage-inducible *recA* gene regions from Gram-positive bacteria, the results suggest that the regulation of the *C. perfringens* *recA* gene also involves the binding of a LexA-like protein to this site.

Keywords: RecA, SOS operator site, DNA damage, *Bacillus subtilis*, regulation, Cheo box

INTRODUCTION

Clostridium perfringens is an anaerobic, Gram-positive bacterium which causes human gas gangrene and food poisoning and several animal diseases. *C. perfringens* strains produce a large number of extracellular toxins which play an important role in disease (Rood & Cole, 1991; Songer, 1996). The genetics of *C. perfringens* is well developed but little is known about the recombination mechanisms or DNA repair systems that operate in this organism (Rood & Cole, 1991; Cole *et al.*, 1995).

In most prokaryotes the *recA* gene encodes the major protein involved in homologous recombination, DNA repair, and the SOS response (Walker, 1984; Roca & Cox, 1990). RecA proteins may also be involved in other processes such as the expression of extracellular proteins (Ball *et al.*, 1990; McEnvoy *et al.*, 1992), and heat shock and oxidative stress (Duwat *et al.*, 1995). Although *recA* genes have been cloned from many different bacteria, only a relatively small number of Gram-positive

organisms are represented (Karlin *et al.*, 1995), and to our knowledge, no clostridial *recA* genes have been cloned.

This study describes the cloning, sequence analysis, and characterization of the *C. perfringens* *recA* gene. The gene is shown to be induced upon treatment with methyl methanesulphonate (MMS) and to complement an *Escherichia coli* *recA* mutant. Comparative sequence analysis suggests that it is regulated by a similar mechanism to the *recA* gene from *Bacillus subtilis*.

METHODS

Bacterial strains and plasmids. All *E. coli* strains were derivatives of DH5 α (Bethesda Research Laboratories), which carries the *recA1* mutation. Cultures were grown at 37 °C in 2YT medium (Vieira & Messing, 1982) with ampicillin (100 μ g ml⁻¹) or erythromycin (150 μ g ml⁻¹). *C. perfringens* strain 13 (Mahony & Moore, 1976) was grown at 37 °C under anaerobic conditions on nutrient agar (Rood, 1983) in an atmosphere containing 10% H₂, 10% CO₂ and 80% N₂, or in fluid thioglycollate broth (Difco) or TPG broth (Rood *et al.*, 1978). The properties of the recombinant plasmids used in this study are described in Table 1.

DNA techniques. *C. perfringens* chromosomal DNA was prepared by the method of Abraham & Rood (1985). DNA

Abbreviation: MMS, methyl methanesulphonate.

The GenBank accession number for the nucleotide sequence data reported in this paper is U61497.

Table 1. Recombinant plasmids used in this study

Plasmid	Characteristics	Reference
pUC18	Ap ^R <i>lacZ'</i>	Norlander <i>et al.</i> (1983)
pWSK29	Ap ^R <i>lacZ'</i>	Wang & Kusher (1991)
pBSKS(-)	Ap ^R <i>lacZ'</i>	Stratagene
pJIR774	pUC18 Ω (<i>EcoRI</i> / <i>Bam</i> HI: <i>C. perfringens</i> strain 13 PCR product #414-#415, 0.7 kb) Ap ^R	This study
pJIR828	pWSK29 Ω (<i>EcoRI</i> : <i>C. perfringens</i> strain 13, 5.2, 3.3, 2.7, 1.4 kb fragments) Ap ^R	This study
pJIR1120	pBSKS(-) Ω (<i>ermQ</i> cassette, 1.3 kb) Ap ^R Em ^R	This study
pJIR1197	pWSK29 Ω (<i>EcoRI</i> : pJIR828, 5.2 kb) Ap ^R	This study
pJIR1232	pJIR1197 <i>Sph</i> I Ω (<i>Sma</i> I/ <i>EcoRV</i> : pJIR1120, 1.3 kb) Ap ^R Em ^R	This study

sequencing was performed with an Applied Biosystems 373A automated DNA sequencer using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems). *E. coli* plasmid DNA was prepared using the Applied Biosystems protocol. Oligonucleotide primers were made using an Applied Biosystems 392 DNA/RNA synthesizer. The nucleotide sequence was compiled using Sequencher 3.0 (Gene Codes Corporation). Other molecular techniques were carried out by standard methods (Sambrook *et al.*, 1989).

Isolation of the *C. perfringens* *recA* gene. A fragment of the *C. perfringens* *recA* gene was amplified using degenerate oligonucleotide primers designed from conserved regions of the RecA protein. The low mol% G+C content of *C. perfringens* DNA was taken into account in the design of the primers. *Bam*HI and *EcoRI* restriction sites were engineered into the primers #414 and #415, respectively, to facilitate cloning of the PCR product. Primer #414 [TTTGG-(A/T)AAAGGATCCAT(A/T)ATGA] corresponded to the *E. coli* RecA peptide FGKGSIM and primer #415 [CCATAGAT(A/T)ATATCGAATTC(A/T)GCTTG] corresponded to amino acids QAEFQILYG. PCR reactions were performed on a Hybaid Intelligent Heating Block, for 30 cycles using the following parameters: 1 min denaturation at 94 °C, 2 min annealing at 48 °C and 2 min extension at 72 °C.

A *C. perfringens* gene bank was constructed in the low-copy-number vector pWSK29 from an *EcoRI* digest of *C. perfringens* strain 13 chromosomal DNA. The library was screened by colony hybridization using the *recA*-derived PCR product labelled with digoxigenin (Boehringer Mannheim) as a probe.

RNA extraction and primer extension analysis. RNA was extracted by a modification of the method of Oelmuler *et al.* (1990) from *C. perfringens* cells grown to mid-exponential phase in either the presence or absence of 0.1% MMS. Primer extension reactions were performed on 50 µg *C. perfringens* total RNA using the Primer Extension System and protocol outlined by Promega.

Sensitivity to MMS and UV light. *E. coli* strains were tested for sensitivity to MMS or UV light over a range of doses. Viable counts of serially diluted stationary-phase cultures were determined following UV irradiation under a germicidal lamp (Fyfe & Davies, 1990). Sensitivity to MMS was determined by plating onto medium containing 0–0.02% MMS. Note that *E. coli* is more susceptible than *C. perfringens* to inhibition by MMS.

RESULTS AND DISCUSSION

Cloning and complementation analysis of the *recA* gene from *C. perfringens*

PCR using degenerate oligonucleotide primers has been used successfully to amplify and clone *recA* genes from a number of Gram-positive bacteria and mycoplasmas (Quivey *et al.*, 1992; Nußbaumer & Wohlleben, 1994; Duwat *et al.*, 1992; Dybvig *et al.*, 1992; King *et al.*, 1994; Tao *et al.*, 1995). Therefore, a similar approach was used to clone the *C. perfringens* gene. The degenerate

Fig. 1. Construction of an inactivated copy of *recA* in pJIR1232. The *recA* gene is indicated by the striped arrow. The extent and orientation of the *ermQ* gene within *recA* is shown by the shaded region. The *Ap*^R gene is indicated by a black arrow. Only relevant restriction sites are shown.

oligonucleotide primers #414 and #415 were designed from conserved regions of the RecA protein. A 711 bp DNA fragment was PCR-amplified from the *C. perfringens* strain 13 chromosome using these primers and cloned into pUC18 to form pJIR774. Nucleotide sequence analysis of the insert from pJIR774 and comparison with the GenBank database revealed that it encoded a putative product with a high level of amino acid sequence identity to RecA proteins from other bacterial species, which indicated that the PCR product contained a portion of the *C. perfringens* *recA* gene.

To isolate the entire *recA* gene from *C. perfringens*, a genomic library was constructed from *Eco*RI-digested *C. perfringens* strain 13 chromosomal DNA. The low-copy-number vector pWSK29 was used because the *recA* genes of some species appear to be unstable on high-copy-number plasmids (Fyfe & Davies, 1990; Dybvig & Woodard, 1992; Bayles *et al.*, 1994; Marrero & Yasbin, 1988). The 711 bp PCR fragment was digoxigenin-labelled and used to probe the *C. perfringens* gene bank. A recombinant clone was detected which hybridized to the *recA* probe and produced a PCR product of the expected size on amplification with #414 and #415. Restriction analysis of the resultant plasmid, pJIR828, revealed the presence of four cloned *Eco*RI fragments. Southern hybridization experiments localized the *C. perfringens* *recA* gene to the 5.2 kb *Eco*RI fragment within pJIR828 (data not shown). This fragment was subsequently subcloned into pWSK29 to yield pJIR1197 (Fig. 1).

The *C. perfringens* *recA* gene complements the *E. coli* *recA1* mutation

To determine if the *C. perfringens* *recA* gene could complement a *recA* strain of *E. coli*, viable counts were performed following exposure of cells to UV irradiation or after growth in the presence of different concentrations of MMS. The strains used were DH5 α derivatives carrying pJIR828 or pJIR1197, which had a complete copy of the *C. perfringens* *recA* gene, or pJIR1232, which carried an insertionally inactivated *recA* gene (Table 1). The inactivated *recA* gene was constructed by insertion of the erythromycin resistance (*ermQ*) cassette from pJIR1120 into the *Sph*I site of pJIR1197 (Fig. 1). *E. coli* DH5 α strains carrying either pJIR828 or pJIR1197 showed increased resistance to MMS (Fig. 2a) and increased survival following UV irradiation (Fig. 2b) when compared with the vector control or the *recA::ermQ* derivative. These results show that the *C. perfringens* *recA* gene is functional in *E. coli* and suggest that the clostridial RecA protein can substitute for the *E. coli* RecA protein in conferring resistance to MMS and survival following UV irradiation.

Sequence analysis of the *C. perfringens* *recA* gene and its upstream region

Outward-firing oligonucleotide primers for sequencing of the upstream and downstream regions were designed

Table 2. Comparison of putative Cheo boxes from the *recA* genes of several Gram-positive bacteria

Organism	Putative Cheo box
<i>Clostridium perfringens</i>	GAAC TTAT GTTC
<i>Bacillus subtilis</i>	GAATAT GCGTTC
<i>Staphylococcus aureus</i>	GAACAAATATTC
<i>Streptococcus pneumoniae</i>	GAAC TGAGGTTA
<i>Mycobacterium tuberculosis</i>	GAACAGGTGTTC
<i>Mycobacterium leprae</i>	GAACAGATGTTC
<i>Streptomyces lividans</i>	GAACATCCATTC
<i>Streptomyces ambofaciens</i>	GAACATCCATTC
<i>Lactococcus lactis</i>	GAAAAAT-GTTC
	GATAGAAAGTTC
	GTGACAAAGTTC
<i>Corynebacterium glutamicum</i>	GAATTTTCGTCC
Consensus	GAACNNNNGTTC

from known sequences within the 711 bp *recA* fragment. The entire *recA* gene then was sequenced on both strands in an overlapping manner using pJIR1197 and pJIR828 as the templates. The *recA* gene encoded a putative 362 amino acid protein that had 69% identity to the product of the *B. subtilis* *recA* gene, with the most highly conserved residues being located in the N-terminal region. The *C. perfringens* gene was most closely related to *recA* genes from *B. subtilis*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Karlin *et al.*, 1995). For a complete comparative alignment of the RecA amino acid sequence see Roca & Cox (1997). A 28 bp inverted repeat, which may act as a factor-independent transcriptional terminator, was found directly downstream of the end of the gene and an imperfect inverted repeat structure, which could have regulatory significance, was located upstream of the gene (Fig. 3). A putative σ^{70} promoter sequence was identified upstream of the *recA* coding region.

In *B. subtilis* a conserved motif (GAAC-N₄-GTTC), called a Cheo box, has been identified in the upstream regions of damage-inducible genes (Cheo *et al.*, 1991). Deletion analysis of the *B. subtilis* *recA* promoter region has shown that this motif is required for the regulation of *recA* expression in *B. subtilis* (Cheo *et al.*, 1992, 1993). This sequence has also been identified in the promoter regions of the *recA* genes from other Gram-positive bacteria such as *M. tuberculosis*, *M. leprae*, *Staphylococcus aureus*, *Streptomyces lividans*, *Streptomyces ambofaciens* and *Streptococcus pneumoniae* (Davis *et al.*, 1991, 1994; Bayles *et al.*, 1994; Nußbaumer & Wohlleben, 1994; Pearce *et al.*, 1995). Similar sequences also exist upstream of the *recA* genes from *Lactococcus lactis* and *Corynebacterium glutamicum*, although these sequences are less well conserved (Duwat *et al.*, 1992). Analysis of the *C. perfringens* *recA* sequence revealed that there was a consensus Cheo box (Table 2) located

Fig. 2. Complementation of the *E. coli* *recA1* mutation. Viable counts were carried out on DH5 α strains carrying pWSK29 ($\square$), pJIR828 ($\blacktriangle$), pJIR1197 ($\blacksquare$) or pJIR1232 ($\circ$) following treatment of cell cultures with (a) MMS or (b) UV radiation. Values represent the means of two independent experiments.

8 bp downstream of the putative -10 promoter sequence (Fig. 3).

In *Escherichia coli*, the SOS regulon is controlled by both LexA and RecA. LexA acts as a repressor of SOS genes by binding to a consensus sequence, the SOS box, in the upstream region of these genes. Induction is achieved as a result of DNA damage, by activation of the *E. coli* RecA protein, which then mediates auto-proteolytic cleavage of the LexA repressor (Walker, 1984). Regulation of the SOS response in *B. subtilis* occurs via a similar mechanism. A LexA-like protein has been identified from *B. subtilis* and shown to bind specifically to the Cheo box or SOS operator site in the promoter regions of damage-inducible genes (Lovett *et al.*, 1993). This binding is abolished by activated *B. subtilis* RecA protein. The nature of the inducing signal which results in activation appears to be conserved in both *B. subtilis* and *E. coli* (Lovett *et al.*, 1994). The presence of similar operator sites in the upstream regions of the *C. perfringens* *recA* gene and *recA* genes from other Gram-positive bacteria suggests that the mechanism of regulation of these genes has been conserved between these organisms.

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1  AATAAAATGTTATAAACTTATAAAATATGTTTAAAAAGTCAAAAAATAGTCAATTAT
61  TAAGACATGAGGTTCTTTAAGAACTTCATGTTGATGTTGACTTTTTTTTGTATATATT
121  ATAATAATAGTACAACTTATGTCGGTTATTTAAGGAGTTACGTATGGCAATATAGAT
                                     M A N I D
181  AAAGATAAATTAAAGCTATTGAGATGGCTATGGGTCAAATAGAGAAGCAATTGGAAAG
      K D K L K A I E M A M G Q I E K Q F G K

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Fig. 3. Nucleotide sequence of the region upstream of the *recA* gene. Consensus -10 and -35 promoter regions are shown in bold. The putative Cheo box is shown in outline. The putative RBS and the transcriptional start site are underlined. The imperfect inverted repeat is indicated by the arrows.

Fig. 4. Mapping of the *C. perfringens* *recA* transcript. Primer extension experiments were performed on 50 μ g *C. perfringens* RNA samples isolated from mid-exponential-phase cells grown in TPG broth (lane 1) or TPG broth with 0.1% MMS (lane 2). The sequence of the coding strand is shown. The transcriptional start point is indicated by the asterisk.

Determination of the transcription start site of the *recA* gene

To map the transcription start point of the *C. perfringens* *recA* gene, and to determine whether the *recA* transcript was induced following DNA damage, RNA was extracted from *C. perfringens* cells grown in the presence or absence of 0.1% MMS. Primer extension experiments (Fig. 4) were used to map the beginning of the transcript to the A residue located immediately upstream of the Cheo box (Fig. 3). Transcription of *recA* appeared to be induced following growth of the *C. perfringens* cells in the presence of 0.1% MMS, suggesting that the *recA* gene is derepressed by DNA damage. To confirm this observation, dot-blots were carried out on RNA purified from cultures grown in the presence and absence of MMS. The results showed that

the *recA* gene, but not the control *gyrA* gene, was induced by exposure to MMS. It is likely that in *C. perfringens* there is a regulatory system similar to the SOS system of *B. subtilis*, whereby DNA damage results in activation of the RecA protein and leads to cleavage and inactivation of a LexA-like repressor protein which is normally bound at the SOS operator site. The net result would be induction of the *recA* gene and other potential damage-inducible genes from *C. perfringens*.

In summary, this study has led to the cloning and genetic analysis of the *recA* gene from *C. perfringens* and provided evidence that it is induced upon response to DNA damage. The data suggest that this gene has a similar function in DNA repair to that of the *E. coli* *recA* gene and is regulated via a similar mechanism to the *recA* gene from *B. subtilis*.

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Received 1 July 1996; revised 27 October 1996; accepted 31 October 1996.