Molecular Cloning and Isolation of a Cyanobacterial Gene Which Increases the UV and Methyl Methanesulphonate Survival of *recA* Strains of *Escherichia coli* K12

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The unicellular cyanobacterium *Gloeocapsa alpicola* contains both photoreactivation and excision repair mechanisms for correcting UV-induced damage to its cellular DNA. An 11.5 kb *EcoRI* fragment was isolated from a cosmid bank of *G. alpicola* and was shown to complement a *recA* deletion in *Escherichia coli* S.17 and JC10289. These *recA* strains showed increased survival to UV and methyl methanesulphonate (MMS) when transformed with the cyanobacterial DNA fragment, and also showed filamentation in response to UV irradiation. Preliminary analysis of the protein encoded by the cyanobacterial DNA fragment indicated a major protein of 39000 Da; this is very similar in size to the *recA* protein of *E. coli*.

INTRODUCTION

Cyanobacterial DNA repair systems have been poorly studied in the past. Kumar (1963) demonstrated that *Anacystis nidulans* responded to UV light in a similar manner to other organisms. The existence of enzymic photoreactivation was demonstrated in *Plectonema boryanum* by Werbin & Rupert (1968). A photoreactivating enzyme was purified from *Anacystis nidulans* by Saito & Werbin (1970) and shown to cleave pyrimidine dimers *in vitro* (Saito & Werbin, 1970) and *in vivo* (Tang & Asato, 1978). The efficiency of the photoreactivating system of *Gloeocapsa alpicola* was demonstrated by Williams *et al.* (1979), who showed that 60 min exposure to blue light restored the viability of UV-irradiated cells to pre-irradiation levels. A dark repair system for pyrimidine dimer removal has also been demonstrated in *G. alpicola* (O'Brien & Houghton, 1982*a*). Pyrimidine dimers were induced in a fluence-dependent manner and chromatographic analysis of labelled DNA extracted from cells grown in non-photoreactivating conditions showed that the dimers had been removed by an excision repair system. This DNA degradation was inhibited by caffeine and acriflavine (O'Brien & Houghton, 1982*b*), further confirming the existence of a dark excision repair system. O'Brien & Houghton (1982*b*) also reported that cyanobacteria may have a damage-inducible repair function. Cultures of *Synechocystis* PCC 6308 (*G. alpicola*) were exposed to a sublethal UV dose (19.2 J m⁻²) and incubated for 24 h under non-photoreactivating conditions. Half of each culture was then exposed to a challenge UV dose (198 J m⁻²) and the other half untreated. During a 48 h period after the challenge dose, there was a 40% loss of radioactivity from DNA in the induced culture, compared to a 60% loss in the absence of induction. In *Escherichia coli*, this limitation of DNA degradation is mediated by the *recA* protein (Marsden *et al.*, 1974). Satta *et al.* (1979) have also shown that synthesis of *recA* protein is necessary for limitation of DNA degradation. The damage-inducible repair function observed in *G. alpicola* may be similar to the SOS system of *E. coli*, since both the limitation of DNA degradation and its removal by inhibition of protein

Abbreviation: MMS, methyl methanesulphonate.

synthesis have been demonstrated by O'Brien & Houghton (1982*b*). The aim of this study was to clone and identify a gene from *G. alpicola* which may be involved in inducible DNA repair. The approach was to isolate a cyanobacterial gene similar to the *recA* gene of *E. coli* and study its effects on a variety of *E. coli* mutants. *E. coli* was chosen as a host since a complete range of mutants is available to study the pleiotropic effects of the *recA* gene, whereas no such mutants are available in *G. alpicola*.

METHODS

Organisms. *Gloeocapsa alpicola* 1430/1 (Lyngbye) Bornet was obtained from the Culture Centre of Algae and Protozoa, Cambridge, UK [Rippka *et al.* (1979) reclassified *G. alpicola* as *Synechocystis*]. *Escherichia coli* S.17, which has a deletion in its *recA* gene (Simon *et al.*, 1983), was obtained from the University of Bielefeld, FRG. *E. coli* strains AB1157 (a wild-type strain for repair functions) and JC10289 (a *recA*-deleted strain) were obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, USA. *E. coli* strains BHB 2688 and BHB 2690 (Hohn, 1979) were used for the preparation of *in vitro* packaging mixes as described in Maniatis *et al.* (1982).

Growth of bacteria. Liquid cultures of *G. alpicola* were grown in a modified medium of Allen (1968) as described by Williams *et al.* (1979), incubated in an illuminated orbital incubator at $30 \pm 2^\circ\text{C}$ at 100–120 oscillations min^{-1} . Light (2000 lx) was provided by white fluorescent tubes.

E. coli was routinely grown at 37°C on Luria broth (LB) or Luria agar (1.5%, w/v) containing the appropriate antibiotic when required.

Plasmids. pJC79 (Ap^r Tc^r cos) was used as a cosmid vector for the construction of the gene bank. pTM-2 (Ap^r *recA*⁺) was used as a source of *E. coli recA* and pACYC184 (Cm^r Tc^r) was used to subclone DNA from recombinant cosmids. Plasmid DNA was routinely prepared by standard *E. coli* cleared lysis procedures and isopycnic CsCl centrifugation. A rapid method for screening plasmids was also used (Birnboim & Doly, 1979).

Lysis of *G. alpicola*. Due to the highly resistant outer sheath and cell wall of *G. alpicola* (Jensen & Sicko, 1973) standard lysing procedures were not suitable. A rigorous lysis method was developed involving EDTA treatment of the external sheath, Sarkosyl NL-35 treatment of the proteins in the outer cell layer, osmotic and temperature shock of the outer membrane, followed by lysozyme and Sarkosyl treatment of the peptidoglycan and internal membrane. The procedure was as follows. A 50 ml volume of a late-exponential phase culture was centrifuged and the cells were resuspended in 5 ml 0.25 M-EDTA pH 8.0. After 15 min incubation at 37°C , 0.1 ml Sarkosyl NL-35 was added and mixed for 10 min at 37°C . The mixture was centrifuged at 4000 *g* for 10 min and then the cells were resuspended in 4 ml 1.6 M-sucrose, 50 mM-Tris, 1 mM-EDTA, pH 8.0, and incubated for 15 min at 37°C , followed by centrifugation at 20000 *g* for 15 min. The cells were resuspended in 4 ml distilled water and immersed in liquid N₂ for 60 s; after thawing, 2 ml lysozyme (5 mg ml⁻¹) was added and the mixture was incubated at room temperature for 2.5 h, followed by addition of 2 ml 10% (v/v) Sarkosyl NL-35 in 50 mM-Tris, 1 mM-EDTA, pH 8.0. The lysate was extracted twice with phenol, and the aqueous phase was centrifuged at 20 000 *g* for 20 min. The total DNA so released was purified by standard extractions and CsCl isopycnic centrifugation.

Construction of cosmid bank. Target DNA was prepared by digesting total *G. alpicola* DNA with varying concentrations of *Sau*3A over a fixed time period. Reaction conditions using 0.5 U *Sau*3A per 0.5 µg DNA h⁻¹ provided the maximum number of DNA molecules in the 35–40 kb region. The cosmid vector pJC79 was digested to completion with *Bam*HI and the target DNA was ligated into the tetracycline resistance gene of the vector at a molar ratio of 10:1 (target:vector). The final DNA concentration in the ligation mixture was 750 µg ml⁻¹. After ligation, the DNA was checked on a 0.7% (w/v) agarose gel to confirm the formation of concatamers. The concatameric DNA was packaged and transfected to the *recA* host S.17. Positive recombinant clones were selected by insertion inactivation of the tetracycline resistance gene of pJC79. The number of recombinant clones required to represent the entire genome of *G. alpicola* was calculated to be 500 by the method of Maniatis *et al.* (1982).

MMS enrichment for selection of *recA*-complementing ability. The cosmid bank was spotted onto 10 master plates, each containing 50 colonies. These colonies were replica plated, grown and the cells subsequently washed from the agar with 4 ml sterile saline. Samples (100 µl) from each plate were spread on MMS gradient plates, prepared by placing 100 µl 2% (w/v) MMS in the centre of LB-ampicillin plates and allowing it to diffuse into the medium to form a concentration gradient. The plates were incubated at 37°C for 48 h. Colonies that grew near the centre of the MMS gradient plates were deemed to have a higher level of DNA repair potential than the host strain S.17/pJC79. Since these colonies had been treated with the mutagen MMS, the apparent increase in survival could have been due to an up mutation in some other gene. To disprove this, the colonies from the relevant original master plates were individually screened for increased UV survival, using a rapid UV screening test.

Selection for *recA*-complementing ability using UV irradiation. The colonies from the relevant master plates were analysed for an increase in their UV resistance compared to the S.17/pJC79 *recA* host. Each colony was resuspended in 300 µl 0.85% sterile saline, and the cells were then streaked across an LB-ampicillin plate in one

direction only. The plates were masked into three sections and UV irradiated (253.7 nm) at 0, 4 and 8 J m⁻² under gold light conditions (500–700 nm) to prevent enzymic photoreactivation of the UV damage. The cells were incubated in the dark overnight at 37 °C and the plates examined and scored for increase in UV resistance.

Confirmation that the increased UV and MMS survival observed in E. coli S.17 was cosmid associated. DNA was isolated from the relevant strains and retransformed into another *recA*-deleted strain of *E. coli*, JC10289. The transformed clones of JC10289 were analysed for increase in UV resistance by the rapid UV survival screening method.

Quantitative analysis of the complementary effect of recombinant cosmids on the UV survival of E. coli S.17. *E. coli* S.17 containing putative *recA*-complementing cosmids was analysed quantitatively for UV survival. Overnight cultures of the strains were subcultured into LB with the selective antibiotic and grown to an OD₆₀₀ of 0.3–0.4. The cells were left in 0.85% saline for 30 min before irradiation to allow DNA replication to reach completion. Irradiation was done by exposing the culture (with agitation) to a mercury discharge lamp, emitting mainly at 253.7 nm. Dose rates were measured with a UV radiometer (UV Photoproducts). All irradiations were done under gold light to prevent enzymic photoreactivation of the UV damage. After irradiation, serial dilutions were plated onto LB agar with the selective antibiotic. After overnight incubation at 37 °C in the dark, the number of viable cells was counted and survival expressed as a percentage of the initial cell number. S.17/pJC79 was used as a negative and S.17/pTM2 as a positive control.

Quantitative analysis of the complementary effect of recombinant cosmids on the MMS survival of E. coli S.17. The response of putative *recA*-complementing cosmids in S.17 was determined by plating the cells on increasing concentrations of MMS. Cultures were grown overnight in LB with the selective antibiotic. Serial dilutions were made in 0.85% saline and 100 µl quantities of each dilution were spread onto LB plates containing MMS at 0, 50, 100, 150 and 200 µg ml⁻¹. The plates were incubated at 37 °C and colonies were counted after 12–24 h. The number of surviving cells was expressed as a percentage of the initial cell number.

Filamentation analysis of E. coli in response to UV irradiation. Cultures were grown overnight in LB, subcultured and grown to an OD₆₀₀ of 0.5. Cells were collected by centrifugation and resuspended in 0.85% saline, and the suspension irradiated at 5 J m⁻² under gold light conditions. The cells were collected again by centrifugation, resuspended in fresh LB medium and grown in the dark for 40–60 min at 37 °C. Samples were examined for filamentation by phase-contrast microscopy.

Restriction analysis of plasmid DNA. Enzyme reactions were routinely done in 30 µl volumes in which a ratio of 1 µg DNA to 2–3 U enzyme was used. A low-salt buffer (10 mM-Tris pH 7.5, 6 mM-MgCl₂, 1 mM-DTT) or a high-salt buffer (10 mM-Tris pH 7.5, 5 mM-NaCl, 6 mM-MgCl₂, 1 mM-DTT) was used depending on the enzyme requirement. When modifications from these conditions were required, NaCl, KCl and pH were adjusted according to the manufacturer's specifications. For double digestions, the enzyme requiring the lowest salt concentration was used first, and the salt and/or pH requirements were then changed for the second reaction. Molecular masses of DNA fragments were determined using *Hind*III and *Xho*I digestions of λ DNA as reference standards.

Ligation of DNA. The two preparations were combined at a molar ratio of 10 : 1 (target : vector). ATP was added to 0.5 mM and ligase reaction buffer was added from a 10 × stock (666 mM-Tris/HCl pH 7.6, 66 mM-MgCl₂, 100 mM DTT). One unit of T4 ligase was used and the reactions were incubated overnight at 12–14 °C.

Analysis of plasmid-encoded proteins by SDS-PAGE. Cultures (10 ml) were grown overnight in LB with the relevant antibiotic. The cells from a 2 ml sample of culture were collected by centrifugation, resuspended in 100 µl water and 100 µl PAGE loading buffer (10%, v/v, glycerol, 2%, w/v, SDS, 0.0625 M-Tris pH 6.8, 0.005% bromophenol blue, 5%, v/v, 2-mercaptoethanol) and heated at 100 °C for 2 min. Samples (30 µl) were loaded onto an SDS-polyacrylamide gel (5%, w/v, stacking, 12%, w/v, separating) and the gel was run at 60 V for 4–5 h. The gel was stained for 2 h in Coomassie staining solution, containing, per litre, 300 ml methanol, 628 ml water, 70 ml acetic acid, 2 ml Triton X-100 and 1.15 g Coomassie blue R250, and destained overnight with gentle agitation in methanol/acetic acid (3 : 7, v/v).

RESULTS AND DISCUSSION

Total DNA preparation

Standard lysing procedures for Gram-negative bacteria were not suitable for *G. alpicola*. Consequently, a method was developed which facilitated the extraction and purification of DNA from this organism (see Methods). One of the problems with the purification of the DNA was the separation of the photosynthetic pigments. Despite repeated proteinase K treatment, phenol and phenol/chloroform extractions, the DNA was not sufficiently pure to provide a high ligation frequency in subsequent gene cloning. The inclusion of a CsCl purification step removed the inhibitor(s) of ligation.

Fig. 1. UV streak test analysis on cosmid bank clones showing variation in the degree of UV resistance. The irradiation dose is shown at the top of the photograph. Clones S.17/4, S.17/9 and S.17/11 were chosen for further analysis.

Selection of recA-complementing cosmid clones by MMS enrichment

Examination of the MMS gradient plates after overnight incubation of the pooled S.17/cosmid clones revealed that growth had occurred near the centre of three of the 10 plates (the equivalent of master plates 1, 4 and 5). This indicated that the cosmid bank may have contained clones which were complementing the *recA* mutation of the host. To ensure that the MMS selection procedure had not induced a mutation which would alter the survival of *E. coli* S.17 to the alkylating agent, the original recombinant clones from the master plates were examined for *recA*-complementing ability by UV survival analysis.

Each *E. coli* S.17/cosmid clone was individually examined for its UV survival characteristics by the rapid UV survival test. Six clones had a higher level of UV resistance than the S.17/pJC79 *recA* parent control strain; they varied in their degree of resistance (Fig. 1). This provided evidence for the existence of a function in the cosmid bank of *G. alpicola* which was capable of increasing the UV and MMS survival of the *recA* strain *E. coli* S.17. The three clones (S.17/4, S.17/9 and S.17/11) that showed the highest level of UV resistance were chosen for further analysis. The cosmid DNA was purified from these strains and retransformed to *E. coli* JC10289. This strain has 90% of its structural *recA* gene deleted and is consequently very sensitive to UV radiation. The cosmids purified from S.17/4, S.17/9 and S.17/11 were designated pRCG4, pRCG9 and pRCG11 respectively. UV survival analysis (data not shown) of the JC10289 derivatives demonstrated that the increased level of UV resistance which had been shown with *E. coli* S.17/4, S.17/9 and S.17/11 (Fig. 1) was also observed when pRCG4, pRCG9 and pRCG11 were present in JC10289. This directly demonstrated the association between the *recA*-complementing ability observed in *E. coli* S.17 and JC10289 and the recombinant cosmids pRCG4, pRCG9 and pRCG11.

Restriction analysis of pRCG4, pRCG9 and pRCG11

The three cosmids with *recA*-complementing activity were analysed with a range of restriction enzymes to determine if any restriction fragments were common to all three. The most suitable enzyme for comparison purposes was *EcoRI*, which had one site in pRCG4 and two sites in both pRCG9 and pRCG11. The three cosmids had an 11.5 kb *EcoRI* fragment in common. Cosmids pRCG9 and pRCG11 had the same restriction patterns and were regarded as being the same clone. Since the size of pRCG4 (11.5 kb) was too small for *in vitro* packaging during the construction of the gene bank, this cosmid must have been deleted *in vivo* prior to its purification. Stability studies on pRCG4 and pRCG9, using varying levels of antibiotic selection and repeated transformations of both plasmids, indicated that pRCG9 was more stable than pRCG4. Consequently, pRCG9 was selected for further analysis.

Fig. 2. UV (a) and MMS (b) survival analyses of *E. coli* S.17/pJC79 (○), S.17/pTM-2 (●) and S.17/pRCG9 (□). UV irradiations were done under gold light conditions to prevent enzymic photoreactivation.

Quantitative effect of pRCG9 on the UV and MMS survival of *E. coli* S.17

Fig. 2(a) shows the effect of pRCG9 on the UV survival of the *recA* strain *E. coli* S.17. *E. coli* S.17/pJC79 was included as a negative control for *recA* and S.17/pTM-2 was used as a positive *recA*⁺ control. A typical unshouldered survival curve for S.17/pJC79 was obtained, indicating the absence of complete repair capacity, whereas S.17/pTM-2 displayed a large shoulder over the dose range 0–10 J m⁻², demonstrating the ability of the *recA*⁺ gene (from pTM-2) to increase the level of survival in the *recA* strain S.17. The effect of pRCG9 on the UV survival of S.17 was intermediate between the *recA* mutant strain (S.17/pJC79) and the repair-proficient strains (S.17/pTM-2). At 10 J m⁻² the survival of S.17 was less than 1%, whereas when this strain was transformed with pRCG9, UV survival increased to approximately 50%.

To examine the effect of the recombinant cosmid pRCG9 on the MMS survival of a *recA* strain of *E. coli*, the responses of S.17/pJC79, S.17/pTM-2 and S.17/pRCG9 were determined by plating the cells onto increasing concentrations of MMS (Fig. 2b). S.17/pJC79 was extremely sensitive to the alkylating agent, with a survival of approximately 2% at 50 μg MMS ml⁻¹. The introduction of the *recA*⁺ genotype to S.17 rendered the cells almost completely resistant. The presence of pRCG9 in S.17 enabled the cells to grow up to 200 μg MMS ml⁻¹. At this concentration, S.17/pRCG9 had a 52% survival rate, compared to 93% survival for a wild-type strain. The *recA* mutant showed no growth at this level of MMS. Above 50 μg MMS ml⁻¹, the colonies appeared very small after 24 h growth.

Effect of pRCG9 on filamentation in *E. coli* S.17

Chromosomal damage caused by treatment of *E. coli* with a range of mutagens is alleviated by the induction of the SOS response (Witkin, 1976). This effect is controlled by a finely-tuned balance between the products of the *recA* and *lexA* genes and is the major response to DNA-damaging treatment (Little & Mount, 1982). Under normal growth conditions, the *lexA* protein represses expression of the SOS genes by binding to a specific recognition sequence at the operator, the SOS box (Little *et al.*, 1981). The *sfiA* gene, which codes for cell division inhibition, is one of the genes under the control of *lexA*. After DNA damage or the perturbation of replication, *sfiA* is expressed, causing cell division arrest and the formation of long filaments. Filamentation is completely dependent on functional *recA*–*lexA* circuitry. Since *E. coli* S.17 has a deletion in the *recA* gene, it has lost the ability to induce the SOS system, and thus filamentation, in response to DNA damage. The ability of pRCG9 to complement the *recA* mutation of *E. coli* S.17 and to promote the expression of filamentation was analysed with S.17/pJC79, S.17/pRCG9 and S.17/pTM-2. No microscopic evidence of filamentation in S.17/pJC79 was seen, whereas S.17/pRCG9 showed definite cellular filamentation (Fig. 3). S.17/pRCG9 showed no filamentation when non-irradiated cells were examined. The positive

Fig. 3. Effects of UV irradiation (5 J m^{-2}) on S.17/pJC79 (a) and S.17/pRCG9 (b). The presence of pRCG9 in S.17 enables the cells to form filaments in response to UV irradiation, implying that the cyanobacterial gene is capable of complementing the *recA* mutation of S.17 (see text).

control strain S.17/pTM-2 also showed cellular filamentation in response to UV irradiation, suggesting that a gene from pRCG9 was being expressed and synthesizing a protein capable of inducing filamentation either directly or indirectly. Although this effect could be explained by the existence of a pRCG9-associated gene which was involved in filamentation or the regulation of cell division in *G. alpicola*, neither UV nor mitomycin C induced filamentation in the cyanobacterium. It was therefore assumed that the filamentation effect was associated with the cosmid-linked *recA*-complementing function.

Subcloning the recA-complementing function from pRCG9

Digestion of pRCG9 with *EcoRI* yielded two fragments (11.5 kb and 23.5 kb). Since the 11.5 kb fragment was common to the original *recA*-complementing isolates from the gene bank, it was subcloned into pACYC184 and *recA*-complementing activity in S.17 and JC10289 was examined. Both *EcoRI* fragments from pRCG9 were successfully cloned into pACYC184. The transformants were screened for a chloramphenicol-sensitive, tetracycline-resistant phenotype and such clones were subsequently screened for increased UV survival characteristics. Eight clones showing higher UV resistance than S.17 alone were isolated and their plasmids were purified by CsCl centrifugation. These plasmids were retransformed into JC10289 and a UV survival analysis was performed to confirm their ability to increase the UV survival of *recA* *E. coli* hosts. A low rate of plasmid instability was found among the JC10289/pA-CYC184::pRCG9 transformants, and clones which had lost their *recA*-complementing phenotype had *in vivo* deletions in their plasmid DNA, as shown by alterations in the restriction patterns of the unstable plasmids. This loss of *recA*-complementing ability accompanied by *in vivo* deletions in the plasmid DNA provided further evidence to assign a *recA* function to the cyanobacterial cosmid clone pRCG9.

Restriction analysis of the recA-complementing pACYC184::pRCG9 subclone

Preliminary restriction analysis of the eight *recA*-complementing subclones demonstrated that they all contained the 11.5 kb *EcoRI* fragment of pRCG9. The pACYC184::pRCG9 hybrid plasmid selected for further analysis was designated pCL4. A restriction map of this plasmid is shown in Fig. 4.

An initial screening of the protein(s) encoded by the plasmid pCL4 was performed by transforming S.17 with pCL4, checking for a *recA*-complementing phenotype of the resulting transformants and preparing total proteins from an overnight culture for examination by SDS-PAGE (Fig. 5). A major protein of approximately 39 kDa was synthesized in the S.17/pCL4 strain, but was absent from the two control strains, S.17 and S.17/pACYC184. This protein, which is similar in molecular mass to the *E. coli* *recA* protein (Sancar *et al.*, 1980) and is coded for

Fig. 4. Restriction map of the *recA*-complementing plasmid pCL4. —, The vector pACYC184; —, the *EcoRI* fragment subcloned from pRCG9.

Fig. 5. SDS-PAGE analysis of total proteins synthesized in (A) S.17/pCL4, (B) S.17/pACYC184 and (C) S.17. Molecular mass standards were bovine serum albumin (67 kDa), ovalbumin (45 kDa) and trypsinogen (24 kDa). The putative cyanobacterial '*recA*' protein with an approximate molecular mass of 39 kDa is indicated by the arrow in lane A.

by the recombinant pCL4, may be the protein responsible for the increase in UV survival observed when this plasmid is present in the *recA* strain S.17.

The results of this study clearly show a strong conservation in the functional domains of the *recA* proteins from two diverse organisms, *G. alpicola* and *E. coli*.

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