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Characterization of Several Transformation-deficient Mutants of *Streptococcus pneumoniae* in DNA Damage

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Seventeen transformation-deficient mutants of *Streptococcus pneumoniae*, which are defective in competence induction(*com*$^-$), DNA uptake(*ent*$^-$) or recombination(*rec*$^-$), were investigated to determine sensitivity to ethylmethane sulfonate(EMS), methylmethane sulfonate(MMS), UV and mitomycin C. In ethylmethane sulfonate assay, the viability of most *com*$^-$, *rec*$^-$ and *ent*$^-$ mutants was decreased about 2-10 times and the viability of *ent-9* and *ent-13* mutant was decreased about 33 and 25 times, respectively. On the other hand only half of the transformation-deficient mutants tested was sensitive to methylmethane sulfonate about 2 times and ent-12 mutant was sensitive to 2.0% MMS about 8 times. After UV and mitomycin C treatment, most of the mutants are not sensitive to UV and mitomycin C, although the viability of some transformation-deficient mutants was decreased slightly. Especially none of the *com*$^-$ mutants were sensitive to DNA damage suggesting that competence is not involved in DNA repair. Also DNA uptake and recombination gene might be related to DNA repair function.

Key words : *S. pneumoniae*, DNA damage, Transformation

INTRODUCTION

Streptococcus pneumoniae, a highly transformable Gram positive organism, has a special physiological state, referred to as competence. Competence occurs simultaneously in all cells of a competent culture. The timing of competence is known to be controlled by an excreted protein; the "competence factor", which induces competence and a dramatic change in the pattern of proteins synthesized by a culture. At competence, DNA is degraded into oligonucleotide, taken up into the cytoplasm, and recombined by homologous recombination resulting in transformant formation. A set of new proteins, competence specific proteins (CSPs), are synthesized during competent period and have been considered to play a role in DNA uptake and recombination (Morrison & Baker, 1979; Pearce *et al.*, 1995). Although many transformation-deficient mutants of *S. pneumoniae* have been isolated (Morrison *et al.*, 1983; Morrison *et al.*, 1984) and several genes involved in the transformation process have been cloned, only part of them have been characterized (Chandler & Morrison, 1987; Clave & Trombe, 1989; Hui & Morrison, 1991; Lopez *et al.*, 1989; Martin *et al.*, 1985; Martin *et al.*, 1992; Prats *et al.*, 1985; Radnis *et al.*, 1990; Rhee & Morrison, 1988). In this study, the sensitivity of transformation-deficient mutants of *S. pneumoniae* to DNA damage was determined to characterize which mutant is sensitive to DNA damage and to get information on relationship between transformation and DNA damage.

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MATERIALS AND METHODS

The bacterial strains used in this study are listed in Table I and insertion sites of *com*$^-$ mutants are illustrated in Fig. 1. All *S. pneumoniae* strains were grown in broth derived from CAT either in liquid or on solid medium containing 1.5% agar. Broth was composed of 5 mg of choline, 10 g of casein hydrolysate (Difco), 5 g of tryptone (Difco), 5 g of NaCl and 1 g of yeast extract (Difco) per liter. It was sterilized for 50 min at 121°C and then brought to 0.2% glucose and 16.6 mM dipotassium phosphate before use. Cultures were grown in broth at 37°C without aeration, and cells were diluted and washed in the same medium. Cultures to be frozen were first brought to 10 to 15% glycerol and then stored at -70°C. Turbidity was measured in the Spectronic 20 spectrophotometer at 550 nm with 13×100 mm Pyrex screw cap culture tube used directly as a cuvette. Under these conditions, cultures in broth usually grew exponentially to an optical density of 0.2 (OD 0.2).

Table I. Bacterial strains used in this study

Strain	Genotype	Source	Reference
CP1200[a]	*Xfo*$^+$	CP1015×BP272DNA	Morrison *et al.*, 1983
CP1206	*rec*-6	CP1200×MNNG[b]	Morrison *et al.*, 1983
CP1208	*rec*-8	CP1200×MNNG	Morrison *et al.*, 1983
CP1209	*ent*-9	CP1200×MNNG	Morrison *et al.*, 1983
CP1211	*ent*-11	CP1200×MNNG	Morrison *et al.*, 1983
CP1212	*ent*-12	CP1200×MNNG	Morrison *et al.*, 1983
CP1213	*ent*-13	CP1200×MNNG	Morrison *et al.*, 1983
CP1214	*ent*-14	CP1200×MNNG	Morrison *et al.*, 1983
CP1216	*ent*-16	CP1200×MNNG	Morrison *et al.*, 1983
CP1217	*ent*-17	CP1200×MNNG	Morrison *et al.*, 1983
CP1218	*rec*-18	CP1200×MNNG	Morrison *et al.*, 1983
CP1219	*rec*-19	CP1200×MNNG	Morrison *et al.*, 1983
CP1220	*rec*-20	CP1200×MNNG	Morrison *et al.*, 1983
CP4101	*comB*$^-$	*ermB* insertion[c] at 3660::*Taq* I>	Chandler and Morrison, 1987
CP4102	*comA*	*ermB* insertion at 3335::*Taq* I>	Chandler and Morrison, 1987
CP4103	*comA*	*ermB* insertion at 3090::<*Taq* I	Chandler and Morrison, 1987
CP4104	*comA*	*ermB* insertion at 2150::<*Cla* I	Chandler and Morrison, 1987
CP4105	*com*	*ermB* insertion at 5300::<*Taq* I	Chandler and Morrison, 1987

[a]wild type.
[b]N-methyl-N'-nitro-N-nitrosoguanidine.
[c]Restriction site used for *ermB* insertion (Chandler & Morrison, 1987); distance from the HindIII site, in base pairs, as indicated by the scale in Fig. 1

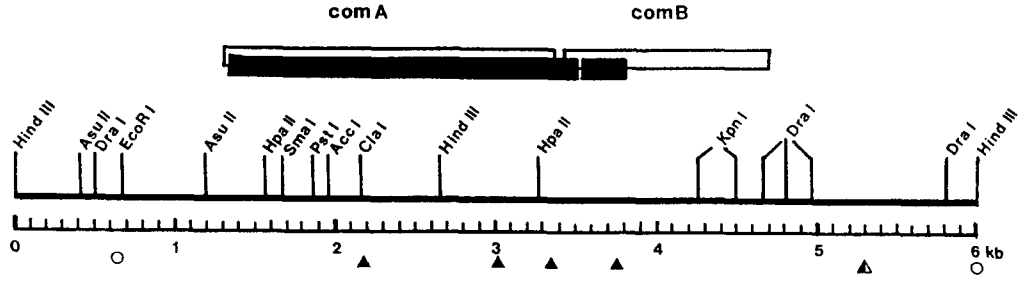

Fig. 1. Map of the *S. pneumoniae com* locus (Hui & Morrison, 1991). Open boxes show genes *comA* and *comB* as deduced from gene truncation studies(Chandler & Morrison, 1988). Solid boxes show genes deduced from DNA sequence (Hui & Morrison, 1991). Triangles indicate the portions of insertion mutations, and circles indicate the outer ends of insertion-duplication mutations. Mutant phenotypes Com$^-$ and Com$^+$ are indicated by closed and open symbols, respectively.

Cultures grown to OD 0.2 in CAT broth were exposed to the mutagen ethylmethane sulfonate (EMS; 0.1%, 0.5%, 2.0%), methylmethane sulfonate (MMS; 0.1%, 0.5%, 2.0%) or mitomycin C (0.1 mg/ml, 0.5 mg/ml, 2.0 mg/ml) for 30 min at 37°C. The mutagen-treated cells were washed twice in CAT broth, diluted in the same medium. Each dilution of mutagen-treated cells was plated on CAT agar and grown for 24 h at 37°C. In UV irradiation experiment, 2 ml samples of exponential-phase cultures were poured into a Petri dish of 5cm diameter and irradiated with stirring at a distance of 30 cm from 15 W germicidal lamp (Sam-Kong Co. Japan) giving an incident dose rate of 0.75 mW/cm^2/sec as measured with a short-wave UV meter (UV Products, Inc., USA). Samples were withdrawn at various times and plated in CAT agar medium after appropriate dilutions. Colonies were scored after 24 h at 37°C. All these operations were made in the dark to avoid possible photoreactivation.

RESULTS

Sensitivity of transformation-deficient mutants to EMS

The results of the EMS sensitivity experiment are shown in Fig. 2 [A]. *com*$^-$ mutants were not sensitive to EMS treatment. The *ent*$^-$ mutants showed wide range of sensitivity to EMS: The survival rates of *ent*-9 and *ent*-13 were 3% and 4%, respectively, of the wild type, however survival rates of *ent*-14 and *ent*-16 were 16% and 9%, respectively, of the wild type at 2.0% EMS concentration. *ent*-11, *ent*-12 and *ent*-17 had survival rates similar to the wild type.

The survival rates of two *rec*$^-$ mutants, *rec*-8 and *rec*-20, were 5 to 9% of the wild type at 2.0% EMS

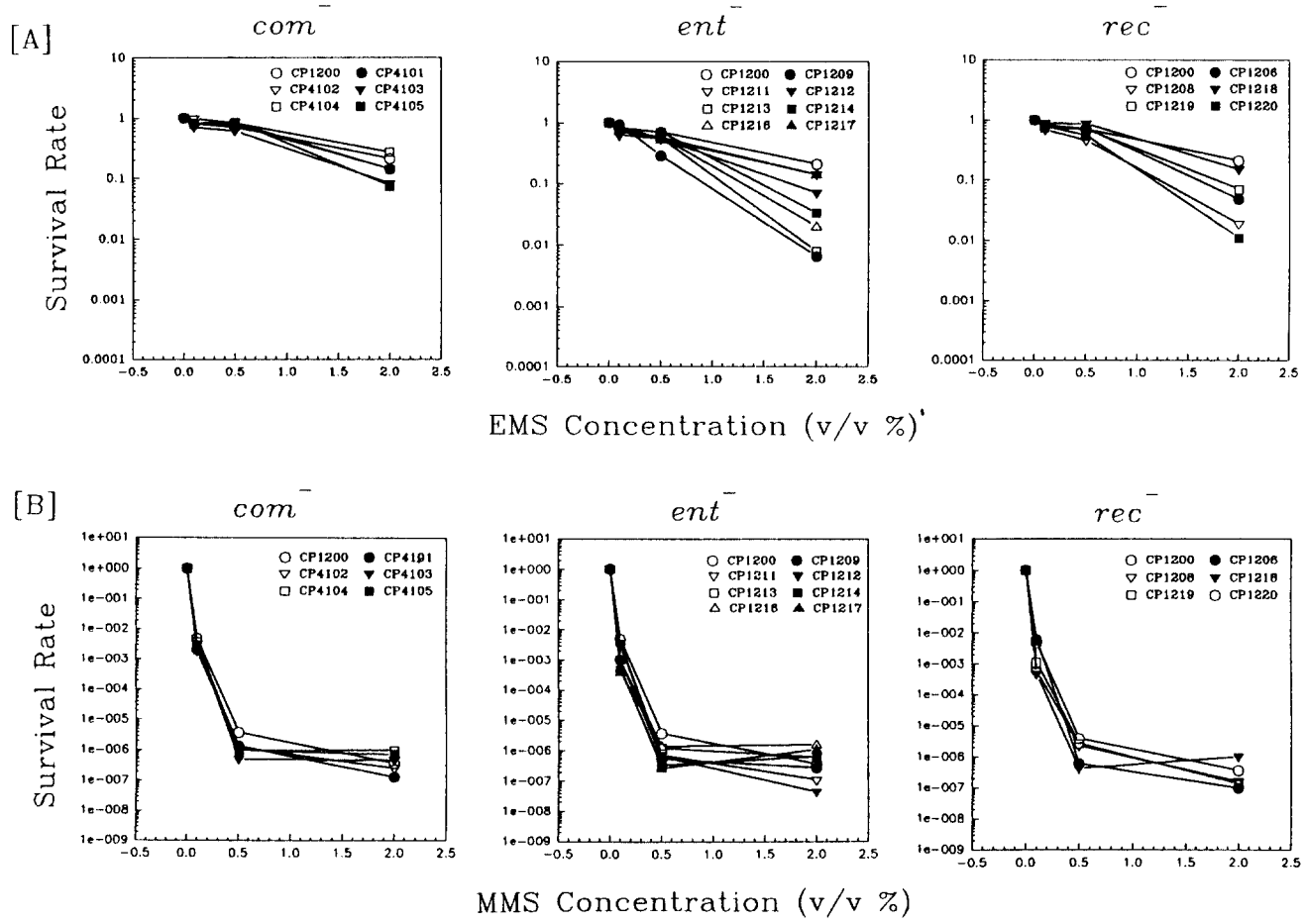

Fig. 2. Survival rates of *S. pneumoniae* strains after EMS (top row) and MMS (bottom row) treatment. Cultures were grown in casein hydrolysate (CAT) broth at 37°C without aeration(Morrison *et al.*, 1983), and 2×10^8 cells in CAT broth were incubated for 30 min at 37°C with the specified concentration of EMS and MMS, washed twice, diluted appropriately with the CAT broth and plated on CAT agar. Number of cells survived were scored after 24 hr incubation at 37°C. Survival rate was measured in duplicate for three times, and presented as geometric mean. [A] EMS treatment. [B] MMS treatment.

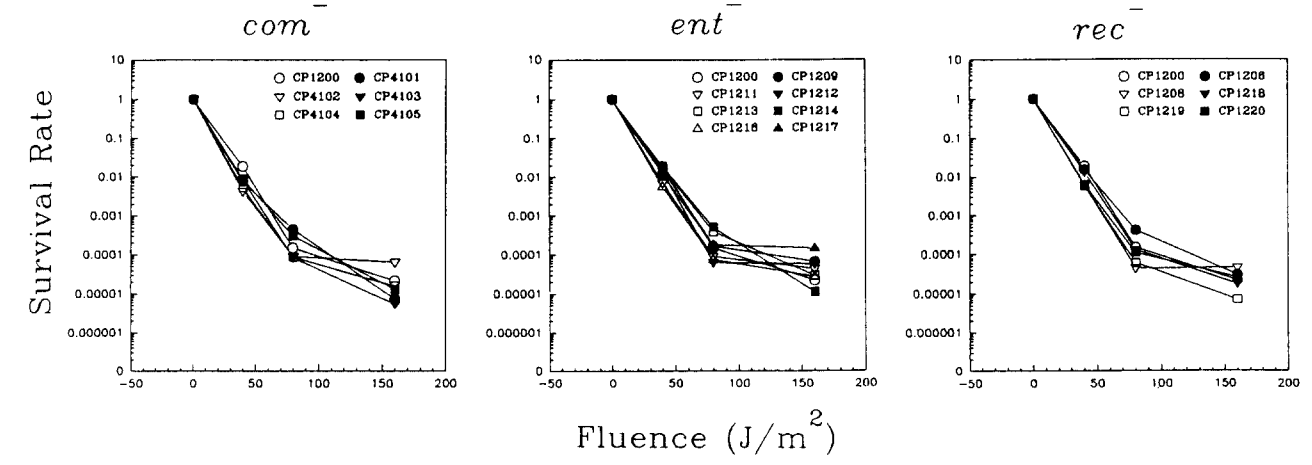

Fig. 3. Survival rates of *S. pneumoniae* strains after UV irradiation in balanced salt solution. 2×10^8 exponentially growing cells were harvested, washed and resuspended in 1.5 ml balanced salt solution (Sicard, 1964), and poured into a Petri dish of 5 cm diameter and irradiated with UV producing an incident dose rate of 0.75 mW/cm²/sec. After irradiation, the cell suspensions were diluted and plated on CAT agar medium. Colonies were scored after 24 hr incubation at 37°C. All these operations were made in the dark to avoid possible photoreactivation. Survival rate was measured in duplicate for three times, and presented as geometric mean.

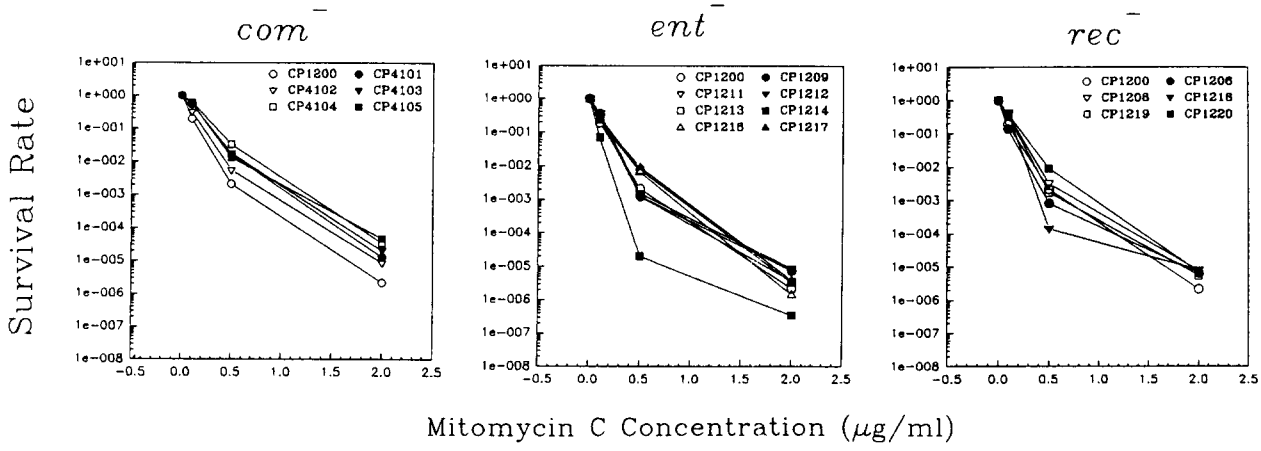

Fig. 4. Survival rates of *S. pneumoniae* strains after mitomycin C treatment. Cultures were grown in casein hydrolysate (CAT) broth at 37°C without aeration (Morrison *et al.*, 1983), and 2×10^8 cells in CAT broth were incubated for 30 min at 37°C with the specified concentration of mitomycin C, washed twice, diluted appropriately with the CAT broth and plated on CAT agar. Number of cells survived were scored after 24 hr incubation at 37°C . Survival rate was measured in duplicate for three times, and presented as geometric mean.

concentration. But the remaining 3 *rec*⁻ mutants, *rec*-6, *rec*-18 and *rec*-19, had survival rates of 23%, 71%, and 33%, respectively, of the wild type.

Sensitivity of transformation-deficient mutants to MMS

Fig.2 [B] shows the sensitivity of transformation-deficient mutants to MMS. All *com*⁻ mutants had no significant changes in viability after MMS challenge. *ent*-13, *ent*-14, *ent*-16, and *ent*-17 mutants showed similar survival pattern; *ent*-13, *ent*-14, *ent*-16, and *ent*-17 had the survival rates of 20%, 9%, 8%, and 11%, respectively, of the wild type at 0.1% MMS concentration, but they had higher survival rates than the wild type at 2.0% MMS concentration. But the survival rate of *ent*-12 was close to the wild type at 0.1% MMS concentration, but it was decreased to 12% of the wild type at 2.0% MMS concentration. The survival rates of *ent*-9 and *ent*-11 were close to the wild type or slightly sensitive to MMS treatment.

The survival rate of *rec*-18 was 9% of the wild type at 0.1% MMS concentration, but it was higher than the wild type at 2.0% MMS concentration. The other *rec*⁻ mutants, *rec*-6, *rec*-8, *rec*-19, and *rec*-20, were slightly sensitive to MMS treatment.

Sensitivity of transformation-deficient mutants to UV and mitomycin C

Fig. 3 and Fig. 4 show the sensitivity of transformation-deficient mutants to UV and mitomycin C, respectively. In these experiments, virtually none of the transformation-deficient mutants were sensitive to UV. All transformation-deficient mutants except *ent*-14 and *rec*-18 mutants were marginally resistant to mitomycin C treatment: The survival rate of *ent*-14 mutant was 1% of the wild type at 0.5 μg/ml of mitomycin C. *rec*-18 mutant showed 7% viability at 0.5 μg/ml of mitomycin C challenge, but it showed slight resistance to 0.1 μg/ml and 2 μg/ml of mitomycin C.

DISCUSSION

Our results revealed that the viability of *S. pneumoniae* was 10^2 to 10^6 times more sensitive to MMS treatment than EMS treatment. Although both EMS and MMS are DNA alkylating agents, a distinct difference in sensitivity to EMS and MMS was observed. DNA methylation caused by MMS seems to be more detrimental than DNA ethylation caused by EMS. One possible explanation for this observation may be that ethylated DNA damage is much more efficiently repaired than methylated DNA.

It is known that the development of competence in *B. subtilis* is accompanied by the transcriptional activation of DNA damage-inducible operons and other SOS-like responses (Love *et al.*, 1985; Yasbin, 1977). The *recA* mutant of *B. subtilis* is sensitive to UV irradiation and mitomycin C treatment, and the SOS repair system is induced by certain forms of DNA damage (Love *et al.*, 1985; Yasbin, 1977). But in *S. pneumoniae*, the relationship between competence and SOS response has not been resolved. It was proposed that *S. pneumoniae* does not exhibit SOS response because UV irradiation and thymidine starvation does not induce any increase in the mutation rate (Gasc *et al.*, 1980; Grist & Butler,1983) although the *S. pneumoniae recA* null mutant is UV sensitive and deficient for chromosomal transformation (Martin *et al.*, 1992). There is neither a photoreactivation (Goodgal

et al., 1957) nor an efficient inducible repair process similar to the SOS repair system in *E. coli* (Gasc *et al.*, 1980), however *S. pneumoniae* does have the capacity to carry out excision repair when exposed to UV light (Estevenon & Sicard, 1989). In *S. pneumoniae*, UV-induced pyrimidine dimers might be repaired by a system similar to the *uvr*-dependent system in *E. coli* (Sicard & Estevenon, 1990; Sicard *et al.*, 1992).

The *com* mutants (Chandler & Morrison, 1987) we used were constructed by an *ermB* insertion mutagenesis (Fig. 1). These mutants were defective in competence induction but could be complemented by competent culture supernatant. If competence were induced upon DNA damage, CSPs might be involved in DNA repair and the viability would be decreased in the *com* mutants relative to the wild type. But our results showed that none of the *com* mutants were sensitive to DNA damage which suggests that competence is not involved in the DNA repair process.

ent mutants were defective in DNA entry, but not defective in competence development: DNA is degraded at competence, but uptake is blocked or severely reduced. These mutants showed wide range of sensitivity to EMS and MMS, but no sensitivity to UV and mitomycin C except *ent*-14 mutant. Of 7 *ent* mutants, *ent*-14 mutant was the most sensitive to mitomycin C treatment: its survival rate was only 1% of the wild type on 0.5 μg/ml of mitomycin C treatment but was same as the wild type on UV irradiation. This information would be invaluable for *ent*-14 gene cloning.

Recombination deficient mutants were defined as mutants which are defective in chromosomal integration, but they are normal in competence development as well as DNA uptake. They were divided into two classes (Morrison *et al.*, 1983): The first class, *rec*-8 and *rec*-20 mutants, have deficiency in chromosomal recombination but not in plasmid reassembly. These mutants were sensitive to EMS but not to other DNA damaging agents. Mutants of the second class, *rec*-6, *rec*-18, and *rec*-19 mutants, were defective both in plasmid reassembly and chromosomal recombination. Of these 3 mutants, *rec*-18 mutant revealed different sensitivity to MMS and mitomycin C treatment from *rec*-6 and *rec*-19 mutants. It was sensitive at 0.1% MMS concentration but became resistant to 2.0% MMS concentration. Also *rec*-18 revealed sensitivity to 0.5 μg/ml mitomycin C concentration, but not to other mitomycin C concentration.

Although the *rec* mutants (*recA*, *recH*, *recR*, *recN*, *recF*, *recL* etc.) of *B. subtilis* are known to be sensitive to UV (Alonso *et al.*, 1993a,b; Ceglowski *et al.*, 1990), the *rec* mutants of *S. pneumoniae* were resistant to UV than the standard wild type or had the same sensitivity as the wild type. The absence of sensitivity towards UV and mitomycin C for *rec* mutants of the *S. pneumoniae* suggests, indirectly, that the *rec* alleles of *S. pneumoniae* employed here are not involved in DNA repair, especially in a *recA*-like function. This conclusion is based on the fact that the *recA* null mutant of *S. pneumoniae* is UV sensitive and defective in chromosomal transformation (Martin *et al.*, 1992). Further studies on back crossing of sensitive mutants to wild type should be done to verify which gene in the sensitive mutant is responsible for the DNA repair function.

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