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Chemical and UV mutagenesis in *Zymomonas mobilis*

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Abstract

Mutagenesis of the facultative anaerobe *Zymomonas mobilis* was accomplished by three different mutagens. Ultra-violet (UV) irradiation, whose effectiveness relies on misrepair of damaged DNA via an error-prone pathway, was a poor mutagen for this organism. Ethyl methane sulphonate (EMS) gave results very similar to UV-irradiation. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), which is believed to act by multiple mutagenic mechanisms, was the most powerful mutagen, always resulting in a large number of mutants of all types examined (i.e. auxotrophs, antibiotic resistant, heavy metal resistant and ultraviolet sensitive). Reversion frequencies of MNNG-induced mutants were very low. Evidence is provided that mutagenesis of *Z. mobilis* is affected by photoreactivation, adaptive response and error-prone repair mechanisms. Moreover, cells treated with alkylating agents and allowed to recover under anaerobic conditions clearly demonstrated that anaerobiosis plays a significant role in repair, but not in the induction of mutants.

Introduction

The effects of UV irradiation and simple alkylating agents, such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or ethyl methane sulphonate (EMS), on mutagenesis and DNA repair have been studied extensively in *Escherichia coli* and other aerobic bacteria (Walker, 1984; Friedberg, 1985). Mutants of obligate anaerobes are, in general, very difficult to obtain (Droffner & Yamamoto, 1983; Abratt, Jones & Woods, 1985). The bacterium *Zymomonas mobilis* is not a strict anaerobe. However, its specific growth rate is 25-40% lower in the presence of oxygen than under anaerobic conditions (Bringer-Meyer, Finn & Sahm, 1984), thus indicating a preference of the organism to grow anaerobically. Although some antibiotic resistant mutants of *Z. mobilis* have been produced by different research groups employing UV-irradiation and/or alkylating agents (Skotnicki, Tribe & Rogers, 1980; Goodman, Rogers & Skotnicki, 1982; Walia *et al.*, 1984), there is still a distinct lack of a thorough search in the mechanisms of mutagenesis and repair in this organism. Moreover, there is an increasing number of reports raising the problem of marker stability in both spontaneous and induced mutations (Walia *et al.*, 1984; Bringer-Meyer, Scollar & Sahm, 1985; Buchholz & Eveleigh 1990).

Thus, this study was undertaken to achieve two goals: firstly, to produce stable mutants suitable for further biochemical and genetic analysis, and secondly to establish the optimum conditions for chemical and UV mutagenesis in *Z. mobilis*, in the account of aerobiosis and the possible repair mechanisms of this bacterium.

Materials and methods

Bacterial strains and growth conditions

Z. mobilis wild-type strains CP4, NCIB 11163, ATCC 10988 and its derivative CU1, which apparently lacks at least one plasmid species with concomitant reduced ethanol production (Drainas, Typas & Kinghorn, 1984), were used throughout

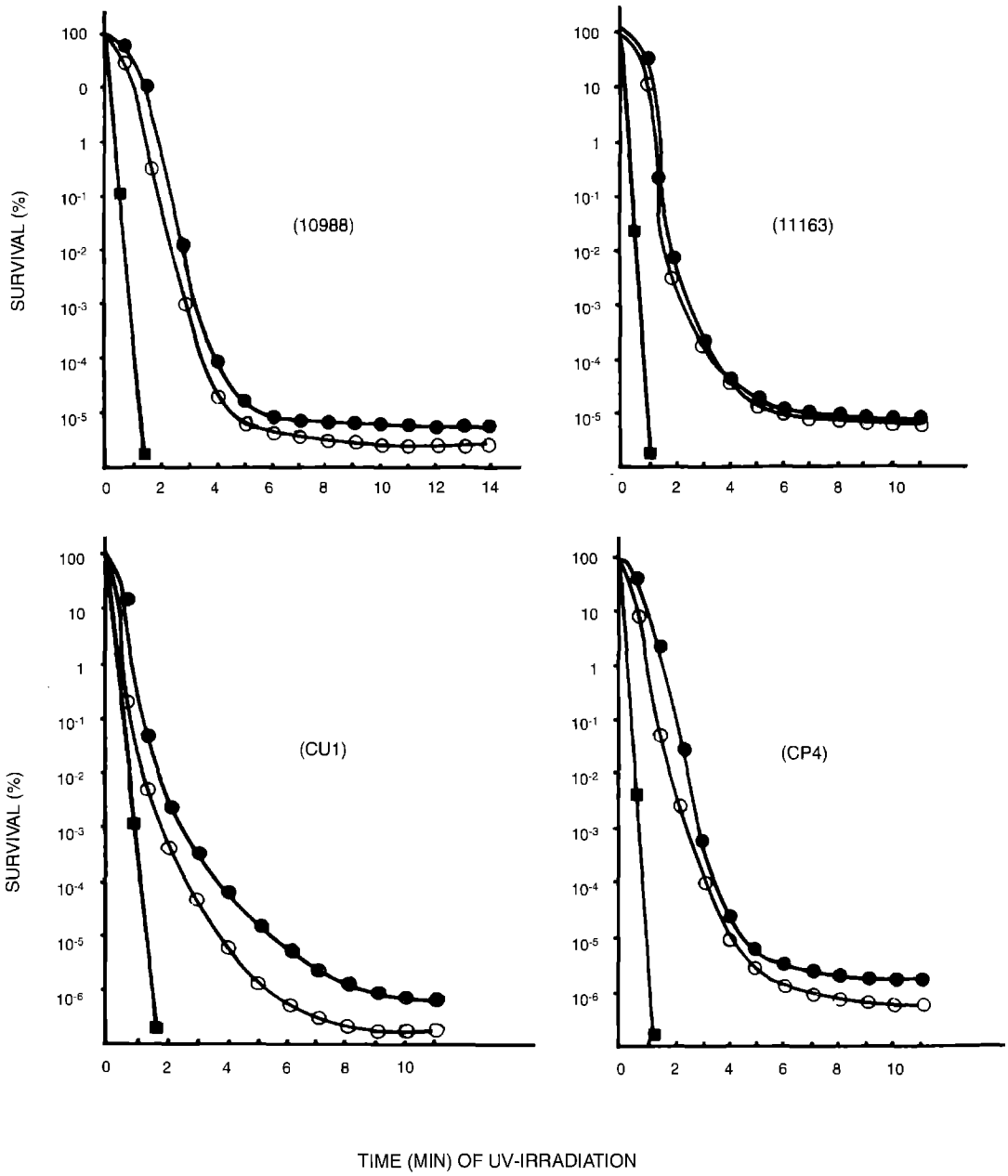

Fig. 1. The effects of UV-irradiation on survival of *Z. mobilis* strains ATCC 10988, CU1, NCIB 11163 and CP4 following incubation in the dark (○—○) or light (●—●). *Uvs* mutants of each strain (■—■) were incubated only under photoreactivation conditions.

this work. UV-sensitive mutants (*uvs*) from each of these strains, produced by MNNG mutagenesis (see below), were isolated and used in all experiments in which the relative repair mechanisms were to be examined.

A chemically defined minimal medium (MM), which satisfies the growth requirements of all *Z. mobilis* strains (Galani, Drainas & Typas, 1985), was used for the isolation of auxotrophic mutants. Solid media were prepared with the addition of 2% agar, and complete media (CM) were made up by adding 1% yeast extract to the MM. All cultures were grown at 30 °C in the dark. Anaerobiosis was achieved by cooling the culture medium from the

sterilization temperature (120 °C, 15 lb · inch^{-2}) to 30 °C under a stream of nitrogen and CO_2. Liquid media were grown into capped vials and agar plates were grown in anaerobic jars (Gas-Pack system, Oxoid) or in an atmosbag (Sigma) filled with a mixture of N_2 and CO_2.

UV-irradiation and chemical mutagenesis

Cells from liquid cultures in late exponential phase were pelleted by centrifugation, washed three times in Ringer's solution, resuspended into either Ringer's or CM and placed into glass petri dishes at a maximum density of 10^7 cells/ml. They were then irradiated for various periods of time, at 254 nm, with a General Electric lamp (G 15TB, with an ozone filter) at 12 J · m^{-2} (as measured by a Jagger meter). Similarly, to a series of 10 ml suspensions, prepared as above, different concentrations of ethyl methane sulphonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were added and incubation was continued for 3 h, both aerobically and anaerobically. EMS and MNNG treatment was performed by standard methods (Gerhardt *et al.*, 1981). The treated cells were harvested as above, washed three times, resuspended in CM, and viable counts were performed on the appropriate plates following serial dilutions.

Isolation of mutants

In preliminary experiments, the minimum inhibitory concentrations (MICs) of the inhibitors used in this work were established [Table 1]. At these MICs, naturally occurring and induced resistant mutants were isolated and further examined for stability after 50 cell cycles. Surviving colonies from any treatment (UV-irradiation, MNNG or EMS) were replica plated on CM plates containing methyl methane sulphonate (MMS) or Mitomycin C at their MICs. Colonies unable to grow on mitomycin C or MMS were further tested for UV sensitivity.

Auxotrophs were isolated by replica plating large numbers of mutagenized colonies from CM onto MM plates. Mutant enrichment was achieved by treatment with ampicillin 250 μg · ml^{-1} for 18 h. In all later experiments, to make results comparable, concentrations of each compound and UV-irradiation fluence used were those causing a 99.9% decrease in cell viability of wild-type strains. Similarly, once MNNG was established as the best mutagen, 250 μg · ml^{-1} of this were used together with ampicillin to obtain auxotrophic mutants. The treated cells were pelleted after 3 h, washed three times in CM and resuspended in rich CM (containing 5% yeast extract) in which they were allowed to recover for 12-15 cell cycles in order to minimize unstable mutants.

Detection of repair mechanisms

The effects of post-irradiation treatment on the survival of *Z. mobilis* cells in the dark or under visible light was examined by the immediate incubation of UV-irradiated cells under these conditions (Fig. 1). Caffeine and arsenite, specific inhibitors for photoreactivation and recA-dependent excision repair respectively, were used at MICs shown in Table 1. Their effect on survival of UV-irradiated cells was

Table 1. Minimum inhibitory concentrations of various inhibitors used for the isolation of resistant mutants.

	ATCC 10988	CU1	NCIB 11163	CP4
Amikacin (Amk^R)	40	30	40	30
Chloramphenicol (Cml)^R	30	30	50	70
Furazolidone (Fur^R)	35	35	50	40
Rifampicine (Rif^R)	12	12	14	18
Tetracycline (Tet^R)	15	15	15	15
Mitomycin C	5	5	5	5
MMS	3	3	3	3
Caffeine	5	2.5	5	2.5
Arsenite	0.02	0.02	0.5	0.02

* antibiotics and mitomycin C in μg · ml^{-1} and all others in mg · ml^{-1}.

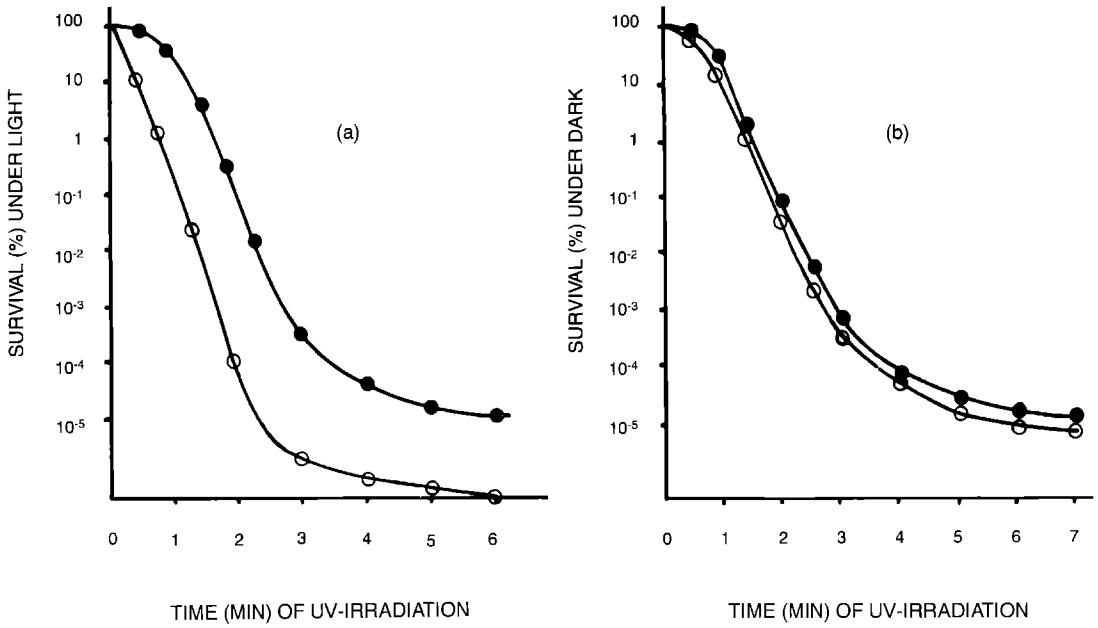

Fig. 2. The effects of caffeine (a) and arsenite (b) on a representative strain of *Z. mobilis* (CP4) following incubation under light for the former and dark for the latter [treated cells (●—●), non-treated (○—○)]. Mutation frequencies were very similar for treated and non-treated cells and are omitted for clarity.

compared with untreated cells (Fig. 2). Adaptive response, a mechanism which renders *E. coli* cells more resistant to the killing and the mutagenic effects of alkylating agents (Jeggo, 1979) was detected in *Z. mobilis* as follows: cells were grown in MM until they reached ~10^8 cells · ml^{-1}. EMS or MNNG were added in the culture at concentrations which did not affect survival (5 μg · ml^{-1} and 10 μg · ml^{-1} respectively), and the treated cells were allowed to grow for another 3 h. Cells were harvested by centrifugation, washed once with MM containing 2% $Na_2S_2O_3$ to inactivate residual MNNG or EMS and resuspended in a series of vials containing CM with various concentrations of the mutagens and incubated for 3 h, both aerobically and anaerobically. ($Na_2S_2O_3$ itself affected neither survival nor mutagenesis). Viable counts and mutation frequencies were estimated by standard methods. All data presented in the figures are the average of at least four independent experiments.

Results

The effects of UV-irradiation and the isolation of uvs mutants

Direct methods for *uvs* mutant isolation are time-consuming and often result in colonies showing only a slight sensitivity to UV. Thus, the suitability of an indirect selection procedure for UV sensitivity based on the use of mitomycin C or MMS was investigated. Mitomycin C seemed to be somehow inactivated under anerobic conditions. All *Zymomonas* strains had a MIC of ~5 μg · ml^{-1} when plates were stored first aerobically for 24 h and then exposed to anaerobic conditions for 4 days. In plates kept under strict anaerobic conditions for more than 24 h the compound lost its activity and MIC was greater than 25 μg · ml^{-1}. From a large number of MNNG treated colonies (3,000) which were examined for sensitivity to mitomycin C, only 6 mutants were isolated (0.2% of total, i.e. 4 isolates of NCIB 11163 and 2 of CP4), all of which were extrasensitive to mitomycin C (MIC 0.5 μg · ml^{-1}); none were sensitive to

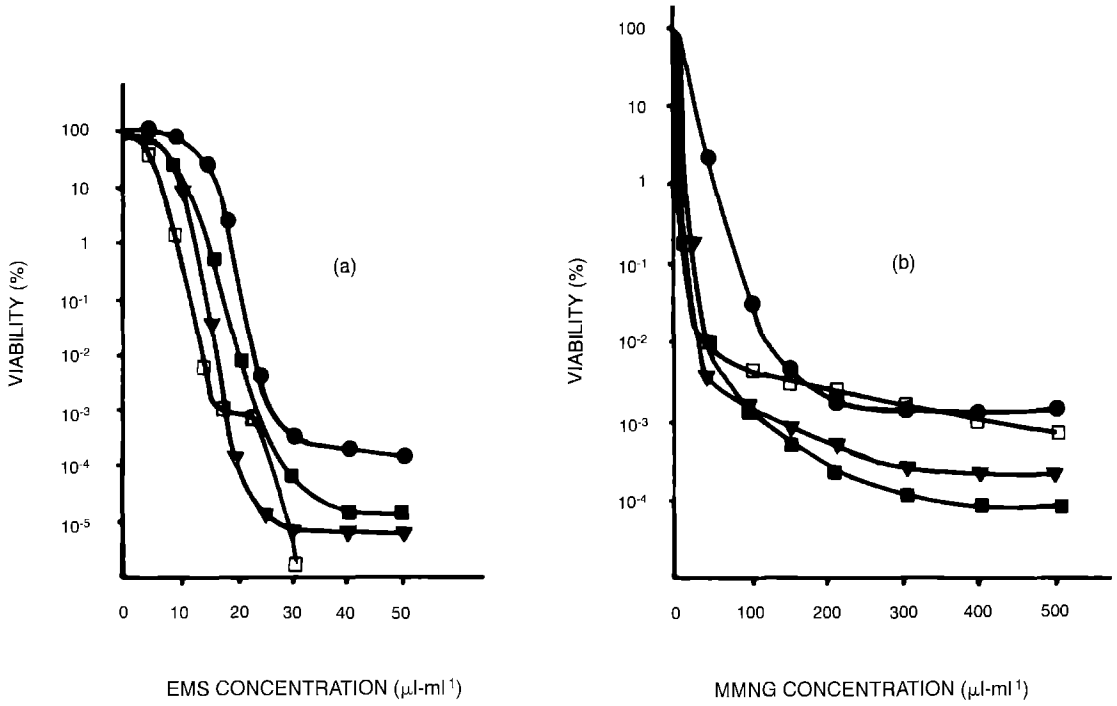

Fig. 3. Effects of EMS (a) and MNNG (b) on *Z. mobilis* strains ATCC 10988 (○—○), CU1 (□—□), NCIB 11163 (●—●) and CP4 (■—■).

UV. On the contrary, MMS was an excellent agent to screen for *uvs* mutants. From over 5,000 MNNG treated colonies which were examined, 32 *uvs* mutants (0.64%) were isolated. Several such mutants were isolated from each strain, as well as some mutants which were sensitive to this mutagenic agent but resistant to UV-irradiation. Most of the former mutants showed a remarkable sensitivity to UV-irradiation, in contrast with the wild-type strains.

The four wild-type *Z. mobilis* strains and one representative *uvs* mutant from each of them were used in further experiments; their reaction to UV-irradiation following incubation in the dark or light is shown in Fig. 1. All data presented in the figures are the averages of four independent experiments in each case. With the exception of strain NCIB 11163, it can be seen that the other three strains show a constant (~10-fold) increase of survival under photoreactivation conditions, whereas post-irradiation incubation in the dark had no effect on any of the eight strains. In general, forward mutation frequencies with UV-irradiation were very low for all types of mutants tested. Moreover, these mutants exhibited a characteristic instability as most of them reverted back to wild-type phenotype when examined for more than 50 generations. Post-irradiated cells were incubated under aerobic or anaerobic conditions but no accountable differences in survival or forward mutation frequencies were observed.

UV-irradiated cells from all strains were transferred to CM and CM-caffeine containing liquid media which were subsequently incubated in the dark or light for 24 h. The cells were then plated onto the appropriate CM and CM-rifampicine containing plates on which the survival and mutation frequencies were estimated. A marked decrease (10-fold to 10^3-fold) in the survival levels was observed for caffeine-treated cells incubated under light conditions in comparison with the survival on the control plates (Fig. 2a). As expected, when caffeine-treated cells were incubated in the dark no difference in survival was observed. Similarly, the post-irradiation treatment with caffeine did not seem to affect the mutation frequencies in treated cells, both under light or dark. Arsenite had a much weaker effect and only on plates incubated in the dark. Under these conditions a 2-fold to 10-fold decrease in survival was observed, with strains

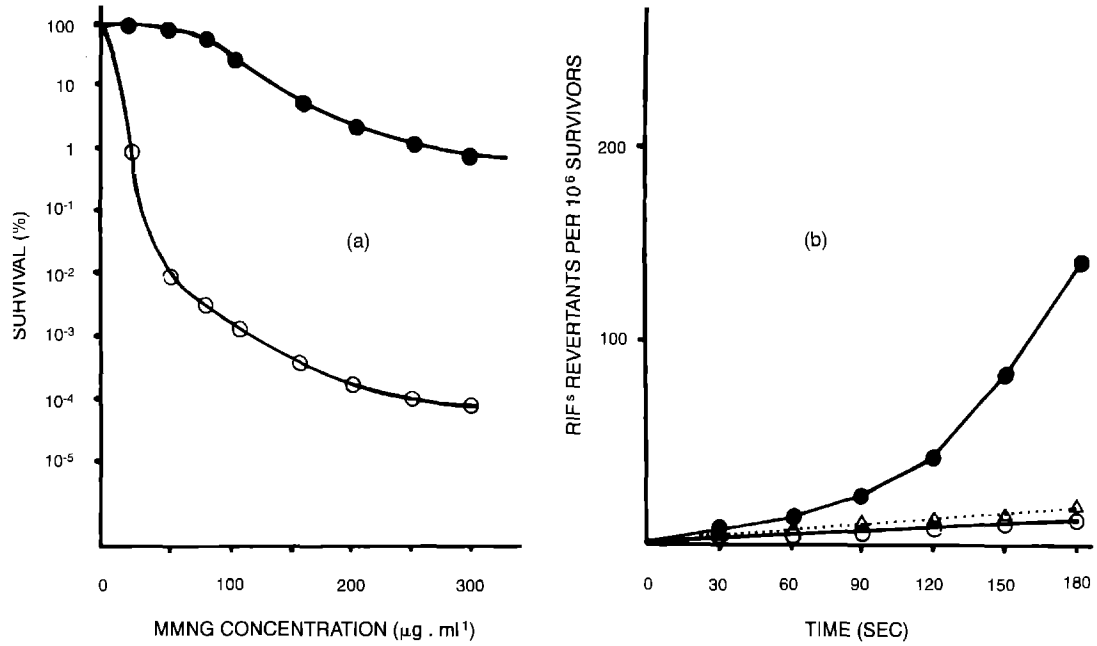

Fig. 4. (a) Survival of a representative strain of *Z. mobilis* (CP4) at various concentrations of MNNG, with (●—●) or without (○—○) prior exposure to 10 μg · ml^{-1} MNNG. (b) Reversion frequencies of CP4 Rif^R to wild-type (Rif^S) in 50 μg · ml^{-1} EMS (△—△) or 100 μg · ml^{-1} MNNG (●—●) for varying times under potentially adaptive and non-adaptive (○—○) conditions.

ATCC 10988 and CP4 always showing the higher decrease in viability (Fig. 2b). Forward mutation frequencies of treated cultures remained unchanged for all strains (data not shown for clarity). It should be pointed out here that neither caffeine nor arsenite, at their MICs, reduced colony-forming ability of any of the *Z. mobilis* strains, and similarly no obvious effects on the overall metabolism of this bacterium were observed at these concentrations.

Treatment with EMS and MNNG

The survival curves of all wild-type *Z. mobilis* strains treated with EMS or MNNG are presented in Fig. 3. It is evident that at concentrations of EMS higher than 20 μg · ml^{-1} all strains show a great reduction of viability (6-7 orders of magnitude), after which all curves reach a plateau. Strain CU1 shows a further dramatic loss of viability at concentrations above 30 μg · ml^{-1} with no surviving colonies even when more than 10^9 cells were plated. The mutagenic effects of EMS were very similar to those of UV-irradiation; only the frequencies for antibiotic resistant mutants were slightly higher. On the contrary, treatment with MNNG had a surprisingly weaker effect on the survival of all strains tested. The respective plateau was reached at high concentrations of MNNG (150 μg · ml^{-1}), with a reduction in viability of 4-5 orders of magnitude (Fig. 3). All *Z. mobilis* strains show a remarkable resistance to MNNG, and there is some viability even at concentrations of 500 μg · ml^{-1} that no other bacteria are known to tolerate. In spite of the fact that all strains of the bacterium exhibited high levels of resistance to MNNG, it was the most powerful mutagen. As far as stability of mutants is concerned, EMS-induced mutants were relatively unstable showing high reversion rates (with the exception of Rif^R mutants), whereas MNNG-induced mutants exhibited a high degree of stability with extremely low reversion rates for all types of mutants produced.

Using concentrations of EMS or MNNG at levels which already give maximum lethal effect, e.g. 30 μg · ml^{-1} and 100 μg · ml^{-1} respectively, treated cells were examined for viability every 30 min for up to 6 h. Duration of exposure to these mutagens did not seem to have any effect, as no obvious differences in survival or mutagenesis frequencies

were observed for all strains. On the contrary, anaerobiosis of treated cultures seemed to have a marked effect, especially on the production of mutants conferring antibiotic resistance. In most cases, at least, a 10-fold to 100-fold improvement of survival rates was observed on antibiotic-containing plates incubated anaerobically. This effect was particularly strong for amikacin and kanamycin, for which a 1,000-fold increase on the survival rates of treated cells was observed. Out of five antibiotics used, only furazolidone resistance was not affected by conditions of incubation; survival was the same for both aerobically and anaerobically grown cultures of the same strains. Back mutation rates were totally unaffected by these incubation conditions, giving similar numbers of revertants in almost all cases.

A wide variety of mutants have been produced from all strains tested in this work. Almost all types of auxotrophs have been produced (14 amino acid requiring, 6 vitamin requiring and 2 nitrogen base requiring), but their distribution was not completely random. The most predominant types comprised over 85% of the total number of auxotrophs and were those requiring cysteine, methionine, histidine, serine, arginine and alanine (given in order of numbers obtained). No aspartate, glutamine, glutamate, isoleucine, lysine or threonine requiring auxotrophs were obtained from any of the strains tested. The vitamin requiring auxotrophs were very rare (0.6% of the total) and only one isolate of each of the following types were obtained: p-amino benzoic acid, biotine, nicotinic acid and choline for strain ATCC 10988, and pantothenate and inositol for strain CP4. From preliminary experiments (data not shown) we knew that all *Z. mobilis* strains have a natural high tolerance against several antibiotics, e.g. they can tolerate (almost 100% viability) concentrations of 60 $\mu g \cdot ml^{-1}$ ampicillin, 100 $\mu g \cdot ml^{-1}$ neomycin, 250 $\mu g \cdot ml^{-1}$ nalidixic acid, and 750 $\mu g \cdot ml^{-1}$ for either streptomycin or trimethoprim. Thus, to avoid selection at extremely high concentrations of antibiotics, resistant mutants were isolated only on those antibiotics which seemed to have a strong effect on wild-type cells at low concentrations (Table 1). Even then, the MIC values are still remarkable when compared with the relative concentrations at which mutants of other bacteria are isolated. The stability of all the MNNG mutants was examined for a long period of time and the majority of these remained stable for over two years.

Influence of mutagenesis by the adaptive response

It is known that the adaptive response induced by alkylating agents increases resistance to DNA damaging agents, and hence decreases mutagenesis frequencies. This is probably due to interference with the induction of the SOS response. To ascertain whether high concentrations of alkylating agents such as MNNG or EMS could interfere with the expression of the SOS genes through the adaptive response, the survival levels of all wild-type strains were examined following prior treatment of cells at low levels of both EMS and MNNG (see methods). EMS failed to induce the adaptive response at significant levels; only in the case of strain NCIB 11163 a slight reduction of the killing effect of EMS was observed. Similarly, the same mutagen also failed to cause an induction on forward mutation frequencies. On the contrary, MNNG proved to be an excellent inducer of this repair system, affecting drastically the survival levels of all strains tested. The survival of pretreated cells was increased by 10^4-10^6 at concentrations of MNNG up to 250 $\mu g \cdot ml^{-1}$, both aerobically and anaerobically. The effects of adaptation on a representative strain (CP4) are given in Fig. 4a (the others are omitted for clarity). Pretreatment with MNNG had a much weaker effect on mutation frequencies of all strains. Under both aerobic and anaerobic conditions, a slight increase of forward mutation frequencies (varying between 4-16% from strain to strain) was observed with MNNG pretreatment. Results for the most representative strain (CP4) are presented in Fig. 4b.

Discussion

Although UV light and EMS are fairly potent mutagens for *E. coli* and other microorganisms, they were only poorly mutagenic for *Z. mobilis*. On the contrary, MNNG which, like EMS, induces mutations primarily by the error-prone DNA repair pathway, was highly effective in producing various types of stable mutants at much higher frequencies. The high instability of UV- and EMS-induced mutants, as well as the strong mutagenic activity of

MNNG, may be due to the nature of these mutagens; the former ones are known to produce mainly point mutations, whereas the latter may also produce multiple mutations or larger lesions (Schnedel *et al.*, 1978). Defining the optimum dose of the alkylating agent accurately is usually important to ensure efficient mutagenesis. However, this was not adequate for *Z. mobilis*, as the mutagenic effect of the alkylating agents used increased only slightly with the increase of their concentration. Moreover, duration of treatment did not increase mutation frequencies. Concentrations of alkylating agents and dose of UV-irradiation required for killing of cells or production of mutants were all remarkably higher than those reported for *E. coli* or any other gram$^-$ bacteria (Witkin, 1976; Schnedel *et al.*, 1978; Vericat, Guerrero & Barbe, 1986); thus indicating putative highly effective repair systems in *Z. mobilis* which decrease drastically the mutation frequencies.

Although the production of auxotrophs in *Z. mobilis* has been previously reported (Goodman, Rogers & Skotnicki, 1982), neither this research group nor any other group has used auxotrophs in subsequent genetic experiments with this organism so far, possibly due to mutant instability. Furthermore, the high natural antibiotic resistance levels of *Z. mobilis* strains observed in our work and also reported by several others (Skotnicki, Tribe & Rogers, 1980; Walia *et al.*, 1984; Buchholz & Eveleigh, 1986) restricts the isolation of appropriately marked resistant mutants for genetic analysis. In order to overcome these difficulties, a wide variety of mutants which fulfill the needs for basic biochemical and genetic analysis have been produced in this work. Several stable auxotrophs have been produced for the first time in this bacterium, including vitamin-requiring mutants. In particular, previous researchers failed to obtain the latter class of mutants, as they have used for selection a medium which contained a mixture of vitamins (Goodman, Rogers & Skotnicki, 1982). It should be pointed out that most of the mutants isolated in the present work were very stable and could be maintained safely for a period of over two years.

Facultative anaerobes have both aerobic and anaerobic respiratory systems. Moreover, their repair mechanisms should be equally effective in order to replicate and survive under both environments. In *Salmonella typhimurium*, Droffner & Yamamoto (1983) reported the inhibition of mutagenesis under anaerobic conditions. In spite of the fact that survival always increased under anaerobiosis, forward mutagenesis frequencies in *Z. mobilis* were inhibited under the same conditions, especially for mutants concerning resistance to antibiotics. This is easily explained as it has long been known that anaerobiosis increases the minimal inhibitory concentrations of various antibiotics such as aminoglycosides, affecting the membrane potential for their uptake (Davis, 1987). However, back mutation rates of the same mutants were totally unaffected, thus indicating that anaerobiosis has an effect on repair rather than on mutagenesis itself.

Repair systems such as the visible light-induced photoreactivation, the uvrA, B, C excision repair system and certain glycosylases are known to remove photolesions prior to replication (Miller, 1985). Three out of four *Z. mobilis* strains examined in this work, i.e. ATCC 10988, CU1 and CP4, exhibited a small but constant increase of survival following incubation under light conditions immediately after UV-irradiation. This was possibly due to the operation of a photoreactivation repair system in this organism. Caffeine is a specific inhibitor of 'error-free' repair in *E. coli* (Rothman, 1980). Its effect on treated cells, i.e. a significant decrease of survival levels, as well as a less distinct increase of mutation frequencies, provided a strong indication that mutation rates are also effected by a putative excision repair mechanism of *Z. mobilis*. Arsenite, which is an inhibitor of 'error-prone' recA dependent repair (Rossman, Meyn & Troll, 1975), had only a weak effect on *Z. mobilis*. However, a strong adaptive response was expressed by all *Z. mobilis* strains. As it has been shown in other bacteria that the adaptive response is due to the induction of several genes involved in repair of alkyl adducts in DNA and is part of the SOS system (Jeggo, 1979; Evensen & Seeberg, 1982; Mitra, Pal & Foote, 1982), this effect may suggest a high recA activity. MMS sensitivity proved to be an accurate criterion for the isolation of *uvs* mutants; several such mutants from each strain have been isolated. The availability of UV-MMS sensitive mutants may prove to be useful for a thorough study of the recA repair system in *Z. mobilis*.

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