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UV and Ethyl Methanesulfonate Effects in Hyperthermophilic Archaea and Isolation of Auxotrophic Mutants of *Pyrococcus* Strains

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Abstract. The lethal and mutagenic effects of ethyl methanesulfonate (EMS) and UV on nine archaeal strains belonging to each of the two described genera of Thermococcales, *Pyrococcus* and *Thermococcus*, were investigated. To test the efficiency of the EMS and UV mutagenesis under a variety of experimental conditions, we chose *Pyrococcus abyssi* strain GE5 as a model strain. We observed a strong induced mutagenicity in both cases, since the spontaneous mutation frequency (expressed as the frequency of resistance to 5-fluoroorotic acid) increased up to 150-fold with EMS and 400-fold with UV, after mutagen exposure. Although a heterogeneous response to the induced effects caused after EMS and UV exposures was detected for all the other sulfothermophilic archaea tested, an efficient mutagenicity of *Pyrococcus*-like isolates GE27, GE23, and GE9 was observed. Optimal procedures described for UV mutagenesis yielded a number of useful uracil auxotrophic mutant strains of *Pyrococcus abyssi*.

Sulfothermophilic archaea have several unusual characteristics, such as their capacity of growth at high temperatures (≥80°C) with short doubling times. Members of the genera *Pyrococcus* and *Thermococcus* can be studied now more easily with the improvement of different techniques such as plate cultivation, growth on simple compounds as the sole carbon or nitrogen source, or amenability to routine biochemical analysis. In addition, different cryptic plasmids have been described, and some genes have been cloned and sequenced [2, 3, 11, 15, 16, 18]. Successful experiments have been performed to isolate spontaneous mutants and specific mutant phenotypes of Sulfolobales or methanogens; however, in hyperthermophilic archaea, *Pyrococcus furiosus* and other *Pyrococcus* strains have been chosen by many researchers as model strains; thus, the development of genetic tools in hyperthermophilic archaea is now a priority. Despite the absence of a transformation system in Thermococcales, studies on genetic transfer systems are generally frustrated owing to the lack of stable genetic markers such as auxotrophic mutants to score for successful recombinant events, since these microorganisms are resistant to most antibiotics [20]. Therefore, studies that focus on the utilization of different mutagens in Thermococcales are clearly needed to overcome such difficulties.

Few studies with EMS treatments have been carried out in archaea, and the result is that few mutants have so far been produced [8, 12, 14], and none in Thermococcales. No data on the response of the Thermococcales to the lethal or mutagenic effects induced by EMS and UV were reported; thus, the aim of this study has been to examine these effects on nine Thermococcales strains, representatives of the two genera *Pyrococcus* and *Thermococcus*. To establish the optimal mutagenesis experimental conditions, we have studied the influence of several variables on mutagenesis by using the best characterized strain in our laboratory, *Pyrococcus abyssi* strain GE5. We have also used these data to produce mutants by mutagenesis in the uracil biosynthetic pathway, to compare the action of mutagens with spontaneous selection. To our knowledge, this is the first report on the optimization of EMS and UV treatments for general mutagenic purposes in hyperthermophilic archaea, and on the selection of auxotrophic mutants in Thermococcales.

Materials and Methods

Bacterial strains and growth conditions. Nine Thermococcales strains were selected for this study: *Pyrococcus abyssi* strain GE5 and

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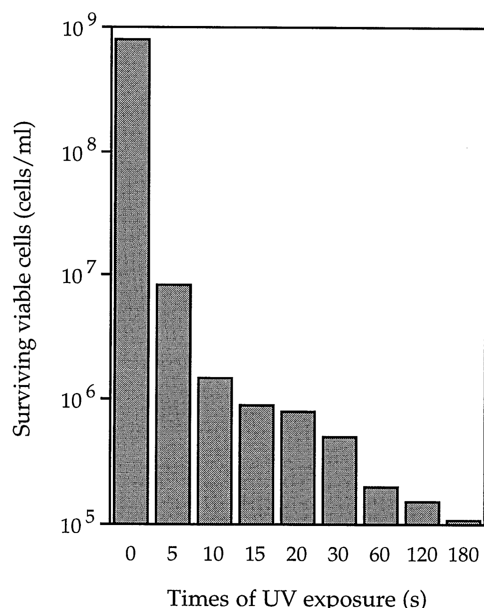

Fig. 1. Killing action of UV in *Pyrococcus abyssi* strain GE5 for various times.

the isolates GE1, GE7, GE9, GE23, GE27, and GE31 were isolated in our laboratory [13]; *Pyrococcus furiosus* (DSM 3638) and *Thermococcus stetteri* (DSM 5262) were obtained from the German Collection of Microorganisms (Göttingen, Germany). Strain GE5 and the isolates GE27, GE23, GE7, GE9, and GE1 were compared with *Pyrococcus*-like, and strain GE31 with *Thermococcus*-like [13].

The microorganisms were cultured in a saline medium (SW) supplemented with yeast extract (1 g/L) and bacto-peptone (4 g/L), (VSM medium) [4]. Furthermore, other sulfur and salt concentrations were also used in experiments in which we studied the effects of different salt concentrations and sulfur sources on the lethality of EMS and UV. The pH of each of these media was always adjusted to 6.8 (unless otherwise stated), and incubation was at 85°C in an oven. When necessary, solid media were prepared by adding 1% (wt/vol) gelrite (Sigma) [20]. Plates used for the detection of mutants were inoculated inside an anaerobic tent (Calhene, Velizy, France) containing an atmosphere of N_2:CO_2:H_2 (90:5:5) [6]. The inoculated plates were sealed in glass jars (Le Parfait, Familia Wiss, Reims, France) and incubated at 85°C, with a plating efficiency varying between 30% and 50%. For a test of the possible auxotrophy of the EMS- or UV-treated cells, a 20-amino acid medium was used as a minimal medium, supplemented with a vitamin solution [19].

Chemicals. Ethyl methanesulfonate and 5-fluoroorotic acid (5-FOA) were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA).

UV treatment. A mid-exponential-phase culture containing about 2×10^8 cells/ml was centrifuged and resuspended in SW. Fractions of this bacterial suspension were serially diluted, and portions (0.1 ml) were dispensed onto appropriate gelrite plates to measure the number of viable cells per milliliter prior to irradiation. The bacterial suspension was placed in glass Petri plates and constantly stirred during irradiation. The effect of UV irradiation (254 nm) on cells was studied in the anaerobic tent with a 15W germicidal lamp (Poly Labo, Paul Block & Cie, Paris, France) at a distance of 8 cm and at different exposure times. The number of viable cells in control dishes remained unchanged in the course of irradiation. After irradiation, portions of cells were removed, diluted serially, and spread onto appropriate solid media to determine the surviving fraction of viable cells according to plating efficiency, initial cellular concentration, and dilution factor. The CFUs (colony-forming units) were counted 2–5 days after incubation at 85°C. To avoid possible photoreactivation, all operations during UV irradiation and plating were carried out in semidarkness.

Auxotrophic mutants were obtained by centrifuging and resuspending the UV-treated cells in 10 ml of 20-amino acid medium supplemented with uracil (50 μg/ml) (complete medium), and by incubating at 85°C for one day. The viable cell count was determined by diluting the one-day culture and plating portions onto plates without 5-FOA. These data should provide a quantitative estimate of the incidence of UV-induced mutations to 5-FOA resistance. Then, 100 μl of UV-treated cells was dispensed onto 20-amino acid plates containing 5-FOA and uracil, and incubated at 85°C for 4 days. 5-FOA-resistant mutants were tested for uracil auxotrophy by replica-plating onto complete and minimal media, and stable auxotrophic mutants were selected.

Ethyl methanesulfonate treatment. Cells were mutagenized under anaerobic conditions by the following procedure: a log-phase culture containing approximately 2×10^8 cells/ml was centrifuged at 8000 *g* for 20 min, washed, and finally resuspended in half the original volume in SW. An appropriate volume of EMS (or anoxic water in the controls) was added to obtain final concentrations of 0.01, 0.05, 0.1, and 0.2 M respectively. The treatment was carried out at 37°C in a thermobloc (Bioblock Scientific, Paris, France) for 15, 30, 45, 60, 90, or 120 min. To stop the mutagenic treatment after different times of exposure, we resuspended 0.5 ml of the treated culture in 0.5 ml of sodium thiosulfate (12% wt/vol). The cells were harvested by centrifugation, washed with SW, and finally resuspended in the same solution. This bacterial suspension was serially diluted, and portions (0.1 ml) were dispensed onto suitable gelrite plates, which were incubated at 85°C for 2 days.

The survival level was defined as the fraction of the initial number of cells able to grow in VSM medium after the mutagenic treatment, whereas the induced mutagenicity was measured as the frequency of appearance of resistant mutants to 5-FOA in the surviving population. Resistance to 5-FOA was chosen as the most suitable resistance marker on the basis of its relatively low frequency of spontaneous mutation shown by Thermococcales strains, and because of its utilization in the search for uracil auxotrophs. Auxotrophic mutants were obtained by exposing the cells under the optimal mutagenesis conditions, and then as described with UV mutagenesis in the presence of 5-FOA (800 μg/ml) and uracil (50 μg/ml).

Results

Killing action of UV and EMS. Results on survival of *P. abyssi* strain GE5 after UV and EMS exposures are shown respectively in Figs. 1 and 2. Exposure of this strain to UV or EMS led to a time-dependent decrease in the number of viable cells. Since a UV dose during 5 s of exposure yielded a survival level of about 1%, and a concentration of 0.1 M of EMS from 60 min of exposure yielded a similar fraction of strain GE5 survivors, these data were used in further mutagenesis experiments with Thermococcales strains.

Influence of several variables on survival after UV and EMS treatments. The dependence of the lethality on cell concentration was determined by exposing samples

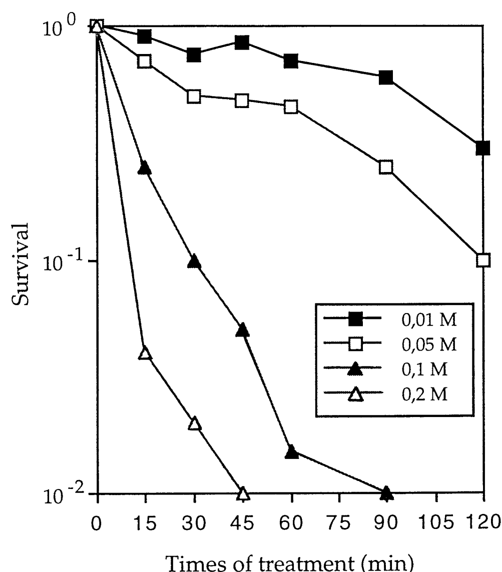

Fig. 2. Lethal action of EMS in *P. abyssi* strain GE5 for various times at different concentrations of the mutagen.

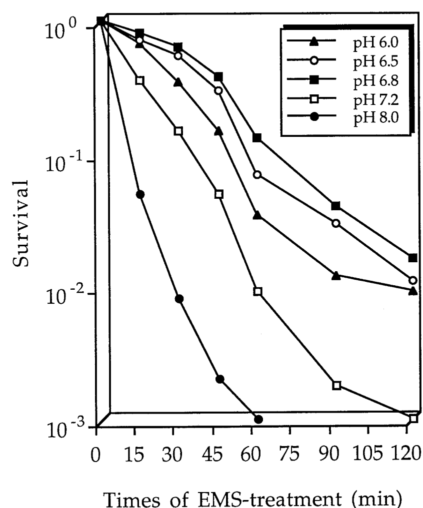

Fig. 3. Influence of pH on EMS-induced lethality of *P. abyssi* strain GE5.

of strain GE5 containing 10^7, 10^8, or 10^9 log-phase cells/ml to UV for 5 s. The results showed a noteworthy enhancing of the activity of the mutagen with the lowest cell titer, whereas survival levels were significantly increased at higher cell titers. Similar results were obtained with 0.1 M of EMS; therefore, a common cell titer of 10^8 cells/ml was chosen for further studies. The addition of different sources of sulfur (elemental sulfur, cystine and polysulfides) stimulated growth of *P. abyssi* strain GE5 but was not absolutely required [5]. Hence, elemental sulfur, cystine, or polysulfides were omitted from VSM medium before mutagenic treatment, and in comparison with cultures not mutagenized, their absence appeared to have no significant effect on the sensitivity of archaeal cells to mutagen agents (data not shown). Since an optimal growth was obtained with cystine for all the other Thermococcales used in this study, this compound was selected for further experiments.

The influence of salt concentration of the medium on the killing action of UV and EMS was tested. Despite an enhancement of the lethal action of UV and EMS at the highest salt concentration (4.5%), only slight differences in viability were detected at 2, 3 or 4% salt concentrations. Thus, since 3% NaCl was optimal for growth of strain GE5 and many other strains used in this study, this salt concentration was selected for further studies.

It has been reported that pH can affect the efficiency of the killing action of EMS [17]. We therefore studied the survival of *P. abyssi* strain GE5 cells after treatment with 0.1 M EMS at different pH values in VSM medium. Figure 3 shows that a general enhancement of induced lethality occurred both at lower and higher pH values than the optimum pH for growth (6.8). The most marked lethal effects were observed at the higher pH values. pH 7.2 was selected, since this allowed us to obtain useful fractions of survivors with shorter times of exposure to EMS. The viability of cells in VSM medium without the mutagen at the different pH values was determined, and a decrease in the cell titer was observed only at pH 8.0.

Relationship between mutant frequency and survival after EMS and UV treatments. Survival counts and frequency of 5-FOA-resistant mutants were obtained when cells of strain GE5 were exposed to 0.1 M EMS under optimal conditions (Fig. 4). EMS and UV treatments resulted in a strong induced mutagenicity, since the spontaneous mutation frequency was increased about 150-fold after EMS treatment (60 min of exposure) and about 400-fold after UV exposure (Table 1).

EMS and UV effects in other Thermococcales. A comparative study of the relationship between lethality and mutagenicity after EMS and UV treatments in eight strains corresponding to *Pyrococcus*- and *Thermococcus*-like strains was carried out. Four strains of the genus *Pyrococcus*, GE1, GE7, GE9 and *P. furiosus*, and all those of the genus *Thermococcus* showed a lower sensitivity to the killing action of EMS when compared with that exhibited by strain GE5; in contrast, GE27 and GE23 demonstrated similar survival levels after exposure (data not shown). It was interesting to observe the same phenomena for these microorganisms with UV-induced lethality, except *P. furiosus*, which showed a higher sensitivity to UV in comparison with strain GE5. Otherwise, GE23 and GE9 exhibited a significant increase (about 100-fold with UV treatment and 80-fold with EMS treatment) in the mutation frequency, which was, in any

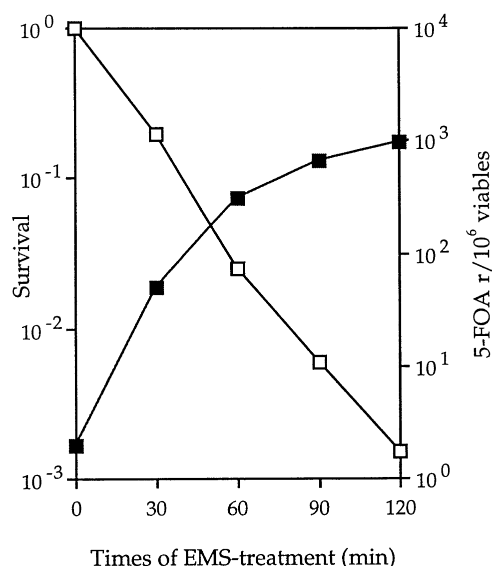

Fig. 4. Relationships between mutant frequency at 800 μg/ml 5-FOA (filled squares) and survival (open squares) after EMS treatment of *P. abyssi* strain GE5. Cells were exposed to 0.1 M EMS in SW medium (3% NaCl), pH 7.2, for different times.

Table 1. Mutant frequency (at 800 μg/ml 5-FOA-resistant/10^6 viables) of 9 hyperthermophilic archaea after exposure to 0.1 M EMS or UV for different times

	Mutant frequency			
Microorganisms	UV 0 s	UV 5 s	EMS 0 min	EMS 60 min
P. abyssi strain GE5	3.2	1300	2.0	310
P. like GE1	2.0	9.5	1.5	9.0
P. like GE7	2.5	18	3.0	16
P. like GE9	3.0	310	2.4	190
P. like GE23	3.5	360	3.5	280
P. like GE27	3.2	1300	2.2	330
P. furiosus	2.0	13	2.6	9.5
T. stetteri	2.8	14	1.0	7.8
T. like GE31	1.0	8.0	1.4	11

case, always lower than observed for *P. abyssi* strain GE5, and only GE27 showed similar high mutant frequency with strain GE5 after UV and EMS treatments (Table 1).

As a consequence of these results, we chose as the optimal experimental conditions of mutagenesis the exposure of cellular suspensions containing about 10^8 log-phase cells/ml to 0.1 M EMS in SW medium (3% NaCl) with cystine, at pH 7.2, for 60 min (or longer), and to UV for 5 s, in the search for auxotrophic mutants for strains GE5, GE27, GE23, and GE9.

Isolation of uracil auxotrophic mutants of strains GE5, GE27, GE23, and GE9 by mutagenesis. The effects of 5-FOA on the growth of strains GE5, GE27, GE23, and GE9 were studied by using concentrations in the range of 0.25–1 mg/ml. Plates containing 0.8 mg/ml of 5-FOA showed no growth after 6 days, whereas there was a lawn of growth after 2 days in unsupplemented control plates; thus, this concentration was chosen in the search for uracil auxotrophs; 20-amino acid medium was selected for this purpose because all the other media tested (tryptone, peptone, and VSM) held uracil sources (data not shown).

The number of uracil auxotrophs among 5-FOA-resistant mutants was higher among mutants obtained with UV mutagenesis under the optimal conditions mentioned above than among spontaneous mutants. Only 1% of spontaneous 5-FOA-resistant mutants were complete uracil auxotrophs compared with about 10% of UV-treated, 5-FOA-resistant mutants; for example, among 40 5-FOA-resistant colonies of strain GE5, 30 of strain GE27, 12 of strain GE9, and 20 of strain GE23, only 10 clones were complete uracil auxotrophs, 6 with strain GE5 and 4 with strain GE27. Of each of the other 5-FOA-resistant mutants, 5% could be considered as "leaky" uracil auxotrophs because of their slow growth and different morphology compared with wild-type strains (data not shown). Thus, complete uracil auxotrophs can be considered as stable mutants with reversion rates lower than 10^{-7}.

EMS optimal conditions mentioned previously were used for obtaining auxotrophic mutants in three separate mutagenic treatments. Three cultures were individually exposed to 0.1 M EMS, one for 60 min, another for 90 min, and the last for 120 min. 5-FOA-resistant colonies were obtained after different EMS exposures of strain GE5, GE9, GE23, and GE27, and the following "leaky" uracil auxotrophs were detected: two colonies with strain GE5 (60 min of exposure) plus another with 90 min of exposure, one colony with strain GE9 (120 min of exposure), and two colonies with strain GE27 (90 min of exposure). No complete uracil auxotroph was selected.

Discussion

Thermococcales and especially *Pyrococcus* strains are considered now as model strains for genetic studies in hyperthermophilic archaea. However, very few resistant mutant or auxotrophic mutants are so far available in archaea, and most of them in methanogens or Sulfolobales [1, 7–9, 12]. The purpose of our study, therefore, was to optimize mutagenic treatments for Thermococcales in order to isolate mutants. Ethyl methanesulfonate was selected with UV as mutagenic agents, since EMS is a monofunctional ethylating agent with mutagenic activity demonstrated in a wide variety of bacterial genera. Thus, this chemical agent is able to produce transition

mutations as well as base-pair insertions or deletions [17]. UV has the advantage of producing a greater range of substitutions; most occur in runs of pyrimidines, particularly T-T pairs, and include both transitions and transversions. In addition, UV induces a significant frequency of frameshift mutations, almost exclusively of the single nucleotide deletion variety. Together, one or other of EMS and UV are likely to satisfy most experimental needs, and it may be an advantage to induce mutants with each of these agents.

A significant decrease in viability was observed both at lower and higher pH values than at the optimum pH (6.8) for cell growth. This might be owing to differences between the ionic strength and/or chemical character of the mutagen. In the case of the influence of salinity on the killing activity of the mutagenic agents, we observed a strong influence of decreasing salinity of the induced lethality. This might be owing to osmotic cellular changes that result in more EMS and UV entering the cells.

Survival profiles of Thermococcales with EMS and UV (between 2 and 40 s of exposure at a distance between germicidal lamp and samples of 60 cm; data not shown) have a distinct shoulder to the beginning of the curve (a little less distinct with EMS) followed by an exponential loss of viability. These data provide evidence for the existence of some DNA protection mechanisms in archaea cells. Apparently, archaea DNA is poorly protected from the effect of pyrimidine dimers formed after UV irradiation [10]. This shoulder might also be owing to progressive accumulation of cell damage before cell death occurs. The mutagenicity studies revealed that the increase in spontaneous mutation frequencies (to 400-fold) with UV treatment was significantly greater than that reported for the same strain GE5 when using EMS (only a 150-fold increase), and, therefore, UV seems to be more effective than EMS in producing 5-FOA-resistant mutants in Thermococcales under our experimental conditions. However, cells of *Pyrococcus* strains seem to be a little more resistant to the lethal action of EMS, maybe because of a more effective impermeability of the *Pyrococcus* cells.

Some 5-FOA-resistant mutants were classified as ‘‘leaky’’ uracil auxotrophs, but, although morphology might suggest changes occurred owing to mutagenesis, it does not provide evidence of a leaky phenotype. In any case, complete uracil auxotrophs are the first mutants of Thermococcales isolated to date. These mutagenesis protocols might be helpful for the isolation of other useful mutants for further genetic and physiologic studies in hyperthermophilic archaea. Moreover, the complete auxotrophic mutants of strain GE5 and GE27 could provide an interesting help in the development of selection markers, and in the elaboration of a transformation system in Thermococcales.

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