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The Mutation Range of *Brevibacterium* sp. M27

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ABSTRACT. The mutation range was studied in *Brevibacterium* sp. M27 after UV irradiation and after treatment with N-methyl-N′-nitro-N-nitrosoguanidine. The induction of auxotrophic mutants and mutants resistant to streptomycin and tetracycline was investigated. A collection of auxotrophic mutants for the studies of genetic transfer in this model was prepared.

Brevibacteria are a group of microorganisms that holds promise for the future because of their metabolism. A study of mutagenesis was performed as the first step in the investigation of the genetic variability of this model. UV radiation and N-methyl-N′-nitro-N-nitrosoguanidine, mutagens that are highly effective in related microorganisms, were used, and changes of the phenotype, particularly induction of auxotrophy and resistance to antibiotics, were tested. It was the aim of this study to evaluate mutability of the model and to prepare a collection of strains with changed phenotypes that could be used for the study of genetic transfers in brevibacteria.

MATERIALS AND METHODS

Bacterial strain. Brevibacterium sp. M27 (Collection of Microorganisms, Institute of Microbiology, Czechoslovak Academy of Sciences). A culture from the exponential growth phase, grown statistically in a complete liquid medium at 28 °C was used in all mutation experiments. The grown culture was always washed with buffer or physiological saline and adjusted to a cell concentration of about 1/pL.

Cultivation media (composition in g): Complete medium: Beef extract Difco 3, peptone 5, Yeast extract Difco 10, Bacto casitone 5, distilled water added to 1 L, pH 7.5; minimal medium: $K_2HPO_4.3H_2O$ 7, KH_2PO_4 2, trisodium citrate (pentahydrate) 0.5, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1, distilled water to 1 L, pH 7.2, 20 mL of 50 % glucose were added after sterilization. Solid media contained agar at a 2 % concentration.

Effect of UV radiation. UV lamp (30 W, germicidal lamp TESLA) with a current stabilizer served as a source of UV radiation. The cell suspension was irradiated in a very thin layer in a Petri dish under permanent mixing from a distance of 250 mm and fluence rate of 5 W/m². Time of irradiation was 5–25 min. Samples were taken at 1-min intervals, diluted and inoculated on the solid complete medium and incubated 3 d at 28 °C.

Effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Solution of MNNG (Koch-Light, England) at a concentration of 1 mg/mL was prepared always fresh and sterilized by filtration. Tris-maleate buffer in combination with the minimal medium ($Na_2HPO_4.7H_2O$ 8.2 g, KH_2PO_4 2.7 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4.7H_2O$ 100 mg, $Ca(NO_3)_2$ 5 mg, $FeSO_4.7H_2O$ 0.25 mg, distilled water to 1 L), in which phosphate salts were substituted by components of the Tris-maleate buffer (2-amino-2-hydroxymethyl-1,3-propanediol and

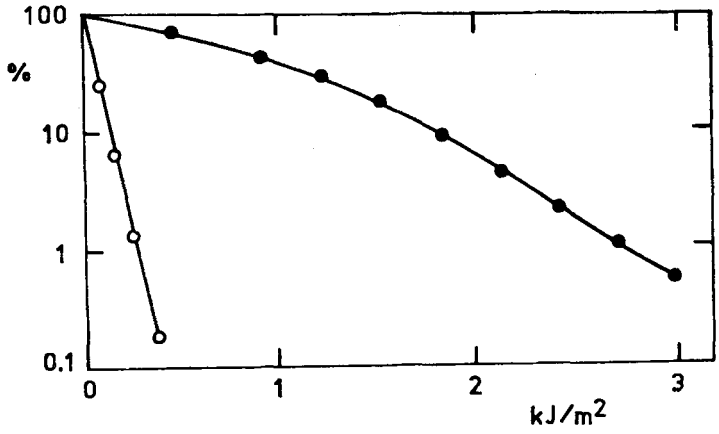

FIG. 1. Survival curve (%) of *Brevibacterium* sp. M27 (*closed symbols*) and *Mycobacterium phlei* PA (*open symbols*) after UV irradiation (energy fluence, kJ/m^2).

maleic acid at 0.2 M final concentration and pH 6.0, was used to prepare the MNNG solution (Adelberg *et al.* 1965; Koníčková-Radochová and Málek 1969*a*). The culture was incubated in the MNNG solution in a water bath for 60 or 90 min at 28 °C. Samples were taken at 10-min intervals, diluted with 10—15 volumes of cold buffer (4 °C), washed, concentrated to the original volume, diluted, inoculated on the solid complete medium and incubation for 3 d or more at 28 °C.

Auxotrophy was tested by replica-plating on the minimal medium. Nutritional requirements of the obtained auxotrophic mutants were identified by the auxanographic method on the minimal medium. Stability of the mutation changes was evaluated by determining the frequency of spontaneous reversion in cultures from the exponential growth phase.

Induction of resistant mutants was examined with respect to the UV radiation dose and the time of MNNG treatment (MNNG concentration — 1000 μg/mL). Samples were taken at regular intervals, transferred to a complete liquid medium and incubated for 2 d at 28 °C to allow for the phenotypic expression of the induced mutations. After the incubation the samples were suitably diluted and inoculated in the complete medium and further on the same medium containing either streptomycin (Streptomycin sulfate, Medexport, USSR) at a final concentration of 0.5 μg/mL or tetracycline (Tetracyklin, Spofa, Czechoslovakia) at a concentration of 9 μg/mL. The frequency of spontaneous and induced resistant mutants was evaluated on the basis of the numbers of colonies grown on the above mentioned solid media.

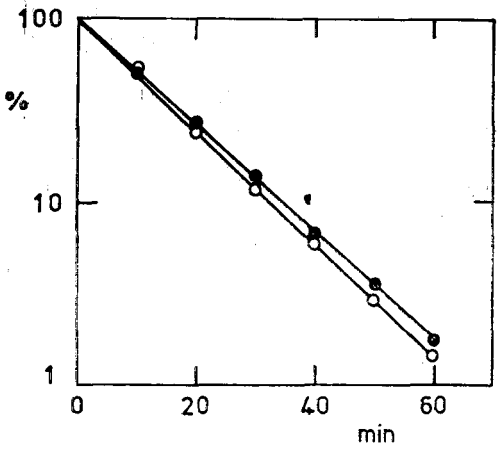

FIG. 2. Survival curve (%) of *Brevibacterium* sp. M27 (*closed symbols*) and *Mycobacterium phlei* PA (*open symbols*) after treatment with N-methyl-N′-nitro-N-nitrosoguanidine at a concentration of 1 mg/mL (time of treatment, min).

RESULTS AND DISCUSSION

The lethal effect of UV radiation was very low as the survival of cells of the basic suspension of about 1 % was reached only when applying energy fluence as high as 3 kJ/m^2 (Fig. 1). The yield of mutants of auxotrophic phenotypes also corresponds to the lethal effect. Seven suspected auxotrophic mutants were isolated when testing 20 000 colonies after the UV treatment. After a detailed verification only a single strain requiring cystine or cysteine, in which the genetic change is sufficiently stable and can hence be used for further genetic work, was chosen. The remarkably high degree of resistance of *Brevibacterium* sp. M27 to lethal effects of UV radiation and mutagenic effect in the induction of auxotrophy might well be due to a high content of pigments acting as a protective factor, as shown for instance in the case of carotenoid pigments (Jensen 1965). When these results are compared with the sensitivity of a culture of *Mycobacterium phlei*, the dose of UV radiation causing the same survival (1 %) is about ten times lower than in the culture of *Brevibacterium* sp. M27, UV light being a highly effective mutagen for the induction of auxotrophic mutants (Koníčková-Radochová and Málek 1969*b*). The response of the organisms to the effect of UV radiation with respect to DNA structure can be compared in both models, as microorganisms closely related according to the GC content are involved (Keddie and Cure 1978; Rytíř *et al.* 1968).

The lethal effects of MNNG varied within limits usual for this mutagen in related microbial species (Koníčková-Radochová and Málek 1969*a*) (Fig. 2). With prolonged time of MNNG treatment the formation of auxotrophic mutants increases in such a way that the yield is equal to 0.37 % at the survival of 45 % cells of the basic suspension and up to 10 % when the survival is lower than 1 %.

However, when the lethal effect of MNNG is high the growth of cells is also influenced, the colonies grow very slowly on a complete solid medium, older colonies have a skin-like surface, so that their replica-plating is very difficult and individual colonies must be usually isolated. In parallel, we introduced the method of induction of auxotrophic mutants with the aid of a MNNG crystal placed on the supplemented minimal medium. The mutagen

TABLE I. The range of auxotrophic phenotypes of *Brevibacterium* sp. M27 obtained after treatment with UV radiation and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)

UV radiation	MNNG		
	amino acids	bases	multiple auxotrophic phenotypes
cystine (cysteine)	arginine	purines	leucine, cystine
	histidine	adenine	isoleucine, valine
	cystine (cysteine)	adenine (hypoxanthine)	serine, adenine
	leucine	guanine	tryptophan, adenine
	isoleucine	guanine (xanthine)	lysine, guanine
	serine	guanine (xanthine; hypoxanthine)	glutamic acid, hypoxanthine, thiamine
	proline	xanthine (hypoxanthine)	adenine, hypoxanthine
	tryptophan	uracil	arginine, uracil
	lysine (arginine; histidine)		
	aspartic acid		
	norleucine		

acts under completely different conditions (high mutagen concentration, pH 7.0, long-term effect on cells) than those of the classical method of treatment with the MNNG solution. This procedure is also inexpensive as to the amount of material required and both qualitative and quantitative effects

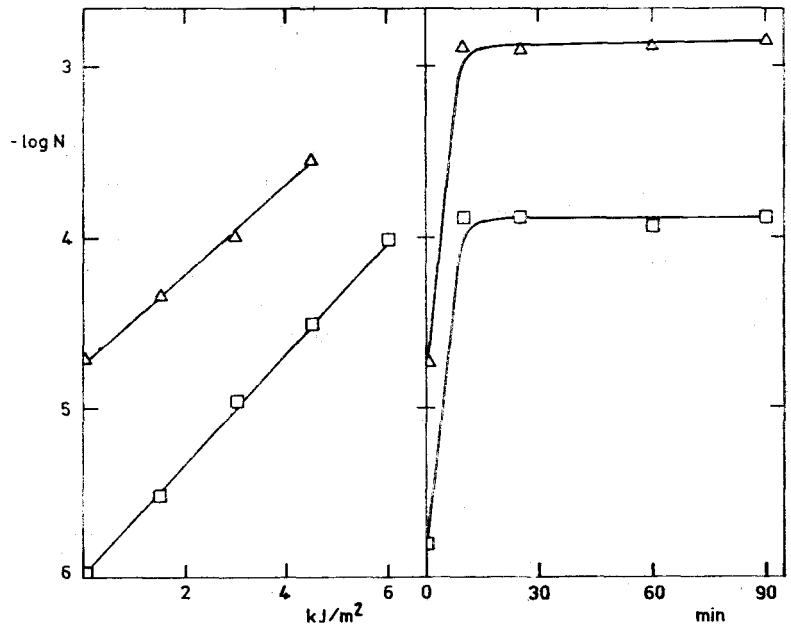

FIG. 3. Frequency (N) of induced streptomycin-resistant (*triangles*) and tetracycline-resistant (*squares*) mutants in *Brevibacterium* sp. M27 after treatment with UV radiation (*left*) and N-methyl-N′-nitro-N-nitrosoguanidine (*right*).

of the two techniques are similar on the condition that the lethal effect is high. It was the aim of the present work to obtain the highest possible number of auxotrophic mutants and a wide spectrum of mutant phenotypes.

The auxotrophic mutants exhibit nutritional requirements within the range of amino acids and nucleic acid bases. Requirements for arginine, cystine, cysteine, leucine, isoleucine and histidine predominate among amino acid deficient mutants. These phenotypes occurred at higher frequencies whereas mutants requiring proline, serine and lysine and further aspartic acid, 2-aminobutyric acid and norleucine were rare. Genetics changes in strains of the last three phenotypes were highly unstable. More genetic blocks originated in the area of purine biosynthesis, so that a spectrum of mutants with different requirements for purine bases was obtained. Double auxotrophic mutants were mostly dependent on an amino acid and a base. The total mutation range of auxotrophic phenotypes is presented in Table I. Most auxotrophic strains, except for phenotypes requiring the last three amino acids mentioned above were of satisfactory stability of the acquired genetic changes with a frequency of spontaneous reversions varying within the range of 10^{-6} to 10^{-10}.

The induction of mutants resistant to streptomycin and tetracycline was positive, both after UV irradiation and MNNG treatment. After UV irradiation the frequency of streptomycin-resistant and tetracycline-resistant mutants increases linearly with the applied dose, as compared with the spontaneous frequency. At a total energy fluence of 4.5 kJ/m^2 and cell survival of 0.01 % the maximal frequency increase in the tetracycline-resistant mutants was by more than one order of magnitude, whereas in the streptomycin-resistant mutants by merely one order. The efficiency of UV radiation can be evaluated in such a way that a fluence of 1.5 kJ/m^2 increases the frequency of induced tetracycline-resistant mutants by about a half of the order of magnitude, whereas in streptomycin-resistant mutants the induction is roughly 50 %. When using MNNG the kinetics of the induction of mutants is different. At the beginning of mutagenic treatment the frequency of mutants of both types of resistance increases roughly by two orders of magnitude at a 45 % cell survival but on further exposure the maximum frequency reached does not increase any further (Fig. 3).

Brevibacterium is a difficult model, mainly due to the negligible level of knowledge about its genetics and physiology and further due to its growth and other biological characteristics, resulting in difficulties associated with the execution and reproducibility of some types of experiments. At the beginning of this work we utilized the experience obtained with a rather closely related model of mycobacteria, which is also quite complicated (Konířková-Radochová *et al.* 1970). In *Brevibacterium* sp. M27 we evaluated the mutation range of auxotrophy and prepared a collection of mutant strains bearing markers of auxotrophy and resistance to streptomycin or tetracycline, which is a necessary prerequisite for studies of genetic transfers in this model.

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