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A Study of Some Mutations in a Strain of *Rhizobium trifolii*

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SUMMARY

Mutants of a strain of *Rhizobium trifolii* were obtained by selective action of bacteriophage and streptomycin and by exposure to ultraviolet (u.v.) radiation. All mutants resistant to bacteriophage were streptomycin-susceptible and all resistant to streptomycin were bacteriophage-susceptible; no survivor after exposure to u.v. radiation resisted either the bacteriophage or streptomycin. Mutation to bacteriophage resistance was closely correlated with inability to fix nitrogen in symbiosis with red clover. Some streptomycin-resistant mutants used streptomycin as a nutrient supplement, enabling them to grow on a mineral medium without growth factors. All but two mutants remained serologically indistinguishable from the original strain.

INTRODUCTION

Previous work on mutation among nodule bacteria was mostly concerned with changes in the ability to form nodules on the leguminous host and in the effectiveness of nitrogen fixation in the nodules (briefly reviewed by Kleczkowska, 1950). Kleczkowski & Thornton (1944) found that an ineffective mutant of *Rhizobium trifolii* and its effective parent were indistinguishable serologically. Kleczkowska (1950) found that phage-resistant mutants also tended to differ from parental strain in nitrogen-fixing efficiency. It seemed, therefore, that mutation in susceptibility to a bacteriophage is correlated with the symbiotic effectiveness, although the degree of correlation varied from one bacterial strain to another. Some of the mutants also differed in colony morphology and pigmentation, and analysis showed that changes in colony morphology, pigmentation or effectiveness were independent. This work investigates the problem further by comparing mutants selected by bacteriophage or streptomycin treatment with those caused by u.v. irradiation. The nutrient requirements and serological specificity of all strains were also examined.

METHODS

The strain of *Rhizobium trifolii* used in this work, strain A, has been continuously used over many years and has always been effective in fixing nitrogen. It was periodically re-isolated from a single colony.

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Two different media were used: (1) Yeast water mineral salts mannitol agar (YWA); (2) a mineral salts medium with galactose (MSG). The first medium was the same as used by Kleczkowska (1950). The composition of the MSG medium is: 0·3 g. KH_2PO_4; 0·7 g. Na_2HPO_4; 0·3 g. $(NH_4)_2SO_4$; 0·1 g. $MgSO_4 \cdot 7H_2O$; 0·06 g. $CaCl_2$. The salts were dissolved in 800 ml. H_2O and the solution adjusted to pH 7·2. The solution was sterilized at 121° for 15 min. and mixed with 200 ml. of a separately sterilized 1%(w/v) solution of galactose. To both media 1·2%(w/v) agar was added and plates were prepared each containing 10 ml. medium. The MSG medium was used to study nutrient requirements of mutants. The original *Rhizobium trifolii* strain A grows well on the YWM medium but on the MSG medium only after adding biotin and thiamine.

The bacteriophage was a mutant of phage S_2P_{11} originally isolated from soil (Kleczkowska, 1945). Filtered lysed rhizobium cultures in liquid YWM medium were used for stock. They contained about 10^9 phage particles/ml. (by plaque count). To obtain phage-resistant and streptomycin-resistant mutants, plates containing phage or streptomycin were inoculated with water suspension of bacteria. Phage stock was added to the liquid media at 42° in the amount corresponding to 1·0 ml./plate just before the medium was poured into Petri dishes. In addition to this, phage stock was mixed in equal volumes with bacterial suspension before inoculating the plates. Streptomycin sulphate was added to the media at 0·5 mg./plate. The concentration of bacteria in the suspension, usually about 2×10^8 bacteria/ml., was determined by haemocytometer and by plating. To inoculate the plates, 0·1 ml. of the bacterial suspension was placed on the surface of agar medium and spread evenly with a platinum rod.

Bacterial colonies that developed on plates with bacteriophage or with streptomycin after several days incubation at 25°, were counted and isolated for further work.

Ultraviolet irradiation was done by placing 5 ml. of a thin water suspension of bacteria (about 10^5 bacteria/ml.) on a Petri dish of 9 cm. diameter and exposing the dish for 1 min. to a Hanovia low-pressure mercury lamp. The intensity of the radiation was about 1500 μW./cm.^{2}. The lamp was fitted with a filter to exclude radiation below 240 mμ, so that most of the radiation was of a wavelength of 254 mμ. The fluid was rocked during irradiation to equalize mutual shading. After irradiation a number of YWM agar plates were inoculated with 0·1 ml. of the irradiated suspension spread evenly with a platinum rod. The colonies that developed after incubation for a few days at 25° were counted and isolations made for further work. About 0·5% of the bacteria treated survived irradiation.

The effectiveness in nitrogen fixation in root nodules was tested on late-flowering Montgomeryshire red clover as previously described (Kleczkowska, 1950).

The nutrient requirements of bacteria were tested on MSG agar plates supplemented with test nutrients. One ml. of bacterial suspension (containing about 10^9 bacteria) was added to 10 ml. melted agar medium previously cooled to 42° and the mixture poured into a Petri dish. The plates were then divided into radial segments and a drop of solution of a supplementary nutrient was placed in the middle of each segment. The following solutions were tested: 0·01% (w/v) biotin; 0·01% (w/v) thiamine; 0·01% (w/v) yeast nucleic acid; a solution containing 0·1% (w/v) each of asparagine and glutamine. When mixtures were used each component was present at the above concentrations.

RESULTS

Table 1 shows that none of the strains obtained by the selective action of streptomycin resisted bacteriophage and none selected by bacteriophage resisted streptomycin. These two kinds of mutation are, therefore, independent. Mutation to inability to fix nitrogen was much more frequent among phage-selected than among streptomycin-selected mutants and the χ^2 test showed that the incidence of changes in effectiveness in the two kinds of mutant differs significantly, $P < 0{\cdot}001$.

No similar comparison can be made with survivors after u.v. irradiation, as it is not known what proportion of them were mutants. There were certainly 3 mutants out of the 23 isolates, 2 of which were ineffective in nitrogen fixation and the third altered in a nutrient requirement (see below). Any of the others may or may not have been mutants in some other respects that were not tested. None of the tested strains become resistant either to the bacteriophage or to streptomycin. Certainly u.v. irradiation did not prove to be an efficient method for obtaining mutants in the characters under test.

Table 1. *A comparison of some properties of the original bacterial strain A of* Rhizobium trifolii *with those of survivals after three different treatments*

			Streptomycin		Bacteriophage		Symbiotic effectiveness in nitrogen fixation	
			R	S	R	S	E	I
Pretreatment	Proportion of survivals	Total numbers of isolates tested	No. strains isolated from single colonies that fell into indicated categories					
No treatment (original strain A)	—	20	0	20	0	20	20	0
Streptomycin	2×10^{-8}	28	28	0	0	28	27	1
Bacteriophage	3×10^{-3}	20	0	20	14	6	6	14
U.v. irradiation	5×10^{-3}	23	0	23	0	23	21	2

R = resistant; S = susceptible; E = effective; I = ineffective.

Table 1 shows that 6 isolates after phage treatment proved on subcultivation to be susceptible to the phage, whereas 14 remained resistant. Similar results were obtained and discussed previously (Kleczkowska, 1950). Four ineffective mutants occurred among the 6 reverted susceptible strains, and 9 among the 14 which remained phage resistant. The ineffective mutants were about equally distributed between these two groups.

Neither the original bacterial strain A nor those isolated after these treatments grew on the MSG medium, and nor when this was supplemented with yeast nucleic acid, a mixture of amino acids, or with biotin. All strains grew on the YWM medium and in the MSG medium supplemented either with yeast extract or with biotin + thiamine. Only one mutant obtained after u.v. irradiation grew on the basal medium supplemented with thiamine only. Thus the search for mutants in nutrient requirements by means of the set of supplements used in the work proved unfruitful, but 10 mutants were obtained for which streptomycin appeared as a nutrient supplement in the presence of which they could grow on the MSG medium.

The mutants which grew on the basal medium + streptomycin were amongst those that were obtained by exposing the original strain to streptomycin. Streptomycin-resistant mutants were obtained both on the YWM medium and on the MSG medium and the proportion of survival was about the same on each medium. Ten of the streptomycin-resistant mutants shown in Table 1 were obtained on MSG medium and 18 on the YWM medium. The single ineffective mutant was isolated from YWM medium.

The mutants obtained on the MSG medium grew normally on this medium with streptomycin but not without. They retained this property after several subcultures on YWM medium without streptomycin. The mutants obtained on YWM medium with streptomycin grew normally only on this medium (with or without streptomycin). They did not grow on MSG medium without streptomycin; in its presence they formed incipient colonies.

All strains were tested with an antiserum prepared against the original strain A. All agglutinated except two obtained by phage treatment. One of these remained permanently phage-resistant and was also ineffective in nitrogen fixation, the other reverted to phage susceptibility and was effective.

DISCUSSION

The strain A of *Rhizobium trifolii* used in this work was effective in nitrogen fixation, required biotin and thiamine as nutrients and was susceptible to streptomycin and phage. The study of changes in its properties showed a high degree of correlation between mutation to phage resistance and to ineffectiveness in nitrogen fixation. (The phage-resistant mutants contained 71% ineffective strains.) Previous work on two ineffective and phage-susceptible strains showed no effective mutants among the phage-resistant strains of one strain. The other produced 7% of mutants which were both phage-resistant and effective; but the degree of correlation was much lower (Kleczkowska, 1950) than with strain A. The problem now is whether the apparent ease with which effective strain mutated to ineffectiveness is the general rule; obviously too few strains have yet been tested to allow any general conclusion.

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