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Isolation of *Streptomyces rimosus* Mutants with Reduced Actinophage Susceptibility

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The infection of *Streptomyces rimosus* by the virulent actinophage RP1 was partially characterized. RP1 infection of the host cells results in a dramatic decrease in viable cell count, followed by reduced antibiotic production. Phage-resistant mutants were isolated after mutagenic treatment and RP1 selective pressure. Characterization of the isolated mutants has revealed that RP1 infection had no influence on their growth and antibiotic production. However, multiplication of the phage particles in the lawns of resistant mutants was detected. Since these strains differ from the wild type in RP1 relative efficiency of plating, plaque morphology, and the time necessary for plaque appearance, they are considered to be semiresistant mutants. The propagation of RP1 on semiresistant strains is characterized by lower adsorption of phage particles and longer latent and rise periods. As a consequence, the multiplication of the phage is slower than that of their host, which consequently reduces the ratio of phage to its host, thus diluting out the phage.

Since many actinophages are capable of infecting industrial cultures, efforts have been made to select antibiotic-producing strains resistant to their action. Phage-resistant strains have been obtained in a number of cases (14), although with much greater ease for some strains than for others (8). Mutants possessing a partial degree of resistance to the virus often appear. Such strains, when plated with a given actinophage preparation, give rise to a much lower number of plaques than the original, sensitive organism. These strains were designated semiresistant (13). Although considerable knowledge exists concerning typical resistance (2, 11), almost no studies have been devoted to phenotypes that do not fall neatly into this category of actinophage-actinomycete interaction.

In this study we describe mutants of an oxytetracycline (OTC)-producing *Streptomyces rimosus* strain which are semiresistant to a virulent actinophage.

MATERIALS AND METHODS

Microorganisms and media. The organism used in this study was *S. rimosus* strain R6, obtained from the Culture Collection of the Faculty of Technology in Zagreb. Host techniques, including maintenance, propagation, and preservation of stocks, were described by Alačević et al. (1). Actinophage RP1, a soil isolate, was kindly supplied by V. Adamović.

Complete agar for maintenance of stock cultures and for phage assay, as well as liquid medium for growth and propagation of the host, were described previously (7). Liquid medium for antibiotic biosynthesis contained (in grams per liter of distilled water): starch, 55; corn steep solid, 7.5; $CaCO_3$, 7; $(NH_4)_2SO_4$, 8.5; NH_4Cl, 2; $CoCl_2 \cdot 6H_2O$, 0.14; soy bean oil, 10.

Phage techniques. Conventional methods were adapted and used for phage propagation, purification, and assay. Adsorption, a one-step growth experiment, electron microscopy, nucleic acid determination, and preparation of RP1 antiserum were done as described by Hranueli et al. (7).

Isolation of mutants. Phage-resistant mutants were isolated after 1 h of treatment with 1 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in 0.05 M tris(hydroxymethyl)aminomethane-maleic acid buffer, pH 9 (4).

The enrichment of mutants was done by RP1 selective pressure. The mutagen-treated cells were subcultured in liquid medium containing RP1 infective particles at a multiplicity of infection of about 10. The successive subcultures were incubated for 48 h at 28°C with shaking. Samples were taken from each subculture and screened for phage-resistant mutants by replica plating on plates seeded with RP1 particles. Each colony growing on a plate seeded with RP1 was tested in an RP1-containing liquid culture for ability to overcome phage infection.

Determination of OTC concentration. The production of OTC was determined in uninfected and RP1-infected cultures at a multiplicity of infection of about 10. For optimal OTC production, the liquid medium for antibiotic biosynthesis was inoculated with mycelial fragments (10%, vol/vol) grown in liquid medium for 48 h at 28°C and incubated for 6 days on a rotary shaker (220 rpm). The concentration of OTC was determined by the colorimetric method of Monastero et al. (9).

The relative OTC yield was calculated in relation to the production of an uninfected *S. rimosus* R6 culture.

RESULTS

Morphological and chemical characteristics. The actinophage RP1 produced regular clear plaques when plated on a sensitive strain of *S. rimosus* (Fig. 1a). Plaques typically appeared after 1 to 2 days of incubation at 28°C. On prolonged incubation the plaques increased in size, reaching an average diameter of about 10 mm.

An electron micrograph of the RP1 particle from a purified preparation is shown in Fig. 2. The phage consists of a hexagonal head and a long, flexible, noncontractile, striated tail. Measurements taken from 70 particles indicated an average head diameter of 75 ± 3.0 nm, a tail length of 220 ± 1.5 nm, and a tail width of 9.5 ± 1.5 nm.

Fluorescent staining of RP1 nucleic acid with acridine orange revealed a double-stranded deoxyribonucleic acid structure.

Conditions for storage and assay. Samples of RP1 lysate were diluted 10-fold in various salt solutions and buffers, and after 24 h the optimal storage and assay conditions were determined. Survival was >80% in nutrient broth, 5 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4), and 1 to 10 mM $MgSO_4$, $Ca(NO_3)_2$, or NaCl solution. In distilled water, 5 mM phosphate buffer (pH 7.4), and a 100 mM solution of $MgSO_4$, $Ca(NO_3)_2$, or NaCl, rapid and irreversible inactivation of >75% of the phage was observed. Tris(hydroxymethyl)aminomethane-hydrochloride buffer supplemented with 1 mM $MgSO_4$, 10 mM NaCl, and 10 μg of gelatin per ml was selected for storage of the phage.

The efficiency of plating (EOP) of RP1 was

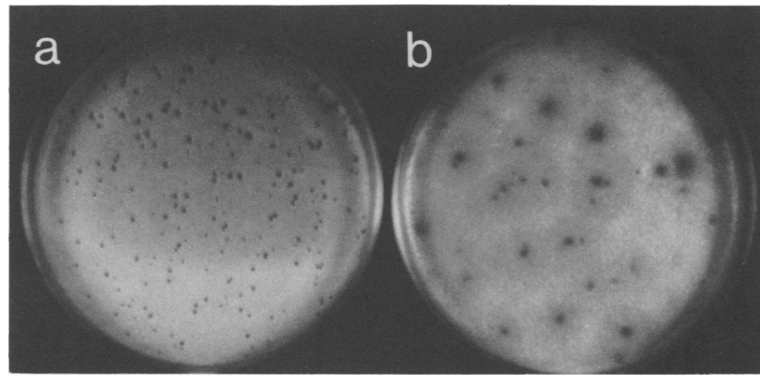

FIG. 1. *Morphology of RP1 plaques on (a) S. rimosus R6 after overnight incubation and (b) S. rimosus R6ø'$_{10}$ after incubation for 4 days. ×0.5.*

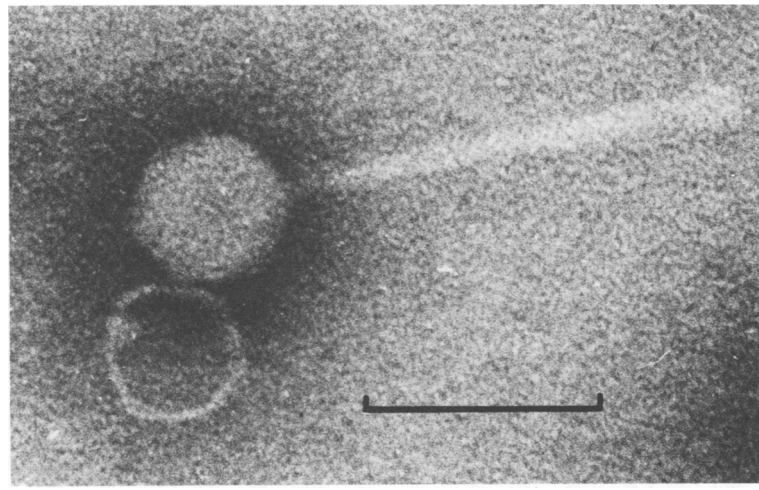

FIG. 2. *Electron micrograph of actinophage RP1 negatively stained with potassium phosphotungstate. Bar, 100 nm.*

dramatically influenced by the divalent cation concentration in the medium. The relative EOP obtained in the presence of 1 to 10 mM $Ca(NO_3)_2$ was 1. In the absence of added Ca^{2+}, the EOP decreased to 0.4, whereas in the presence of an excess (100 mM), the EOP averaged 0.07. The effects of Mg^{2+} were less dramatic. In the presence of 10 mM $MgSO_4$ the relative EOP was 0.6. Therefore, all further experiments were done with a medium containing 5 mM $Ca(NO_3)_2$.

Life cycle. The adsorption curve for RP1 obtained with mycelial fragments from the exponential phase of growth is shown in Fig. 3. The slope of the curve was linear for the first 30 min (90% adsorption at 30 min; adsorption constant, 4.2×10^{-10} ml/min). After 30 min the adsorption rate began to decrease.

The one-step growth curve of RP1 at 28°C is shown in Fig. 4. It showed a value of 2 h for the minimum latent period and 1.5 h for the rise period. The average burst size was about 50 phage particles.

Isolation of phage-resistant mutants. To isolate phage-resistant mutants, the mycelial fragments of R6 were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine giving 10^{-3} to 10^{-2} survival. The survivors of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment were exposed to RP1 selective pressure by successive subculturing. A typical experiment is presented in Table 1. Despite the presence of the phage, no decrease in viable count occurred after a few subcultures.

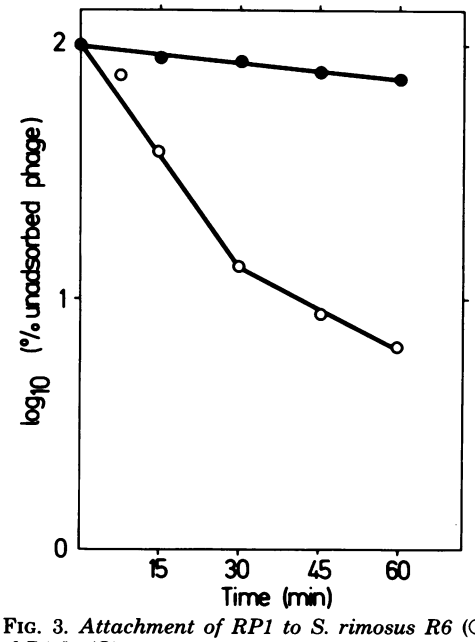

FIG. 3. *Attachment of RP1 to S. rimosus R6* (○) *and R6ø$^r_{10}$* (●).

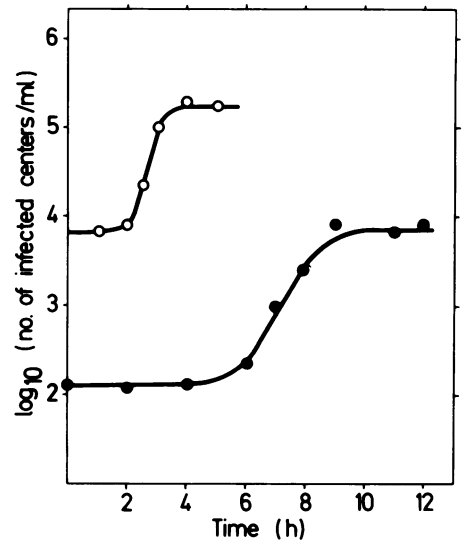

FIG. 4. *One-step growth curve of RP1 on S. rimosus R6* (○) *and R6ø$^r_{10}$* (●) *at 28°C.*

TABLE 1. *Isolation of phage-resistant mutants of S. rimosus R6 after nitrosoguanidine treatment and enrichment by RP1 selective pressure*

No. of subcultures	CFU/ml in 48-h shaking cultures[a]	No. of colonies tested	No. of resistant isolates after:	
			Replica plating on RP1	Testing in RP1-containing liquid culture
1	1.0×10^4	579	5	0
2	1.0×10^5	2,177	18	0
3	6.0×10^8	776	10	0
4	8.0×10^8	774	11	0
5	2.0×10^8	309	40	0
6	9.0×10^8	845	43	1
7	9.0×10^8	412	26	2
8	1.3×10^9	792	20	3
9	1.0×10^9	825	61	0
10	1.3×10^9	1,387	80	5

[a] CFU, Colony-forming units.

Colonies obtained from these subcultures appeared to contain phage particles. Before replica plating, they were cured from the phage either by successive restreaking or by growing them in the presence of RP1-specific antiserum.

The replica plating of colonies from different subcultures showed that only 1 to 10% of them were potentially phage resistant. However, by testing them in RP1-containing liquid culture, the resistance to actinophage was confirmed only with 0.1% (11 of 8,876) of the colonies. The actinophage resistance of these mutants was retained for many generations, confirming its ge-

netic nature. One of them, R6ø$^{r}_{10}$, was chosen for further study.

Characterization of phage-resistant mutants. RP1 infection of exponentially growing R6 cells resulted in a dramatic decrease in viable cells (survival of about 10^{-3} in <24 h [Fig. 5A]). The number of infective particles increased, reaching a maximum (2×10^9 to 4×10^9 plaque-forming units/ml) at between 24 and 48 h of incubation. Growth of the resistant isolates was not affected by RP1 infection in liquid cultures (Fig. 5B). Phage-resistant strains exhibited no loss of viable cells and supported almost no growth of RP1 particles. Moreover, relative OTC yields of resistant strains in uninfected and RP1-infected cultures showed an average production of 100% OTC (Table 2). The OTC yield of the sensitive strain R6, after infection with phage, was always <10%.

Large turbid plaques with clear centers were observed when phage-resistant isolates were used as indicator strains (Fig. 1b). Plaques formation was delayed for 4 to 5 days after incubation at 28°C. When compared with the sensitive strain R6, the relative EOP of RP1 on resistant isolates was about 0.2. Since these strains could at least partially support phage growth, we considered them to be semiresistant mutants. The partial resistances suggested heterogeneity of either phage or host populations.

To test whether RP1 lysates contained a constant proportion of mutant particles, or whether some sort of restriction modification system was operating, the material from 10 turbid plaques was scraped and suspended in nutrient broth. Lysates obtained in this way were then assayed on both sensitive and semiresistant strains. Phages from these lysates exhibited the same host-dependent morphology (Fig. 1a and b), whereas the EOP on semiresistant strains remained low (0.15). Efforts were also made to isolate a fully resistant or lysogenized variant from the survival fraction from RP1 plaques. Mycelial fragments of R6ø$^{r}_{10}$ obtained from turbid growth were treated with antiserum to remove exogenous phage and plated for single colonies. Sixty colonies were submitted to five serial streak isolations and tested at each stage for phage resistance. All 300 isolates exhibited the same semiresistant phenotype. Therefore, the frequency of any subpopulation, if present, would be <1 in 300 (<0.3%).

Nature of the semiresistant phenotype. The characteristics of RP1 adsorption and the one-step growth curve were determined by using semiresistant strains as hosts. Only about 10% of RP1 particles were adsorbed on mycelial fragments of the strains tested (Fig. 3). This was in good agreement with a lower RP1 relative EOP. The one-step growth experiment revealed poor recovery of the infective centers and prolonged latent and rise periods. At the same time, the recovery of infective centers on semiresistant

TABLE 2. *Relative OTC yields of S. rimosus R6 and three resistant isolates in uninfected and RP1-infected cultures*

Strain	Relative OTC yields in cultures	
	Uninfected	RP1 infected
R6	1.00	0.06
R6ø$^{r}_{6}$	0.95	0.96
R6ø$^{r}_{10}$	1.00	0.96
R6ø$^{r}_{12}$	0.93	0.98

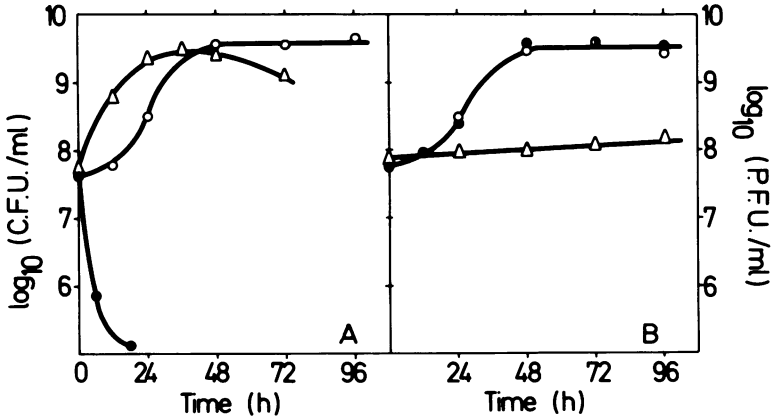

FIG. 5. *Kinetics of growth and actinophage propagation of S. rimosus R6 (A) and R6ø$^{r}_{10}$ (B). Uninfected and RP1-infected (multiplicity of infection, ~1) cultures (1% inoculum) were grown with shaking at 28°C. The number of colony-forming units (CFU) in uninfected (○) and RP1-infected (●) cultures, as well as free phage particles (△), were assayed at 12-h intervals. PFU, Plaque-forming units.*

strains was about 90% lower than that on R6 when the same multiplicity of infection was used. The latent and rise periods reached values of 5.5 and 3.5 h, respectively, whereas the burst size was 70 phage particles (Fig. 4).

DISCUSSION

To isolate *S. rimosus* mutants capable of overcoming phage infections, actinophage RP1 was partially characterized. RP1 appears to be very similar to temperate actinophage RP2 in its morphology, dimensions, host range, and nucleic acid type (7). However, RP1 has a shorter life cycle, it depends on divalent cations for its attachment, and, most important, it forms large, regular, clear plaques, which confirms its virulent nature.

The actinophage-resistant mutants were isolated from mutagenic treatment and RP1 selection. It appeared that an enrichment by selective pressure was not very efficient for obtaining fuly resistant mutants. After a few subcultures in RP1-containing liquid medium, the population predominantly consisted of phenotypically resistant cells. The majority of them could be reverted to the sensitive state by restreaking mycelial fragments or by growing the cells in the presence of RP1-specific antiserum. A pseudolysogenic condition was established, similar to that described by Welsch (13). However, 0.1% of potentially resistant isolates when tested in RP1-containing liquid culture exhibited the following characteristics: (i) there was no loss of viable cells upon RP1 infections; (ii) resistant cells supported almost no growth of the actinophage; and (iii) relative OTC yields remained the same irrespective of the presence of the phage. Still, multiplication of phage particles in lawns of resistant strains was detected. These characteristics opened the possibility of heterogeneity of the phage population or of the involvement of some sort of restriction-modification system. However, phage particles isolated from turbid plaques did not show the above-mentioned characteristics when assayed on a sensitive strain. Thus, the heterogeneity and restriction-modification of the phage population were excluded. Efforts were also made to isolate a fully resistant or lysogenized variant from the turbid growth of RP1 plaques. Since all our attempts failed, the possibility that the resistant population is heterogeneous or partially lysogenic is very unlikely. Because the isolated strains could support the phage growth to some extent, we considered them semiresistant mutants.

Mutants with similar phenotypes were reported in *Escherichia coli* and *Shigella paradysenteriae* and were described as variably resistant (3), partially resistant with inhibition (6), and semiresistant (12) mutants. In these cases, the populations consisted of a constant proportion of phenotypically sensitive and resistant cells. The alternative responses to phage appear to be determined at the adsorption level of phage-host interaction. At present, we cannot rule out entirely that poor adsorption of RP1 to semiresistant mutants to *S. rimosus* R6 was due to the presence of a sensitive subpopulation or, as postulated by Garen and Kozloff (5), to a subtle surface modification which can reduce the rate of phage attachment to all cells in the population. Considering the low adsorption and poor recovery of the infective centers reflected in a low relative EOP, one might expect that sensitive cells would represent at least 10% of the entire population. In contrast to this expectation, we were not able to detect the presence of a sensitive subpopulation. Moreover, poor adsorption of RP1 to semiresistant mutants is not the only way by which resistance to infection can arise. It is well known that phage infection does not necessarily lead to lysis of the culture. The fate of the infected population depends on the relative concentration of phages to bacteria and is dependent on their actual rates of proliferation (10). In our semiresistant strains the propagation of RP1 was characterized by approximately 2.5-fold-longer latent and rise periods. As a consequence, the multiplication of the phage is much lower than that of its host, which considerably reduces the relative actinophage to actinomycete ratio and dilutes out the phage. Thus, semiresistant mutants of *S. rimosus* R6 retained their ability to produce the same amount of OTC even in the presence of the phage. Therefore, they could prove useful in the industrial production of the antibiotic.

All attempts to isolate fully resistant mutants of *S. rimosus* R6 were unsuccessful. Inefficient selective pressure and the relatively frequent appearance of semiresistant strains could explain the difficulties in detection of fully resistant mutants.

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