Characterization of Temperate Actinophage φC31 Isolated from *Streptomyces coelicolor* A3(2)

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Actinophage φC31 isolated from *Streptomyces coelicolor* A3(2), the only strain among actinomycetes for which a genetic map had been constructed, appears to be a typical temperate phage. After φC31 infection, true lysogenic cultures arose which liberated phage and were immune to infection with homologous phage after repeated single-colony isolations and treatment with phage-specific antiserum. Clear-plaque (c) mutants were derived from φC31 phage which failed to lysogenize sensitive cultures. Actinophage φC31 has a temperature-sensitive stage of reproduction. A phage which reproduces with the same effectiveness at high (37 C) and low (28 C) temperatures has also been obtained. Heat-inducible (ct) mutants were isolated from this phage which were able to lysogenize sensitive cultures at 28 C but failed to do so at 37 C. Properties of ct mutants suggest that ct mutations involve a gene controlling maintenance of the lysogenic state in actinomycetes and synthesizing repressor, which may become heat-sensitive as a result of mutation.

The occurrence of true lysogeny in actinomycetes was reported and the general method for the screening of lysogenic actinomycetes was described by Welsch (14).

Many papers on various aspects of lysogeny in actinomycetes were published soon afterward. This interest was due to the fact that the most industrial cultures of antibiotic-producing actinomycetes were lysogenic ones. The distribution of lysogenic *Streptomyces* in collections of strains was studied, especially in relation to antibiotic production (4, 15). The spontaneous induction of phage liberation and the efficiency of various agents that induce phage liberation were also studied. The measure of spontaneous induction appeared to vary with the composition of the nutrient medium (9). Studies of the efficiency of inducing agents, namely, ultraviolet irradiation and some chemical agents, have given somewhat conflicting results (1, 12). The existence of a defective lysogen was shown on the basis of electron microscope observations (10).

There seems, however, to be not a single report in which the genetic control of lysogeny in actinomycetes was investigated. A detailed genetic study of actinophages and their relationship with host cultures has not been available until recently.

To study the genetics of actinophages in a suitable host and to enlarge the possibilities of genetic analysis of actinomycetes by using a temperate phage, an actinophage designated φC31 was isolated from *S. coelicolor* A3(2) (6). This culture is the only representative of the actinomycetes whose genetics have been extensively studied (2, 11). The phage φC31 appeared to be a temperate phage which produces large turbid plaques on the indicator strain *Actinomyces coelicolor* 66. We obtained variants of A3(2), which had been cured of prophage after treatment of strain A3(2) with high doses of ultraviolet light. In crosses between lysogenic and nonlysogenic (cured of prophage) A3(2) strains prophage behaved just like any other marker on the A3(2) genetic map. Hence, prophage chromosomal location was suggested (6). The possibility of genetic recombination in φC31 was described in our previous report (8).

Some characteristics of actinophage φC31 isolated from *S. coelicolor* A3(2) and its mutants affecting the lysogenization process are presented in this report. The results described suggest that φC31 is a most suitable actinophage for study of the genetic control of lysogeny in actinomycetes as well as the genetic structure of the actinophage itself.

MATERIALS AND METHODS

Actinomycete strains. *S. coelicolor* A3(2) was kindly supplied by D. A. Hopwood. *A. coelicolor* 66 (Russian name for *Streptomyces*) obtained from the collection of Institute of Microbiology AN USSR was used as indicator culture for phage platings. To determine the efficiency of phage adsorption, a 66 str^r strain was obtained on medium containing 100 μg of streptomycin per ml.

Nonlysogenic strains were designated as ly⁻ and lysogens as ly⁺ cultures. A3(2)ly⁻ cured of prophage, A3(2)ly⁺, and 66ly⁺ were obtained in our laboratory. A3(2)ly⁺ was prepared from A3(2)ly⁻ cured of prophage, since A3(2) itself liberated phage with a very low spontaneous rate, possibly owing to a defective lysogenic nature of this culture (7), which made it not a very suitable lysogen to work with.

Phages. Temperate actinophage φC31, was isolated from *S. coelicolor* A3(2) on indicator strain *A. coelicolor* 66 (7). Spontaneous clear-plaque (c) mutants were obtained. A recombinant phage which, unlike wild-type φC31, reproduced normally at 37 C was prepared by the cross of φC31 with a c mutant which had the ts⁺ phenotype at 37 C. It was designated φC31R. Spontaneous heat-inducible (ct) mutants were obtained from φC31R phage.

Media. Corn steep liquor medium used for phage assay plates contained, per liter: corn steep liquor (ca. 50% solid), 5.0 g; peptone (Spopha, Prague, Czechoslovakia), 5.0 g; glucose, 10 g; NaCl, 5.0 g; CaCl₂, 0.5 g; and agar (Difco), 15 g for the basal layer and 7 g for the top layer. The medium was adjusted to pH 7.0 to 7.2. When streptomycin was used, the basal layer contained 70 μg of streptomycin per ml; an equivalent amount of streptomycin was added to the soft agar. Peptone broth contained (per liter): peptone, 10 g; NaCl, 5 g; CaCl₂, 0.5 g; glucose, 10 g; NaHPO₄, 1.25 g; KH₂PO₄, 0.42 g; MgSO₄, 0.25 g; and tryptophan, 0.02 g. The broth was adjusted to pH 7.0 to 7.2.

Germination of spores and measurement of actinophage adsorption. Since the most optimal adsorption is generally obtained with a young mycelium aged from 4 to 18 hr (13), young germinated spores were used in our experiments. A spore suspension was incubated with aeration in peptone broth for 6 hr with addition of 0.2% agar to prevent cohesion of germinated spores. The spore concentration was estimated with the aid of a microscope and by plating.

The adsorption was carried out in a mixture with KCN (final concentration, 10⁻⁴ M) for 30 min. A phage was added to a 66 strˢ culture at a low multiplicity of infection (MOI), and the mixture was kept at 28 C. Samples were removed and assayed at requisite times on plates containing streptomycin and seeded with strain 66 strʳ to determine the number of unadsorbed phage particles.

One-step growth experiment. Phage were added to a sensitive 66 culture at a low MOI. After an adsorption period, phage-specific antiserum was added; 10 min later, the infected cells were diluted properly into fresh prewarmed peptone broth and incubated at 28 or 37 C. Samples were then assayed at intervals for plaque-forming units.

Phage crosses. Germinated spores of the sensitive strain were infected with five of each of a pair of parents to be used in a cross. After antiserum treatment and appropriate dilutions, the mixture was incubated for one cycle of phage growth and then assayed for total phage and recombinants as described in the text.

Preparation of lysogens. Actinophage φC31 formed turbid plaques in sensitive lawns of 661y⁻ and A3(2)-ly⁻. Survivors from infection with phage φC31 were selected by picking turbid centers of plaques at 28 C.

Colonies of survivors were streaked across a line of 10⁷ c mutants to test their resistance or sensitivity to phage. Resistant variants were plated to obtain single colonies. Spore suspensions of these colonies, after phage-specific antiserum treatment, were plated to obtain single colonies, which were then transferred onto the slants. The variants obtained were studied for lysogenicity as follows: single colonies of the variant tested were covered with a layer of soft agar containing indicator strain spores. All of the colonies of lysogens were surrounded by turbid halos of lysis formed by the phage spontaneously liberated during growth of a lysogenic colony.

Measurement of frequency of lysogenization. Germinated spores were infected at an MOI of 0.1 at 28 C. After antiserum treatment and appropriate dilutions, infected cells were incubated for an additional 40 min to allow establishment of a lysogenic condition. The culture was then spread on plates with 3 × 10⁷ c mutants to prevent growth of sensitive cells and to detect lysogenic colonies.

Isolation of heat-inducible (ct) mutants. The mutants were isolated from φC31R phage propagated at 37 C. The plaques were selected for c phenotype at 37 C and for c⁺ phenotype at 28 C. Each ct mutant has been derived from a different lysate.

RESULTS

Characteristics of lysogens. *S. coelicolor* A3(2)-ly⁺ and *A. coelicolor* 66ly⁺ lysogens were obtained (see section on preparation of lysogens). The lysogens were observed to adsorb φC31 phage with the same efficiency as the sensitive indicator strains did. However, φC31 failed to produce either lysis areas or single plaques in a lawn of lysogens. Infection of the lysogens with φC31 phage in one-step growth experiment did not lead to an increase in the number of infective centres above the spontaneous level. Thus, lysogens appeared to be immune to homologous phage.

The lysogens were also shown to yield phage after spontaneous induction. When single colonies of lysogens were covered with a layer of soft agar containing indicator culture, they always gave a turbid halo of lysis formed by the phage. This capacity was maintained by subsequent generations, and among a great number of the colonies tested we did not find a single one which failed to yield the phage.

Assay of the A3(2)ly⁺ spore suspension containing 3.2 × 10⁸ spores per ml showed that it contained 4.8 × 10³ free phage particles per ml (the free phage titer was determined in the supernatant fluid after centrifugation of the suspension). A3(2)ly⁺ proved to be resistant to this free phage. To determine a concentration of phage-producing spores in this suspension, the sediment obtained after centifugation was treated with phage-specific antiserum and plated onto plates seeded with sensitive culture. It appeared to contain 2.7 × 10⁶

plaque-forming units. This means that in a lysogen spore suspension containing 3.2×10^8 spores per ml, 2.7×10^6 spores produced phage. Thus, during about 14 hr of growth on a lawn of the sensitive culture, approximately 1 of 100 germinated spores of lysogen yielded phage progeny as a result of spontaneous induction.

General properties of actinophage ϕC31. Production of ϕC31 phage at 28 and 37 C was determined in one-step growth experiments, with adsorption carried out at 28 C. It should be noted that the rate of phage adsorption was low and not highly efficient ($K_{28} = 7.8 \times 10^{-10}$ ml/min). There were no profound differences in the kinetics of adsorption at 28 and 37 C. As is shown in Fig. 1, at 28 C the first free phage appeared between 50 and 60 min after infection; the burst size at this temperature was usually 20 to 50 phage particles per infected cell whereas it was only 0.8 to 1 at 37 C.

The limits of temperature compatible with phage reproduction are, as a rule, closely correlated with the limits of tolerance of the host. ϕC31 phage, however, failed to reproduce at 37 C despite the fact that the host culture grew normally at that temperature. The similar failure to repro-
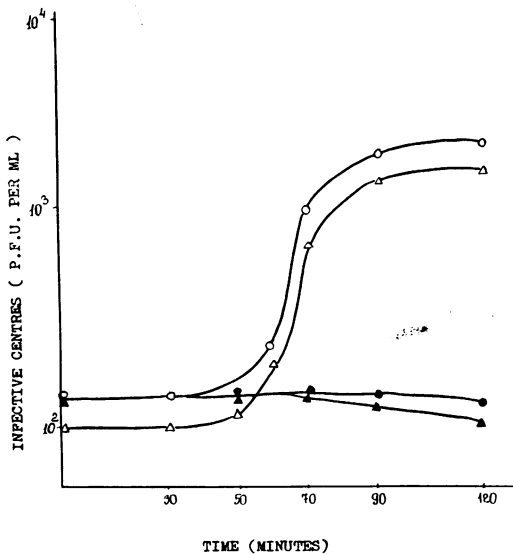
duce at a high temperature still optimal for host cells was reported for virulent actinophage SAP-2 (5). The high temperature was found to affect an early stage of actinophage SAP-2 replication and formation of the late protein.

Further evidence concerning the effect of temperature on phage reproduction was sought in temperature shift-up (28 to 37 C) experiments (Fig. 2). Germinated spores were infected at 28 C, and during the latent period samples were removed at certain intervals and incubated at 37 C. It was found that transfer from 28 C to 73 C during 20 min after infection led to inhibition of phage yield. Shift-up at subsequent intervals resulted in partial but decreasing inhibition of the yield. The data suggest that an elevated temperature affected a certain step of intracellular phage development which took place between 20 and 25 min after infection.

The frequency of lysogenization by ϕC31 at 28 C was 20 to 40% of the number of infected germinated spores.

Clear-plaque mutants. Clear-plaque mutants arise spontaneously with high frequency from ϕC31 wild-type stock. The frequency of lysogenization for these mutants was $< 10^{-5}$; we failed to obtain lysogenic variants among survivors from infection. Apparently they were not able to lysogenize sensitive cultures and might therefore be connected with mutations affecting the lysogenization process.

The mutants as well as c^+ phage have the ts phenotype, but unlike c^+ phage they form plaques

FIG. 1. *One-step growth experiments. Phage (final concentration, 4×10^7 to 5×10^7 plaque-forming units/ml) were added to a culture of A. coelicolor 66 containing 2×10^8 germinated spores per ml (final concentration). After adsorption at 28 C, mixtures were treated with antiserum (K-20), diluted, and incubated at 28 or 37 C. Samples were assayed at intervals for plaque-forming units. Symbols: $\triangle$, ϕC31 at 28 C; $\blacktriangle$, ϕC31 at 37 C; $\bigcirc$, c mutant at 28 C; $\bullet$, c mutant at 37 C.*
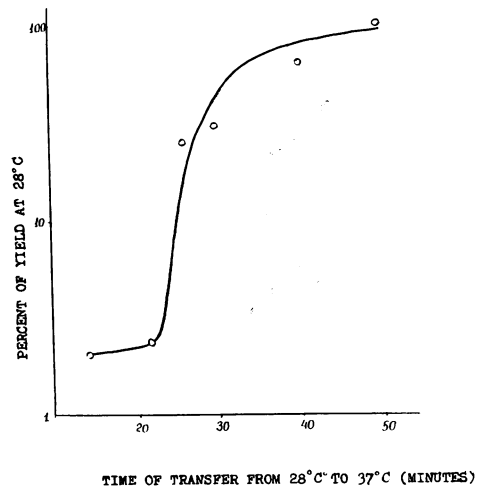

FIG. 2. *Effect of shift-up (28 C to 37 C) on ϕC31 reproduction. A one-step growth experiment was conducted at 28 C. At certain intervals in the latent period, samples were removed, diluted into broth at 37 C, incubated at that temperature, and assayed for phage.*

at 37 C with a frequency of 10^{-5} to 10^{-6}. The phage isolated from these plaques at 37 C formed clear plaques without differences in efficiency of plating at temperatures ranging from 28 to 37 C.

φC31 phage with the ts$^+$ phenotype at 37 C was isolated in the progeny of the two-factor cross between c$^+$ts and cts$^+$ phages. Two classes of recombinants, c$^+$ ts$^+$ and cts, which were expected to arise from a single crossover event were obtained. Recombinants of the cts type were scored by testing clear plaques produced at 28 C for their inability to grow at 37 C, and c$^+$ ts$^+$ recombinants were selected at 37 C. Thus, the c$^+$ ts$^+$ recombinant which reproduced normally at a high temperature was obtained and was designated φC31R in contrast to φC31 wild-type actinophage. φC31R also appeared to lysogenize the host culture with a high frequency.

Heat-inducible mutants. φC31R produced c and ct mutants. The latter formed turbid plaques at 28 C and clear plaques at 37 C. They retained the ability to lysogenize sensitive strains at 28 C; under these conditions, about 30% of infected germinated spores were lysogenizeo. No lysogeny was established for ct mutants at 37 C.

The *S. coelicolor* A3(2)ly$^-$ strain was lysogenized with the ct$_1$ mutant at 28 C. Heat induction occurred when A3(2)ly$^+$ was kept at 40 C for 5 min before incubation at 28 C. As can be seen in Fig. 3, the number of plaque-forming units immediately after heat induction was eight times higher than the number of plaque-forming units producing spontaneously and was 31% of the total number of germinated spores. The latent period was as long as that of the phage during infection of the sensitive culture. In 90 min, all of the induced germinated spores yielded the phage. The number of free phage particles in the induced sample was higher by two orders of magnitude than the number of phages produced during the same period as a result of spontaneous induction. These properties of ct mutants were considered to mean that the mutations affect a gene responsible for repressor protein synthesis.

The number of heat-inducible germinated spores was strongly determined by the stage of spore germination at the moment of induction and by the physiological state of germinated spores. Ungerminated spores displayed a complete absence of heat induction. As spores germinated, the number of induced spores increased and reached a maximum in germinated spores aged from 6 to 8 hr. However, heat induction was never observed in all of the germinated spores. Absence of heat induction in part of the germinated spore population may be due to the restoration of the repressor activity which occurred after transfer from a high temperature to a low
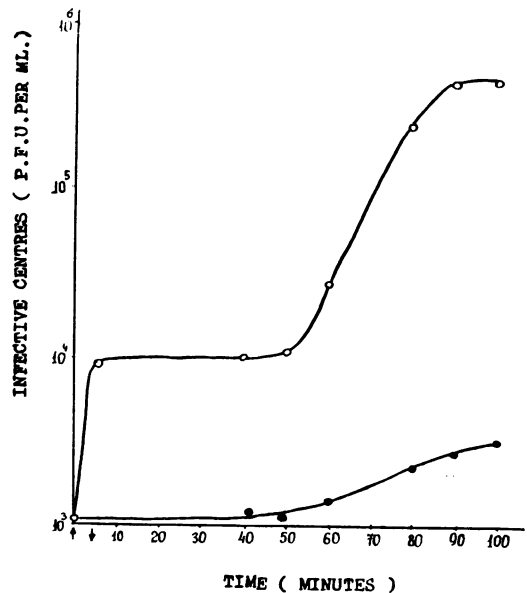

FIG. 3. *Heat induction of S. coelicolor A3(2) lysogenic for ct$_1$. Germinated spores of a lysogenic culture were treated with phage-specific antiserum and diluted (final concentration, 2.9 × 10^4 germinated spores/ml). One sample was incubated at 28 C for 100 min (●). A second sample was incubated at 28 C after heat induction (○) at 40 C for 5 min (↑ ↓).*

temperature. Nevertheless, more prolonged heat induction followed by subsequent incubation at 28 C in the complete medium, i.e., in the conditions unfavorable for the renatured repressor to function, did not increase the number of induced spores. Studies on the reversibility and irreversibility of the repressor inactivation are in progress.

DISCUSSION

The actinophage φC31 isolated from *S. coelicolor* A3(2) appeared to be a typical temperate phage. The survivors of infection with φC31 were true lysogens, immune to infection with homologous phage. During incubation, spontaneous phage production occurred in part of the population of lysogens. The prophage was located on the A3(2) linkage map (6). The maintenance of the lysogenic state is controlled, therefore, by a prophage integrated into the actinomycete chromosome.

Properties of ct mutants suggest that mutations affect a gene involved in maintenance of the lysogenic state. The gene responsible for maintenance of the lysogenic state seems to synthesize the repressor protein, which becomes thermosensitive as a result of mutation.

The fact that clear-plaque mutants of φC31 lost

the ability to lysogenize sensitive cultures suggests, by analogy with temperate phage λ (3), that these mutations affect some steps of the lysogenization process. Further genetic investigations of c mutants are in progress. Unfortunately, the study of c mutants is not sufficiently advanced to elucidate their role in the lysogenization process because of the peculiarity of this system. A great number of unsolved problems concerning the biology of actinophages and their hosts makes it difficult to apply methods available for the study of classic coliphages. Actinophages lyse hosts which are very specialized bacteria. They have multinucleated cellular organization, a complex differentiated colonial structure, and a reproduction cycle involving the stage of sporulation. Owing to these peculiarities, we have had some difficulties in attempts to obtain precise estimations in experiments with mixed infection.

However, the isolation of actinophage φC31 mutants with impaired lysogenizing ability, the possibility of using genetic recombination between actinophage mutants, and the fact that the host's genetics have been extensively studied make actinophage φC31 a suitable model for studies of the genetic control of lysogeny and of the genetic structure of the actinophage itself.

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