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

N. D. LOMOVSKAYA, N. M. MKRTUMIAN, N. L. GOSTIMSKAYA, AND V. N. DANILENKO

The Institute of Genetics and Selection of Industrial Microorganisms, Moscow, USSR

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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 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, Czecho-

**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 φC31 phage.** Assay of the A3(2)-ly+ spore suspension containing 3.2 × 105 spores per ml showed that it contained 4.8 × 105 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 centrifugation 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 $3.2 \times 10^8$ spores per ml, $2.7 \times 10^4$ 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 ($K_m = 7.8 \times 10^{-10}$ 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 reproduce 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°C 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 37°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.

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**Fig. 1. One-step growth experiments.** Phage (final concentration, $4 \times 10^9$ to $5 \times 10^9$ plaque-forming units/ml) were added to a culture of A. coelicolor 66 containing $2 \times 10^8$ 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: Δ, φC31 at 28°C; ▲, φC31 at 37°C; ○, c mutant at 28°C; ●, 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 lysogenized. No lysogeny was established for ct mutants at 37 C.

The S. coelicolor A3(2)ly⁻ strain was lysogenized with the ct 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 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.

LITERATURE CITED