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Isolation and Characterization of a Lysogenic Strain of *Nocardia erythropolis*

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A stable phage-carrying strain of *Nocardia erythropolis* was isolated from an infection with the nocardiophage φEC. Growth of the strain in phage-specific antiserum for 48 hr produced cured organisms at a frequency of about 0.5%. Spontaneous curing, determined by serial single-colony isolations, was less than 0.4%. The strain could not be infected by phage φEC nor by a closely related phage, φC, although the cells were able to adsorb these phages. In cell populations, a frequency of 2.5×10^{-4} cells spontaneously induced. The growth rate of the strain was comparable to that of the uninfected wild-type *N. erythropolis*. Ultraviolet irradiation or treatment with mitomycin C induced the strain to produce larger numbers of phage. It was concluded that the isolated strain was lysogenic.

The phenomenon of lysogeny has been recognized for many years and has been thoroughly studied, particularly in the enterics (11, 12, 18). Reports have been made concerning lysogeny among various *Actinomycetales* including the *Streptomyces*, the *Actinomyces*, and the *Mycobacterium* (17, 19). In the genus *Nocardia* there have been reports of only one lysogenic strain (*N. fructiferi*) by Rautenshtein et al. (15, 16). The following communication concerns the first characterization of the bacteriophage-host relationship in a lysogenic *Nocardia*. Since many strains of *Streptomyces* have been shown to exhibit the property of pseudolysogeny or the carrier state (20), the possibility that this type of relationship existed was taken into consideration in our study.

Results obtained in our investigation had to be interpreted in light of the unique nocardial life cycle. Adams and McClung (1, 3) studied the developmental cycles of several members of the *Nocardia*. They found that growth of *N. erythropolis* Mat-Ce strains, formerly designated *Jensenia canicruria* and *N. canicruria* (5, 7), in agar cultures began with cell elongation, and nuclear division followed in 12 hr by the formation of mycelial branches. Septum deposition followed branching and, from 18 to 24 hr, fragmentation occurred giving rise to uninucleate and coenocytic cells. Later, Adams (2), studying the developmental cycle in broth cultures, found that fragmentation began at 8 hr and was completed by 30 hr. Fragmentation products included nonviable particles.

Two nocardiophages, φEC and φC, were isolated from the soil and characterized by Brownell et al. in 1967 (10). Phage φEC was found to infect both mating type strains (Mat-Ce and Mat-cE) of *N. erythropolis*. Phage φC infected only Mat-Ce strains of *N. erythropolis*. Both φEC and φC produced clear plaques when plated with the sensitive strains.

In the present study, a stable φEC-carrying strain of *N. erythropolis* Mat-Ce was isolated from broth cultures, and the phage-host relationship was characterized according to a number of criteria. Attempts were made to induce the strain to produce larger numbers of phage by using ultraviolet (UV) irradiation and mitomycin C. Further investigations have involved the location of the φEC prophage on the *N. erythropolis* genophore (Abst. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, p. 74, 1972). Ultimately, phage derived from lysogenic nocardiae will be employed in genetic transduction experiments.

MATERIALS AND METHODS

Strain isolation. The phage-carrying strain, designated Ce-3(φEC), was isolated by making serial transfers of host cell survivors from a lytic infection of the wild-type *N. erythropolis* Ce-3. Peptone-yeast extract (PY) broth (Difco, Detroit, Mich.) was the culture medium employed. Four-hour cell cultures at a density of ca. 1.0×10^6 cells/ml were infected at a multiplicity of infection (MOI) of 10 with phage φEC and were incubated for 24 to 36 hr. Cells were most sensitive to infection when they were cultured less than 6 hr before addition of phage (10). Broth cultures in all experiments were incubated at 30 C in a shaking incubator. Cell survivors (0.1 ml containing ca. 1.0 ×

10^7 cells) from the initial infection were transferred to fresh PY broth, and the culture was reinoculated with phage. This same procedure was repeated until 10 transfers had been made. A sample from each transfer was centrifuged, the supernatant fluid was assayed for phage, and the cells were streaked onto PY agar for single colonies. Three serial streak isolations were made from each transfer of cells. Isolated colonies, obtained after 36 hr of incubation, were suspended in PY broth and assayed for phage. All phage assays were done by the soft-agar overlay technique of Adams (4).

Production of antiserum. The specific antiserum was obtained by inoculating a rabbit subcutaneously with ca. 1.0×10^9 plaque-forming units (PFU) per ml. Intravenous booster inoculations with the same titer of phage were made after 1 and 2 wk, and the serum was collected after another week. The K value of the serum was 21.2/min.

Phage curing. In the antiserum curing experiment, Ce-3(ϕEC) cells cultured on PY agar were inoculated into PY broth containing 10% (v/v) phage-specific antiserum to give ca. 1.0×10^7 cells/ml. Likewise, Ce-3 cells cultured on PY agar were inoculated into PY broth at the same density and were infected with phage ϕEC at a MOI of one. After allowing for adsorption, phage-specific antiserum was added to the Ce-3 culture to give a final concentration of 10% (v/v). Both of these cultures were incubated for 48 hr with transfers to fresh broth plus antiserum at intervals. At the end of 48 hr, the cells were transferred to fresh broth without antiserum and were incubated for 24 hr. The cultures were assayed for free phage at each transfer and at the end of incubation. In this and other experiments, the number of free phage was determined by filtering the supernatant fluid through a 0.45-μm pore size Swinnex filter (Millipore Filter Corp., Bedford, Mass.) before assaying it. Ce-3(ϕEC) and Ce-3 cells were also cultured in the same manner without antiserum for the entire 72 hr to serve as controls.

Phage adsorption. When strain Ce-3(ϕEC) was tested for its ability to adsorb phages ϕC and ϕEC, known amounts of each phage were added to 4-hr broth cultures, and the suspensions were allowed to incubate. After 10 min, the suspensions were assayed for free phage. A suspension of cells without added phage was treated in the same manner to serve as a control. The number of free phage in the test cultures minus the number in the control was considered the number of phage added which did not adsorb.

Induction experiments. In UV induction experiments, 8- to 12-hr PY broth cultures (active growth phase) of Ce-3(ϕEC) cells were centrifuged and suspended in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) to a density of ca. 1.0×10^8 cells/ml. They were then exposed to UV light at a distance of 30 cm from a pair of Westinghouse Sterilamps (G15T8) for either 60 or 70 sec, and were diluted into PY broth and assayed at 30-min intervals for viable cells, total phage, and free phage. The length of exposure to UV light was predetermined by assaying for viable cells, total phage, and free phage after exposing a suspension of cells to UV light at 30 cm for intervals from 0 to 120 sec. The intervals of 60 and 70 sec gave the highest production of phage. Cell survival at these UV dosages was 26% and 9.0%, respectively.

The procedure for mitomycin C induction was the same as that for UV induction except that Ce-3(ϕEC) cells were incubated in PY broth with either 1.0 μg or 10 μg of mitomycin C per ml (final concentration) (Nutritional Biochemicals Corp., Cleveland, Ohio) for 15 min. Almost 100% of the cells survived when treated with 1.0 μg/ml, but only 7.0% survived when treated with 10 μg of mitomycin C per ml.

RESULTS

Strain isolation. When phage ϕEC infected strain Ce-3, an apparently lytic response was produced, i.e., no stable phage-carrying cells could be isolated, and clear rather than turbid plaques were observed even after 48 hr of incubation. However, after serial broth transfers, 99% of the population was eventually composed of cells which were immune to infection. Phage-producing isolates were obtained from the seventh transfer of host cell survivors; 99% of the colonies tested from this transfer produced phage. Strain Ce-3(ϕEC) was derived from one of these single colonies that continued to produce phage after three successive streak isolations. Phage produced by strain Ce-3(ϕEC) also produced clear plaques when plated with strain Ce-3. When mixed cell suspensions of strains Ce-3(ϕEC) and Ce-3 were plated in soft agar overlays, clear plaques were formed after 12 to 18 hr of incubation from induction of Ce-3(ϕEC) cells. After 24 to 36 hr, the plaques were turbid due to growth of immune Ce-3(ϕEC) cells.

Phage curing. The frequency of spontaneous curing was obtained by making serial single-colony isolations. Ce-3(ϕEC) cells were either antiserum-treated to remove exogenous phage or untreated, and five serial streak isolations were made from each kind of suspension. Twenty-five colonies were tested from the untreated cells and from the antiserum-treated cells at each serial isolation. All 250 of these colonies produced phage. Therefore, the frequency of spontaneously cured organisms was less than 1 in 250 ($<0.4\%$) for strain Ce-3(ϕEC).

Efforts were made to cure the isolated strain of its phage. Growth of strain Ce-3(ϕEC) in PY broth containing 10% (v/v) phage-specific antiserum for 48 hr decreased the free phage titer to zero, whereas free phage in the Ce-3(ϕEC) control reached 7.0×10^8 PFU/ml (Fig. 1). The free phage titer in the lytically infected Ce-3 culture was also decreased to zero by growth in antiserum for 48 hr. The lytic culture was employed as a control to determine whether or not the antiserum could completely rid a nonlyso-

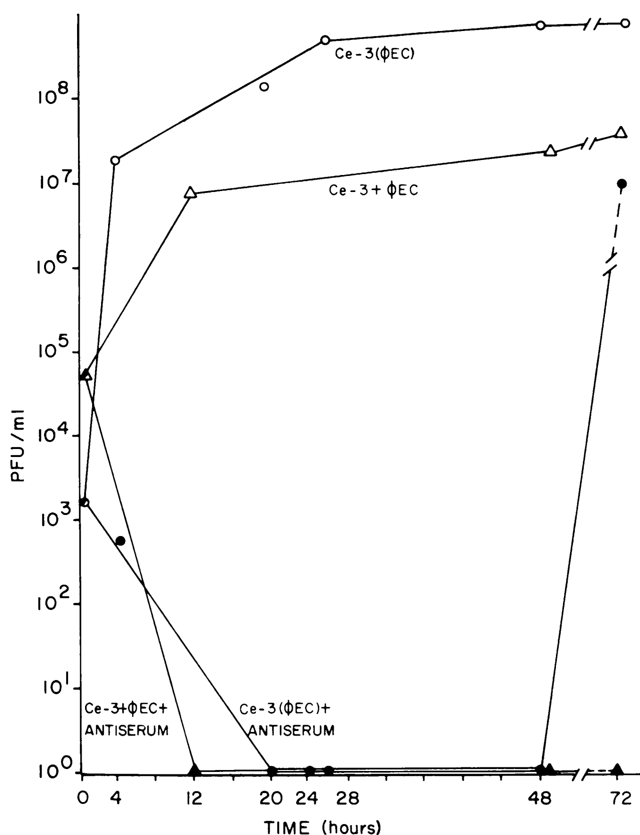

FIG. 1. *Free phage production in Nocardia erythropolis wild-type Ce-3 infected with ϕEC (▲—▲) and in strain Ce-3(ϕEC) (●—●) during growth in PY broth plus phage-specific antiserum. The last point on each of these two curves represents the free phage titer after transfer of the culture to broth without antiserum for 24 hr. The same two strains were grown in broth not containing antiserum for the entire 72 hr; Ce-3 infected with ϕEC* (△—△) *and strain Ce-3(ϕEC)* (○—○). *Each culture was transferred to fresh broth or fresh broth plus antiserum at the intervals plotted.*

genic strain of infecting phage. Free phage produced by the untreated lytically infected Ce-3 control reached a titer of 1.0×10^7 PFU/ml at the end of 48 hr. After both antiserum-treated cultures were transferred to broth not containing antiserum and were incubated for 24 hr, strain Ce-3(ϕEC) produced a free phage titer of 5.0×10^6 PFU/ml whereas the Ce-3 culture still contained no free phage.

When strain Ce-3 (ϕEC) was grown in phage-specific antiserum, as before, and was plated for single colonies, one colony was found which did not produce phage out of 200 tested. The strain derived from this colony was sensitive to infection by phages ϕEC and ϕC. The frequency of cured cells obtained after growth of strain Ce-3 (ϕEC) in antiserum, then, was approximately 0.5%.

Phage sensitivity. Neither ϕEC nor ϕC was able to produce plaques when plated with strain Ce-3 (ϕEC). Attempts to lytically infect the strain with either phage in broth cultures also failed. The phage titer in these cultures was not greater than the phage titer produced by sponteneous induction in a control culture. However, both ϕC and ϕEC were able to adsorb to Ce-3 (ϕEC) cells. With 10 min of incubation, 99.9% of phage ϕC and 86.4% of phage ϕEC had adsorbed.

Spontaneous induction. Spontaneous phage production by strain Ce-3 (ϕEC) and phage production in the lytically infected Ce-3 are depicted in Fig. 2. The first 210 min of incubation of the lytic culture gave the results of one-step phage growth. Under the conditions indicated, the latent period of ϕEC extended for 90 min and the rise period for another 90 min. The burst size was about 100 particles. When incubation was continued, the phage titer reached 5.0×10^6 PFU/ml at the end

of 24 hr. The total number of phage produced spontaneously in strain Ce-3 (ϕEC) was considerably lower; at the end of 210 min, the phage titer was 1.5×10^5 PFU/ml. After about 6 hr, the titer continuously decreased as the culture was incubated. The viable cell count from the Ce-3 (ϕEC) culture remained the same, i.e., ca. 6.0×10^6 cells/ml, for the first 210 min. The burst size from the lytic infection had to be used to calculate the frequency of spontaneous induction since the burst size from a lysogen was not known. Since the burst size from the lytic infection was 100 particles, the spontaneous induction frequency was 2.5×10^{-4} cells at 210 min.

The frequency of spontaneous lysis in individual Ce-3 (ϕCE) cells was determined by the following cloning experiment. A 0.1-ml portion of a cell suspension was added to each of 200 tubes to give approximately 1 cell/tube. An additional 0.4 ml of Trypticase soy broth (Difco, Detroit, Mich.) was added to each tube, and the tubes were incubated for about 4 days. They were then scored for bacterial growth and assayed for phage. From the number of viable cells in the suspension used to inoculate the tubes, the calculated average number of bacteria per tube was 0.5. On this basis, the number of tubes that should have contained bacteria (Poisson expectation) was 79. The number of tubes that actually did contain bacteria was 73. All 73 tubes also produced confluent plaques when assayed for phage. No tubes contained only bacteria or only phage. Tubes that would have contained only phage with a number in the range of the burst size would have been considered to have spontaneously lysed within the length of time required for a burst. From these data, less than 1 in 73 ($<1.3\%$) individual Ce-3 (ϕEC) cells was undergoing a lytic cycle.

Cell growth. The growth rate of strain Ce-3 (ϕEC) was compared to that of uninfected Ce-3 and to that of Ce-3 infected with ϕEC at a MOI of 1. The curve obtained from strain Ce-3 (ϕEC) was practically identical in shape to that obtained from the uninfected Ce-3 strain (Fig. 3). Cell numbers began to increase after 6 hr, reaching a plateau at the end of 24 hr. In contrast, the curve of Ce-3 infected with ϕEC showed a dramatic decrease in cell numbers from 90 to 210 min. This

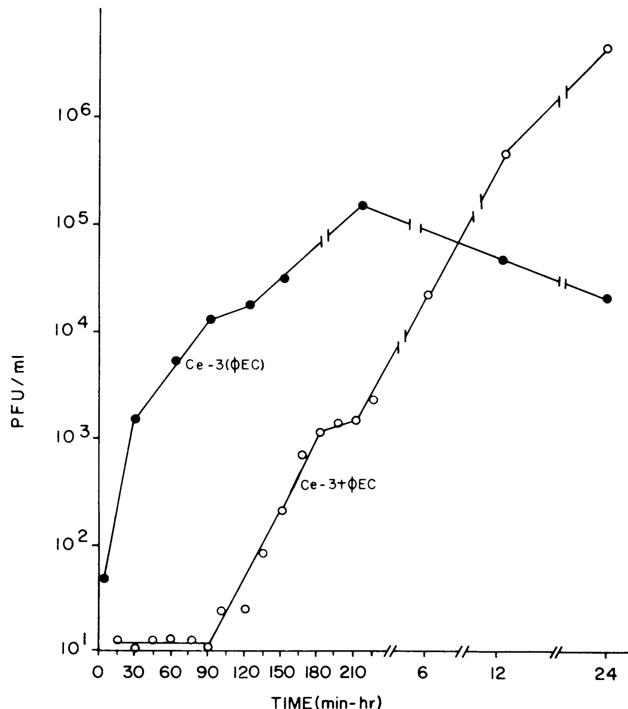

FIG. 2. *Spontaneous phage production in Nocardia erythropolis Ce-3(ϕEC) (●—●) and phage production in strain Ce-3 infected with phage ϕEC (○—○). Both strains were cultured for 4 hr in PY broth after which strain Ce-3 was infected with ϕEC at a multiplicity of 1. Ten minutes later, specific antiphage serum was added to both cultures to remove exogenous phage. The cells were then diluted into PY broth and samples were assayed for phage at the indicated intervals.*

time interval corresponded to the rise period of phage infection. After 6 hr, cell numbers in the lytic culture began to increase.

Induction experiments. Ce-3 (ϕEC) cells were UV irradiated or treated with mitomycin C in attempts to induce the carried phage. When cells were UV irradiated for 60 sec (Fig. 4), there was an immediate increase in the total number of PFU (representing induction) followed by a latent period of 2 hr and a rise period of another 2 hr. The burst size was about 10 particles. The free phage titer, undetectable for the first 2 hr, began to increase during the rise period of infection, finally paralleling the total phage titer. Viable cell numbers at this UV exposure decreased from 2.3×10^6/ml to 5.9×10^5/ml, of which 4.6×10^5 cells/ml were lysed by induction. Thus, cell survival was 26% and induction was 20%. After the initial decrease in viable cells, their number did not change appreciably during the experiment. The total phage titer in the control culture increased slowly and continuously throughout the sampling time. The control free phage titer also increased continuously throughout the experiment, although the titer for the first 2 hr was less than 1.0×10^3 PFU/ml and therefore is not represented on the graph. Total phage in the control culture had reached 5.0×10^4 PFU/ml at the end of the sampling time in contrast to a total phage titer of 6.0×10^6 PFU/ml in the UV-irradiated culture at the same time.

With 70 sec of exposure to UV irradiation, cell survival was 9.0% and induction increased to 46%. The burst size, again, was about 10 particles. Otherwise, the curves were comparable to those obtained with 60 sec of irradiation.

The curves obtained after mitomycin C treatment of Ce-3 (ϕEC) cells (Fig. 5) were similar to those from UV induction. However, in these experiments, the latent period was only 1 hr and the rise period extended for 3 hr. With 10 μg of mitomycin C per ml (Fig. 5), cell survival was about 7.0% and there was 6.0% induction. The burst size was 50 particles. Again, the free phage titer increased during the rise period. In the control culture, the total phage titer and the free phage titer were parallel throughout the experiment. The total phage titer, at the end of sampling, was 1.0×10^5 PFU/ml in contrast to 2.0×10^6 PFU/ml total phage in the mitomycin C-treated culture. Viable cells increased very little above 6.5×10^5/ml in the control culture and, after the initial decrease to 5.0×10^4 cells/ml, they increased only slightly through 5 hr in the mitomycin C-treated culture.

When cells were treated with 1.0 μg mitomycin C per ml, the curves were similar to those in Fig. 5 except that cell survival was almost 100%. The amount of induction was about 1.0%, and the burst size was 20 particles.

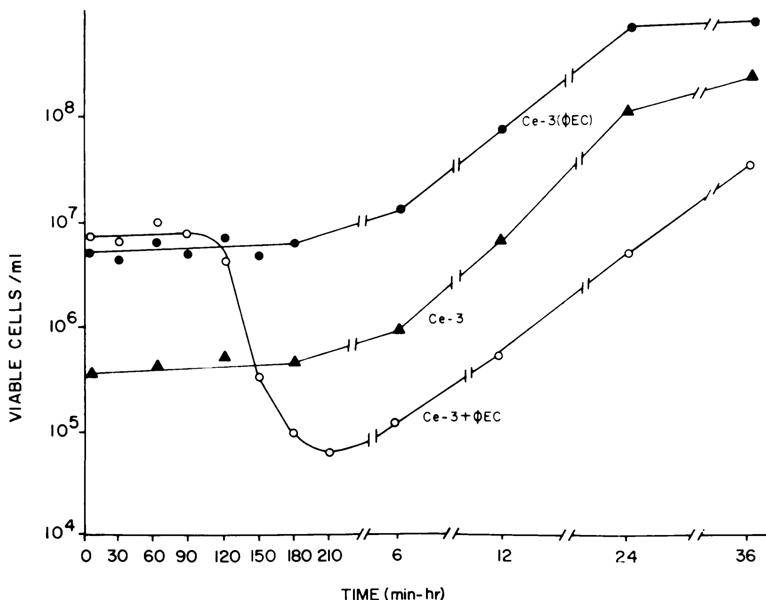

FIG. 3. *Cell growth of Nocardia erythropolis strains Ce-3 infected with phage ϕEC at a multiplicity of 1* (○—○), *Ce-3(ϕEC)* (●—●), *and Ce-3* (▲—▲). *Cells were inoculated into PY broth, and samples were assayed for viable cells at the indicated intervals.*

DISCUSSION

Less than 1 colony in 250 tested from the phage-carrying strain was spontaneously cured. Although the free phage in cell populations was eliminated by growing the phage-carrying strain in specific antiphage serum, this treatment was not effective in curing the cells. When antiserum was removed from the growth medium, free phage again became detectable. The inability to cure strains of their carried phage by this method indicates that they are lysogenic. Nonlysogenic phage-carrying strains, e.g., strains in the carrier state, are quite efficiently cured of their phage by growth in phage-specific antiserum (8, 20).

The percentage of curing (about 0.5) obtained by culturing strain Ce-3 (ϕEC) in antiserum may represent the actual spontaneous curing frequency. Due to the filamentous nature of the organism, the transfer of colonies from an untreated cell suspension on agar may not have given cured cells an opportunity to segregate. Growth of the cells in antiserum greatly reduced the number of free phage, and the length of incubation allowed the filaments to fragment before being plated on agar. These conditions may have enhanced the isolation of spontaneously cured cells. In any case, the spontaneous curing frequency was very low and was another indication that strain Ce-3 (ϕEC) was lysogenic. Nonlysogenic phage-carrying strains readily segregate cured colonies. Spontaneous curing frequencies as high as 30 to 90% have been reported for pseudolysogenic mycobacteria (6). Bott and Strauss (8) reported carrier state spontaneous curing frequencies as high as 40 to 50% in *Bacillus subtilis*. Derivation of a new, stable phage-carrying strain, resistant to antiserum curing, from infection of a cured isolate was further evidence that the original strain, Ce-3 (ϕEC), was lysogenic.

The ability of strain Ce-3 (ϕEC) to effectively adsorb phages ϕC and ϕEC and the inability of either phage to productively infect the strain suggested that Ce-3 (ϕEC) may be exhibiting lysogenic superinfection immunity, although conclusive evidence was not obtained. These results were not definitive since carrier cultures have been found to be unable to plaque the same phage that they carry (8).

In various lysogenic systems, 10^{-2} to 10^{-5} cells/generation undergo spontaneous induction (4). In comparison, a frequency of 2.5×10^{-4} Ce-3 (ϕCE) cells spontaneously induced in the

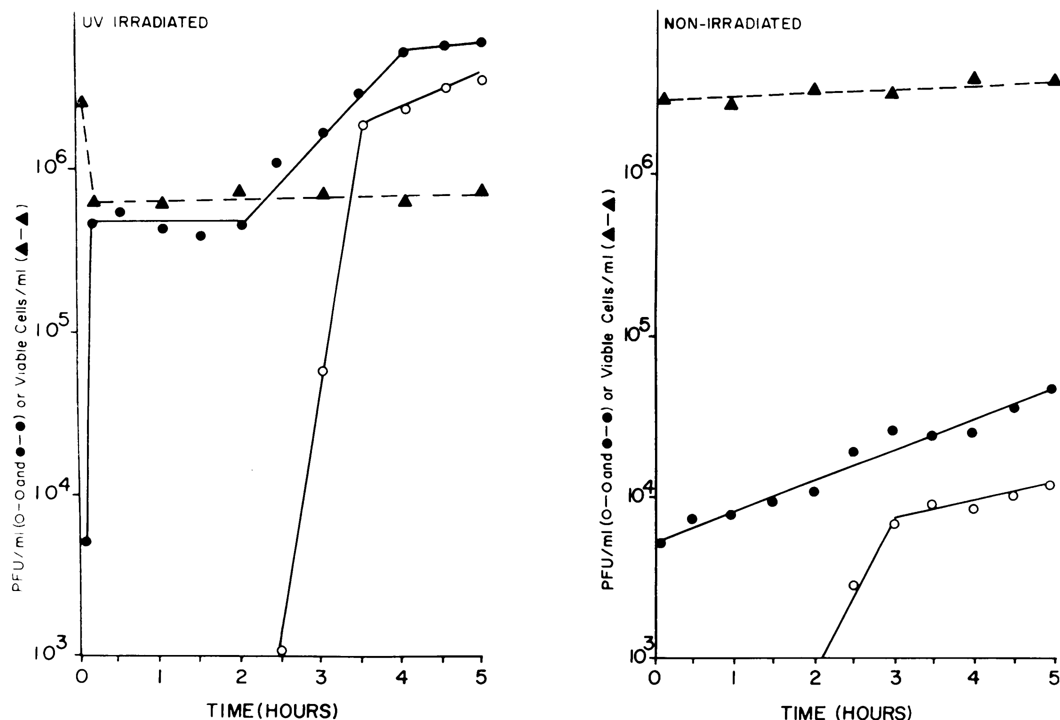

FIG. 4. *Ultraviolet (UV) induction of Nocardia erythropolis Ce-3(ϕEC). The response in the UV-irradiated culture is illustrated by the graph on the left and that in the nonirradiated control by the graph on the right; viable cells* (▲—▲), *total phage* (●—●), *and free phage* (O—O).

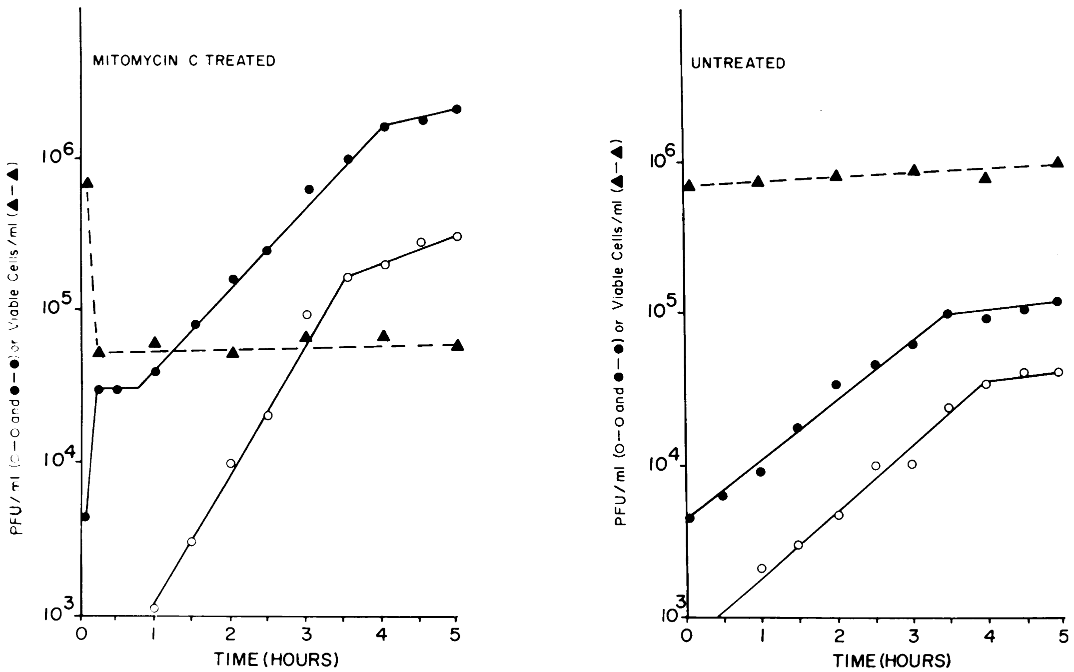

FIG. 5. *Mitomycin C induction of Nocardia erythropolis Ce-3(φEC). The response in the mitomycin C-treated culture is presented on the left of the figure and that in the untreated control is presented on the right; viable cells (▲—▲), total phage (●—●), and free phage (O—O). The cells were incubated at time zero with 10 μg of (final concentration) mitomycin C per ml for 15 min, diluted into PY broth, and assayed at the times indicated.*

first 210 min. Spontaneous induction is usually calculated as the number of cells that lyse per generation. Since the nocardiae do not reproduce by binary fission and thus do not have a generation time defined as such, spontaneous induction was calculated as the frequency of lysis among viable cells during the period of increase in phage production (i.e., during the first 210 min). This period of sampling time was too short to detect an increase in viable cell numbers, since the culture was not in the fragmentation phase (2). The highest titer of phage was seen from about 210 min to 6 hr; after this, it continuously decreased. This phenomenon has been reported to occur in lysogenic strains in which it constituted readsorption of the phage by the cells (9). The one-step growth curve of the lytically infected Ce-3 showed a continuous increase in phage production through 24 hr. Since the culture was sufficiently diluted to prevent readsorption of released phage, the only plausible explanations are that infection continued to occur through septa that were forming in the growing filaments or that phage released by one cell in the filament immediately infected other cells in the same filament. The phage titer in the lytically infected culture was not as great as that in the phage-carrying culture, after the first 210 min, because the number of lytically infected cells was not as great as the number of phage-carrying cells that induced during that time. Cell density did not affect the general characteristics of the respective curves. The curves representing phage production by strain Ce-3 (φEC) in Fig. 1 and 2 were different due to different cultural conditions in the two experiments. The culture in Fig. 2 was antiserum treated, diluted, and maintained in the same broth throughout the experiment. Ce-3 (φEC) control cells in Fig. 1 were not antiserum treated and were transferred to fresh broth a number of times. When cells were transferred to fresh broth, they either produced more phage or did not as readily readsorb the phage produced.

The cloning experiment was performed to determine whether there was a significant difference in the frequency of spontaneous lysis in individual phage-carrying cells as compared to that in populations of these cells. Spontaneous lysis in individual Ce-3 (φEC) cells was less than 1.3%, supporting the data obtained from populations of cells. In contrast, 10 to 15% spontaneous lysis has been reported for individual cells in the carrier state (8).

Cell growth of strain Ce-3 (φEC) was not distinguishable from that of the uninfected wild-type *N. erythropolis*. There was no detectable lysis in

phage-carrying populations as was expected from the low spontaneous induction rate. Although there was a decrease in viable cell numbers in the lytically infected strain, the culture was not completely lysed. Even at higher multiplicities of infection, the same pattern of cell growth was observed. Since there presently is no way to synchronize nocardial cultures, they may contain, at the time of phage infection, mostly uninucleate and short coenocytic cells and fewer coenocytic filaments. Organisms in the later stages of development may be physiologically resistant to phage infection whereas those in earlier stages are sensitive. The ratio of physiologically resistant cells to sensitive cells becomes progressively larger as the culture is incubated, and growth of the resistant cells overshadows any lysis of sensitive cells.

UV light was much more effective than mitomycin C in eliciting induction. The percentage of induction was considerably less than that reported for other systems in which cell survival was comparable to or less than that in our system. Exposure of *Salmonella typhimurium* LT-2(P22) to 1.0 μg of mitomycin C per ml for 15 min gave 6.0% induction and 75% cell survival, whereas exposure to 10 μg/ml for 15 min produced 60% induction and 30% cell survival (13). In *Escherichia coli* K-12 (λ), UV induction was more than 99% when cell survival was less than 1.0% (14). When Ce-3 (ϕEC) broth cultures were as much as 12-hr old, the free phage titer was never higher than ca. 10^3 PFU/ml. Centrifuging the suspensions eliminated some free phage, and UV irradiation killed the remainder. After the cells were inoculated into broth, free phage were detectable in all cultures within a few minutes except in those that were UV irradiated. The reason for lack of appearance of free phage in the UV irradiated culture until the rise period of infection is not known. Since suspensions were assayed for free phage after they were passed through a membrane filter (0.45 μm pore size), the free phage titer did not ever appear to be as great as the total phage titer. Even though the filters retained a portion of the free phage, the pattern of increase in titer was valid. The number of viable cells had increased only slightly after 5 hr even in the control cultures. In a normal broth culture, these cells would have started to fragment and an increase in number would have been expected. The manipulations (i.e., centrifuging and resuspending of cells) involved at the beginning of the experiments may have facilitated fragmentation. When the cells were then reinoculated into broth, the majority of them may have been starting a new developmental cycle. No decrease in viable cell numbers was seen in the control cultures even with an increase in total phage due to the small proportion of cells which were lysing.

Phage characterization experiments including host range determinations, adsorption kinetics, antiserum inactivation kinetics, and one-step growth characteristics gave evidence that the phage produced by strain Ce-3 (ϕEC) was identical to phage ϕEC.

It was concluded that Ce-3 (ϕEC) is a lysogenic strain based on exhibition of the following characteristics: (i) resistance to curing with phage-specific antiserum, (ii) a spontaneous curing frequency of approximately 0.5% (iii) a spontaneous induction frequency of 2.5×10^{-4} in populations of cells, (iv) less than 1.3% spontaneous lysis of individual cells, (v) no detectable lysis of cell populations, (vi) inducibility with UV light and mitomycin C, and (vii) production of the same phage used in the original isolation of the strain.

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