Effect of Chloramphenicol on Host-Bacteriophage Relationships in the Lactic Streptococci

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Chloramphenicol (CM)-resistant mutants of Streptococcus lactis strain ML3 were obtained either as a consequence of continuous transfer of the bacteria in broth containing increasing amounts of CM or by selecting for high-level resistant derivatives after mutagenic treatment of the bacteria. Some CM-resistant cells obtained by the first method were also resistant to the homologous bacteriophage. Cells trained to grow in the presence of CM developed resistance to some heterologous attacking phages but not to phage ml3. Mutants selected for phage resistance were not resistant to CM. There appear to be two different loci for CM resistance on the bacterial chromosome: the one for high-level resistance is associated with the phage-resistance locus and the other is independent of it. A concentration of CM (280 μg/ml) that was bacteriostatic for ML3 inhibited the intracellular growth of ml3 phage in strain ML3-CM-l, which had been trained to grow in the presence of that CM concentration, despite the fact that cells of this strain were not phage-resistant per se. The drug had no direct virucidal action and did not prevent adsorption or penetration of phage into the bacterium. Lysogenization did not occur. It is concluded that the block in phage development probably involves inhibition of synthesis of phage components, either involving deoxyribonucleic acid at an early stage or the phage coat protein at a later one.

STUDIES OF BACTERIOPHAGE RESISTANCE IN THE LACTIC STREPTOCOCCI

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MATERIALS AND METHODS

Bacterial strains. Streptococcus lactis strain ML3, from the collection held by the New Zealand Dairy Research Institute, was maintained by daily transfer (1% inoculum) in lactose-yeast-phosphate (LYP) broth (16). Bacteriophages. In phage infection experiments with S. lactis ML3 and its homologous phage, a whey suspension of phage ml1 containing 2.4 × 10^10 plaque-forming units per ml was used. In testing mutants of ML3 for their spectrum of resistance to heterologous phage types, the collection of S. lactis and S. cremoris phages held by the New Zealand Dairy Research Institute was used. Phage assays were carried out by the agar overlay method (1) on LYP agar (16) plates. To test the reaction of a bacterial strain to a particular phage, a sample of the bacteria was layered in semisolid agar on an LYP agar plate, and the phage suspension was spotted on.

Reagents. CM was obtained from Parke, Davis and Co., Sydney. N'-methyl-N'-nitro-N-nitrosoguanidine (NG), from Aldrich Chemical Co. Inc., Milwaukee, Wis., was used for mutagenic treatment of bacteria. Isolation of phage-resistant mutants. Mutant colonies of S. lactis ML3 resistant to phage ml1 were isolated from plates layered with semisolid agar containing about 5 × 10^8 cells and phage at a multiplicity of 5. These were streaked on plates to purify,
they were grown in broth, and the cultures were checked for phage resistance.

Isolation of CM-resistant mutants. Mutants of *S. lactis* ML3 resistant to CM were obtained during the process of continuous transfer of ML3 in LYP broth containing increasing amounts of CM. Other CM-resistant mutants of ML3 were isolated from LYP agar plates containing CM which had been spread with about 10⁸ colony-forming units from an ML3 culture previously subjected to mutagenic treatment with either NG (15) or ultraviolet (UV) irradiation. Treatment with NG was carried out as follows. A log-phase LYP broth culture received 1,000 μg of NG/ml before incubation at 30 C for 20 min. The cells were centrifuged, resuspended in LYP broth, and incubated at 30 C for 3 hr before they were spread on CM-containing plates. For UV treatment, the cells of a log-phase culture were centrifuged, washed in Ringer solution, and finally resuspended in this medium. A sample (3 ml) of the suspension was placed at a distance of 29 cm from a Hanovia UV-lamp and irradiated for 30 sec. Samples were then withdrawn, incubated at 30 C for 3 hr, and spread on CM-containing plates.

Bacterial growth curves. The growth or lysis of log-phase LYP broth cultures of *S. lactis* ML3 and derivative CM- and phage-resistant mutants, in the presence or absence of CM and phage, was determined by viable cell counts and turbidity measurements in a Spectronic 20 colorimeter/spectrophotometer set at 560 nm.

One-step growth experiments were carried out as described by Adams (1). Premature lysis of cells was achieved by diluting infected cells into 1,0-ml samples of Ringer solution, held at 4 C, containing two drops of chloroform and 25% (v/v) Chance no. 12 Ballotini beads, followed by shaking on a Mickel disintegrator for 2 hr at 4 C.

To determine whether phage penetration of bacterial cells occurred after phage adsorption in the presence of CM, cultures of CM-treated and untreated cells were infected with phage and, after allowing time for adsorption, the bacteria were separated from unadsorbed phage by low-speed centrifugation (4,000 × g for 5 min). The cells were then suspended in a solution containing (per liter): 1 mM MgSO₄, 0.1 mM CaCl₂, and 0.1 g of gelatin (12). This suspension was agitated in a Waring Blender for 2 min at 10,000 rev/min before cooling in ice water. Samples were removed for assay of infective centers or for further centrifugation to collect released phage.

RESULTS

Mutagenesis studies with *S. lactis* ML3. The killing effects of NG and UV irradiation were examined initially to determine suitable mutagenic doses for treatment of *S. lactis* ML3. The effect of NG treatment on ML3 is presented as a function of dose (Fig. 1) and as a function of time (Fig. 2). ML3 was found to be very resistant in comparison with gram-negative bacteria (15). The cells of an NG-treated culture, examined with a phase-contrast microscope, were more elongated than those of an untreated culture. Chain length remained the same, and the appearance of plated colonies was normal. The mutagenic dose chosen for subsequent treatment of ML3 cells (1,000 μg/ml for 20 min) was one that gave about 90% killing of cells.
is 2 \mu g/ml. A culture of ML3-CM\textsuperscript{1}, isolated after 66 transfers, grew normally in the presence of 210 \mu g of CM/ml and was chosen for examination in subsequent growth curve studies in the presence or absence of CM and phage ml\textsubscript{1}. ML3-CM\textsuperscript{1} did not revert to CM susceptibility when subcultured in the absence of the antibiotic. During the period of training, after addition of each CM increment, the growing culture was tested for its susceptibility to 25 stock S. lactis and S. cremoris phages, seven of which, under normal conditions, are able to form plaques when spotted on an ML3 culture plated in semi-solid agar on LYP agar. The phage relationships of ML3 and the culture of ML3 enriched for CM\textsuperscript{r} mutants are shown in Table 1. In the course of transfer in CM-containing broth, the ML3 culture lost its resistance to hp phage for a period of 29 transfers; it acquired resistance to z8 phage after 14 transfers and to sk2 phage after 72 transfers; and it became susceptible to p1 phage after 50 transfers. Apart from phage-relationship changes, other significant observations made during the course of training were as follows. At a level of 16 \mu g of CM/ml, there was a distinct change from slow growth to normal growth in the presence of increasing CM concentrations. At 42 \mu g of CM/ml and thereafter, there was a marked increase in the number of phage-resistant colonies appearing in the phage ml\textsubscript{1} lytic areas. At levels between 175 and 190 \mu g of CM/ml, cultures required 48 hr of incubation at 30 C to yield cell numbers previously obtained in 24 hr. Above 190 \mu g of CM/ml, it became necessary to alternate subculture in LYP broth plus the appropriate CM increment, with subculture in normal LYP broth to achieve satisfactory growth in the presence of the increased CM concentration.

Single CM-resistant colonies were isolated from plates, containing a range of CM concentrations, that had been spread with NG-treated and UV-irradiated S. lactis ML3 cultures. Each colony was suspended in LYP broth, grown to log phase, and then tested for resistance to both the homologous phage and to CM. In this way, nine mutants resistant to CM concentrations ranging up to 200 \mu g/ml were isolated from the NG-treated culture. All of the mutants tested were susceptible to phage ml\textsubscript{1}. Sixteen mutants, resistant to similar CM concentrations and sensitive to ml\textsubscript{1}, were isolated from the UV-irradiated culture and, in addition, four mutants were isolated at a level of 500 \mu g of CM/ml after examination of 200 plates. These four mutants were resistant to phage ml\textsubscript{1}. In the course of daily subculture in LYP broth in the absence of CM, they lost their resistance to CM after about 4 days, but retained their resistance to phage ml\textsubscript{1} for

The results of a UV survival experiment (Fig. 3), carried out with a suspension of ML3 cells in Ringer solution, indicated that irradiation at a distance of 29 cm for 30 sec gave about 90% killing of cells, and this was subsequently chosen as the mutagenic dose.

**CM resistance and phage resistance in S. lactis ML3.** Continuous transfer of ML3 in LYP broth containing increasing concentrations of CM produced, after 73 transfers, a culture able to grow slowly in the presence of 280 \mu g of CM/ml. At levels of CM above this value, no bacterial growth could be obtained. The natural level of resistance

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**TABLE 1. Phage relationships of S. lactis ML3 during the course of daily transfer in broth containing increasing increments of CM**

<table>
<thead>
<tr>
<th>CM concen\textsuperscript{a} (\mu g/ml)</th>
<th>Response of CM-treated S. lactis ML3 to phages\textsuperscript{b}</th>
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\textsuperscript{a} The concentrations given are those at which changes were observed. The increments were 1 to 50 \mu g of CM per ml; thereafter, they were 10 \mu g/ml.

\textsuperscript{b} L = L, plaques formed on plated bacteria.
about 6 weeks before reversion to phage sensitivity was observed in two of the cultures. An identical number of plates spread with the NG-treated culture and containing the same high concentration of CM failed to yield any CM-resistant mutants.

A total of 100 phage-resistant forms of *S. lactis* ML3 were examined for resistance to CM by subculturing to tubes of LYP broth containing a range of CM concentrations; none of the strains tested had acquired CM resistance in addition to their phage resistance.

**Effect of CM on the multiplication of *S. lactis* phage m13.** The mutant *S. lactis* strain, ML3-CM'I, trained to grow in the presence of 210 μg of CM/ml, was compared with normal ML3 in a number of growth curve experiments to determine the effect of CM on phage multiplication. Each strain was grown to early log phase, and its subsequent growth was examined both when untreated and after receiving additions of (i) an m13 phage suspension, (ii) CM solution at two different concentration levels, and (iii) m13 phage suspension plus CM solution at the two different concentration levels (Fig. 4 and 5). At a concentration which did not inhibit bacterial growth, CM clearly had no effect on phage multiplication in ML3, and the same concentration did not inhibit phage lysis in ML3-CM'I. On the other hand, at a concentration of 50 μg of CM/ml, growth of ML3 was halted, whereas ML3-CM'I continued to grow normally. As would be expected under these conditions, ML3 was not lysed by the phage. However, ML3-CM'I was also not lysed by phage m13 in the presence of 50 μg of CM/ml, despite the fact that normal cell growth occurred at this concentration.

As a control experiment, the effect of CM on phage per se was tested, and no decrease in viability was observed.

**Adsorption experiments.** To determine whether phage was adsorbed to ML3-CM'I in the presence of CM, the titer of free phage in an adsorption mixture of ML3-CM'I plus 50 μg of CM/ml was compared with that in ML3-CM'I during the course of an incubation period of 19 min. There was a rapid adsorption by both strains of approximately 45% of the plaque-forming units within 2 min. Adsorption continued at a decreased rate until about 90% of the particles were adsorbed by 19 min.

**Phage penetration.** An experiment was carried out to determine whether addition of phage m13 to ML3-CM'I in the presence of CM resulted in phage adsorption but no penetration. Adsorption mixtures of ML3-CM'I plus phage and ML3-CM'I plus 50 μg of CM/ml plus phage were examined, after a 10-min adsorption period, for phage penetration. A phage multiplicity of 0.1 was used. The mixtures were assayed for infective centers at the end of the 10-min adsorption period.
and then subjected to blendor treatment before determining survival of infective centers by plaque assay. At the same time, the infected bacteria in both mixtures were centrifuged at 5,000 rev/min, and the supernatants were assayed for any free phage that may have been separated from the bacteria by the blendor treatment.

Approximately 94% of the infective centers in the two adsorption mixtures survived the blendor treatment. The free phage titers in the two supernatants, after the final low-speed centrifugation, were: ML3-CMrI plus phage, $1.9 \times 10^3$/ml; ML3-CMtI plus CM plus phage, $1.4 \times 10^5$/ml. That is, similar small amounts of phage, probably representing unadsorbed phage, were found in the two mixtures, indicating that adsorption of phage leads to penetration in both cases.

Premature lysis and one-step growth curves. The results of one of four such experiments for each of the strains ML3 and ML3-CMtI are depicted in Fig. 6. Strain ML3-CMrI was tested both with and without 50 μg of CM/ml added to the culture before addition of phage. In the one-step growth curves for ML3, and ML3-CMtI without CM added, phage ml₁ showed a latent period of about 25 min, followed by a rapid rise in titer which reached its maximum in 6 min. The average burst size in each case was about 43 particles per cell. The other three experiments revealed similar latent and burst periods, although the actual increase in phage titer was variable. In the presence of CM, however, no increase in phage titer was observed for ML3-CMtI during the course of the one-step growth curve.

The premature lysis curves for ML3 and ML3-CMtI revealed similar eclipse periods followed by the usual phage proliferation. No mature phage particles were detected in the ML3-CMtI culture containing added CM during the entire incubation period.

As a further check on the fate of phage penetrating the host cells in an adsorption mixture of ML3-CMtI plus 50 μg of CM/ml plus phage ml₁, a test was made for the appearance of lysogenic forms. The mixture was incubated for 4 hr after infection, and samples were then withdrawn and spread on LYP agar plates. Single colonies were selected and tested for resistance to ml₁ phage. One hundred colonies were examined in this way, and none was phage resistant, indicating that no lysogenic association resulted from infection of ML3-CMtI with ml₁ phage in the presence of CM.

DISCUSSION

At least three types of mutation have been shown to affect resistance to CM in S. lactis ML3. The stepwise mutation associated with training host cells to grow in the presence of increasing increments of CM produced forms resistant to CM up to a maximum concentration level of 280 μg/ml. In association with this stepwise mutation, there was a change in the response of the strain to infection with heterologous phage types, generally in the direction of increased resistance to these phages. However, it remained susceptible to the homologous phage. After treatment with NG or UV irradiation, both potent mutagenic agents for many bacterial genera, at levels sufficient to give 90% inactivation of the cells, mutants showing resistance to concentrations of CM up to 200 μg/ml could be selected. These one-step mutants remained susceptible to attack by the homologous phage. The third type of mutant was selected at low frequency, and again involved a one-step mutation event. In this case, ML3 yielded, after UV irradiation, mutants resistant to 500 μg of CM/ml; these mutants were also resistant to phage ml₁. During the course of subsequent daily subculture, some of these mutants reverted to CM sensitivity after four transfers and to phage sensitivity after 43 transfers. Mutants selected for phage resistance were not CM resistant.
It seems likely that there are at least two different loci for CM resistance on the ML3 chromosome: the one for high-level resistance (500 μg/ml), although not identical with the phage-resistance locus, is associated with it, whereas the one or more concerned in the stepwise mutation are independent of it. The locus affected by the single-step mutations conferring resistance to CM levels up to 200 μg/ml may not be the same as that involved in the stepwise mutational events.

It has been shown that resistance to CM in Escherichia coli may be due to one of three possible mechanisms (17): (i) resistance of the protein-synthesizing machinery; (ii) decreased permeation to the site of antibiotic action; (iii) inactivation of the drug. The demonstration that CM interferes with the multiplication of phage in S. lactis ML3-CM'I indicates that the second of these mechanisms does not operate in this case. If ability to inactivate the drug resulted from the mutation to CM-resistance, the mutation would be one in which the necessary enzyme was formed in greater amount or had a higher affinity for the CM substrate. In either case, after addition of CM to the growing culture, the growth rate would be decreased until inactivation was completed. No reduction in growth rate was observed when normally bacteriostatic concentrations of CM were added to strain ML3-CM'I. It seems likely, therefore, that in this strain the mutation to CM resistance confers resistance on the protein-synthesizing ability of the host cells.

At or below a concentration of 2 μg/ml, CM had no effect on the growth of ML3 in LYP broth, and lysis occurred after the usual period of incubation in the presence of phage m1s. The same was true for strain ML3-CM'I which had been trained to grow in the presence of 280 μg of CM/ml. When the concentration of CM in each adsorption mixture was raised to 50 μg/ml, growth of ML3 was inhibited and no phage lysis occurred; phage lysis of ML3-CM'I was also inhibited by this concentration of CM, but cell growth remained unaffected. In analogous experiments (R. J. Richards, Ph.D. thesis, Ohio State University, 1960), it was shown that subinhibitory concentrations of penicillin decreased the time required for lysis initiation by a S. lactis host-phage system in broth, whereas oxytetracycline caused a delay in the onset of lysis, and streptomycin had no effect. The magnitude of the penicillin and oxytetracycline effects increased as the antibiotic concentration increased.

Since it could be demonstrated that CM had no effect on phage m1s per se, the possibility existed that the CM affected the phage-host interaction in one or more of the following ways. (i) Phage adsorption was inhibited; (ii) phage penetration was inhibited; (iii) penetrating phage established a lysogenic relationship with the host. When cells are infected with temperate phages, either a lytic response or a lysogenic response can occur. In some bacteria, CM brings about an increase in the lysogenic response, apparently by inhibiting the protein synthesis that is required for the lytic response (4). This effect is not specific but is brought about also by other conditions that retard biosynthetic operations.

The adsorption and penetration experiments carried out with ML3-CM'I provided evidence that CM at a concentration of 50 μg/ml did not affect adsorption to and penetration of the host cells by phage m1s. In addition, it was shown that lysogenic forms did not arise as a result of the phage infection, since surviving cells were not resistant to the homologous phage. The one-step growth and premature lysis experiments confirmed that no intracellular multiplication of phage took place even though phage adsorption and penetration occurred. Thus, any infection that occurred may be termed abortive.

It may be concluded that, in an adsorption mixture of ML3-CM'I plus phage m1s plus 50 μg of CM/ml, the CM interferes with the synthesis of mature phage particles without affecting the synthesis of host cell material, or that phage is synthesized but is nonviable (defective) when synthesized in the presence of CM. When abortive infection occurs, phage development is halted before the completion of mature phage particles. With a variety of chemical manipulations, it is possible to allow phage development to proceed to completion with the formation of structurally mature phage which, however, are non-infective. This results from the incorporation of an unnatural amino acid or pyrimidine into the protein or nucleic acid of the phage. No defective phage of phage components were detected with an electron microscope after artificial disruption of the phage-infected ML3-CM'I.

Phage production is dependent on the host cell metabolism for energy and for the synthesis of raw materials. Therefore, any interference with the energy metabolism or the synthetic enzyme system of the host cell may be expected to have an effect on phage production. However, it is possible to interfere with bacterial multiplication without affecting phage growth, and vice versa, showing that the nutritional requirements for the two processes are not identical. CM inhibition of deoxyribonucleic acid (DNA) synthesis has been observed in phage-infected bacteria (7). Several new enzymes, which are necessary for synthesis of phage DNA, are produced in the first few minutes after phage infection. It is possible that
CM inhibits phage DNA synthesis indirectly by blocking the formation of these enzymes.

It is concluded that the block in phage development in ML3-CM*I probably involves inhibition of phage component synthesis so that, although phage penetrates the cell, either the genome is not replicated or phage coat proteins are not synthesized. Thus, no mature phage particles are formed.

ACKNOWLEDGMENT

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LITERATURE CITED