Acriflavine-Resistant Mutant of *Streptococcus cremoris*

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Selection for resistance to acriflavine in *Streptococcus cremoris* resulted in cross-resistance to the drugs neomycin, streptomycin, ethidium bromide, mitomycin C, and proflavine. Furthermore, the mutants showed resistance to lytic bacteriophages to which the parental strain was sensitive, and, unlike the parent, the mutants grew well at higher temperatures (40°C). Revertants selected independently either for temperature sensitivity or for acriflavine sensitivity lost resistance to all the drugs and dyes but retained the bacteriophage resistance phenotype. The acriflavine-resistant mutation resulted in an increase in resistance by the bacterial cells to sodium dodecyl sulfate, a potent solvent of lipopolysaccharide and lipoprotein. It is suggested that the acriflavine resistance mutation determines the synthesis of a membrane substance resistant to higher temperatures.

Acriflavine (AF) has been used for a variety of biological and biochemical purposes, especially to eliminate various extrachromosomal genetic elements (10, 11, 19, 20, 33). AF has also been shown to be mutagenic to bacteria and bacteriophages (27, 34). Resistance of *Escherichia coli* to AF and other basic dyes was found to be controlled by a gene located near the lac region of the chromosome (22), and Nakamura (24) has shown that AF resistance in *E. coli* was transmitted together with phenethyl alcohol resistance.

During a study of the elimination of plasmids in *Streptococcus cremoris*, it was initially observed that a mutant isolated on the basis of resistance to AF also showed resistance to a number of drugs. This observation was not in itself surprising, since cross-resistance to AF, proflavine, and ethidium bromide have been shown in *Bacillus subtilis* (5), and cross-resistance to such drugs and basic dyes have been well documented in *E. coli* (22, 24). What was of prime interest, however, was that the AF-resistant mutant, unlike the parent, grew normally at a higher temperature (40°C) and showed resistance to virulent bacteriophages. This prompted a detailed examination of the mutant, since there has been no reference in the literature of such correlation between AF resistance mutation and resistance to higher temperatures. The present paper describes the isolation and properties of one such resistant mutant. The possible involvement of the cell membrane in this resistance is discussed.

MATERIALS AND METHODS

**Bacterial and bacteriophage strains.** Strain ML1 of *S. cremoris* and bacteriophages ml-1, dr-7, br-4, and ml-3 used in the experiments were obtained from the culture collection of the Food Research Institute. The mutant strain ML1-11 was isolated by AF treatment of ML1, and the revertant ML1-11R was isolated from ML1-11 as described below.

The cultures were maintained by biweekly transfer in sterile 10% reconstituted nonfat milk solids. Incubation was at 32°C overnight in litmus milk, and the cultures were held at 4°C between transfers. Lactic broth (LB; 9) was used for growth of the organisms in all experiments unless otherwise indicated. Strains were also maintained after being grown in LB overnight, centrifuged, and suspended in 0.1 volume of fresh broth plus 15% glycerol and kept at -20°C. The bacteriophages were maintained in both whey suspension and in broth containing 10% or more plaque-forming units per ml.

**Media and growth of cultures.** LB and agar were purchased from Difco Laboratories Ltd. Streptococci were grown routinely at 32°C without shaking, unless otherwise indicated. The turbidity was followed by a Klett-Summerson colorimeter equipped with a no. 54 filter from an overnight broth culture (14 to 16 h, 32°C) diluted (1 in 100) in fresh broth. Total counts were made by diluting the bacterial culture in saline (0.85%) and spreading 0.1 ml of an appropriate dilution on LB agar (LBA) plates and incubating overnight or for 48 h at 32°C.

**Chemicals.** The chemicals were purchased from the following sources: neomycin and dihydrostreptomycin sulfate, Nutritional Biochemicals Corp.; ethidium bromide and mitomycin C, Sigma Chemical Co.; AF, Allied Chemicals; radioactive compounds, New England Nuclear Corp.

**AF treatment.** Two procedures for AF treatments were used. In the first, an overnight culture was
diluted in fresh broth (0.1 in 10) and grown for 5 h. The culture was diluted again (1 in 10), 15 μg/ml of AF per ml was added, and the culture was incubated at 32°C for 48 h in the dark. The treated culture was then centrifuged, suspended in 0.2 ml of broth, and plated out on LBA plates. All surviving colonies were picked on master plates and then tested for resistance to drugs and bacteriophages.

The second method employed the use of LBA plates containing the desired concentration of AF. Cells were grown overnight in LB at 32°C and centrifuged, and concentrated cells were plated out. After 48 h at 32°C, surviving colonies were picked on master plates and tested for resistance to drugs and phages.

Determination of drug resistance. The sensitivity to drugs was first determined by point inoculation of a loopful of a cell suspension (about 10^6 cells/ml) on LBA containing the desired concentration of drugs. Strains that gave confluent growth on the inoculation point were judged to be resistant, whereas strains having none or a few isolated colonies at the point of inoculation were considered to be sensitive. This was subsequently confirmed by plating out 0.1 ml of an overnight culture on LBA containing the concentrations of drugs to be tested with appropriate controls. The minimal inhibitory concentrations were those at which no colonies were formed on plating 5 × 10^5 cells.

Determination of phage resistance. The sensitivity of the bacterial cultures to the phages was assayed by the soft-agar method (1), using LBA as the bottom layer, with a top layer containing 0.16% CaCl_2 and 0.6% agar and also using M17 medium (31). Duplicate plates were incubated at 32°C overnight, and the plaques were counted.

Isolation of deoxyribonucleic acid (DNA) and dye-buoyant density centrifugation. Cells were labeled with radioactive thymidine by growing the cultures in LB containing (per ml) 250 μg of deoxyadenosine and 20 μCi of [methyl-3H]thymidine (6.7 Ci/mmol, New England Nuclear Corp.) for 6 to 8 h at 32°C. Cells were chilled for 10 min, harvested by centrifugation, washed twice with TES buffer [tris(hydroxymethyl)aminomethane, 0.05 M; disodium ethylenediaminetetraacetic acid, 0.005 M; NaCl, 0.05 M], pH 8.0 (3), and suspended in 1 ml of TES containing 20% sucrose. Two-tenths milliliter of lysozyme (10 mg/ml in 0.25 M tris(hydroxymethyl)aminomethane, pH 8.0) was added, and the mixture was incubated at 37°C with shaking for 40 min. Lysis of cells was accomplished by adding 0.2 ml of 25% sodium lauryl sulfate and incubating for an additional 10 min at 37°C. NaCl was then added to a final concentration of 1.0 M, and the suspension was mixed well and left overnight at 4°C. The cell lysate was then centrifuged at 11,000 × g for 20 min, with the clear supernatant fraction retained and the pellet discarded. The clear lysate was adjusted to 2.5 ml with TES buffer, and 3.5 g of solid CsCl was added. The resulting solution was mixed well with 1.5 ml of ethidium bromide (4 mg/ml in 0.1 M phosphate buffer, pH 7.0), and the refractive index was adjusted to 1.3390 at room temperature with a saturated solution of CsCl. The mixture was placed in polyallomer centrifuge tubes, topped with paraffin oil, and centrifuged in a 50 Ti rotor (Beckman LS 65B centrifuge) at 40,000 rpm and 17°C for 40 to 42 h. Five-drop fractions were collected on Whatman 3MM filter paper disks, by puncturing the bottom of the tubes with a needle, and air dried. The disks were then washed with cold trichloroacetic acid according to the method of Bol- lum (6). Radioactive samples were counted in a liq- uid scintillation spectrometer (Beckman LS 250).

Isolation of revertants from strain ML1-11. The bacterial cells were grown for 2.5 h in broth from an overnight culture using a 1% inoculum. The cells were collected by centrifugation, washed, and suspended in citrate buffer (pH 4.5) to the original volume. The cell suspension was then divided into two tubes: one contained 1 mg of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) per ml, and other served as a control. The cells were then incubated for 1 h at 32°C with shaking. The treatment yielded 0.5 to 1.0% survivors. Survivors were then picked onto master plates and tested by replica plating for growth at 40°C. Colonies that failed to grow at 40°C were picked from master plates, grown in broth, and then tested for sensitivity to other drugs and dyes or temperature.

A spontaneous revertant that had lost AF resistance was also obtained by replica plating a large number of colonies of strain ML1-11 on LBA and LBA plus 15 μg of AF per ml.

RESULTS

Isolation of AF-resistant mutants. AF treatment either in liquid broth or on solid agar plates gave similar results. AF-resistant mutants occurred at a frequency of about 10^{-7} on LBA containing 15 μg of AF per ml. A total of 252 such AF-resistant colonies were analyzed. Of the survivors, 5.8% were not only AF resistant but, unlike the parental strain, grew well at a higher temperature (40°C). All such temperature-resistant mutants were also markedly resistant to the virulent bacteriophages. One such mutant, referred to as ML1-11, was chosen for further use in the present study.

Effect of temperature on growth of strains ML1 and ML1-11. Both strains were grown at two different temperatures to study the effects of mutation to AF resistance on growth and cell physiology. Since the bacterium grows in chains, the growth pattern was investigated by both the turbidity (Fig. 1) and total viable count (Fig. 2) at 32 and 40°C with a 1% inoculum from an overnight culture. The higher temperature was lethal to the parental strain ML1, whereas the mutant strain ML1-11 grew normally (Fig. 2). Microscopic observation of the cultures grown at 32 and 40°C consistently revealed the predominance of short chains in the mutant strain as compared with the parent. The mutant strain ML1-11 showed an average
of 2.79 ± 0.195 and 2.78 ± 0.193 cells per chain at 32 and 40°C, whereas the parental strain, ML1, gave an average of 6.8 ± 1.60 and 6.6 ± 1.39 cells per chain at 32 and 40°C, respectively.

In another set of experiments, the parental strain ML1 was grown at 32°C, and part of the culture was shifted to 40°C at 2 and 4 h to see the effect of high temperature at different stages of growth. Figure 3 shows that the growth was unaffected for 1 h at 40°C when shifted from 32°C at 2 h. There was also an increase in turbidity for 1 h, and thereafter the viable cells and turbidity decreased. However, when the culture was shifted to 40°C after 4 h of growth at 32°C, the growth ceased immediately, followed by decreases in total viable cell counts and turbidity. No colonies were formed on lactic agar plates when the culture growing at 32°C was plated out at 2- and 4-h intervals and the plates were incubated at 40°C. Similarly, when the cells growing at 40°C for 1 h from a 2-h shift were plated out and incubated at 40°C, no colonies were formed.

Growth of strains ML1 and ML1-11 in presence of AF. Growth of strain ML1 was inhibited by 5 μg of AF per ml and completely blocked by 10 μg of AF per ml (Fig. 4A), but the growth of mutant strain ML1-11 was unaffected by 5 μg/ml and only slightly inhibited by 10 μg/ml (Fig. 4B).

Cross-resistance to ethidium bromide, mitomycin C, and proflavine. Acridine dyes are known to bind DNA by intercalation (16). It was thus of interest to see whether the AF-resistant mutant was cross-resistant to other chemicals known to bind DNA by intercalation (14, 32). Table 1 summarizes the results of such experiments. Strain ML1-11 grew well on LBA containing 10 μg of ethidium bromide per ml,
whereas the parental strain was susceptible. The mutant strain was also resistant to 1 μg of mitomycin C per ml and 4 μg of proflavine per ml, whereas strain ML1 was susceptible to 0.5 μg of mitomycin C and proflavine per ml.

Relative resistance to the antibiotics neomycin and streptomycin and resistance to lytic bacteriophages. Table 2 shows the antibiotic resistance of strain ML1, its mutant ML1-11, and the revertant bearing the AF-sensitive phenotype. The level of resistance of the mutant to the drugs was much higher than that of the parent, as revealed by the minimal inhibitory concentration of each drug. The revertant strain showed minimal inhibitory concentrations of the drugs similar to those of strain ML1.

The AF-resistant mutant ML1-11 was also found to be resistant to several lytic bacteriophages (Table 2). The biochemical basis for the ability of AF-resistant mutants to develop resistance to lytic phages is unclear. The possibility that the strain ML1-11 had an altered phage adsorption was examined. Bacterial cells growing in broth were infected with a multiplicity of infection of 0.5 with the phage to be studied, and the adsorption process was allowed to proceed for 2 min without shaking at 32°C followed by 5 min of shaking. The cells were then centrifuged at room temperature for 10 min at 5,000 rpm. The supernatants obtained were assayed for plaque-forming units with appropriate hosts. Under similar conditions, 93.9% of phage ml-1 adsorbed to the parental strain, and 84.3% adsorbed to the mutant strain resistant to phage ml-1. The phage resistance must therefore result from a cause other than defective adsorption.

To explore the possible relation between AF resistance and the phage resistance phenotype, phage-resistant mutants were isolated by infecting strain ML1 with phage ml-1 at a multiplicity of infection of 10 and incubating for 6 h.

| Strains* | AF (μg/ml) | Ethidium bromide (μg/ml) | Mitomycin C (μg/ml) | Proflavine (μg/ml) |
|----------|------------|--------------------------|---------------------|-------------------|
| ML1      | 8          | 10                       | 12                  | 15                |
| ML1-11   | +          | –                        | –                   | –                 |
| ML1-11R  | +          | +                        | +                   | +                 |
| ML1-ml-1' | +          | –                        | –                   | –                 |

* ML1, parental strain, AF sensitive; ML1-11, AF-resistant mutant; ML1-11R, revertant of AF-resistant mutant; ML1-ml-1', ml-1 phage-resistant mutant, AF sensitive.

** Growth was scored on LBA plates containing the indicated concentrations of each chemical. Symbols: –, no growth; +, growth.

Table 2. Drug resistance and bacteriophage and temperature sensitivity of strains ML1, ML1-11, ML1-11R, and ML1-ml-1'

| Strains* | MIC of antibiotics (μg/ml) | Bacteriophage growth at 40°C |
|----------|---------------------------|------------------------------|
|          | Nm | Sm | ml-1 | ml-3 | dr-7 | br-4* |
| ML1      | 40 | 25 | S   | S   | S   | S   |
| ML1-11   | 100| 100| R   | R   | R   | R+  |
| ML1-11R  | 40 | 25 | R   | R   | R   | R-  |
| ML1-ml-1' | 40 | 25 | S   | S   | S   | S   |

* For designations, see Table 1.

** Minimum inhibitory concentrations (MIC) were the lowest antibiotic levels at which no colonies were formed on plating 10⁶ cells. Nm, Neomycin; Sm, streptomycin.

† +, Growth; –, no growth.

The cultures were then centrifuged, and the pellets were suspended in fresh broth containing phage ml-1 and grown overnight. One hundred individual colonies were regrown and purified several times and then tested for phage and AF resistance. Since such phage-resistant mutations do not alter the AF resistance detectably (Table 1), it is unlikely that plating a population of S. cremoris cells on LB medium containing AF selects for phage-resistant mutants. One possibility is that, under the experimental conditions used, AF induces mutations and/or loss of plasmid-bearing genes for successful infection of bacterial cells. AF has been shown to be mutagenic for bacteria (34), and the loss of plasmids by the dye is well documented in a number of bacterial species (10, 11, 19, 20, 33).

AF- and temperature-sensitive revertants and their phenotypic characteristics. To investigate whether the reversion to temperature sensitivity and drugs occurs simultaneously,
three independent revertants that had lost the ability to grow at higher temperature (40°C) were also isolated after NTG treatment. These revertants were again not only sensitive to higher temperature but also sensitive to AF and other drugs. The spontaneous revertant strain referred to as ML1-11R was then used in detailed experiments. This strain showed all the characteristics similar to the original strain ML1 but retained the resistance to lytic bacteriophages characteristic of the mutant strain (Tables 1–3, Fig. 4C).

One of the properties of plasmid DNA is that once it is lost from the cell, the characteristics associated with it cannot be regained by means of reverse mutation. The mutant cells were therefore treated with NTG and checked for reversion of phage resistance. NTG treatment failed to produce phage-sensitive revertants out of more than 10,000 colonies tested, whereas, under the same conditions, about 4% temperature-sensitive revertants were obtained.

DNA analysis. The cell lysates of the parental, mutant, and the revertant strains were analyzed for the presence of covalently closed circular DNA by dye-buoyant density centrifugation. A typical elution profile of DNA is shown in Fig. 5. Mutations in R plasmids of E. coli cause increases in the level of resistance to antibiotics due to increased numbers of plasmid copies per cell (26), and therefore an attempt was made to examine for the amount of putative plasmid DNA in each of the three strains. Such analysis showed 4.72, 4.40, and 4.42% of the total counts in DNA to be present in the plasmid bands of the strains ML1, ML1-11, and ML1-11R, respectively. This indicates that AF has not caused plasmid loss in strain ML1-11, which leads to increased resistance to antibiotics or to mutations leading to an increase in the number of plasmid DNA copies per chromosome.

Sensitivity to sodium dodecyl sulfate (SDS). A number of studies have shown that there is less binding of dyes in AF-resistant strains

| Strains* | Survival (%) at SDS concn (µg/ml): |
|----------|-------------------------------------|
|          | 0* 25 50 75 100 125                  |
| ML1      | 100.0 20.8 0.36 0.015 0.001 0.001    |
| ML1-11   | 100.0 73.91 28.91 0.41 0.069 0.016    |
| ML1-11R  | 100.0 73.91 0.29 0.011 0.002 0.001    |

* For designations, see Table 1.
† Cells were incubated at 32°C from an overnight culture inoculated 1 in 100 for 4 h. The cultures were then treated with different concentrations of SDS in broth for 0.5 h at 32°C with shaking.

**Fig. 4. Growth of strain ML1 and ML1-11 at 32°C with and without AF. Symbols: ■, ▲, and ○ represent 0, 5, and 10 µg/ml, respectively. (A) ML1; (B) ML1-11; (C) ML1-11R.**
than in sensitive ones (23, 29, 30), suggesting the involvement of the cell membrane. However, Kushner and Khan (16) found no difference in the binding of proflavine to resistant and sensitive E. coli and showed that there is an active excretion system for the dye. However, a more recent study by McKeller et al. (18) showed cross-resistance of proflavine and penicillin resistance in E. coli without any \( \beta \)-lactamase activity, suggesting permeability changes in the cell envelope. A different approach, therefore, was made to study the involvement of the cell membrane in the AF resistance mutation. Mutations affecting the cell membrane might be expected to show alterations in response to chemicals specific to cell wall constituents. SDS is a potent solvent of lipopolysaccharide and lipoprotein (2). Thus, it is possible that the difference in SDS sensitivity reflects a difference in membrane structure of cells. Freshly grown cells of strains ML1, ML1-11, and ML1-11R were treated with different concentrations of SDS in broth for 0.5 h at 32°C with shaking. Total viable cells were obtained by plating out appropriate dilutions of treated and control cultures. The strains ML1 and ML1-11R were much more sensitive to SDS than was the mutant strain ML1-11 (Table 3).

**Fig. 5. Elution profile of cesium chloride-ethidium bromide gradient of DNA from cleared lysate of S. cremoris strains.**

**DISCUSSION**

The foregoing results pose the interesting problem of a mutation that shows alterations not only for the selected marker but also pleiotropically for other characteristics. This could be due either to structural modifications of the cell membrane affecting the interactions that occur between different molecules in the cell surface or to multiple mutations. Attempts to investigate whether all the characteristics associated with the AF resistance mutation were due to a single mutation by employing genetic recombination have been hampered by the difficulties in transferring the genome of one strain of the group N streptococci to another. Nevertheless, evidence has been obtained by reverse mutation that the AF resistance phenotype can be lost together with resistance to higher temperature and other drugs without the loss of phage resistance. This suggests that there may be two discrete mutations, one conferring resistance to higher temperature and drugs, and the second conferring resistance to lytic phages. The revertant strain ML1-11R showed a growth pattern in the presence of different concentrations of AF similar to that of strain ML1 (Fig. 4A and C). This suggests that the revertant has undergone reverse mutation at the site of the AF resistance mutation. However, the possibility of a suppressor cannot be ruled out. If the reversion from AF resistance to AF sensitivity occurred by means of a secondary suppressor mutation, this suppressor would have to be very efficient and specific to account for simultaneous loss of temperature resistance and other associated properties in all the spontaneous and NTG-induced revertants. Siegel and Bryson (28) earlier reported the mutator effect of a azaserine-resistant strain in E. coli, showing higher mutation frequencies of resistance to bacteriophages T1, T3, and T7 and the antimicrobial agents. This observation is analogous to the present observations, insofar as the presence of the additional mutations became apparent only after the detection of the mutation that was selected for.

The growth of the parental strain at 40°C as revealed by turbidity did not show a decrease (Fig. 1) parallel to the viable counts (Fig. 2). This may be due to the fact that cells fail to form colonies on solid-agar plates but stay in chains in liquid broth for 7 to 8 h at 40°C.

Previous studies have shown that a large proportion of bacterial mutants with an altered cell surface exhibit a pleiotropic phenotype (4, 12, 13, 17, 21). The phenotype of the AF-resistant mutant in this report appears to show such similarity. It is not unreasonable to believe...
that an AF-resistant mutation may have caused structural modification of the cell membrane resulting in the temperature-resistant phenotype. Increased resistance of the mutant strain to SDS treatment supports such a belief. Nakamura and Suganuma (25) have shown modification of the cell membrane in an AF-sensitive mutant of E. coli K-12. The demonstration of the pleiotropic phenotype of the AF-resistant mutant indicating the involvement of cell membrane could have valuable implications in the understanding of the functions of different molecular species of cell surfaces and their interactions.

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