Loss of Lactose Metabolism in Lactic Streptococci

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Lactose-negative mutants occurred spontaneously in broth cultures of Streptococcus lactis C,F. Instability of lactose metabolism was noted in other strains of S. lactis, in strains of S. cremoris, and in S. diacetylactis. Colonies of S. lactis C,F grown with lactose as the carbohydrate source also possessed lac^- cells. Treatment of lactic streptococci with the mutagen acriflavine (AF) increased the number of non-lactose-fermenting variants. The effect of AF on growth and on loss of lactose-fermenting ability in S. lactis C,F was consequently further examined. The presence of AF appears to favor competitively the growth of spontaneously occurring lactose-negative cells and appears to act in the conversion of lactose-positive to non-lactose-fermenting cells. The lactose-negative mutants partially revert to lactose-positive variants which remain defective in lactose metabolism and remain unable to coagulate milk. The lactose-negative cells become dominant in continuous culture growth and provide evidence that alterations in the characteristics of starter strains can be produced by continuous culture, in this case, the complete loss in ability to ferment lactose.

During studies of lactose metabolism in lactic streptococci (11, 12), it was observed that acriflavine (AF) treatment of Streptococcus lactis C,F resulted in the appearance of lactose-negative (lac^-) cells. In addition, spontaneous lac^- mutants were isolated from stock cultures of the same organism. This variation in lactose metabolism by certain lactic streptococci was noted by earlier workers. In 1937, Yawger and Sherman (22) isolated four variants of S. lactis from milk which did not ferment lactose. Okulitch and Eagles (16) observed that the successive transferring of S. cremoris 142 in a glucose, mannose, fructose, or salcin medium caused the organism to lose the ability to ferment lactose. Galactose and lactose were the only fermentable carbohydrates studied which failed to induce a complete loss of lactose fermentation. These authors suggested that the inhibitory power of glucose or one of its metabolic products was the cause for the sudden or gradual loss of lactose metabolism in starters. In 1939, Okulitch (15) described the microbiol dissociation of lactic acid streptococci. He suggested that the organism must be in a susceptible condition before dissociation could be induced. The dissociation was accompanied by loss of lactose metabolism. His attempts to repeat the experiments at a later date were unsuccessful. Hunter (8) isolated variants of S. cremoris which failed to ferment lactose and which were defective in galactose metabolism. The occurrence of these variants was spasmodic, and all attempts to define the precise conditions leading to the change were unsuccessful. Many attempts resulted in the production of 100% of the lac^- cells, but even then the actual factor(s) responsible for the change could not be determined. Hirsch (6), in 1951, observed that S. lactis 354 lost the ability to ferment lactose after repeated subculturing in glucose broth. The question remains, however, as to how the loss of lactose metabolism is induced in cultures of lactic streptococci.

Lactose metabolism and its possible instability seem particularly important at the present time since there is widespread interest in production of concentrated starter cultures in which the cells are grown by continuous culture techniques (9). It is only assumed that in the continuous culture there is no selection of cells which might be lacking in enzymes essential for acid production when the cells are grown in milk. In fact, little or no evidence is

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available concerning possible mutagenic changes in starter strains produced by continuous culture. It was the purpose of this investigation to establish whether lactose metabolism is unstable in lactic streptococci and to examine the possible cause(s) for this instability.

**MATERIALS AND METHODS**

**Cultures.** All lactic streptococci, except *Streptococcus cremoris* B, and *S. cremoris* Wg1, were obtained from W. E. Sandine of the Department of Microbiology, Oregon State University. *S. cremoris* B, and *S. cremoris* Wg1, were provided by J. Strawhouders, Netherlands Institute for Dairy Research, Ede, The Netherlands. The cultures were maintained by biweekly transfer in sterile (121 C, 12 min) 11% reconstituted nonfat milk (Matrix Mother Culture Medium, Galloway-West, Fond du Lac, Wis.) with incubation at 21 C until milk coagulation. Cultures were held at 5 C between transfers. Lactic broth (4) was used to propagate the organisms in a liquid media.

**Detection of lac- mutants.** Lactic agar (4), containing 1% lactose as the primary carbon source and supplemented with 0.004% bromocresol purple, served as the indicator medium. Plates were spread with 0.1 ml of the appropriate cell dilution and incubated at 32 C for about 48 hr. On this medium, lactose-fermenting colonies were yellow in contrast to the white non-lactose-fermenting variants. To avoid the selection of contaminants, only catalase-negative lac- mutants were quantitated, and, for *S. lactis* C2F, the lac- mutants were examined for lysis by the host phage.

**Treatment of lactic streptococci with AF.** Two loopsful of a 24-hr-old culture in lactic broth of each organism were inoculated into 2.0 ml of lactic broth containing various concentrations of AF. The AF was separately sterilized at 121 C for 15 min. After 24 hr at 21, 32, or 37 C, each culture was diluted and spread over the surface of the indicator agar for total bacterial count and for scoring of the lac- mutants. For the determination of the direct effect of AF on lactose-fermenting cells of *S. lactis* C2F, a culture was diluted to contain 50 to 100 colony-forming units (CFU) per 0.1 ml of the test solution. The test solution consisted of 0.2 ml of lactic broth containing 1.2 µg of AF. The tubes were incubated at 32 C for 3 hr, and the 0.2-ml samples were spread over the surface of the indicator agar. Control tubes, plated after 1.5 hr at 32 C, lacked AF and initially contained 10 to 20 CFU per 0.1 ml. Comparative growth rates of lac- *S. lactis* C2F and three lac- variants isolated from this strain were determined in lactic broth containing 6 µg of AF per ml. Cultures were incubated at 32 C after receiving an inoculum to give 10^4 CFU per ml. Samples were removed at periodic intervals and diluted, and the total count was determined by using pour plates of lactic agar. The plates were incubated at 32 C for 48 hr.

**Occurrence of lac- cells within lactose-fermenting colonies of *S. lactis* C2F.** To obtain evidence for instability of lactose metabolism in lactic streptococci, *S. lactis* C2F was cloned several times on the lactose indicator agar. A typical colony was placed into a tube of lactic broth and incubated at 21 C for about 16 hr. The culture was diluted and spread over the surface of the indicator agar to obtain individual colonies. The plates were incubated at 32 C for 65 hr after which 10 lac+ colonies were separately picked into individual tubes containing 1.0 ml of 0.85% saline. The tubes were mixed thoroughly and scored for the presence of any lac- cells by diluting and spreading over the surface of the lactose indicator agar.

**Reversion of lac- mutants.** For each of the various isolates, 10 cultures, each containing about 10 organisms per ml, were prepared and incubated at 21 C for 16 hr. A 0.1-ml sample from each culture was then spread onto the lactose indicator agar and inoculated for 2 days at 32 C. The number of colonies utilizing lactose was then counted. At the time of sampling, equal portions from each of the 10 cultures were pooled, and the resultant bacterial suspension was plated to give the basis for a reversion frequency.

**Continuous culture growth of *S. lactis* C2F.** Continuous culture studies were made in a culture vessel designed by H. M. Tsuchiya, Department of Chemical Engineering, University of Minnesota. In this system, hyponeedle wire (gauge 21) transferred nutrient from the nutrient reservoir to the growth vessel. The height of the nutrient reservoir was adjusted to deliver the nutrient at a continuous feed rate of approximately 0.17 ml per min. The vessel with a culture volume of 100 ml was water-jacketed for constant temperature control. The circulating water temperature was maintained by using a Beckman Thermocirculator model 1818. Mixing of the cells was accomplished by introduction of air from the bottom of the culture vessel. Air was sterilized by passing through field monitor disposable plastic membrane filter holders (Millipore Corp., Bedford, Mass.) and then by bubbling through sterile water before entering the culture vessel. Lactic broth containing defoamer (Marshall, Division of Miles Laboratories, Inc., Madison, Wis.) at 10 µg/ml to control foaming served as the nutrient medium. Continuous cultures were initiated by inoculating the culture vessel with 2 to 5 ml of *S. lactis* C2F. Samples were periodically removed from the culture vessel for determination of the presence of lac- mutants.

**RESULTS**

**Effect of AF.** The effect of AF concentration at several temperatures on appearance of lac- mutants from *S. lactis* C2F is shown in Table 1. No lac- mutants were observed in the absence of AF; yet, in the presence of AF, lac- mutants were readily isolated at 21, 32, and 37 C. The highest frequency was noted at 32 C in the presence of 6 µg of AF per ml. In some experiments, spontaneous lac- mutants were observed in the control tubes; however, AF always increased their number. Whether AF
increased the frequency of conversion from lac⁺ to lac⁻ directly without appreciable selective growth of any naturally occurring lac⁻ cells was unknown.

Figure 1 illustrates that the number of viable S. lactis C₂F cells decreased when exposed to AF. No cells could be recovered after 24 hr of incubation. On the other hand, the lac⁻ mutants derived from S. lactis C₂F were resistant to the mutagen. The lac⁻ culture isolated in the presence of AF exhibited a lag period of 4 hr before growth occurred. The other lac⁻ cultures also grew after an extended lag and reached maximum populations in 24 to 48 hr. This shortened lag period of the AF mutant could be due to the cells prior exposure to AF. Figure 2 indicates the spontaneous lac⁻ mutants did exhibit a shortened lag if they were first grown in the presence of AF. It therefore appears that the lac⁻ mutants are more resistant to AF than are the lac⁺ cells and that the lac⁻ variants can mutate more readily to AF resistance, thus increasing their selective advantage in the presence of AF.

**Conversion of lac⁺ cells of S. lactis C₂F to lac⁻ cells by treatment with AF.** The results obtained in Fig. 1 indicate that AF selectively allows growth of lac⁻ cells; however, it does not rule out the possibility that AF is involved in the direct conversion of lac⁺ cells to lac⁻ variants. To obtain evidence for this possibility, a small number of cells were inoculated into lactic broth containing AF, and 0.2-ml samples were dispensed into 100 tubes. After 3 hr of incubation at 32 C, the contents were spread over the surface of the indicator agar. In the presence of AF, 56 tubes contained evidence of lac⁻ cells. Some were pure lac⁻ clones, but many lac⁺ colonies were found to contain outbursts or papilliae consisting entirely of lac⁻ cells which formed at the edge of the colony. In the absence of AF, only 10 tubes of the 100 inoculated were found to contain lac⁻ cells, suggesting that the mutagen was involved in the conversion of lac⁺ to lac⁻.

AF treatment of S. lactis C₂F, as shown above, increased the occurrence of lac⁻ variants. Table 2 illustrates the effect of AF treatment on other strains of lactic streptococci. The appearance of lac⁻ mutants was increased.

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**Table 1. Effect of acriflavine on conversion of Streptococcus lactis C₂F to lac⁻ variants at several temperatures**

| Acriflavine concn (µg/ml) | Viable count | No. of colonies examined | No. lac⁻ | Viable count | No. of colonies examined | No. lac⁻ | Viable count | No. of colonies examined | No. lac⁻ |
|---------------------------|--------------|--------------------------|----------|--------------|--------------------------|---------|--------------|--------------------------|---------|
| 0                         | 5.5 x 10⁸    | 55                       | 0        | 1.2 x 10⁸    | 252                      | 0       | 1.3 x 10⁹    | 13                       | 0       |
| 2                         | 3.0 x 10⁸    | 626                      | 0        | 1.1 x 10⁸    | 127                      | 0       | 1.6 x 10⁹    | 329                      | 1       |
| 4                         | 1.6 x 10⁸    | 347                      | 14       | 1.1 x 10⁸    | 117                      | 3       | 9.6 x 10⁹    | 207                      | 34      |
| 6                         | 7.4 x 10⁸    | 84                       | 5        | 2.7 x 10⁷    | 552                      | 147     | < 10⁷        | 0                        | 0       |
| 8                         | 3.6 x 10⁸    | 369                      | 12       | 2.0 x 10⁷    | 20                      | 2       | < 10⁷        | 0                        | 0       |
| 10                        | 4.4 x 10⁸    | 50                       | 0        | 4.0 x 10⁶    | 40                       | 0       | < 10⁷        | 0                        | 0       |
| 15                        | 2.5 x 10⁸    | 25                       | 0        | 7.2 x 10⁵    | 75                       | 1       | < 10⁷        | 0                        | 0       |
| 20                        | 7.0 x 10⁴    | 82                       | 0        | 4.0 x 10³    | 40                       | 0       | < 10⁷        | 0                        | 0       |

**Fig. 1. Comparative growth at 32 C of S. lactis C₂F (○) and three lac⁻ mutants in lactic broth containing 6 µg of AF per ml. The lac⁻ mutants were selected from S. lactis C₂F by treatment with AF (□) or as spontaneous variants which occurred when the parent strain was subcultured at 25 C (△) or at 37 C (■). The control curve (●) represents the growth of S. lactis C₂F in lactic broth without AF.**
by AF treatment in *S. lactis* C₄F, *S. cremoris* B₁, *S. cremoris* Wg₁, and *S. diacetilactis* 18-16. Spontaneous lac⁻ mutants were also isolated from *S. lactis* b, *S. cremoris* C₄, and *S. cremoris* W, as well as *S. lactis* C₄F, *S. cremoris* B₁, and *S. diacetilactis* 18-16. Thus, it appears that the loss of lactose metabolism is a general phenomenon among the lactic streptococci.

In addition to certain chemical agents, elevated temperature is also known to increase the loss of metabolic functions from bacteria (14). When cultures of *S. lactis* C₄F were incubated at 37 C for 72 hr and subsequently held at 25 C, a high proportion of the cells which grew out were lac⁻. The percent of lac⁻ cells in the total population ranged from 19 to 64 depending upon the experiment.

**Occurrence of lac⁻ variants within lactose-fermenting colonies of *S. lactis* C₄F.** To obtain further evidence for the instability of lactose metabolism in lactic streptococci, we examined lactose-fermenting colonies of *S. lactis* C₄F to determine whether any lac⁻ cells were naturally present. Table 3 indicates that lac⁻ cells were obtained from presumably pure lac⁺ colonies. From the 10 lac⁺ colonies examined, five were found to contain lac⁻ cells.

**Table 2. Elimination of lactose-fermenting ability in lactic streptococci by treatment with acriflavine (AF)**

| Strain              | Viable count | No. of colonies examined | No. of lac⁻ colonies | Viable count | No. of colonies examined | No. of lac⁻ colonies |
|---------------------|--------------|--------------------------|----------------------|--------------|--------------------------|----------------------|
| *S. lactis* C₄F    | 2.9 × 10⁸    | 661                      | 11                   | 8.4 × 10⁸    | 792                      | 4                    |
|                     | 1.4 × 10⁹    | 308                      | 27                   | 2.5 × 10⁸    | 49                       | 0                    |
| *S. lactis* b       | 3.3 × 10⁸    | 36                       | 0                    | 7.4 × 10⁸    | 153                      | 0                    |
|                     | 7.5 × 10¹    | 16                       | 0                    | 8.1 × 10⁸    | 187                      | 1                    |
| *S. cremoris* B₁    | 2.0 × 10⁰    | 425                      | 11                   | 4.1 × 10⁸    | 668                      | 1                    |
|                     | 4.7 × 10⁴    | 94                       | 7                    | 1.6 × 10⁸    | 367                      | 0                    |
| *S. cremoris* C₄    | 2.6 × 10⁹    | 283                      | 0                    | 2.4 × 10⁸    | 517                      | 1                    |
| <10⁹               | 249          | 0                        | 5.2 × 10⁷            | 109          | 3                        |
| *S. cremoris* Wg₁   | 2.8 × 10⁴    | 32                       | 13                   | 1.7 × 10⁸    | 40                       | 0                    |
| <3.0 × 10⁴          | 0            | 0                        | 8.1 × 10⁷            | 183          | 0                        |
| *S. cremoris* W     | 1.1 × 10⁴    | 105                      | 0                    | 1.7 × 10⁸    | 373                      | 1                    |
| <3.0 × 10⁴          | 0            | 0                        | <3.0 × 10⁷           | 0            | 0                        |
| *S. diacetilactis* 18-16 | 7.1 × 10⁴ | 410                      | 24                   | 6.9 × 10⁸    | 156                      | 0                    |
| 1.3 × 10⁵           | 257          | 1                        | 2.9 × 10⁸            | 650          | 1                        |

* Treatment with 4 μg of AF per ml.
* Treatment with 10 μg of AF per ml.
Table 3. Occurrence of lac- cells in lac+ colonies of *Streptococcus lactis* C2F

| Colony no. | Viable count (per ml) | No. of colonies examined | No. of lac- colonies |
|------------|-----------------------|--------------------------|----------------------|
| 1          | 2.3 x 10⁷             | 46                       | 1                    |
| 2          | 2.3 x 10⁷             | 46                       | 3                    |
| 3          | 1.6 x 10⁷             | 317                      | 0                    |
| 4          | 9.1 x 10⁷             | 182                      | 0                    |
| 5          | 4.9 x 10⁷             | 97                       | 0                    |
| 6          | 2.9 x 10⁷             | 57                       | 0                    |
| 7          | 2.9 x 10⁷             | 58                       | 1                    |
| 8          | 2.0 x 10⁷             | 40                       | 0                    |
| 9          | 2.8 x 10⁷             | 56                       | 2                    |
| 10         | 5.1 x 10⁷             | 103                      | 2                    |

Even the others may have contained lac- cells if a larger number of colonies had been examined. It has subsequently been observed that on occasion, when abnormally light yellow colonies are picked from a plate and restreaked, they contain a mixture of lac+ and lac- cells. It should be emphasized that this spontaneous conversion of lac+ to lac- cells must occur at a fairly high rate or it would be difficult to detect any cells by the direct plating techniques used.

Reversion of lac- mutants. Table 4 illustrates the reversion of the lac- mutants to lac+ variants. Mutants selected from the three treatments were examined. Although partial revertants were observed, they did not possess the original *S. lactis* C2F phenotype. They remained defective in lactose metabolism and never regained the ability to coagulate milk. These revertants appeared on the plate only after extended periods of incubation. When *S. lactis* C2F cells were mixed with an excess of a lac- culture and were spread over the surface of the indicator agar, the *S. lactis* C2F cells developed into colonies which produced acid within 24 hr. Thus, the procedure did not inhibit any true lac+ revertants.

Continuous culture growth of *S. lactis* C2F. The spontaneous occurrence of lac- cells in cultures of lactic streptococci suggested that continuous culture growth of starters could present a problem for mass culturing if conditions were selective for dominance by lac- variants. Table 5 shows the continuous culture growth of *S. lactis* C2F and the subsequent occurrence of lac- mutants. After prolonged continuous growth, the lac- mutants slowly became dominant. In one experiment at 37 to 39 C, about 82% of the population consisted of lac- mutants after 85 hr of continuous growth. With continued incubation, the lac- mutants eventually became the only surviving cells.

### DISCUSSION

During studies on lactose metabolism in lactic streptococci, a peculiar loss of lactose metabolism in *S. lactis* C2F was observed. In the isolation of lac- strains from *S. lactis* C2F, over 70% of the survivors were lac- under certain conditions of mutagenesis. In addition,

Table 4. Reversion of the lac- mutants to lac+ variants in *Streptococcus lactis* C2F

| Tube no. | Temp (°C) | 25 Sp (°C) | ACR (%) |
|----------|-----------|------------|---------|
|          | 2 day     | 5 day      | 7 day   | 2 day     | 5 day      | 7 day   |
| 1        | 0         | 64 129    | 0       | 11 54     | 0          | 82 95   |
| 2        | 0         | 83 222    | 0       | 14 102    | 0          | 82 127  |
| 3        | 0         | 105 194   | 0       | 17 48     | 0          | 66 109  |
| 4        | 0         | 22 205    | 0       | 9 17      | 0          | 55 96   |
| 5        | 0         | 22 157    | 0       | 5 48      | 0          | 58 83   |
| 6        | 0         | 28 197    | 0       | 9 46      | 0          | 46 59   |
| 7        | 0         | 25 103    | 0       | 11 32     | 0          | 32 28   |
| 8        | 0         | 10 140    | 0       | 8 58      | 0          | 58 46   |
| 9        | 0         | 31 145    | 0       | 0 78      | 0          | 78 62   |
| 10       | 0         | 16 185    | 0       | 0 83      | 0          | 83 62   |

a Lac- mutants obtained at elevated temperatures. Total number of viable cells per milliliter: initial, 17; final, 2.2 x 10⁴.

b Lac- mutants which occurred as spontaneous mutants at 25 C. Total number of viable cells per milliliter: initial, 15; final, 2.0 x 10⁴.

c Lac- mutants obtained in the presence of AF. Total number of viable cells per milliliter: initial, 17; final, 5.5 x 10⁴.

Table 5. Continuous culture growth of *Streptococcus lactis* C2F at 35 C and the occurrence of lac- variants

| Time (hr) | Viable count (organisms/ml) | No. of colonies examined | No. of lac- colonies | Frequency (%) |
|-----------|----------------------------|--------------------------|----------------------|---------------|
| 0         | 7.8 x 10⁴                  | 168                      | 0                    | 0             |
| 11.5      | 1.3 x 10⁵                  | 283                      | 0                    | 0             |
| 23        | 2.4 x 10⁷                  | 55                       | 1                    | 1.8           |
| 36        | 9.9 x 10⁷                  | 242                      | 1                    | 0.4           |
| 46        | 3.1 x 10⁸                  | 69                       | 0                    | 0             |
| 62        | 1.2 x 10⁹                  | 256                      | 0                    | 0             |
| 71        | 2.0 x 10⁷                  | 647                      | 5                    | 0.8           |
| 84        | 4.2 x 10⁷                  | 85                       | 5                    | 5.9           |
| 98        | 6.7 x 10⁸                  | 134                      | 14                   | 10.4          |
| 107.5     | 1.4 x 10⁹                  | 298                      | 27                   | 9.1           |
| 120       | 6.0 x 10⁹                  | 120                      | 15                   | 12.5          |
| 131.5     | 1.5 x 10⁹                  | 168                      | 91                   | 54.2          |
| 140       | 2.7 x 10⁹                  | 189                      | 137                  | 72.5          |
| 156       | 4.5 x 10⁷                  | 107                      | 73                   | 68.2          |

a Frequency of lac- = no. of lac- colonies/no. of colonies examined.
spontaneous lac⁻ mutants were isolated from stock cultures of this strain after an extended period of daily subculturing in lactose broth at 21°C. Since these variants grew as well as the wild type on glucose, maltose, mannose, and fructose, it was argued that the defect did not involve the glycolytic enzymes but was specific in the metabolism of lactose (11). Their relationship to the parent strain was assured since these mutants retained sensitivity to the bacteriophage specific for its parent. Thus, lactose metabolism in S. lactis C2F, previously believed to be a stable trait, was unstable under certain conditions of cultivation.

The question that arises is how the loss of lactose-fermenting ability is induced in lactic streptococci. Okulitch and Eagles (16) observed that spontaneous transferring in any fermentable carbohydrate medium resulted in the loss of lactose metabolism in S. cremoris 142. Lactose and galactose were the only fermentable sugars which did not result in a complete loss of lactose metabolism. They suggested that the inhibitory activity of glucose or one of its metabolic products may cause this sudden loss. A prime candidate could be lactic acid or H₂O₂ since the latter compound is mutagenic. They also suggested that the specific configuration of the carbohydrate in the medium was important as well as the physiological state of the microorganism.

The data presented here suggest that lactic streptococci could be carrying a genetic element which is responsible for the cells ability to ferment lactose. The loss of this element would cause the cell to become lac⁻. If this were the case, then lactic streptococci would be expected to throw off extrachromosomal-negative variants as a result of occasional errors in its replication (14). This spontaneous loss of lactose metabolism by lactic streptococci was observed. In the present study it was noted in S. lactis C2F, S. lactis b, S. cremoris B₁, S. cremoris C₁, S. cremoris W, and S. diacetilactis 18-16. In earlier work, it was observed in S. cremoris 142 by Okulitch and Eagles (16), in S. cremoris HP by Hunter (8), and in S. lactis 354 by Hirsch (6). The frequency of such variants can often be increased by treatment with certain chemical agents such as AF which selectively inactivates extrachromosomal elements (6). The appearance of lac⁻ variants was increased by AF treatment in S. lactis C2F, S. cremoris B₁, S. cremoris W₉₃, and in S. diacetilactis 18-16. In S. lactis C2F, it was shown that the lac⁻ variants are selected by the presence of AF, but, in addition, it was shown that AF may also be involved in the direct conversion of lac⁺ to lac⁻ cells.

The high incidence of the spontaneous loss of lactose metabolism is presumptive evidence for an extrachromosomal particle's being responsible for lactose metabolism. The effect of AF and elevated temperatures on the occurrence of lac⁻ variants strengthens this hypothesis. However, other sources of genetic alterations such as point mutations, phase variations, and deletions were not ruled out. Point mutations and phase variations are reversible, but deletions or loss of extrachromosomal material are nonreversible. Unlike the earlier workers who considered the change in S. cremoris and S. lactis to be stable (6, 8, 15, 16, 22), we observed a reversion from lac⁻ to lac⁺ to occur. Whether these mutants represent reversions in chromosomal-linked lactose genes (ruling out deletions or loss of genetic material) or represent the ability of the lac⁻ culture to utilize lactose by mutation in alternate genes is not known. Any change in metabolism could mean that a reversion had occurred by changes in a cryptic alternate pathway similar to that reported for mannitol utilization in Aerobacter aerogenes (19). Vakil and Shahani (20) demonstrated that S. lactis UN can utilize lactose by first converting it to lactobionic acid. This could be an alternate pathway. These revertants were considered partial because they lacked the parental lactose-fermenting phenotype and were unable to coagulate milk. They appeared only after prolonged incubation of plates spread with a lawn of lactocilli. Thus, although partial lactose-fermenting variants were observed by the strain of S. lactis C2F, the evidence gained from studies on their spontaneous occurrence and the findings observed with AF strongly suggest the loss of genetic material as being responsible for the instability of lactose metabolism observed in lactic streptococci.

It was previously noted that under certain conditions the successive transferring of S. lactis or S. cremoris resulted in the appearance of lac⁻ cells. This is difficult to interpret, but one explanation is the loss of genetic material. Clark (2) observed that, after 61 successive transfers, Bacillus megaterium lost genetic material and became nonlysogenic. Lwoff (10) also demonstrated the loss of prophage by repeated transfers of the organism. Whether a similar phenomenon is responsible for the loss of lactose metabolism in lactic streptococci is as yet unknown. Lysogenic strains have been reported among the lactic streptococci (17), and it may be that this lysogeny is necessary to obtain lactose metabolism. The loss of the prophage would then result in a lac⁻ variant.

Hirsch (7) proposed the hypothesis that the
lactic streptococci were of recent origin and, although milk is not considered to be a normal habitat of these organisms, he reasoned that they became adapted to this environment. His reasoning included the saprophytic nature of the organism, their lactose-fermenting ability, their habitat, and their antibiotic-producing ability. Could the lactic streptococci have acquired this ability to ferment lactose via transfer of genetic material from another genus? It is known that non-lactose-fermenting Salmonella (3) and Proteus (21) strains become lac+ upon acquisition of extrachromosomal deoxyribonucleic acid-carrying lac genes. Shigella dysenteriae, normally a lactose-negative organism, has also been shown to be converted to a lactose-positive strain by incorporation of prophage (1).

This study also revealed that there can be selection of cells which fail to ferment lactose during continuous culture growth of lactic streptococci. This is the first report providing evidence that alterations in the characteristics of starter strains are produced by continuous culture techniques. Thus, if one is preparing concentrated cultures to be used for direct inoculation into the bulk starter tank or cheese vat, certain precautions must be taken to prevent a high incidence of lac− cells. This is particularly true if continuous culture techniques are used for the preparation of the cells. Spon-
taneous lac− mutants were found at 21, 32, and 37 C so temperature alone was not the primary factor in conversion. The presence of lactose as the sole carbohydrate source in the fermentation media would probably help reduce the frequency of the lac− mutants. If the fermentation media contains glucose, the lac− mutants are capable of growing as well as the lac+ parent cells. The presence of lactose alone, however, does not prevent the appearance of lac− mutants, as was noted in Table 3.

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LITERATURE CITED

1. Barkdale, L. 1959. Lysogenic conversion in bacteria. Bacteriol. Rev. 23:202–212.

2. Clark, N. A. 1962. Studies on the host-virus relationship in a lysogenic strain of Bacillus megaterium. III. The growth of Bacillus megaterium in synthetic medium. J. Bacteriol. 83:187–192.

3. Easterline, S. B., E. M. Johnson, J. A. Wohlhieter, and L. Baron. 1969. Nature of lactose-fermenting Salmonella strains obtained from clinical sources. J. Bacteriol. 100:38–41.

4. Elliker, P. R., A. Anderson, and G. H. Hannessen. 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. 39:1611–1612.

5. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. 46:57–64.

6. Hirsch, A. 1961. Growth and nisin production by a strain of Streptococcus lactis. J. Gen. Microbiol. 5:206–221.

7. Hirsch, A. 1952. The evolution of the lactic streptococci. J. Dairy Res. 19:290–293.

8. Hunter, G. J. E. 1939. Examples of variation within pure cultures of Streptococcus cremoris. J. Dairy Res. 10:464–470.

9. Lloyd, G. T. 1971. New developments in starter technol-
ogy. Dairy Sci. Abstr. 33:411–416.

10. Lwoff, A. 1953. Lysozymes. Bacteriol. Rev. 17:269–337.

11. McKay, L. L., A. Miller III, W. E. Sandine, and P. R. Elliker. 1970. Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analy-
yses. J. Bacteriol. 102:804–809.

12. McKay, L. L., L. A. Walter, W. E. Sandine, and P. R. Elliker. 1969. Involvement of phosphorylase in lactose utilization by group N streptococci. J. Bacteriol. 99:603–610.

13. Morse, M. L. 1967. Reversion instability of an extreme polar mutant of the galactose operon. Genetics 66:331–340.

14. Novick, R. P. 1969. Extrachromosomal inheritance in bacteria. Bacteriol. Rev. 33:210–235.

15. Okulitch, O. 1939. Microbial dissociation of lactic acid streptococci. Can. J. Res. Ser. B 17:171–177.

16. Okulitch, O., and B. A. Eagles. 1936. Cheese ripening studies. The influence of the configurational relations of the hexoses on the sugar-fermenting abilities of lactic acid streptococci. Can. J. Res. Ser. B 14:320–
324.

17. Reiter, B. 1949. Lysozymic strains of lactic streptococci. Nature (London) 164:667–668.

18. Sherman, J. M., and R. V. Hussong. 1937. Fermentative variabil-
ity among strains of Streptococcus cremoris and Streptococcus lactis obtained from pure cultures. J. Dairy Sci. 20:101–103.

19. Tanaka, S., S. A. Lerner, and E. G. Lin. 1967. Replace-
mament of a phosphoenolpyruvate-dependent phospho-
transferase by a nicotinamide adenine dinucleotide-
linked dehydrogenase for the utilization of mannoitil. J. Bacteriol. 93:642–648.

20. Vakil, J. R., and K. M. Shahani. 1969. Carbohydrate metabolism of lactic acid cultures. V. Lactobionate and gluconate metabolism of Streptococcus lactis UN. J. Dairy Sci. 52:1928–1934.

21. Wohlhieter, J. A., S. Falkow, R. V. Citarella, and L. S. Baron. 1964. Characterization of DNA from a Proteus strain harboring an episome. J. Mol. Biol. 9:576–588.

22.Yawger, E. S., and J. M. Sherman. 1937. Variants of Streptococcus lactis which do not ferment lactose. J. Dairy Sci. 20:83–86.