ABSTRACT
A sterile, nonfat, dry-milk substrate with beta glycerophosphate was used to differentiate proteinase-positive and proteinase-negative lactic streptococci. Fifteen pairs of both cell types were incubated in this medium 24 h at 22°C. Proteinase-positive cells produced from 6 to 27 times the cell mass of the proteinase-negative variants. *Streptococcus cremoris* proteinase-negative isolates produced a significantly larger cell mass than did *Streptococcus lactis* isolates. Cell mass production from proteinase-negative variants did not correlate with those from parent strains producing high cell mass. When mixtures of the two cell types were grown in the medium, from 10 to 50% of proteinase-positive cells were required for growth and acid production to equal that of 100% proteinase-positive cells. It was possible to detect 2% proteinase-positive cells in a proteinase-negative culture.

INTRODUCTION
With the suggestion for exclusive use of proteinase-negative (Prt-) lactic streptococcal variants in cheese making (10), a method is needed to differentiate them from their proteinase-positive (Prt+) counterparts (4, 12) and to detect when Prt+ cells are in Prt- cultures. Various media and methods of differentiating Prt- and Prt+ isolates exist but are time consuming and are not reliable with some strains (1, 2, 3, 4, 7). A simple and reliable method of differentiating between Prt- and Prt+ isolates was investigated with a buffered substrate that contained only the nitrogenous matter available in sterilized nonfat dry milk (NDM).

MATERIALS AND METHODS

Cultures
The Prt+ and Prt- cultures of *Streptococcus lactis* and *S. cremoris* were from the frozen stock maintained at the Department of Nutrition and Food Sciences at Utah State University. The Prt- variants had been isolated on glycerophosphate-milk agar (8) and by test of the ability of the isolates to coagulate sterile 10% NDM within 24 h (10). The Prt+ cultures were propagated in sterile, 10% NDM. The Prt- cultures were propagated in NDM fortified with .5% yeast extract (YNDM). The media were sterilized at 121°C for 15 min. Frozen cultures were stored at -40°C, and working cultures were transferred weekly by inoculation of .1 ml of coagulated culture into 10 ml of the appropriate media and storing at 4°C. They were incubated 24 h at 22°C prior to use or transfer.

Buffered Milk Substrate
A stock solution containing 95 g of Grade II sodium beta glycerol phosphate (Sigma Chemical Co., St. Louis, MO) and 100 ml deionized water was mixed and dispersed in 20-ml quantities into 100-ml dilution bottles and autoclaved (11). Buffered NDM substrate (BNDM) was prepared by .6 ml of the buffer stock solution added to 10.0 ml of NDM (pH = 7.2).

Cell Mass Determinations
Cell mass determinations were made turbidometrically by the modified procedure (10) of Kanasaki et al. (7).
Inoculations

Refrigerated cultures were incubated for 24 h at 22°C before inoculations. The Prt-- cultures were inoculated by .1 ml of coagulated culture added to 10.6 ml of BNDM. Volumes of Prt+ cultures inoculated into BNDM were based on the cell mass of a 1% inoculum of the corresponding coagulated Prt-- culture so that the initial cell numbers of both Prt+ and Prt-- cultures of a given strain would be approximately equal. Because the cell mass attained by the Prt-- cultures was usually larger in YNDM, the inoculum amounts of coagulated Prt+ cultures were raised accordingly. Usually .12 to .17 ml were equivalent to the .1 ml of Prt-- inocula.

Mixed Proteinase-Positive and Proteinase-Negative Cultures

Mixtures of Prt+ and Prt-- cultures were made by determining the initial cell mass of each and adjusting inoculations such that different proportions of Prt+ and Prt-- cells existed in 1% inoculum in BNDM. Mixtures containing varying percentages of Prt+ cells (0 to 100%) were tested in triplicate. Asymptotic curves were calculated to estimate the proportion of Prt+ cells equivalent to 100% Prt+ cells in cell mass and acid production and also to estimate the percentage of Prt+ cells in a Prt-- culture.

RESULTS AND DISCUSSION

Differentiation of Proteinase-Negative Cultures

The cell mass attained by the Prt+ cultures in BNDM was from 6 to 27 times as great as that attained by the corresponding Prt-- cultures (Figure 1). The mean (X) and standard deviation (SD) for cell mass were 6.41 ± 1.83 for Prt+ and .44 ± .15 for the Prt-- variants. They were significantly different at P<.00001. The means (± SD) for the 4 S. lactis Prt-- variants were .30 ± .05 and the 10 S. cremoris Prt-- variants were .49 ± .13. The procedure allowed differentiation of S. lactis from S. cremoris Prt-- strains at P<.00827.

The overall correlation coefficient between the two types of cells was zero; thus, the proportion of proteinase activity after isolation of the Prt-- variant did not relate to the proteinase activity of the parent cells. The slow growth of the Prt-- variants was due to the low concentration of free amino acids and peptides in milk and the unavailability of these by-products (normally released by Prt+ cells) in lactic cultures. Cell mass differences were accentuated by the addition of sodium beta glycerophosphate buffer, which reduced growth inhibition of the Prt+ cultures. The proteinase activity differences among Prt+ strains were much more evident than were the differences among Prt-- cells. This suggests greater uniformity of acid production with Prt-- cultures.

Plating methods for differentiating cell types require 2 to 3 days of incubation. Cell performance in milk is not predicted necessarily by such methods (8), and confirmatory methods require growth in milk for a total analysis time of from 72 to 96 h. Conversely, cell mass production in BNDM provides confirmatory data directly in 24 h.

An improved proteolytic assay, developed by Church et al. (1), was evaluated on nine of these lactic cultures. The method was tedious, but it could differentiate between Prt+ and Prt-- cells at P<.01 with mean readings of A =
.24 and .19, respectively. Cell mass readings in the BNDM substrates were $A = 0.64 \pm 0.18$ and $0.04 \pm 0.02$ for greater differentiation even though they were an indirect measurement of protease activity.

**Mixed Proteinase-Positive and Proteinase-Negative Cultures**

About 1 to 2% of cells in single strains of lactic streptococci gradually lose their plasmid-linked proteinase activity (4, 6). Thus, frequent transfer of a culture and attempts to carry single strains over long times will produce cultures with variable mixtures of Prt+ and Prt− cell types. Because Prt+ cells produce an excess of nitrogenous matter that can be utilized by Prt− cells, no change of acid production rates in a culture are detectable. When the proportion of Prt+ cells drops to where they are unable to sustain the needs of the increased proportion of Prt− cells, however, the rate of acid production is reduced significantly. This has been considered to be 10 to 20% of Prt+ cells (4, 12).

Tests of known mixtures of Prt+ and Prt− cultures of six strains showed that a range of from 10 to 50% of a culture must be Prt+ for growth and acid production to equal that of a Prt+ culture (Figure 2). Some of the strains required higher Prt+ concentrations to restore full Prt+ like activity than had been reported (4, 12). This is indicative of the wide variance of ratios of Prt+/Prt− cells that might exist before measurable losses of culture activity occur. It also suggests that one Prt+ cell can produce sufficient available nitrogen to support the growth from one to nine Prt− cells, depending upon the strain.

For a given strain it should be possible to estimate the proportion of Prt+ cells in a blend below about 50%.

Wright et al. (13) successfully used this type of test to predict inoculum required to produce desired changes of pH during cheese manufacture. Such a technique should find application in cheese culture characterization and selection. Additionally, those strains producing the largest cell mass may be desired where accelerated ripening is desired (5). Conversely, those with lowest growth potential would be usable in stored cheese where ripening is discouraged or in cottage cheese where maximum yield is desired (9).

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**REFERENCES**


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