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# Selection of Protease-Positive and Protease-Negative Variants of *Streptococcus cremoris*

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**Protease-negative variants were shown to outcompete the wild-type strains of *Streptococcus cremoris* $E_8$, HP, and $Wg_2$ at pH values higher than 6.0 in milk. For *S. cremoris* $E_8$ this process was studied in more detail. At lower pH values the wild type had a selective advantage. This pH-dependent selection was not found in all media tested. The poor growth of the protease-negative variant at low pH was not due to lower internal pH values. By growing *S. cremoris* $E_8$ and $Wg_2$ in acidified milk (pH 5.9) the proteolytic activity of the cultures could be stabilized. In continuous cultures under amino acid limitation the wild type *S. cremoris* $E_8$ and HP strains had a selective advantage over the protease-negative variants at low dilution rates ($D < 0.2$) at all pH values of the medium. This was apparently due to a lower affinity-constant ($K_s$) of the protease-positive variants for amino acids. Finally, a high fraction of protease-positive variants could be maintained in continuous cultures by using a growth medium with low concentrations of casein as a nitrogen source. At high dilution rates nearly all cells were protease positive.**

In the Dutch cheese industry mixed cultures of lactic acid bateria are used as starters. The cultures are composed of many different strains, mostly of the species *Streptococcus cremoris*. These strains can vary strongly in their activities relevant to cheese making such as proteolytic activity (6), growth rate (7), and phage sensitivity (23).

The proteolytic activity plays a central role in flavor development during the ripening of the cheese (11). It also enables the streptococci to grow and acidify the milk, which contains casein as major nitrogen source. Many strains form variants which have lost the proteolytic system spontaneously by loss of a plasmid (16). These variants ($Prt^-$) can only grow on milk in the presence of the wild-type strain ($Prt^+$), which supplies the essential amino acids for growth. As long as the percentage of $Prt^-$ variants in the population is low, the culture will grow normally on milk. If, however, the $Prt^-$ variants become dominant the rate of growth and concurrent rate of acidification of milk become low. These so-called "slow" starter cultures were described more than 50 years ago (3). The kinetics of the gradual shift from a normal to a slow starter has been studied by Otto et al. (18; R. Otto, Ph.D. thesis, University of Groningen, 1981). They found that the $Prt^-$ variant of *S. cremoris* $Wg_2$ had a selective advantage over the wild type at pH values above 6.0 in MRS medium (1) and in a yeast extract medium. This is to be expected because less energy has to be invested in biosynthesis of plasmids and enzymes (proteases). At lower pH, however, the $Prt^-$ variant had no selective advantage. Otto et al. suggested that this pH-dependent selection could have practical implications, because it would be possible to maintain a high proteolytic activity in starter cultures when they are grown at appropriate pH values.

In this study, we tested the practical implications of the pH effect by using different *S. cremoris* strains and several growth media, including milk. We also looked for other growth conditions that would select for the $Prt^+$ variants in batch cultures or in continuous cultures.

The data presented in this manuscript suggest that the pH-dependent selection for $Prt^+$ and $Prt^-$ variants is a common phenomenon in strains of *S. cremoris*, although it was dependent on the composition of the growth medium. The $Prt^+$ variants also had a selective advantage in continuous cultures under amino acid limitation and in growth media with low concentrations of casein as a nitrogen source.

## MATERIALS AND METHODS

**Bacterial strains.** *S. cremoris* $Wg_2$, HP, and $E_8$ were obtained from the Netherlands Institute for Dairy Research (Ede, the Netherlands). Two variants of each strain were used: the wild type ($Prt^+$) and the cell wall-associated, protease-negative ($Prt^-$) variant (2, 6). The organisms were routinely stored in 10% skimmed milk at −20°C.

**Batch cultivation.** Cultures of $Wg_2$, HP, and $E_8$ containing small numbers of $Prt^-$ variants were grown for approximately 12 h either in normal or in acidified (lactic acid; pH 5.9) 10% skimmed milk until coagulation had occurred. Samples (100 μl) of these milk cultures were diluted 100-fold with 10 ml of fresh skimmed milk, and growth was continued for another 2 h. This procedure was repeated up to 100 times.

**Continuous cultivation.** Chemostats were used as described previously (10). Complex MRS medium or chemically defined RFP medium (19), both with 0.25% lactose as the growth-limiting substrate, were used for cultivation. The pH of the cultures was automatically controlled at the values indicated in the figures and adjusted with 1 N NaOH.

In the case of amino acid limitation, chemically defined RFP medium was used with 40 mg of sodium glutamate per liter (18) or 12.5 mg of leucine per liter (17) and 1% lactose. When RFP-casein medium was used the amino acid mixture was substituted by sodium caseinate to a final concentration of 0.4%.

**Determination of $\mu_{max}$.** Maximum specific growth rates ($\mu_{max}$ values) were determined in MRS and in RFP medium by batch cultivation at 30°C under pH control with 1 N NaOH. Samples were taken from the cultures every 30 min, and the absorbance at 660 nm was measured in a Vitatron UC200 spectrophotometer (Vitatron Scientific Instruments, Dieren, the Netherlands).

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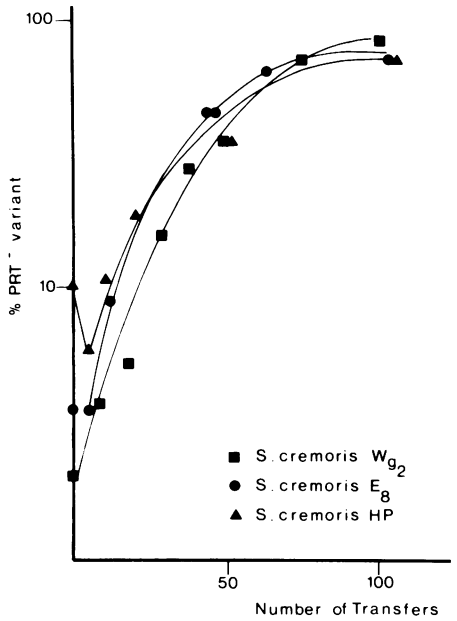


FIG. 1. Increase in numbers of $Prt^-$ variants in pure cultures of *S. cremoris* $Wg_2$, $E_8$, and HP during serial transfer in fresh reconstituted skimmed milk.

**Detection of the number of $Prt^-$ variants.** Samples of the cultures were diluted $10^6$- and $10^7$-fold in a sterile 25 mM potassium phosphate solution (pH 6.5). Samples (100 μl) of these dilutions were spread over a modified agar medium as described by Limsowtin and Terzaghi (12) containing 10% skimmed milk, 1.9% sodium β-glycerophosphate, 0.003% bromocresol purple, and 1.3% Bacto-Agar (Difco Laboratories, Detroit, Mich.). On the agar plates the wild-type ($Prt^+$) *S. cremoris* forms large, yellowish, colonies in 2 to 3 days in contrast to the small white colonies of the $Prt^-$ variant. Each dilution was spread fivefold over agar plates, and average values were used for the figures. Since *S. cremoris* forms chains and only one $Prt^+$ cell within a chain will lead to the development of a $Prt^+$ colony type, this method results in a slight overestimation of the percentage of $Prt^+$ variants in the cultures.

**Acidification rate.** Flasks covering 80 ml of 10% skimmed milk were inoculated with 1 ml of preculture containing a fixed fraction of $Prt^-$ variants. The pH of the cultures was measured every hour during the exponential growth phase. The production of lactic acid by the streptococci was quantitated by comparing the pH values with those of sterile skimmed milk samples titrated with increasing amounts of lactic acid. In some cases control meaurements were done by gas chromatography (5) to check the accuracy. This yielded the same values for lactic acid concentrations.

**Internal pH and proton motive force measurements.** $Prt^+$ and $Prt^-$ cells of *S. cremoris* $Wg_2$ were grown overnight in MRS medium. The cells were washed and suspended in MRS medium of the appropriate pH. The difference between internal and external pH (ΔpH) was determined from the distribution over the cytoplasmic membrane of the weak acid [$^{14}$C]salicylic acid as measured by automated flow dialysis (4). Measurements were done in MRS medium at various pH values.

The membrane potential ($\Delta\psi$) was measured with a tetraphenylphosphonium ($TPP^+$) selective electrode (13). The distribution of this lipophilic cation $TPP^+$ over the cytoplasmic membrane was used as a measure for the electrical potential over the cytoplasmic membrane. Experiments were started by the addition of 0.4% (final concentration) lactose. Accumulation ratios of $TPP^+$ were calculated by using an intracellular volume of 3.8 μl/mg of cell protein (22) and making corrections for binding of $TPP^+$ to the cells as described previously (14).

Accumulation ratios of salicylic acid and of $TPP^+$ were converted into millivolts by using a $Z$ value of 60 at 30°C. The standard error in the measurements was approximately 10 mV for both $\Delta\psi$ and ΔpH.

**Protein determination.** Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as the standard.

## RESULTS

**Slow starter cultures.** The process of starter cultures becoming slow when used repeatedly could be simulated with pure cultures of different strains of *S. cremoris*. The $Prt^-$ variants of strains HP, $E_8$, and $Wg_2$ gradually became dominant in batch culture (Fig. 1). After approximately 100 transfers in fresh medium the $Prt^-$ variants made up 90 to 98% of the total culture. Table 1 shows that these cultures could be considered as real slow starter cultures. Table 1 shows the acidification rates of cultures containing various amounts of protease-negative variants. Cultures containing up to 92% $Prt^-$ variants still acidified milk at the normal rate, but higher percentages of $Prt^-$ resulted in slower acidification rates.

**pH-dependent selection.** Otto (Ph.D. thesis) explained the take-over by the $Prt^-$ variant of $Wg_2$ by its higher maximum specific growth rate. This higher growth rate was only found at pH values above 6.0. At lower pH values the wild type ($Prt^+$) grew faster. These findings suggested that $Prt^+$ or $Prt^-$ variants of $Wg_2$ can be selected by changing the pH of the medium (18).

This pH effect could be very useful in controlling the total proteolytic activity of starter cultures. However, many different strains are used in cheese manufacture all over the world. These strains are known to vary strongly in all kinds of properties (11, 16, 23), including growth parameters (7). We have done growth experiments similar to those described by Otto and co-workers (18) with *S. cremoris* $E_8$ to check whether the pH effect could be a general effect in starter cultures. The strain was cultivated in continuous culture on MRS medium under lactose limitation at three different pH values (Fig. 2). In all cases a considerable fraction (up to 65%) of $Prt^-$ variants of strain $E_8$ was added. At pH 6.3 and pH 6.7 the $Prt^-$ variants quickly became dominant in the

TABLE 1. Specific acidification rate[a] in milk of *S. cremoris* $E_8$ and $Wg_2$ containing various numbers of $Prt^-$ variants

| % of $Prt^-$ variants | Specific acidification rate (per h) | |
|---|---|---|
| | Strain $E_8$ | Strain $Wg_2$ |
| 3 | 0.68 | 0.64 |
| 80 | 0.71 | 0.60 |
| 85 | 0.70 | 0.61 |
| 92 | 0.64 | 0.59 |
| 96 | 0.56 | 0.55 |
| 97 | 0.43 | 0.50 |
| 100 | 0.28 | 0.30 |

[a] The specific acidification rate was calculated as follows: $(\Delta[\text{lactate}]/\Delta t) \times (1/[\text{lactate}])$, where $t$ is the time in hours.

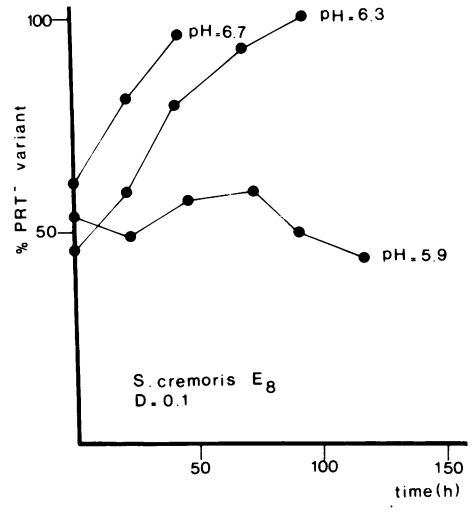


FIG. 2. Percentage of Prt⁻ variants in cultures of *S. cremoris* $E_8$ during continuous cultivation at different MRS medium pH values.

culture, but at pH 5.9 this was not found. At the latter pH the wild type appeared to increase slowly in numbers. These results are comparable to what was found for *S. cremoris* $Wg_2$ (18). When the maximum specific growth rates of Prt⁺ and Prt⁻ variants were compared for strain $E_8$ in MRS medium, the Prt⁻ variant grew at the same rate or slightly faster at pH values higher than 6.0, whereas the Prt⁺ grew faster at lower pH values (Fig. 3). These results show that the pH-dependent selection for Prt⁺ and Prt⁻ in *S. cremoris* $Wg_2$ is also observed in another strain, *S. cremoris* $E_8$, which is quite different with respect to plasmid content (data not shown), specific growth rate (7), proteolytic system (6), and cell wall composition (8).

The observations with MRS medium prompted us to investigate whether the pH effect would also occur in milk cultures. Since continuous cultivation on this medium was not possible with the apparatus used in these experiments, the studies described in Fig. 1 were repeated with acidified milk of pH 5.9. The fraction of Prt⁻ variants seemed to decrease slowly in the cultures of strain $Wg_2$ and remained

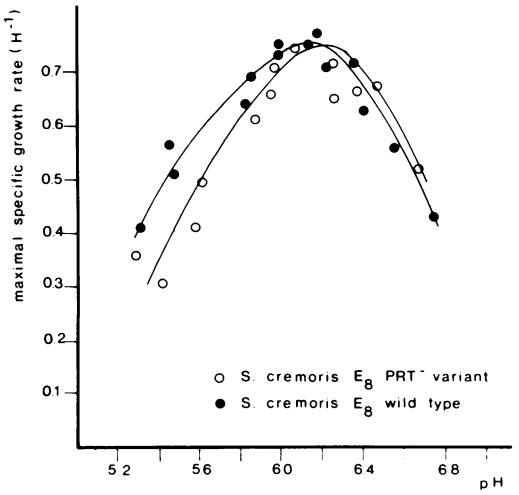


FIG. 3. Maximal specific growth rates of *S. cremoris* $E_8$ of Prt⁺ and Prt⁻ variants in MRS medium at different external pH values.

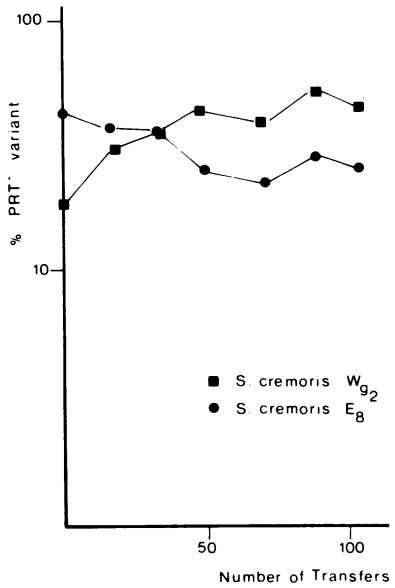


FIG. 4. Ratio of Prt⁻ variants in pure cultures of *S. cremoris* $Wg_2$ and $E_8$ during serial transfer in fresh, acidified, reconstituted skimmed milk.

more or less stable in the cultures of strain $E_8$ during the numerous transfers on this medium (Fig. 4). Although the spread in numbers was occasionally rather large, the Prt⁻ variants never became dominant in all of the experiments performed with acidified milk. This shows that the pH effect also occurs in milk as the growth medium.

**Proton motive force in *S. cremoris*.** The pH effect is the result of the Prt⁻ variants being restricted in growth at low pH values, whereas they grow faster than the wild types at pH values above 6.0. The increased growth rate of the Prt⁻ variants at higher pH values can be expected, since under these conditions these variants do not have to spend metabolic energy for useless plasmids and useless proteases. One reason for restricted growth of the Prt⁻ variants at low pH could be an impaired regulation of the internal pH or of the proton motive force of these cells (9). Therefore, the internal pH and the proton motive force of the Prt⁺ and Prt⁻ cells were measured at two different external pH values (Table 2). No significant differences in internal pH, $\Delta$pH, or $\Delta\psi$ could be measured between the two variants growing in MRS medium.

**Amino acid limitation.** On some growth media pH-dependent selection of Prt⁺ and Prt⁻ variants was not found. In synthetic RFP medium the maximal specific growth rates of the Prt⁻ variant were higher than those of the wild type over the whole pH range (Fig. 5). When both Prt⁻ and Prt⁺ variants were inoculated into a chemostat the Prt⁻ variant

TABLE 2. Internal pH, $\Delta$pH, $\Delta\psi$, and $\Delta p$[a] of *S. cremoris* $Wg_2$ Prt⁺ and Prt⁻ variants in MRS medium at pH 5.7 and 6.4

| Phenotype | Medium pH | Internal pH | $-Z\Delta pH$[b] (mV) | $\Delta\psi$ (mV) | $\Delta p$ (mV) |
|---|---|---|---|---|---|
| Prt⁺ | 5.7 | 6.5 | −46 | −70 | −116 |
| Prt⁺ | 6.4 | 7.0 | −36 | −81 | −117 |
| Prt⁻ | 5.7 | 6.6 | −52 | −76 | −128 |
| Prt⁻ | 6.4 | 7.1 | −40 | −80 | −120 |

[a] $\Delta p = \Delta\psi - Z\Delta pH$.
[b] $Z$, 60 MV at 30°C.

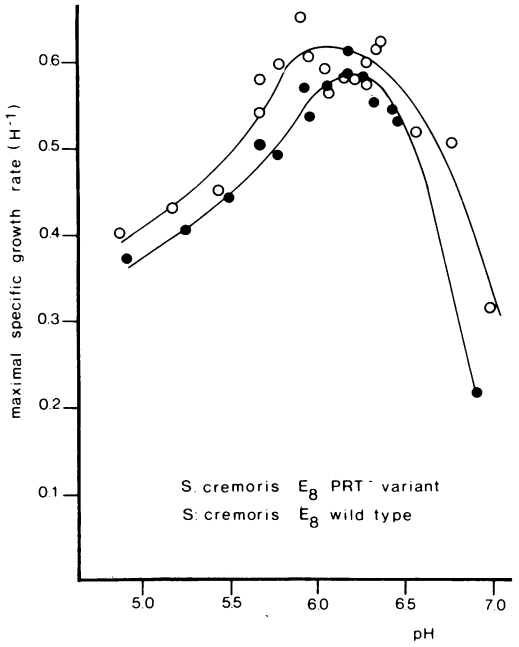


FIG. 5. Maximal specific growth rates of *S. cremoris* $E_8$ $Prt^+$ (●) and $Prt^-$ (○) variants in RFP medium at different external pH values.

became dominant at all pH values of the medium and at all dilution rates under lactose limitation. In Fig. 6 an example is presented for *S. cremoris* strain $E_8$ at a dilution rate of 0.1 and a pH of 5.9.

When *S. cremoris* HP and $E_8$ were cultivated in RFP medium under amino acid limitation the situation was quite different. This is shown for strain $E_8$ under leucine limitation (Fig. 6). A pure culture of the $Prt^+$ variant of this strain was used as starter material. In this culture low numbers of $Prt^-$ variants were already present. During cultivation at a dilution rate of 0.1 the percentage of $Prt^-$ variants remained low (±5%) during the whole period of measurement. In another experiment a pure culture of the $Prt^-$ variant of *S. cremoris* $E_8$ was grown under leucine limitation and then inoculated into a similar $Prt^+$ culture at a ratio of 99 to 1. When this mixture was grown in continuous culture under leucine limitation the ratio of $Prt^-$ variants gradually decreased to, again, 5% (Fig. 6). Similar results were obtained for *S. cremoris* HP under leucine limitation and for *S. cremoris* $E_8$ and HP under glutamate limitation (data not shown). It appears that the $Prt^+$ cells have a lower affinity constant ($K_s$) for amino acids than the $Prt^-$ cells. The $Prt^-$ variants would disappear from the cultures completely if not for a continuous generation of $Prt^-$ variants from the $Prt^+$ cells by loss of plasmids. At the ratio 95% $Prt^+$ to 5% $Prt^-$ an equilibrium was apparently reached between the washout rate and the rate of generation of $Prt^-$ cells.

When the experiments under leucine limitation were carried out at high dilution rates ($D$ = 0.5), the situation was reversed. Since these conditions resemble batch cultivation with all growth substrates present at high concentrations, the $Prt^-$ variants with their higher maximum specific growth rates (Fig. 5) became dominant (Fig. 6).

**Low-casein medium.** Another obvious way to select for $Prt^+$ variants in continuous cultures is by growing the streptococci on media containing casein as the amino acid source. This was done with RFP medium in which the amino acid mixture was replaced by 0.4% casein. The $Prt^-$ variants can only grow on this medium in the presence of $Prt^+$ cells, and so the situation resembles the growth of these mixtures in milk. The actual concentration of casein in this medium (0.4%) was much lower than the concentration in milk (±3%), and a higher percentage of $Prt^+$ cells was expected to persist in the cultures for the necessary proteolysis than in the case of milk cultures (Fig. 1); this was indeed found. For *S. cremoris* strains $E_8$ and HP an apparent steady state was reached, with approximately 30% of the total population being $Prt^-$ during cultivation in a chemostat under lactose limitation at a dilution rate of 0.1 and a medium pH of 6.2. At a higher dilution rate ($D$ = 0.4) the ratio of $Prt^-$ variants remained at an even lower level, ±4%. These results show that the fraction of $Prt^-$ variants can be regulated by changing the dilution rate of the continuous culture.

## DISCUSSION

The proteolytic activity of starter bacteria plays a central role during cheese manufacture (11). It is, however, an unstable property in *S. cremoris*, the main lactic acid bacterium in Dutch starters. Protease-negative variants are generated spontaneously in starter cultures and can reach large numbers under certain growth conditions (3, 18; Otto, Ph.D. thesis). This leads to lower activity of the starters (Table 1). In this paper three different approaches are described to prevent this decrease in acidification rate of the starters by applying culture conditions that favor the growth of $Prt^+$ variants.

The first approach is the pH-dependent selection of $Prt^+$ or $Prt^-$ variants in starter cultures as suggested by Otto et al. (18). They found that for *S. cremoris* $Wg_2$ the $Prt^+$ variant was at a selective advantage over the $Prt^-$ variant when the cultures were grown at pH values lower than 6.0. Under these conditions $Prt^+$ percentages of 100% could be maintained. This pH-dependent selection could offer interesting possibilities for the stabilization of the proteolytic activity in starter cultures if the pH effect would occur in all strains present and with the growth media that are used for cultiva-

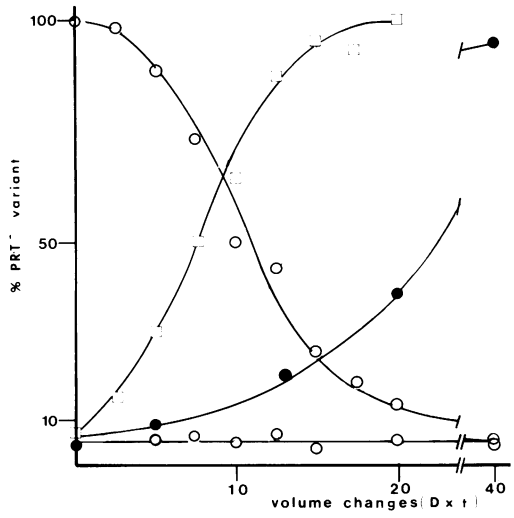


FIG. 6. Ratio of $Prt^-$ variants in cultures of *S. cremoris* $E_8$ during continuous cultivation at a dilution rate of 0.1 (□, ○) or 0.5 (●) in RFP medium under lactose limitation (□) or leucine limitation (○, ●).

tion of starters. We have investigated this by studying the growth of *S. cremoris* $E_8$, which is quite different from strain $Wg_2$ (6, 7), at different pH values in batch and continuous cultures. The selection of $Prt^+$ variants at low pH values was also found in this strain and in several growth media, including milk. However, it was not found in all growth media. In synthetic RFP medium the $Prt^-$ variant grew faster at all pH values (Fig. 5). Differences in the MRS and RFP growth media did not give any indication about the factors which determine the pH-dependent selection in MRS medium and in milk. The potassium ion concentration in MRS (22 mM) is much lower than that in RFP medium (51 mM). This ion has been reported to play an important role in the regulation of the internal pH of bacteria (20). Also acetate, which as a weak acid could dissipate the ΔpH at low pH values in the medium, is present at much higher concentrations in MRS medium (42 mM) than in RFP medium (14 mM). However, even in MRS medium the internal pH (and ΔpH) of the $Prt^-$ variant at lower medium pH values (pH 5.7) was almost identical to that of the wild type. Clearly, both variants have the same problem in keeping the internal pH at a constant level. Other clear differences between MRS and RFP media are the nature and concentration of the amino acids required for growth. RFP medium is a synthetic medium to which all amino acids are added separately. MRS medium is a complex medium containing many peptides and small proteins besides free amino acids. Peptides have a favorable effect on the growth of streptococci (21), but this effect is the same for $Prt^+$ and $Prt^-$ variants (Hugenholtz, unpublished results). The glutamate concentration in MRS medium (20 mM) is much higher than that in RFP medium (5 mM). This amino acid is reported to inhibit growth of streptococci at millimolar concentrations (Otto, Ph.D. thesis), but also this inhibitory effect is the same for $Prt^+$ and $Prt^-$ variants. The physiological background of the pH-dependent selection remains unresolved.

An alternative way to select for $Prt^+$ variants was to grow the streptococci under amino acid-limited conditions. When *S. cremoris* HP and $E_8$ were grown in RFP medium under leucine or glutamate limitation at dilution rates of 0.1 and 0.05, respectively, the numbers of $Prt^-$ variants remained low over extended periods at all medium pH variants. This is in contrast to what was found in RFP medium under lactose limitation. Even when the cultures were inoculated with high numbers of $Prt^-$ variants, the percentage of $Prt^-$ variants still slowly decreased from the original 99% to about 5%. It seems that the $Prt^+$ cells have a higher affinity for amino acids than the $Prt^-$ cells. When the dilution rate was increased to values approaching the $\mu_{max}$ of the streptococci ($D = 0.5$), the situation was reversed. The $Prt^-$ variants with their higher maximum specific growth rates (Fig. 5) now became dominant in the cultures. Here we have, again (6), an example of crossing $\mu$-$S$ curves for *S. cremoris*.

Another growth medium that favors the growth of $Prt^+$ variants in continuous culture is RFP medium with casein as the amino acid source. When low amounts of casein (0.4%) were added to the medium the percentage of $Prt^+$ in the culture remained at a relatively high level (70%). Under lactose limitation the $Prt^-$ variant has a selective advantage which would result in a slow decrease of the percentage of the $Prt^+$ variant. This would, however, lead to an amino acid limitation since too little casein would be hydrolyzed by the remaining $Prt^+$ cells. Under an amino acid limitation the $Prt^+$ variants again are at a selective advantage. This constant variation of amino acid- or lactose-limiting conditions should result in oscillations of the percentages of $Prt^+$ and $Prt^-$; this was not found. The percentages of $Prt^+$ were fixed at 70% at a dilution rate of 0.1 and nearly 100% at a dilution rate of 0.4.

The stability of the protease-positive streptococci at low pH values in several growth media, under amino acid limitation at low dilution rates, or in media containing low concentrations of casein as the nitrogen source makes it possible to study the physiology of these important bacteria in continuous culture without changes in the proteolytic properties of the bacteria. This has not been possible until now. Most studies requiring stable continuous cultures of *S. cremoris* have been performed with $Prt^-$ variants (17, 19). For the study of $Prt^+$ variants, especially with respect to the physiology of the proteolytic system, the described culture methods will be very useful. For the cultivation of starter cultures that are used in the cheese manufacture, continuous cultures are not yet applied in practice. In batch culture the pH-dependent selection described above can be used to stabilize the proteolytic activity of the starters by using acidified milk as the growth medium (Fig. 4).

## ACKNOWLEDGMENTS

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