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Proteinase Activity in Slow Lactic Acid-Producing Variants of *Streptococcus lactis*

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Variants of *Streptococcus lactis* that produce lactic acid slowly in milk were isolated by inducing plasmid loss in the wild type at 39 to 40 C. Such strains had lost most of their surface-bound proteinase activity and were designated *prt*$^-$. The specific proteinase activities of *S. lactis* C10 *prt*$^+$ whole cells and solubilized cell walls were 7 and 18 times, respectively, those of the *prt*$^-$ strain, but spheroplast lysates of *prt*$^+$ and *prt*$^-$ strains contained similar proteinase activity. *S. lactis* H1 showed a similar relative distribution of activity between *prt*$^+$ and *prt*$^-$ cellular fractions, although the overall level was lower. The limited growth in milk, characteristic of *prt*$^-$ strains, can be explained in terms of their low surface-bound proteinase activity.

Streptococcus lactis and *S. cremoris* may spontaneously segregate slow variants that differ from the parent strain by their limited growth in milk (5). Such variants appear in susceptible cultures at high frequency (ca. 1%) and do not revert to the parental type (2, 3, 22). As slow variants can also be induced when the wild-type strain is treated with acridines or grown at high temperatures, this characteristic is believed to arise through loss of a plasmid (14).

Evidence presented by a number of workers strongly suggests that slow variants are deficient in proteinase activity. Growth in milk is stimulated to wild-type levels by the addition of hydrolyzed casein (4), and in broth media both variant and parent strain show the same rates of growth and acid production (2). Milk cultures may contain up to 50% slow variants before the rate of acid production differs from that of the parent. This suggests a growth stimulatory interaction between the parent and the slow variant (14). Although "slowness" and relatively low proteinase activity have been correlated (2), there has been little direct evidence for proteinase deficiency in slow variants. Westhoff et al. (22) compared proteinase activity in whole and fractionated cells of *S. lactis* 3 and a slow acid-producing mutant. Quantitative differences in proteinase activity between the parent and mutant strains, assayed using either whole cells or cell fractions, were low (ca. 1.5-fold) and did not adequately explain the growth characteristics of slow variants in milk. The mutant "intracellular" enzyme, however, did differ from that of the parent, and it was concluded that a different proteinase specificity was responsible for the limited growth of the mutant in milk (21).

This study compares the proteinase activity of two strains of *S. lactis* (*prt*$^+$) with that of slow variants derived from them (*prt*$^-$). The behavior of the *prt*$^-$ strains in milk can be accounted for by the loss of most of their surface-bound proteinase activity.

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MATERIALS AND METHODS

Bacteria and growth media. *S. lactis* H1 *prt*$^+$ and *S. cremoris* R1 *prt*$^+$ were obtained from the New Zealand Dairy Research Institute culture collection. *S. lactis* C10 *prt*$^+$ was obtained from W. E. Sandine, Department of Microbiology, Oregon State University, Corvallis. *S. lactis* 3 (fast and slow isolates) were obtained from M. L. Speck, Department of Food Science, North Carolina State University, Raleigh. *S. lactis prt*$^-$ strains were isolated at high frequency (up to 30%) after growth at 39 to 40 C. *Prt*$^-$ clones developed as tiny colonies on citrate-milk agar (16) and were differentiated from the larger *prt*$^+$ colonies on this medium. *S. cremoris prt*$^+$ and *prt*$^-$ could not be differentiated on this medium. Optimal differentiation was obtained when the medium was autoclaved at 115 C for 15 min. *prt*$^-$ clones selected were *lac*$^+$ on the medium of McKay et al. (9) and were sensitive to the same virulent phages as the parent.

Skim milk was prepared from a single batch of spray-dried nonfat milk powder, reconstituted to 9.5%

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total solids and autoclaved at 10 lb/inch² for 20 min. Plate counts were obtained using M_{16} agar (8) and T_5 broth (20) was used for cell preparation and growth experiments.

Lactate determination. Lactate was measured as the lactate-ferric chloride complex at 400 nm (17).

Cell fractionation and enzyme assay. The methods used were those of Thomas et al. (20). Cells growing logarithmically in T_5 broth were harvested by centrifugation, lysed by enzymatic or mechanical methods, fractionated, and assayed for proteolytic activity using ^{125}I-labeled casein as substrate.

RESULTS

Growth of prt⁺ and prt⁻ in sterile milk and T_5 broth. Doubling times of *S. lactis* C10 *prt*⁺ and *prt*⁻ during exponential growth in sterile milk at 30 C were 60 and 72 min, respectively (Fig. 1). C10 *prt*⁺, however, reached a maximum population of 2×10^9 colony-forming units (CFU)/ml in 7 h, whereas C10 *prt*⁻ ceased exponential growth after 5 h and reached a maximum population density of 5.5×10^8 CFU/ml after 10 h. Both *prt*⁺ and *prt*⁻ strains remained as diplococci throughout growth. Although the viable count of C10 *prt*⁻ remained stationary after 10 h, acid production continued at a slow rate, and the milk reached pH 5.0 after 35 h of incubation. That is, C10 *prt*⁻ cells continue to produce lactate while colony-forming units fail to increase (Fig. 2). The addition of trypsin-hydrolyzed casein (1 mg/ml) to cultures of C10 *prt*⁺ or C10 *prt*⁻ growing in skim milk decreased the doubling time to 54 min for each strain, and both reached a maximum population density of 3×10^9 CFU/ml.

In T_5 broth, C10 *prt*⁺ and *prt*⁻ were indistinguishable and showed the same growth rate (doubling time 38 min, maximum population 1.2×10^9 CFU/ml). This feature of growth was common to all *prt*⁻ strains isolated in this laboratory and can be clearly seen in growth curves for *S. lactis* H1 and *S. cremoris* R1 in T_5 broth (Fig. 3). The slow acid-producing mutant of *S. lactis* 3 grew at a significantly slower rate than the wild type in T_5 broth. Doubling times during logarithmic growth were 78 and 63 min, respectively. A *prt*⁻ derivative of strain 3 was isolated and found to have identical growth characteristics to the parent strain in broth.

Proteinase in whole cells and cell fractions. Intact cells of C10 *prt*⁺ exhibited about seven times the proteinase activity of C10 *prt*⁻, specific activities being 35.8 and 5.2 U per mg dry weight, respectively (Table 1). When the cell wall was removed under conditions that gave insignificant cell lysis (20), the majority of the *prt*⁺ proteinase activity was released. The same treatment removed less than half the *prt*⁻ activity, the relative activity (*prt*⁺:*prt*⁻) being ca. 18:1. No activity could be detected in membranes from either C10 *prt*⁺ or C10 *prt*⁻. The slightly higher intracellular activity de-

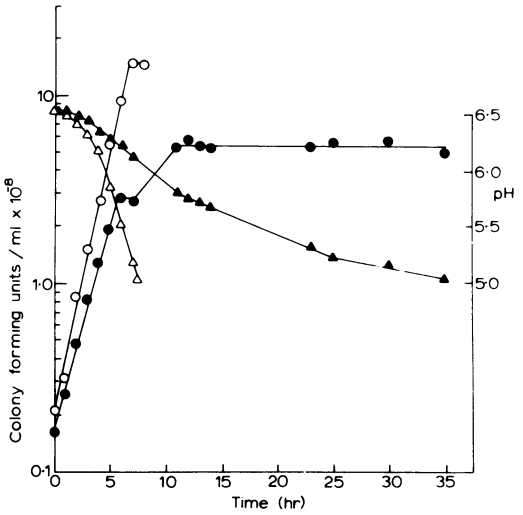

FIG. 1. *Growth of S. lactis C10 prt⁺ and prt⁻ in skim milk at 30 C. Two hundred-milliliter volumes of skim milk were inoculated with 2 ml of C10 prt⁺ and 8 ml of C10 prt⁻, respectively. Inocula were from 16-h, 22 C skim milk cultures. The inoculated milks were divided into portions and incubated. At intervals samples were removed for pH measurement (Δ, prt⁺; ▲, prt⁻); the culture was then chilled, diluted, and plated for colony-forming units (○, prt⁺; ●, prt⁻).*

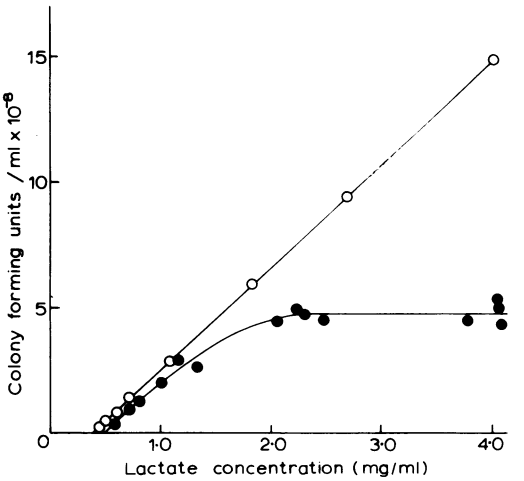

FIG. 2. *Relationship between viable count and acid production of S. lactis C10 prt⁺ (○) and prt⁻ (●) in skim milk at 30 C. Cultures and sampling were as in Fig. 1; lactate and colony-forming units were determined at intervals.*

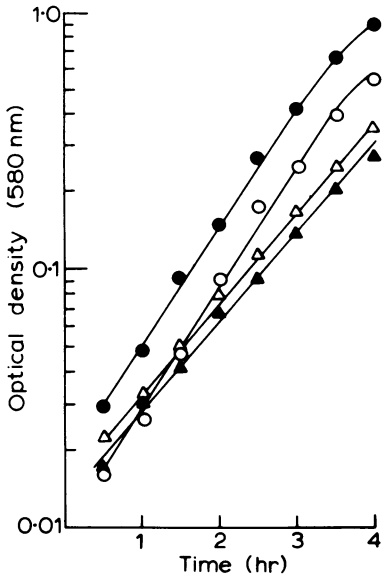

FIG. 3. *Growth of S. lactis H1 prt⁺ (○), prt⁻ (●), and S. cremoris R1 prt⁺ (△), prt⁻ (▲) in T_6 broth at 30 C. Two hundred-milliliter broth volumes were each inoculated with the appropriate strain (1% inoculum from 16-h, 22 C cultures), divided into portions, and incubated. Samples were removed at intervals for optical density measurements using a Bausch & Lomb, Inc., Spectronic 20 colorimeter at 580 nm.*

TABLE 1. *Relative proteinase activity in S. lactis C10 prt⁺ and prt⁻ whole cells and in fractions prepared by osmotic lysis*

Fraction	Specific proteinase activity (U/mg dry weight or equivalent)[a]		Relative activity (*prt⁺*: *prt⁻*)
	prt⁺	*prt⁻*	
Intact cells	35.8	5.2	6.9
Solubilized cell walls[b]	47.8	2.6	18.4
Spheroplast lysate[c]	9.0	5.6	1.6
Plasma membrane	ND[d]	ND	

[a] A unit of proteinase activity is defined as the enzyme concentration that solubilized 1% of the substrate in 6 h.

[b] Mid-log cells were washed twice in 0.2 M phosphate buffer, pH 6.4, suspended in spheroplasting medium (0.5 M sucrose, 20 mM $MgCl_2$, 0.2 phosphate buffer, pH 6.4), and 3 ml phage-associated lysin was added. The suspension was incubated at 30 C for 120 min and centrifuged 35,000 × *g*, and the supernatant was assayed (20).

[c] 35,000 × *g* pellet of solubilized cell walls, resuspended in buffer of equivalent volume to the original suspension.

[d] Not detectable.

tected on lysis of *prt⁺*, as compared with *prt⁻* spheroplasts, is not considered significant.

C10 *prt⁺* and *prt⁻* were also fractionated after mechanical disintegration. The pellet containing cell walls and membranes (35,000 × *g*, 10 min) contained 75% of the *prt⁺* activity recovered in the component fractions but only 27% of the *prt⁻* activity. Specific activities were 18.6 and 1.3, respectively. Half of the *prt⁻* proteinase activity was associated with the cytoplasm (not sedimented at 157,000 × *g*, 120 min), whereas in the parent this fraction contained only one-tenth of the activity.

Intact cells of *S. lactis* H1 *prt⁺* (specific activity, 17.0) had less surface-bound proteinase activity than C10 *prt⁺* (specific activity, 35.8), but the differences between *prt⁺* and *prt⁻* followed an identical pattern to that observed with C10. Specific activities of solubilized cell walls were 16.8 (*prt⁺*) and 3.6 (*prt⁻*); those of the spheroplast lysates were 7.9 (*prt⁺*) and 10.7 (*prt⁻*).

DISCUSSION

The growth of *prt⁻* strains in sterilized milk and in broth follows the pattern established by other workers (2). *prt⁻* strains grow and produce lactic acid slowly in milk, but both characteristics can be restored to normal levels, or better, by supplementing the milk with casein hydrolysate (4). *prt⁺* and *prt⁻* are indistinguishable when grown in rich broth media. The slow lactate increase in milk without increase in viable count was, however, of particular interest. Dissociation of acid production from net growth has been reported in other systems and is probably widespread. The phenomenon appears to be associated with conditions of cellular stress. Cultures of *S. faecalis* approaching the growth-limiting pH have been reported to cease dividing before acid production is inhibited (10). Lowrie et al. (7) have also observed that *S. cremoris* AM2 ceases to divide but continues to produce acid when growth is initiated at 30 C and the incubation temperature is raised to 37.8 C. Depletion of available nitrogen in milk cultures of *prt⁻* bacteria appears to be a further means by which this effect can be induced.

Variants of lactic streptococci that produce acid slowly in milk have been isolated and studied in a number of laboratories (2, 14, 22). Although these can normally be isolated at high frequency, not all slow acid producers are of the *prt⁻* type. It is not uncommon to find mutants that, for some other reason, grow more slowly in milk than the parent. These mutants also grow more slowly in broth, and the *lac⁻* mutants are

one such group (9). It is essential therefore to screen putative *prt*⁻ clones for growth rates in a broth medium where proteolytic activity is not essential for growth. The slow acid-producing strain of *S. lactis* 3 does not appear to be a *prt*⁻ type on the basis of its slow growth in T_5 broth. This was confirmed when a *prt*⁻ derivative of strain 3 was isolated that grew in broth at an identical rate to the parent strain.

The enzyme assays clearly show the fundamental difference in proteinase activity between *prt*⁺ and *prt*⁻ strains of *S. lactis*. The major portion of the proteinase activity in the parent strain has been shown to be localized near the cell surface using two methods of fractionation (20). Mechanical disruption and osmotic lysis both gave similar high levels of activity in fractions derived from the cell wall. In the present study, this activity has been found to be markedly reduced in *prt*⁻ cells. *S. lactis* H1 had less total proteinase activity than strain C10 with a consequent reduction in relative activity between *prt*⁺ and *prt*⁻. The solubilized cell wall fraction from *prt*⁺, however, still had nearly five times the activity of the corresponding *prt*⁺ fraction. The low proteinase activity in *prt*⁻ strains explains their limited growth in milk (see Fig. 1). It is likely that the maximum *prt*⁻ cell count is determined largely by the initial amino acid and small peptide content of the sterilized milk.

The specific activities of C10 *prt*⁺ and H1 *prt*⁺ spheroplast lysates showed that both parent strains carried a portion of their proteinase activity in fractions not associated with surface structures. The slight difference in levels of intracellular proteinase activity between *prt*⁺ and *prt*⁻ in this respect is not considered significant. These intracellular enzymes may be responsible for degradation of peptides resulting from protein breakdown by the surface enzyme, as well as the turnover of endogenous nitrogen. *Escherichia coli*, for example, has at least eight distinct intracellular peptidases (18).

Cells grown in broth were used for the present study due to the difficulty of harvesting bacteria from milk. The higher levels of available amino acids in broth may repress surface-bound proteinase activity, as has been reported with other extracellular proteinase systems (1, 6, 12, 13). Hence, the differences found between *prt*⁺ and *prt*⁻ strains in broth are probably an underestimation of the situation in milk.

Westhoff et al. (22) reported that both "intracellular" and "membrane-associated" proteinase activities in *S. lactis* 3 were reduced by 30 to 35% in a slow acid-producing mutant. As the authors commented, such a difference might be expected to impair rather than prevent growth in milk. The only other difference between the two strains was an altered specificity of the intracellular enzyme (21). The present study shows that their conclusions cannot be applied to all slow lactic acid-producing strains of lactic streptococci.

The loss of most of the surface-bound proteolytic activity accompanying the transition from *prt*⁺ to *prt*⁻ is consistent with plasmid control of this character. Total proteolytic activity in lactic streptococci, however, is low compared with that of corresponding enzymes in some other bacteria (11). The proteinase character has not been recorded as a genetic marker in *E. coli* or *Salmonella* chromosome maps (15, 19), however, and is possibly also plasmid linked in the enterobacteria. The fact that proteinase activity has been recognized and studied in the lactic streptococci is undoubtedly a consequence of the widespread use of milk as a culture medium for this group.

The low level of cell-bound activity remaining in intact cells of C10 *prt*⁻ is presumably determined by chromosomal genes as the possibility of significant cell lysis has been excluded (20). If the surface-bound proteinase is plasmid controlled, it is possible that it controls cellular activities other than proteinase synthesis. The transport of peptides has not been excluded, and further investigation will be required to clarify these points and physically identify the genetic element involved.

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