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Dissociation and Stability of *Lactococcus lactis* Subsp. lactis 357 Culture, with Respect to Sucrose-Fermenting Ability and Exopolysaccharide Production

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Abstract

Strain 347 was found to harbour two subpopulations differing in their ability to utilize and ferment sucrose and to produce an exopolysaccharide on sucrose-containing media. The Suc^+Eps^+ (ts) variant (designated 347-S) grows poorly in milk, is defective in lactose and casein utilization, and does not coagulate milk. In contrast, the STRAIN 347-L, a sucrose metabolism and polysaccharide-deficient variant, (Sud^dEps^d variant 347-L) is an efficient lactic starter. Both variants segregate the opposite type (though at different frequencies), thus exhibiting a phase variation-like behavior. Mixtures of L- and S-strains develop the original stable symbiotic substrain mixture containing about 3–4% of the S-type. Over the course of 3 years propagation in milk, the subpopulation ratio in strain 347 did not change appreciably.

Keywords: dissociation, instability, Lactococci, sucrose fermentation, exopolysaccharide production

1. Introduction

Single-strain, "pure" cultures of lactococci have often been reported to encompass sizeable subpopulations differing in various technologically important properties (Limsowtin et al., 1978). However, only the occurrence of fermentation defective ("slow") subpopulations in the fermentation-proficient ("fast")

starter cultures have been investigated in detail. It has been observed that commercial single-strain starters (used in production) as well as laboratory pure strains may contain up to 80% of metabolically defective (Lac^d, Prt^d or Lac^d/Prt^d) subpopulations (King et al., 1983, Huggins and Sandine, 1984, Stadhouders et al., 1988). The origin of these slow substrains is usually ascribed to the spontaneous loss of the metabolic plasmid(s) (Davies and Gasson, 1981). The subsequent coexistence of the mixed fast/slow population is supposed to be supported by the fast component, which provides the slow one with necessary nitrogenous nutrient factors, such as amino acids and peptides (Hungenholtz, 1986; Hungenholtz et al., 1987).

In this study we describe a different type of a stable symbiotic coexistence of two (fast/slow) subpopulations within a single-strain culture of *Lactococcus lactis* subsp. lactis. One of the substrains is an efficient fast-fermenting lactic starter, whereas the other is a slow-coagulating variant, metabolically deficient when grown in milk. However, it exhibits the ability to efficiently metabolize sucrose and to produce an exopolysaccharide in sucrose containing media. The slow substrain segregates the fast variant and vice versa, thus exhibiting a kind of the "dissociation" or "phase variation" phenomenon.

2. Materials and Methods

Organism: *Lactococcus lactis* subsp. lactis strain 347 (Lactoflora, Collection of Dairy Cultures, MILCOM, Prague).
Media: M17 broth containing 0.5% lactose (M17LB) or 0.5% saccharose (M17SB); Bouillon no. 2 (Immuna, S. Michalany). M17 agar (Difco) containing 0.5% lactose (M17LA), 0.5% sucrose (M17SA). A modified formula of M17 agar from which the β-phosphoglycerate buffer was omitted and 0.025% of bromcresol purple added was used as indicator agar. Glucose-tryptone-yeast extract agar (GTY; MILCOM, Tabor). Reconstituted non-fat dry milk ("Instant skim milk" Dairy Industries, Srakonice).

Growth was measured as OD at 590 nm using Specol EXI (GDR). Colony count was performed by plating appropriate dilutions in peptone-saline onto M17LA and incubating at 30°C for 72 hr. pH was assayed using the pH-meter Radelkis (Hungaria). Colony dimensions were measured under the microscope (Zeiss, Jena, GDR) using a calibrated occular and a calibrated slide micrometer scale (Zeiss, Jena).

The cultures were propagated in reconstituted non-fat dried milk (SM) by transfer at 10 day intervals. Following inoculation (using 1% inocula), the cultures were incubated at 30°C for 16–18 hr, and kept at 5–6°C until the next transfer.

3. Results

Isolation of substrains 347-S and 347-L

Initially, the strain 347 of *L. lactis* subsp. lactis was obtained as a lyophilised specimen from the culture collection. It was revitalised in SM and subsequently propagated as described in Methods. Its intrinsic heterogeneity was revealed when the cells were plated on the surface of M17LA containing 0.25 M saccharose as an osmotic stabilizer. Two types of colonies developed: Large white, opaque colonies, and small, greyish, flat colonies, similar to those formed by other strains on this medium. Previously, heterogeneity of this strain had not been suspected, because it did not show any anomalies in colonial morphology upon regular checks for culture purity using M17LA.

The initial substrain composition of the milk culture, in which the dissociation had been detected, 8% of the large-colony variant, changed over the course of three years passaging to 3–4%. The large-colony variant (designated 347-S) was found to ferment sucrose, whereas the small-colony substrain (designated 347-L) did not express sucrose fermentation in the indicator agar. Isolates from single colonies of 347-S and 347-L were subsequently passaged on M17LA and their growth characteristics in lactose-, sucrose- and glucose containing media and in milk were compared.

Growth of 347-L and 347-S substrains on sugars and in milk

In contrast to their characteristics on sucrose containing agars (M1LA, M17SA or GTY supplemented with 0.5% saccharose), colonies of 347-S on lactose or glucose agars (M17LA, GTY) were indistinguishable from those of 347-L with respect to the colony size and appearance. On sucrose-lactose or sucrose-glucose agars the size as well as the morphology of colonies was the same as on sucrose agar, indicating that sucrose was the sugar predominantly utilised.

Growth characteristic on M-17 broth containing different sugars are shown in Figs. 1–3. Original strain 347 exhibited standard growth characteristics for lactococci. The growth rate was depressed after approximately 6 hr on galactose, glucose, fructose, and lactose. Xylose, manose and starch were not utilized, but in broth containing sucrose the growth continued up to 24 hr (Fig. 1). The partial growth on all media was caused by utilisation of amino acid from the broth. Strain 347-L which is characterised as an efficient lactic starter exhibited similar characteristics for all sugars except sucrose which was not fermented (Fig. 2). On the other hand, strain 347-S's minimal growth in the first 6 hour period may be caused by an inability to utilize amino-acids as

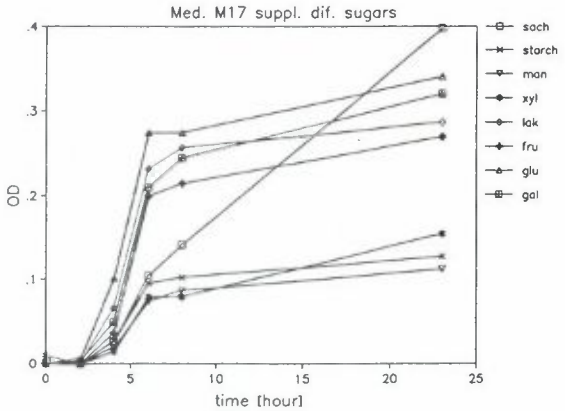


Figure 1. Growth of strain 347 orig. (Med. M17 suppl. dif. sugars)

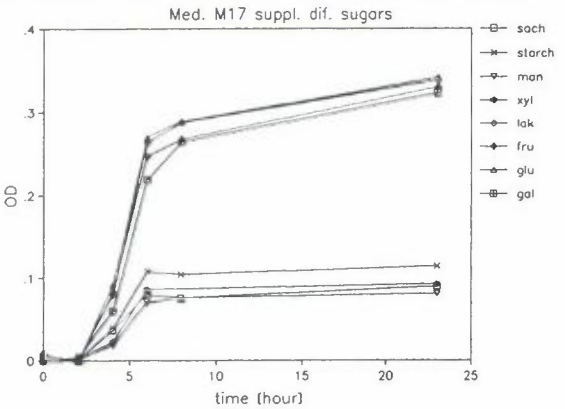


Figure 2. Growth of substrain 347L (Med. M17 suppl. dif. sugars)

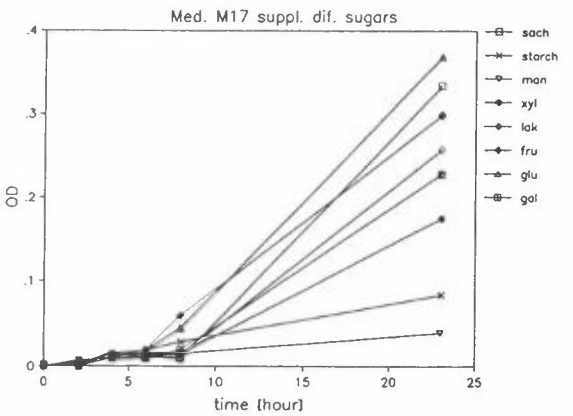


Figure 3. Growth of substrain 347-S (Med. M17 Suppl. dif. sugars)

a carbon source (in correlation with its inability to coagulate milk as shown in Table 1). But strain 347-S is able to stepwise utilize all sugars (sucrose and glucose are utilised efficiently while the utilisation of lactose, fructose and xylose is greatly repressed), starch and manose are not utilised (Fig. 3).

347-L acidified milk rapidly and coagulated milk in less than 24 hr at 21–22°C (Table 1). 347-S did not coagulate milk in 5 days at 21°C or 30°C and exhibited a strongly decreased lactose-fermenting activity. The coagulating activity and growth yield of 347-S could be improved by supplementation of SM with 0.25–0.5% yeast extract. A loose precipitate (but not a firm curd) was formed and the growth yield increased by 50–60%.

Dissociation of substrains and establishment of symbiosis between 347-S and 347-L strains

Cultures of initially "pure" single colony isolates from 347-S and 347-L cultures, and a mixture of two such pure cultures of S and L types were propagated by daily transfers at 30°C in milk. The composition of subcultures was monitored at intervals by plating onto the surface of M17SA. In subcultures of mixed L and S strains, the initially high percentage of the S-variant rapidly declined and through approximately 10 transfers it settled at a value similar to that characteristic of the parent strain 347 (Fig. 4).

Isolates from 347-S propagated either in milk or on M17LA exhibited a relatively rapid segregation of the L-variant, however the dissociative tendency apparently varied among individual isolates. While some colonies contained L-type cells already upon isolation, other isolates showed no segregation until the 2nd–5th subculture (Table 2).

Table 1. Growth of strains 347-L and 347-S on M17 agar and in M17 broth supplemented with lactose and/or sucrose and in milk at 30°C

Strain	Diam. of colony on agar [mm] 48 hr		Growth yield in broth [%]		Growth in milk after 24 hours	
	lactose	sucrose	lactose	sucrose	coagulation at 21°C	CFU/ml
347-orig	4.5	4.2/7.9	100	100	+	2.4E9
347-L	4.8	5.6	104	16	+	3.4E9
347-S	4.7	8.9	23	82	–	4.8E8

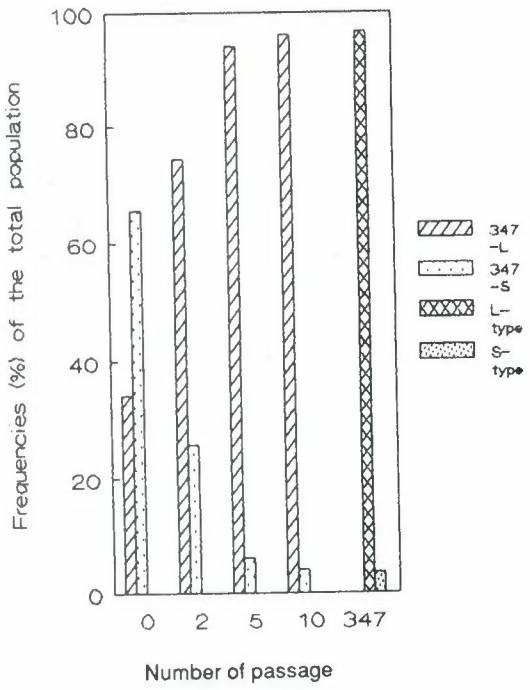


Figure 4. Ratio of strains 347-S and -L in mixture through 10 passages (% of population)

The L-type isolates proved to be considerably more stable, segregating S-type colonies at low frequencies and only after more than 5 passages in milk or on M17LA.

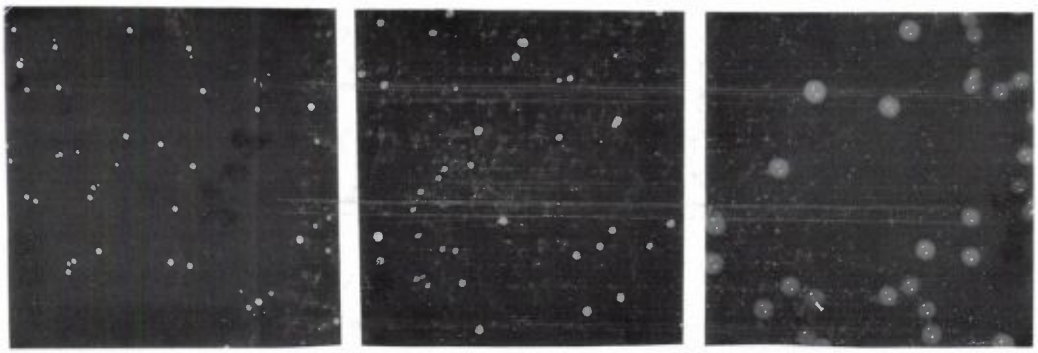
Slime formation on sucrose containing media

Cultures of 347-S on M17SA kept at 5–6°C for some time developed raised mucoid margins and some colonies became slimy. When grown at room temperature on sucrose agar, all colonies were mucoid. Of the five sugars efficiently fermented, only sucrose induced exopolysaccharide (EPS) formation (Fig. 5).

The EPS was of a thin, non-viscous consistency, EPS-producing cells were not encapsulated. EPS formation proved to be a temperature-sensitive process. Two isolates from a large mucoid colony of 347-S were passaged by daily transfers on M17SA at 21°C and 30°C and shifted at intervals to 35°C and 20°C respectively. Following 2,4 and 10 subcultures the non mucoid cells, grown at

Table 2. Dissociation of pure strains 347-S in milk (values are in percent)

	Strain 347-S1		Strain 347-SO	
Passage no.	L-type	S-type	L-type	S-type
0	29.9	70.1	< 1	> 99
7	78.2	21.8	82.2	17.8



(a) (b) (c)

(a) M17LA at 30°C; (b) M17SA at 30°C; (c) M17SA at 21°C

Figure 5. Growth of 347 on M17La and M17SA at 30°C and 21°C

35°C (the 347-S) lineage gave rise to white, slimy growth at 20°C. The 347-S (20°C) mucoid lineage produced no slime when grown at 35°C after an equal number of passages. No evidence for a rapid spontaneous loss of the Suc^+ phenotype or of the EPS-producing ability (Eps^+) was obtained upon plating the 347-S (35°C) subcultures in dilutions on M17SA.

4. Discussion

The Suc^+EPS^+ variant is definitely maladapted with respect to milk as a growth medium. It is metabolically inferior, and apparently also inferior in respect to frequency of segregation, to the L-variant. Nevertheless, it is capable of a stable coexistence with the latter nutritionally proficient and more stable strain. 347-S is capable of some growth in milk and its growth might be further supported by the proteolytic activity of the L-type, as in the case of plasmid-less slow variants mentioned in the Introduction.

From the fermentation experiments with strain 347, it was found that fermentation of sucrose is an inducible system. It is in correlation with results of Thompson and Chassy (1981). Strain 347-L lost its ability to ferment sucrose completely. On the other hand, strain 347-S is able to utilize not only sucrose but also lactose, galactose and xylose with lower efficiency after a lag time

of about 8 hr. Surprisingly, growth in media containing fructose or glucose starts after 6 hr lag-time. There is a question of relationship between long lag-time for glucose and lactose and strongly repressed proteolytic activity. Because of inaccessibility of some essential nutrients the metabolism of the organism is completely changed. It is interesting to note that with the change in metabolism, new ability for growth on xylose is revealed.

From the different lag times of sucrose and fructose growth curves it is possible to conclude that metabolism of sucrose and fructose are not linked, as in the case of *Lactococcus lactis* strain K1 presented by Thompson et al. (1991a). It is also supported by the fact that this strain is unable to produce the antibiotic nisin (Thompson et al., 1991b).

However, we hypothesize that the mechanism mediating the stable maintenance of the deficient variant might be a genetic process. Recently, it has become evident that many bacterial species have evolved genetic mechanisms that prevent extinction and ensure survival following sudden or fluctuating changes in the environment. These mechanisms, which operate at a frequency of one to several orders of magnitude higher than point mutations, enable the organism to simultaneously maintain two or more subpopulations, some of which may be maladapted to the momentarily prevailing conditions and which would be otherwise eliminated by natural selection. The behavior of the 347-S and -L variants upon subculturing on sucrose media is reminiscent of one such mechanism(s) termed phase variation, which has previously been described in other bacterial species. The adaptive value of keeping a subpopulation capable of a rapid and efficient sucrose utilization, and EPS formation, at low temperatures, is not initially obvious, particularly if the strain is passaged in the absence of sucrose, e.g. when grown in milk. However, 347 is an epiphyte (isolated from mountain grasses about 50 years ago), and the Suc^+Eps^+(ts) phenotype may be advantageous under some natural conditions.

In some other strains of Lactococci, the sucrose-fermenting ability was found to be associated with nisin-production and nisin-resistance characters ($Suc^+Nis^+Nis^r$ phenotype), and to be located on a transposon-like chromosomal element or perhaps on an integrative plasmid (Gasson, 1984; Gonzalez and Kunka, 1985; Steele and McKay, 1986; Donkersloot and Thompson, 1990). However, in our studies we were unable to detect any inhibitory activity amongst 20 L and S-type isolates tested against a nisin-testing strain of *Micrococcus luteus* and 5 laboratory strains of *Lactococcus lactis* subsp. cremoris.

It is interesting to note that in the "ropy" and "viili" lactococcal strains which produce a different kind of slime on lactose media and in which the Muc^+ determinant is plasmid-encoded, the production of slime appears to

be temperature sensitive and can be enhanced or repressed by lowering or increasing the temperature of incubation, respectively (Kontusaari and Forsén, 1989; Hunter, 1930).

In conclusion, our findings substantiate the expectations of lactococcal geneticists that technologically useful, essentially non-lactococcal properties could be introduced and stably maintained in lactococci together with classical lactic starter properties. Here we show that one way to realize this, at least in some natural strains, is a stable symbiosis between two subpopulations. These functionally divergent properties exhibited by the two forms may perhaps involve a phase variation-like (genetic) mechanism.

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