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Detection of Specific Strains and Variants of *Streptococcus cremoris* in Mixed Cultures by Immunofluorescence

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Antisera against four different strains of *Streptococcus cremoris* were raised by injecting rabbits with washed suspensions of whole cells. These antisera interacted specifically with the corresponding strain in a mixture of up to nine different *S. cremoris* strains. The antisera could be used for analyzing the composition of mixed cultures containing these strains by immunofluorescence. Competition experiments were performed in batch and continuous cultures under amino acid limitation. A bacteriophage-sensitive variant of *S. cremoris* SK11 (SK1128) could be distinguished from a bacteriophage-resistant variant (SK1143) by the same immunofluorescence technique. The competition between the two variants and the stability of both variants in pure cultures were followed with the specific antibodies. Antibodies against the purified proteolytic system of *S. cremoris* Wg2 were used to determine the presence of proteases by immunofluorescence in several *S. cremoris* strains under different culture conditions. The described immunofluorescence methods can be used to analyze complex mixed starter cultures common in the dairy industry as the strains and variants present in these mixtures can be recognized microscopically.

Starter cultures used in Dutch cheese manufacture are mixed cultures of high complexity. The most important lactic acid bacterium in these starter cultures is *Streptococcus cremoris*, although sometimes *Leuconostoc* species and other *Streptococcus* species can be present in significant quantities for the production of diacetyl and CO_2.

Many different strains and variants of *S. cremoris* make up the Dutch mixed starter cultures. These can vary strongly in the activities relevant for cheese manufacture such as proteolytic activity (11, 19, 22), growth rate (4, 20), phage resistance (14, 23), etc. Changes in the *S. cremoris* strain or variant composition in the starter during cultivation can have drastic effects on the acidification rate, flavor development, and susceptibility to bacteriophages during the process of cheese making.

Only a few attempts have been made to study population dynamics of mixed starter cultures. Most of these studies concentrated on the presence of proteolytically negative variants in the starters (3, 10, 13, 14, 18, 26). The process by which these variants become dominant and the actions which can be taken to prevent this have been described previously (28; J. Hugenholtz, R. Splint, W. N. Konings, and H. Veldkamp, Appl. Environ. Microbiol., in press).

The changes in the population of different strains of *S. cremoris* have not received much attention mainly due to the difficulty in recognizing individual strains in mixed cultures. Strain dominance has been reported occasionally, but a quantitative analysis has never been performed (4, 15, 37). We have reported a study on simple mixtures of different *S. cremoris* strains. All mixtures contained *S. cremoris* HP since this strain could be distinguished from all other strains. In all mixtures tested, one strain became dominant after relatively short cultivation periods (20). These observations demonstrated that drastic changes can occur in starter cultures within a relatively short period of time.

Studies on population dynamics of complex mixed cultures can only be done if a general method is available to distinguish different strains of *S. cremoris* from each other. In this paper we describe a method to recognize specific strains and variants of *S. cremoris* in mixed cultures which is based on the immunofluorescence technique used successfully for the detection of specific strains of the related bacterium *Streptococcus mutans* (16, 21). Three examples of the application of this method in studying starter cultures are described: (i) competition between different strains in batch culture and continuous culture under amino acid limitation; (ii) the occurrence and stability of bacteriophage resistance during starter cultivation; and, (iii) the expression of proteases in *S. cremoris* under different culture conditions.

MATERIALS AND METHODS

Bacterial strains. The *S. cremoris* strains listed in Table 1 were all obtained from the Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. All strains were routinely stored in reconstituted (10%) skimmed milk at −20°C. In the case of proteolytic-negative (Prt^-) variants, 0.1% tryptone (Difco Laboratories, Detroit, Mich.) was added to the milk.

Growth media. Five different growth media were used for cell cultivation: (i) a defined synthetic medium (RFP) with leucine as the growth-limiting substrate (27); (ii) a defined synthetic medium (RFP) with 10 mM $CaCl_2$ and 0.8% sodium caseinate instead of an amino acid mixture (19, 29); (iii) a complex medium (MRS) described by De Man et al. (5); (iv) a complex medium (M_{17}) by Terzaghi and Sandine (34); and (v) 10% reconstituted skimmed milk (Oxoid Ltd., Basingstoke, Hampshire, England). In the clear growth media (RFP, MRS, and M_{17}) 1% lactose was used in all cases.

Isolation of antibodies. *S. cremoris* strains E8, ML1, AM1, SK11, SK1128, and SK1143 were grown overnight in 50 ml of M_{17} medium (34). The cells were washed twice in 50 mM sodium phosphate buffer, pH 6.3, and suspended in 5 ml of the same buffer solution. Rabbits were injected with these antigenic solutions as described previously (9, 35).

Specific antibodies against proteases A and B (19) were obtained by excision of the immunoprecipitates of protease A and B from crossed-immunoelectrophoresis (CIE) gels.

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TABLE 1. List of *S. cremoris* strains used in this study

Strain	Phenotype[a]	Described in reference(s):
S. cremoris Wg2	Prt^+	19, 28; Hugenholtz et al. (in press)
S. cremoris Wg2	Prt^-	11
S. cremoris E8	Prt^+	11, 19; Hugenholtz et al. (in press)
S. cremoris E8	Prt^-	11, 19; Hugenholtz et al. (in press)
S. cremoris AM1	Prt^+	11, 12, 19
S. cremoris AM141	Prt^-	W. M. de Vos (unpublished data)
S. cremoris HP	Prt^+	11, 19, 20
S. cremoris HP	Prt^-	10; Hugenholtz et al. (in press)
S. cremoris ML1	Prt^+	11, 19
S. cremoris TR	Prt^+	11, 19
S. cremoris SK11	Prt^+	7, 8, 19
S. cremoris SK1128	$Prt^- \phi^S$	7
S. cremoris SK1143	$Prt^- \phi^R$	7

[a] Prt^+, Protease positive; Prt^-, protease negative; ϕ^S, phage sensitive; ϕ^R, phage resistant.

These gel pieces were homogenized in 0.9% NaCl and injected into rabbits as described above.

Fluorescence labeling of antibodies. The fluorescent compound fluorescein isothiocyanate (FITC) was used to label the antibodies as described previously (31). Antibody solution (1 ml) in 20 mM barbital-hydrochloride, pH 8.6, containing 20 to 50 mg of antibody was mixed with 1 ml of 50 mM $NaHCO_3$, pH 8.5, containing 15 to 20 mg of FITC on Celite (Johns-Manville Products Corp., Manville, N.J.) (±10% FITC; Sigma Chemical Co., St. Louis, Mo.). The mixture was vigorously shaken for 3 min, and the Celite was removed by brief centrifugation. FITC-labeled antibodies were separated from the free FITC by elution over a Sephadex G-25 column, using 25 mM sodium phosphate, pH 6.5, as elution buffer.

The labeled antibodies (final volume, 4 to 6 ml) were kept frozen at −20°C until use.

Immunofluorescence. Samples of overnight cultures were used for labeling with FITC-immunoglobulins. In the case of milk cultures, 2 ml of a solution containing 0.5 M sodium borate and 10 mM EDTA, pH 8.0, was added to clear the milk. The cells from 1 ml of culture were harvested, washed with 1 ml of 25 mM sodium phosphate, pH 6.3, and incubated with 100 μl of FITC-labeled antibody solution for 30 min at room temperature. Subsequently, unlabeled antibody was removed by centrifugation followed by two washes in 25 mM sodium phosphate, pH 6.3. Finally, the cells were suspended in 1 ml of the same buffer and could be examined under the fluorescence microscope (Zeiss standard microscope, HBO 50 W, KP 490, KP 500, reflector LP 510 [450], LP 528). The photographs were taken at a magnification of ×1,000 with oil immersion, using a Neofluar 100/1.30 objective with a Fuji 1600 ASA color film (exposure time, 5 to 10 s). In some cases samples of mixed cultures were initially incubated with unlabeled antibody against one strain, washed, and then labeled with FITC-antibody against the other strains to intensify the difference in fluorescence between the strains.

Competition in batch culture. Mixtures of *S. cremoris* E8 and HP, E8 and ML1, or SK1128 and SK1143 were grown overnight at 30°C in 10 ml of either 10% reconstituted skimmed milk or MRS medium (5). From these cultures 100-μl samples were transferred to 10 ml of fresh medium, and incubation was continued overnight at 30°C. This was repeated up to 10 times. The composition of the cultures was analyzed regularly by immunofluorescence. Pure cultures of SK1128 and SK1143 were cultivated in the same manner to examine the stability of the phage sensitivity or resistance or both in this strain.

Competition in continuous culture. *S. cremoris* E8 and ML1 were grown in continuous culture under leucine limitation as described previously (27). When both cultures had reached a steady state, they were mixed in different ratios and cultivation was continued in the chemostat for several volume changes. The dilution rate (D) of the cultures was varied from 0.1 to 0.4 by increasing the inflow rate of the growth medium. The composition of the mixed cultures was analyzed regularly by immunofluorescence.

CIE. CIE was carried out as described previously (9, 35). The gels were run at 2.5 V/cm for 90 min in the first dimension and at 1.5 V/cm for 10 to 15 h in the second dimension. Various concentrations of antigens and antibodies were used in all experiments. In absorption experiments the antigen solution (for instance, a cell wall preparation of strain E8) was incubated with increasing amounts of nonspecific antibodies (for instance, antibodies against strain ML1). The precipitate was removed by centrifugation, and the supernatant was used for CIE.

Cell-free extracts and cell wall preparations. Cells were grown overnight in 50 ml of M_{17} medium (34) at 30°C, washed, and suspended in 1 ml of 40 mM sodium phosphate, pH 6.5. Cell-free extracts were prepared by passing the concentrated cell suspensions twice through a French pressure cell (American Institute Corp., Silver Spring, Md. at an operating pressure of 6,000 lb/in^2. The broken cells were centrifuged at 48,000 × g for 45 min, and the supernatant was used for the experiments.

Cell wall preparations were prepared by incubating the concentrated cell suspensions with 5 mg of lysozyme (muramidase, from hen egg white; Boehringer GmbH, Mannheim, Federal Republic of Germany) per ml at 37°C for 30 min. The cells and protoplasts were removed by centrifugation (15 min, 10,000 × g).

Determination of maximum specific growth rates. Maximum specific growth rates were determined as described previously (20) by diluting samples of the milk cultures 10-fold in 0.5 M sodium borate–10 mM EDTA, pH 8.0, and then measuring the A_{660} of the cleared cultures in a Vitatron UC 200 spectrophotometer (Vitatron Scientific Instruments, Dieren,The Netherlands).

Protein determination. Protein was determined by the method of Lowry et al. (24), with bovine serum albumin as the standard.

Isolation of cell wall-bound proteases. The isolation of proteases was performed as described previously (19). The cells were grown in RFP-casein medium with 10 mM Ca^{2+} and in MRS medium with or without Ca^{2+} at a constant medium pH of 6.3.

Determination of proteolytic activity. Proteolytic activity was determined as described previously (19, 33), with fluorescamine-labeled casein as the substrate. The activity was quantitated by comparing the measured relative fluorescence values with those of standard trypsin solutions.

RESULTS

Strain-specific immunofluorescence. Antibodies raised against whole cells of *S. cremoris* E8 (Prt^+) attached readily to cells of this strain, as could be shown by immunofluores-

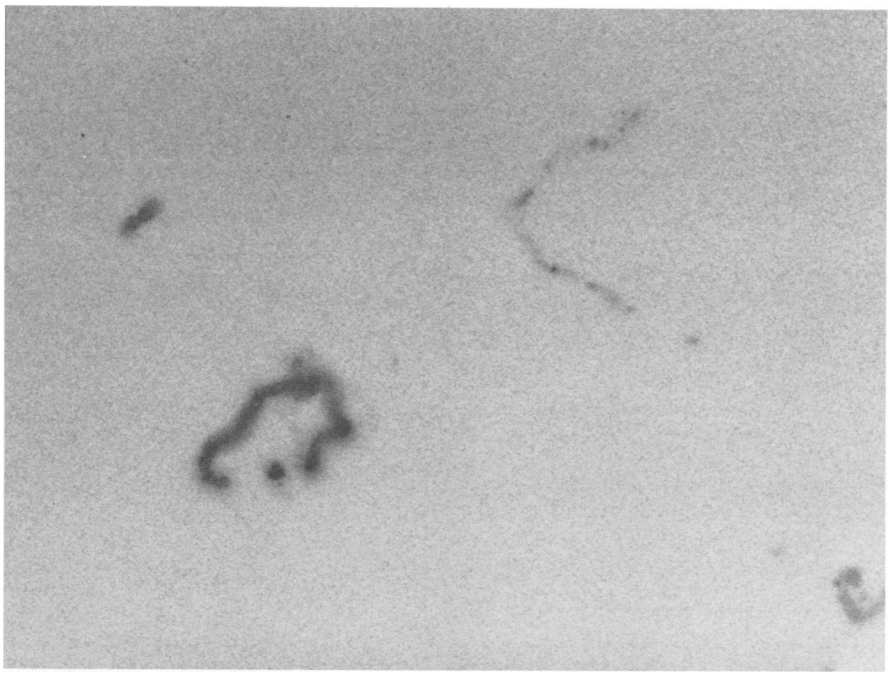

FIG. 1. Fluorescence microscope photograph of a mixed culture of *S. cremoris* strains E8 (Prt$^+$) and ML1. Cells were incubated with FITC-labeled antibodies raised against cells of *S. cremoris* E8, as described in Materials and Methods. A chain of cocci of strain E8 is seen on the left of the picture and a chain of strain ML1 is seen on the (top) right.

cence labeling. The cells became fluorescent when treated with FITC-labeled antibodies, which could be observed with a fluorescence microscope.The edge of the cells was clearly visible under the microscope, indicating that the antibodies attached to the cell surface.

When other strains of *S. cremoris* were treated with the same fluorescent antibodies, the cells were hardly visible with the fluorescence microscope, indicating that the antibodies against *S. cremoris* E8 did not attach to the surface of the cells of the other strains. This is not due to artefacts of the immunofluorescence procedure, as was shown by incubating mixed cultures of *S. cremoris* E8 and another strain (ML1) with the E8 FITC-labeled antibodies. Both strains were grown under the same conditions and still only strain E8 became fluorescent (Fig. 1). The fluorescent labeled chains of cocci had the typical appearance of *S. cremoris* E8 which forms, under these conditions, mainly chains of 5 to 15 cocci. The nonlabeled chains could be observed microscopically with a normal light microscope. These chains consisted, in general, of many more cocci (10 to 40), and the individual cocci were smaller in size, an appearance characteristic of *S. cremoris* ML1 in these cultures. These observations demonstrate that a reliable distinction can be made between strains E8 and ML1 with this immunofluorescence method.

Antibodies against other strains were also tested for their specificity. The same observations were made with antibodies against strain ML1 (Prt$^+$). These antibodies interacted only with strain ML1 when tested in mixed cultures with nine other strains. Antibodies with the same specificity were made against strains HP, Wg2, and SK11 (all Prt$^+$). The latter antibodies could even distinguish between the closely related strains SK11 and AM1 (32).

The protein compositions of ML1 and E8 antigens were compared by CIE. Cell-free extracts and cell wall preparations (by lysozome treatment) were analyzed by CIE with

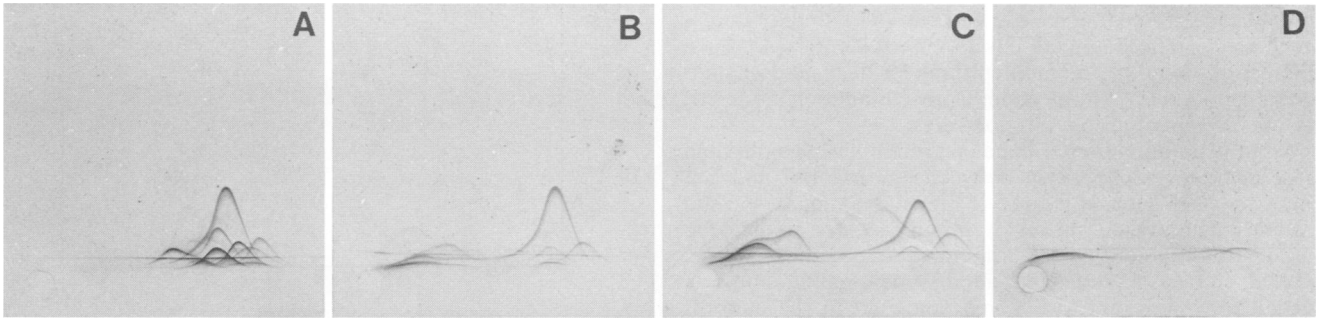

FIG. 2. CIE of a cell wall preparation (by lysozyme treatment) of *S. cremoris* E8 (Prt$^+$) absorbed with increasing amounts of antibodies against whole cells of *S. cremoris* ML1. In the second dimension 50 μl of antibody solution (containing ± 1.5 mg of antibody) against whole cells of strain E8 was used. In the first dimension 2 μl of the cell wall preparation was used, absorbed with 0 (A), 2 (B), 4 (C), and 10 (D) μl of antibody solution against whole cells of strain ML1.

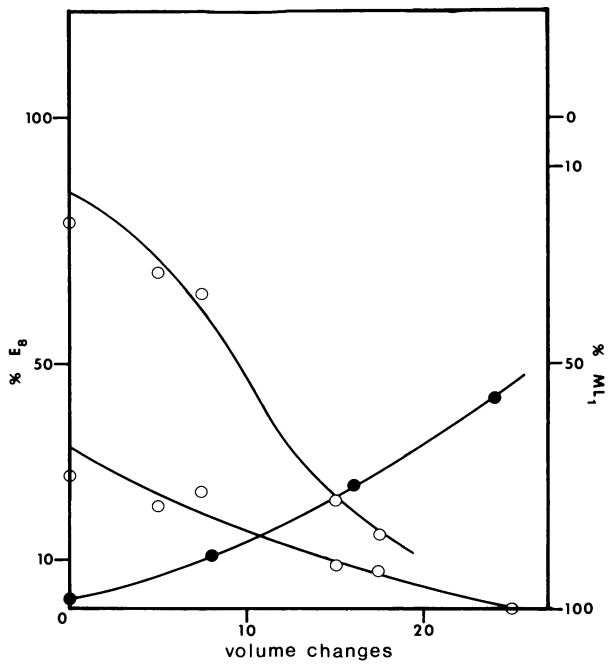

FIG. 3. Competition between *S. cremoris* ML1 and E8 (Prt$^+$) in leucine-limited continuous culture at D = 0.1 (○) and D = 0.4 (●). The composition of the mixed cultures was determined by immunofluorescence, using antibodies against both strains ML1 and E8.

antibodies against whole cells of either strain E8 or ML1. The precipitation patterns obtained with both strains were almost identical (data not shown), and no exclusive precipitation bands could be detected in the CIE patterns of either strain. Absorption experiments as described in Materials and Methods resulted in the disappearance of all precipitates (Fig. 2). These observations indicate that proteins are not responsible for the specific reaction of the antibodies with strains ML1 and E8 and that some other compounds in the cell wall of these two strains are specifically recognized by these antibodies.

Competition experiments. If the described immunofluorescence detection method is reliable, it should be very useful in studying the composition of mixed starter cultures and the population dynamics of these starters. We have tested this possibility by repeating competition experiments between strains E8 and HP (both Prt$^+$). Competition between these two strains has previously been followed with other detection methods (20). The results obtained by using both antibodies against strains E8 and HP in the immunofluorescence analysis of the mixed cultures were identical to those obtained previously. In batch cultures E8 became dominant and in continuous cultures under lactose limitation strain HP became dominant at low dilution rates.

With the immunofluorescence method it was also possible to describe the competition between strains ML1 and E8. The two strains have almost identical maximum specific growth rates, which makes it impossible to predict the outcome of competition experiments between these strains in batch culture. As expected, a long time of cultivation was needed for one strain to become dominant in the culture. After about 10 days (10 transfers), strain ML1 became dominant in batch culture when MRS medium was used.

When RFP medium was used, the outcome of the competition was reversed (data not shown). This medium was also used to study the competition between the two strains in continuous culture. Leucine was chosen as the growth-limiting substrate; it also appears to be growth limiting for streptococci in milk (J. Hugenholtz, M. Dykstra, and H. Veldkamp, submitted for publication). *S. cremoris* ML1 and E8 were cultivated in pure cultures on this medium until steady states were reached. The strains were mixed in two different ratios, and cultivation was continued at a dilution rate of 0.1. Samples of both cultures were taken regularly, and the composition of the mixtures was analyzed by immunofluorescence, using antibodies against ML1 and E8. Both methods yielded the same results in all cases, which again demonstrates the reliability of the immunofluorescence method. As was expected in a competitive interaction of two strains, the outcome of the experiment was independent of the initial composition of the cultures. At low dilution rates (D = 0.1) *S. cremoris* ML1 gradually became dominant in both cultures (Fig. 3). When the competition experiment was repeated at a much higher dilution rate (D = 0.4), starting with a low percentage of E8, the outcome of the experiment was reversed. These results indicate that strain ML1 has a lower affinity constant (K_s) for leucine than strain E8 and, consequently, it has a selective advantage at low dilution rates. At high dilution rates the maximum specific growth rate of an organism is the more important factor in the competition process (36), which explains the dominance of strain E8 under these conditions (Fig. 3). The composition of the mixed culture was therefore determined by the dilution rate of the continuous culture.

Detection of specific variants of *S. cremoris* strains. Mixed starter cultures contain not only many different strains of *S. cremoris*, but also variants within these strains. Most of these are genetic variants caused by loss of one or more of the numerous plasmids present in the streptococci (25). The two most significant types of these variations are discussed

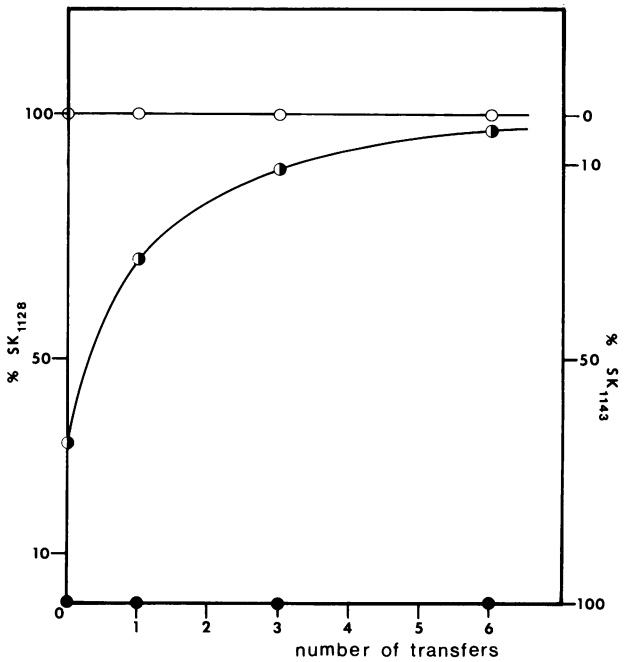

FIG. 4. Percentage of bacteriophage-sensitive *S. cremoris* SK1128 and bacteriophage-resistant *S. cremoris* SK1143 in pure cultures (○, ●) and in a mixed culture (◑) of *S. cremoris* SK1128 and SK1143 during batch cultivation in milk.

here: (i) bacteriophage sensitivity or resistance (ϕ^S/ϕ^R), and (ii) protease positive or negative (Prt^+/Prt^-).

(i) ϕ^S/ϕ^R. Two variants of strain SK11, SK1128 (bacteriophage sensitive) and SK1143 (bacteriophage resistant), have been used to examine whether phage sensitivity and resistance within one strain of *S. cremoris* can be distinguished by immunofluorescence. The only difference in genotype between the two variants is the presence of an extra plasmid (pSK112) in the phage-resistant SK1143 (7, 8). Antibodies raised against both variants were labeled with FITC and used for immunofluorescence studies as described above. With antibodies against SK1143, no discrimination could be made between the bacteriophage-sensitive (SK1128) and bacteriophage-resistant (SK1143) variants. Both variants became clearly fluorescent after incubation with these FITC-labeled antibodies. The antibodies against SK1128, however, interacted more readily with variant SK1128 than with variant SK1143. The difference in fluorescence labeling was not as significant as observed above for strains ML1 and E8. A more clear-cut distinction could be made when cells of both variants were first incubated with nonfluorescent labeled antibodies against SK1143, which interacted equally well with both variants, and subsequently, after thorough washing, with fluorescent antibodies against SK1128. In this way the difference in fluorescence between SK1128 and SK1143 was considerable and allowed the microscopic analysis of mixtures containing both variants (cf. Fig. 1).

The competition between the two variants could be examined in mixed cultures with this method (Fig. 4). This was done by growing mixed cultures of both variants in successive milk (batch) cultures for several days. The mixed culture became dominated by the phage-sensitive variant (SK1128) within 3 days. This can be explained by the large difference in maximum specific growth rates between both variants. The phage-sensitive variant SK1128 has a higher μ_{max} (0.51 h^{-1}) than SK1143 (0.37 h^{-1}) when grown in milk. When each variant was propagated in pure cultures, both remained 100% stable for more than 7 days (Fig. 4), showing that plasmid pSK112 is extremely stable in strain SK1143 under these conditions.

(ii) Prt^+/Prt^- variants. Protease-producing (Prt^+) *S. cremoris* strains AM1, Wg2, E8, HP, SK11, TR, and ML1 were all tested in immunofluorescence experiments that used a mixture of specific antibodies against proteases A, B, and C (19), all labeled with FITC. All strains became clearly fluorescent, indicating that at least one of the proteases was present and located at the outer surface in the strains tested. Proteolysis-negative (Prt^-) variants of strains AM1, Wg2, E8, HP, and SK11 were also incubated with the antibodies against the proteases and did not show any visible fluorescence, as expected. Only under normal light conditions could the Prt^- variants be seen and their numbers be determined microscopically. The immunofluorescence method thus appears to be very suitable for determining the ratio of Prt^+/Prt^- variants in starter cultures. This is of obvious importance for determining the activity of starter cultures (28; Hugenholtz et al., in press).

Expression of proteases in *S. cremoris*. FITC-labeled antibodies against the proteases can also be used for quantitation of these enzymes in the cell wall of *S. cremoris* under different culture conditions. *S. cremoris* strains Wg2, E8, and AM1 were grown in milk, RFP-casein medium, and MRS medium with or without $CaCl_2$ (10 mM) to investigate the role of the nitrogen source and the role of Ca^{2+} ions in the expression of proteases in these cells. The highest fluorescence labeling with the protease-specific antibodies was found when all three strains were grown in milk or RFP-casein medium (Table 2). When the strains were cultivated in complex MRS medium in the presence of 10 mM $CaCl_2$, strain Wg2 was labeled to the same extent as in milk cultures, but under the same conditions the fluorescence labeling of strains E8 and AM1 was clearly reduced. When MRS medium without Ca^{2+} was used, the fluorescence of Wg2 was also reduced considerably.

TABLE 2. Expression of proteases in three *S. cremoris* strains under different culture conditions

Culture condition(s)	Fluorescence (ng/ml)[a]		
	S. cremoris Wg2	*S. cremoris* E8	*S. cremoris* AM1
Milk	+ + +	+ + +	+ + +
RFP-casein + Ca^{2+}	+ + + (75)	+ + + (80)	ND
MRS + Ca^{2+}	+ + + (75)	+ (7)	+
MRS	+ (10)	+ (5)	+
Stationary phase	+ + +	+ + +	+ + +
Logarithmic phase	±	±	±

[a] + + +, Brightly fluorescent; +, fluorescent; ±, slightly fluorescent. ND, Not determined. Value in parentheses is amount of isolated proteolytic activity in trypsin concentration equivalents.

The influence of growth phase on immunofluorescence labeling with protease-specific antibodies was determined by comparing the immunofluorescence of logarithmically growing and stationary-phase cells of the three strains. Stationary-phase cells were brightly and homogeneously fluorescent in all cases, while a weaker and nonhomogeneous labeling was observed in growing cells (Table 2).

The degree of fluorescence of the cells correlated well with the proteolytic activity present in the cells under the same conditions. This is shown by the proteolytic activity which could be isolated from *S. cremoris* Wg2 and E8 when the strains were grown in different media (Table 2). It was also found that cells in the stationary phase contained much more proteolytic activity than logarithmically growing cells (data not shown), which is in agreement with the fluorescence of both types of cells.

DISCUSSION

In this paper we describe a method to identify by immunofluorescence particular strains of *S. cremoris* in mixed cultures. The method is rapid and easy, since it does not involve the usual time-consuming fixation of the bacterial cultures. The method should be very suitable for automation with a cell sorter technique, leading to more exact quantification of cell numbers. All strains tested contain a specific antigenic deteminant(s) at the cell surface. This specific antigen(s) could not be identified by CIE (Fig. 2), and it is possible that these antigens are not proteins but, perhaps, lipopolysaccharides.

An immunological description of the species *S. cremoris* and *L. lactis* was attempted many years ago (1, 2). Some serotypes were distinguished by a rather insensitive agglutination method. These serotypes always included several different strains of *S. lactis* or *S. cremoris*.

In the case of *S. mutans*, many distinct serotypes have been described (6, 16, 30). These serotypes, often specific for one strain or several closely related strains, could be detected by immunofluorescence in mixtures with other bacteria (16, 21). The antigens that determine the specific

serotypes of *S. mutans* were identified as various sugar groups in the cell wall.

The described immunofluorescence method makes it possible to analyze the composition of mixed starter cultures containing different strains of *S. cremoris*. We have used the method to study the changes that occur in the population of mixtures during batch or continuous cultivation. Differences in either maximum specific growth rates or affinity constants (K_s) for the growth-limiting substrates led in all cases to dominance of one strain. In the competition for leucine between *S. cremoris* stains ML1 and E8, crossing μ-S curves were observed, and the outcome of the competition experiment in continuous culture was dependent on the value of the dilution rate (Fig. 3). Amino acids also seem to be growth limiting in milk cultures (Hugenholtz et al., in press). In complex mixed starter cultures competition for amino acids similar to that described for strains ML1 and E8 will take place. This will certainly affect the composition of such starters.

The specific detection of different variants of one strain has not been described before for any bacterial species. The bacteriophage-sensitive variant of *S. cremoris* SK11 (SK1128) could be distinguished from the bacteriophage-resistant variant (SK1143) by using antibodies against SK1128. This indicates that the phage-sensitive variant has one or several extra antigens exposed at the outer surface which are not accessible or are absent in the phage-resistant variant. A possible candidate would be the phage receptor site. Since phage resistance and not phage sensitivity requires the additional genetic information located on a plasmid (7, 8) this aspect is still far from clear.

An interesting application of the immunofluorescence technique is in the detection of proteases in the cell wall of *S. cremoris*. FITC-labeled specific antibodies against the proteolytic system attach readily to the different strains of *S. cremoris*. This is a simple and rapid method for the detection of proteolytically positive and negative variants in starter cultures. The information can be obtained within 1 h instead of the 2 to 3 days required with the traditional plating technique (10, 28; Hugenholtz et al., in press). The method can also be used to examine the expression of proteases in the cell wall of *S. cremoris* under various culture conditions. In all growth conditions proteases were detected in the Prt^+ strains, but the actual amount varied considerably (Table 2). It seems that the external nitrogen source (for *S. cremoris* AM1 and E8) and the Ca^{2+} concentration (for *S. cremoris* Wg2) influences the protease synthesis. This is in agreement with previous reports (12, 22). The results clearly demonstrated that the protease content of *S. cremoris* cells was highest when the cells had reached the stationary phase of growth (Table 2). In conclusion, the described immunofluorescence techniques can be extremely useful in (i) analyzing the composition of unknown mixed starter cultures; (ii) studying the population dynamics of mixed starters; (iii) determining the ratio of bacteriophage-sensitive/-resistant and protease-positive/-negative variants in starters; and (iv) examining protease expression in streptococci.

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