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Slow Milk Coagulating Variants of *Lactobacillus helveticus*

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ABSTRACT. Curing experiments were performed on *Lactobacillus helveticus* strain ATCC 15009 in order to find a correlation between the presence of three extrachromosomal elements and specific phenotypic traits. Mitomycin C treatment of the strain was found to result in an appearance of slow-coagulation variants unable to coagulate milk in 24 h at 42 °C. The effect of mitomycin C on phenotypic and genotypic characteristics of *L. helveticus* was therefore further examined. The presence of mitomycin C appeared to act on the proteolytic system of the strain: slow variants exhibited a poor casein breakdown (50 % less) compared with the parental strain. Aminopeptidase activity and lactose utilization were unaffected. The phenotypic variation is possibly due to a point mutation of genetic patrimony. No loss of plasmid DNA was detected after mitomycin C treatment and the restriction pattern of plasmid and chromosomal DNA of the variants, after digestion with several restriction endonucleases, was identical to that obtained for the parental strain.

Despite the industrial importance of thermophilic lactobacilli as dairy starter cultures, little is known about the behavior of these pure cultures propagated in milk. The ability of lactic acid bacteria to coagulate milk depends mainly on their capacity to metabolize lactose and to break down casein, but these characters are frequently unstable. This instability has been studied chiefly in lactococci and related to the presence of plasmids which can undergo spontaneous or artificially induced curing events (Kok and Venema 1988; McKay 1983). The evidence that plasmid DNA is involved in the phenotypic traits that are important for dairying is not always easy to obtain for lactobacilli. Results of acriflavin-curing experiments on a strain of *Lactobacillus helveticus* spp. *jugurti*, a high acid producer (Smiley and Fryder 1978), suggested a possible relationship between this phenotypic character and a plasmid. Morelli *et al.* (1986) have shown the presence of spontaneous slow coagulating isolates in a pure culture of an *L. helveticus* strain subcultured in milk, and suggested the possibility of a linkage between casein hydrolysis and a 3.5 kbp plasmid. However, it is often difficult to know the plasmid-coded properties, either because many plasmids remain cryptic or because current techniques for plasmid curing are ineffective. In previous studies (Fortina *et al.* 1993), we have isolated and characterized three extrachromosomal molecules from *L. helveticus* strain ATCC 15009. During curing experiments aimed at correlating the presence of the plasmids with specific phenotypic traits, we observed that mitomycin C (MIT) treatment of the strain resulted in the appearance of slow milk coagulating variants, although there was no loss of plasmid DNA.

This report deals with the genotypic and phenotypic characterization of isolate M193, chosen as representative of the slow milk coagulating variants, in comparison with that of the parental strain, to establish the possible cause of this mutation.

MATERIALS AND METHODS

Strains and cultivation. Lactobacillus helveticus strain ATCC 15009 and the derivative strain M193, obtained by MIT treatment of strain ATCC 15009 were used. The MRS broth (De Man *et al.* 1960) and alternatively the reconstituted skim milk medium (10 % in water) (RSM) (*Difco*) were used to propagate the organisms in liquid medium at 42 °C; agar (1.5 %) was added to the former medium for plating. For comparative growth rate studies the MRS was prepared without the sugars. A sterile solution of glucose or lactose was then added to a final concentration of 1 %.

When required the RSM medium was supplemented with: glucose or galactose (final concentration 1 %), yeast extract (0.05–0.5 %), Bacto peptone (0.5–2 %), or a free amino acid mixture con-

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taining (in mg/L): alanine 100, methionine 70, isoleucine 100, phenylalanine 100, threonine 100, tryptophan 100, tyrosine 100, glycine 200, serine 200, leucine 200, cysteine 200, aspartic acid 300, arginine 450, proline 540, histidine 550, lysine 800 and glutamic acid 3200.

Selection and isolation of variants. About 10^4 cells per mL of an overnight MRS culture from strain ATCC 15009 were inoculated in 5 mL MRS containing MIT at a concentration of 0.1–10 mg/L and incubated at 37 °C for 18–24 h. After incubation, the culture containing the highest concentration of curing agent that still allowed visible growth was replicated on MRS agar. After incubation at 42 °C, single colony isolates were randomly selected for plasmid analysis and tested for coagulation in RSM. Isolates were considered as slow milk coagulating variants when no coagulation was observed after 24 h of incubation at 42 °C.

Physiological tests. Phenotypic changes between slow coagulating variant M193 and its parental strain ATCC 15009 were studied using cells collected from overnight cultures in MRS broth. Growth was determined turbidimetrically by measuring absorbance at 600 nm after appropriate dilution in sodium borate buffer containing 10 mmol/L EDTA, in a Perkin–Elmer 550 SE spectrophotometer. Acid production in milk at different incubation times and at 42 °C was evaluated by titration with 0.25 mol/L NaOH and phenolphthalein as indicator (Fortina *et al.* 1990). Lactic acid produced in MRS medium was determined enzymically (*Boehringer*, Mannheim, Germany).

Preparation of cell-free extract. Cell suspension was passed twice through a French Pressure Cell at 138 MPa. Cell debris were removed by centrifugation at 15 000 ***g*** for 10 min. The supernatant was centrifuged at 40 000 ***g*** for 1 h at 4 °C and the resulting cell free extract (CFE) was stored at −20 °C until required.

Protein quantitation. Protein concentrations of the CFE were estimated by the micromethod of Bradford (1976), using bovine serum albumin as standard.

Determination of peptidase and proteolytic activity. The general profiles of whole cell peptidase activities were tested using a wide range of substrates (59 different β-naphthylamide derivatives) (β-NA) with API Peptidase I–VI strips (*BioMerieux*, France) as described by Prost and Chamba (1994).

The aminopeptidase activity was quantitatively assayed in CFE according to El Soda and Desmazeaud (1982) using Ala-*p*-nitroanilide (Ala-*p*-Na), Lys-*p*-NA, Met-*p*-NA, Pro-*p*-NA (*Sigma*) as substrates. The hydrolysis of the substrate was determined by measuring absorbance at 410 nm. Activity was expressed as μmol of substrate hydrolyzed per min per mg of protein of the CFE, based on an absorption coefficient of 8.8 $mmol^{-1}$ for *p*-NA (Exterkate 1975).

Proteolysis was evaluated for milk cultures grown in RSM at 42 °C for different times by using the *o*-phthaldialdehyde (*o*-PA) method developed by Church *et al.* (1983), and expressed as mg/L glycine after comparison with a calibration line.

Caseinolytic activity of the CFE was determined at 37 °C and pH 7 using 0.625 % casein in 0.1 mol/L Tris-HCl as substrate. The reaction mixture contained 0.2 mL of CFE and 0.2 mL of the substrate. After 1 h of incubation the reaction was stopped by addition of 0.8 mL TCA (0.75 mol/L) and casein hydrolysis was determined by the *o*-PA method.

Presence of enzymic activities other than proteolytic activity. The activities of other enzymes were tested using the API ZYM System (*BioMerieux*) containing 19 different substrates, as described by Arora *et al.* (1990).

DNA analysis. Cells used to isolate DNA were grown using the conditions described by Fortina *et al.* (1990). Plasmid DNA was isolated by the alkaline procedure described by Anderson and McKay (1983) and purified by centrifugation to equilibrium in a cesium chloride–ethidium bromide (CsCl–EtBr) gradient. Plasmids were recovered from agarose gels by a procedure of electroelution (Maniatis *et al.* 1982). For the isolation of chromosomal DNA, cells were lysed by the same method without the alkaline denaturation step.

Purified plasmid and chromosomal DNA preparations were digested with the following restriction endonucleases (RE): *Apa*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dII, *Hin*dIII, *Kpn*I, *Mlu*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sph*I, *Ssp*I, *Stu*I, *Xba*I for 2 h at 37 °C and examined by agarose gel electrophoresis. Electrophoresis, staining and evaluation of gels were performed as described by Maniatis *et al.* (1982). Molecular sizes were determined against DNA ladders (Gibco, BRL).

RESULTS AND DISCUSSION

Isolation and growth properties of variants. In an attempt to correlate the presence of three plasmids in *Lactobacillus helveticus* ATCC 15009 with specific phenotypic traits, we conducted curing experiments employing different agents commonly used for this purpose (Vescovo *et al.* 1982; Morelli *et al.* 1983; Ruiz-Barba *et al.* 1991). However, in our experience, acridine orange, acriflavine, ethidium bromide and novobiocin were effective only in the curing of the smallest plasmid contained in *L. helveticus* ATCC 15009 (3.5 kbp) and at low frequency (1–3 %). Since plasmid-free derivatives must be obtained to elucidate the possible role of plasmid DNA, we continued the curing experiments with other substances such as MIT. This compound has not been extensively used as plasmid curing agent in lactobacilli, even though it has been successfully utilized to eliminate plasmids in other bacteria (Trevors 1986).

Growth of strain ATCC 15009 in the presence of MIT determined the appearance of slow milk coagulating variants. The highest frequency was noted at 42 °C in the presence of 0.4 mg/L of MIT. In these conditions the frequency of the mutation was approximately 12 variants per 100 cells. Several variants were isolated and found to show an identical behavior. As opposed to the parental strain, slow milk coagulating isolates were unable to coagulate milk in 1 d except when yeast extract, peptone or free amino acid mixture was added to RSM: the optimal concentration of the additional nitrogen source was 0.4 and 1 % for yeast extract and peptone, respectively. The addition of glucose or galactose did not improve the coagulation in milk of the slow variants. It is well known that *L. helveticus* has complex nutritional requirements and satisfactory growth depends on vitamins, peptides and amino acids (Ledesma *et al.* 1977; Morishita *et al.* 1981). The coagulation data suggested that variant M193, if compared to the parental strain, showed most important nutritional requirements or lower ability to utilize milk components to support its growth.

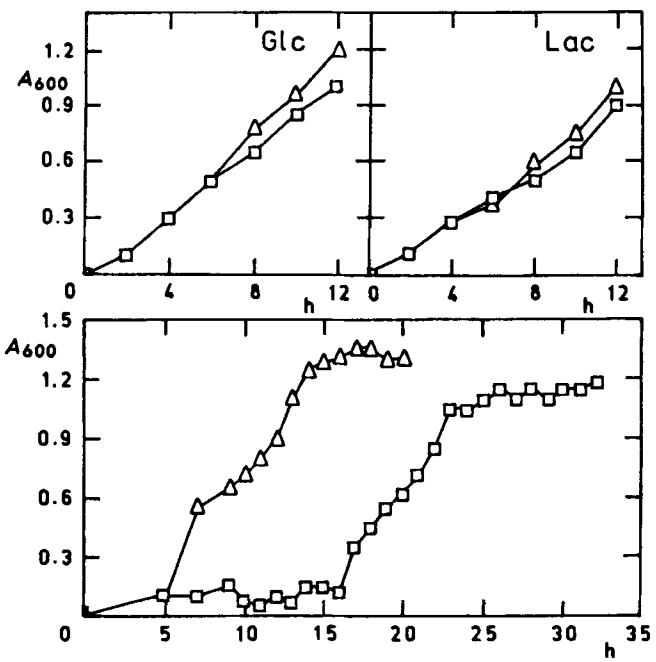

Fig. 1. Comparative growth (absorbance A_{600}) at 42 °C of *L. helveticus* strain ATCC 15009 (*triangles*) and its variant M193 (*squares*) in MRS broth (*top*) supplemented with glucose (Glc) or lactose (Lac) and in RSM medium (*bottom*).

Growth profiles obtained with 1 % inocula show that in MRS medium (Fig. 1) the growth rate of both strains seemed to be similar, even when glucose was replaced with lactose as the only carbon source, suggesting that in slow milk coagulating variants lactose utilization was unaffected. Moreover, in this rich medium the slow variant did not have to hydrolyze proteins to obtain peptides and amino acids already present. When the MRS medium was replaced with RSM the growth rate of the M193 variant significantly decreased. The growth profile of M193 showed a more extensive lag phase (15 h) when compared to that obtained for the parental strain (5 h). After this delay the subsequent growth rate was comparable with that of the parental strain.

Phenotypic characterization of variant M193. Lactic acid production in RSM medium was evaluated after different times of growth at 42 °C. Production of lactic acid was significantly lower for variant M193 than for the parental strain after 16 and 24 h of incubation (Table I). After 48 to 72 h the amount of lactic acid produced by M193 neared that of the parental strain, according to the growth curve in RSM and coagulum formation in this time period. In MRS medium this acidification lag was not observed: both strains produced similar amounts of lactic acid stereoisomers: DL – 7 g/L, D – 2.8 g/L, L – 4.2 g/L (after 24 h of incubation at 42 °C).

Table I. Production of lactic acid (g/L) and proteolytic activity (mg/L glycine) by *L. helveticus* strain ATCC 15009 and its derivative M193 in RSM medium after incubation at 42 °C for 16–72 h

Strain	16 h	24 h	48 h	72 h
Lactic acid production				
ATCC 15009	8.1	11.5	17.6	18.4
M193	0.5	0.99	11.3	17.6
Proteolytic activity				
ATCC 15009	100	120	140	190
M193	40	60	90	160

Table II. Proteolytic activity (mg/L glycine) of cell-free extract obtained by cells grown in MRS medium of *L. helveticus* strain ATCC 15009 and its derivative M193 using casein as substrate and measured by the *o*-PA test

Strain	1 h	4 h
ATCC 15009	170	290
M193	50	100

Table III. Aminopeptidase activity[a] of *L. helveticus* strain ATCC 15009 and its derivative M193

Substrate	ATCC 15009	M193
Ala-*p*-NA	185	172
Met-*p*-NA	176	177
Lys-*p*-NA	162	160
Pro-*p*-NA	1.5	1.1

[a]Measured in a cell-free extract obtained from cells grown in MRS, and expressed as μmol per min per mg protein.

The proteolytic activity of the two strains in RSM, as measured by the *o*-PA test, was clearly different: the variant M193 exhibited a poor casein breakdown and the amount of amino groups released by the parental strain after 24 h of incubation was double that of the slow culture. This difference was lower only after 2–3 d of growth (Table I). When proteolytic activity was measured on CFE obtained by cells grown in MRS medium and employing casein as substrate, a similar behavior was obtained (Table II): the activity of the slow M193 variant was 35 % of that observed for the parental strain. This result suggested that the metabolic defect in the slow variant was a partial loss of catalytic activity on casein, as supported by the requirement of yeast extract, peptone or amino acids for a fast milk coagulation.

The peptidase activity seemed to be unaffected by MIT treatment. Qualitative tests carried out on 59 substrates showed a similar hydrolytic activity for the parental strain and its variant (*data not shown*). The aminopeptidase activity of the two strains, evaluated quantitatively on four substrates reported to be specific for aminopeptidase activity in thermophilic lactobacilli (Cholette and McKellar 1990; Prost and Chamba 1994) (Table III), confirmed these results. Particularly, Ala-*p*-NA, Lys-*p*-NA, Met-*p*-NA were the best substrates for aminopeptidase activity, while little activity was observed when Pro-*p*-NA was used as substrate.

No differences were observed in the enzymic activity of the two strains on the 19 substrates of the API ZYM System. The activity of enzymes such as galactosidases, esterases, arylamidases and phosphatases remained unchanged.

This suggests that the metabolic defect in the slow milk coagulating variant correlates with an altered activity of a cell-surface proteinase. Additional studies are in progress to characterize cell-surface proteinases of *L. helveticus* strain ATCC 15009 and its variant and to compare their catalytic activity toward different kinds of substrates.

Genotypic characterization of variant M193. The selection of Prt$^-$ variants of lactococci and lactobacilli has often been associated with the presence of plasmid DNA. In most cases the loss of the proteolytic activity is accompanied by the loss of plasmid DNA and the mutation frequency could be enhanced by treating the cells with curing agents. This strongly suggests the involvement of extrachromosomal DNA molecules (Otto *et al.* 1982; Morelli *et al.* 1986; De Rossi *et al.* 1989; St-Gelais *et al.* 1993).

As previously reported (Fortina *et al.* 1993), strain *L. helveticus* ATCC 15009 contains three plasmids sized 22.0, 6.0, 3.5 kbp and designated pLH1, pLH2, pLH3, respectively. To determine whether the loss of proteolytic activity in the variant was due to a loss of plasmid DNA, cleared lysates of the two strains were prepared and purified by CsCl–EtBr ultracentrifugation. After analysis by agarose gel electrophoresis the variant showed the same profile of the parental strain and the plasmids were characterized by the same size. To investigate whether the MIT treatment determined an alteration of the nucleotide sequence of plasmid molecules, individual plasmids were eluted from the

agarose gel and further characterized with different RE. Particularly, pLH1 was digested with *Apa*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Pvu*I, *Sac*I, *Stu*I, pLH2 was digested with *Eco*RI, *Eco*RV, *Hae*III, *Hin*dII, *Hin*dIII, *Kpn*I and pLH3 with *Hae*III, *Hin*dII, *Hin*dIII, *Xba*I. In addition, the chromosomal DNA fingerprinting of the two strains was obtained with different RE: *Apa*I, *Bgl*II, *Eco*RI, *Hae*III, *Hin*dIII, *Mlu*I, *Pst*I, *Pvu*II, *Ssp*I, *Sph*I. Comparison of the restriction patterns obtained with both plasmid and chromosomal DNA clearly revealed no visible difference between the two strains (*data not shown*).

Therefore the genetic alteration is probably due to a point mutation of plasmid or chromosomal DNA. This is in agreement with the action of alkylating agents, such as MIT, which can determine base substitution on the nucleotide sequence of DNA. According to a missense mutation, the alteration affecting the ability of the proteolytic enzymes to hydrolyze casein may result in the production of altered proteins still capable of performing this function, but with a partial loss of catalytic activity.

This alteration that reduces proteolytic activity with the maintenance of a high aminopeptidase activity, can be exploited to obtain suitable variants for experimental cheese manufacture. Indeed, the objective of recent studies has been to investigate the performance of proteinase-negative strains in cheese ripening and the influence of high aminopeptidase activity on bitterness reduction (Oberg *et al.* 1986; St-Gelais *et al.* 1992; Prost and Chamba 1994).

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