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Peptide Utilization Encoded by *Lactococcus lactis* subsp. *lactis* SSL135 Chromosomal DNA

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A cloned chromosomal fragment of *Lactococcus lactis* subsp. *lactis* SSL135 on plasmid pVS8 in an *L. lactis* subsp. *lactis* MG1614 background enabled proteinase-negative strain MG1614 to grow in autoclaved milk. The strain (VS230) did not, however, degrade milk proteins and did not grow in pasteurized milk. In contrast, a strain (VS150) carrying pVS9, the proteinase plasmid of SSL135, in an MG1614 background degraded β-casein but did not grow in milk. VS230 was shown to utilize peptides produced by VS150 in growth experiments in pasteurized milk preincubated with the latter strain. The peptide utilization phenotype linked with pVS8 was further confirmed by growth of VS230 on tryptic peptide fractions, on which the plasmid-free but otherwise isogenic strain MG1614 failed to grow. Plasmid pVS8 produced 69-, 42-, 38-, and 36-kilodalton proteins, as determined by in vitro transcription-translation. At least three of these proteins affected the peptide utilization phenotype. We suggest that there could be a coupled peptidase-peptide transport system encoded by the chromosomal fragment.

Dairy starters characteristically produce lactic acid rapidly from lactose and coagulate milk. The amount of free amino acids and low-molecular-weight peptides in milk is too low to support the synthesis of bacterial proteins necessary for high cell densities (11). To achieve good growth and acid production, starter organisms must have several proteinases and peptidases to hydrolyze milk proteins.

Milk proteins are degraded by cell wall-bound proteinases (14). The resulting oligopeptides are further degraded to smaller peptides by peptidases that may be cell wall bound (7, 9). Amino acids and small peptides are transported inside the cell, there being distinct systems for amino acids and di- and oligopeptides (8, 12). In contrast to what is known about amino acid transport in lactococci (2, 3), knowledge about peptide transport is still very limited. It has been suggested that there is either a separate peptide transport system or a peptidase-coupled transport system in the cell wall (16). The internal pH of the cell has been shown to be an important factor in peptide uptake in *Lactococcus lactis* subsp. *cremoris* (17).

The known cell wall-bound proteinases of lactococci are plasmid encoded (5, 6). Eliminating or deleting proteinase plasmids yields proteinase-negative strains that do not grow in milk (5, 10). In our earlier work we reported that a cloned chromosomal DNA fragment of *Lactococcus lactis* subsp. *lactis* SSL135 on plasmid pVS8 enabled MG1614, a proteinase-negative *L. lactis* subsp. *lactis* strain (5), to coagulate autoclaved milk (20). The strain did not grow in pasteurized milk (unpublished observation). Autoclaving is known to partly hydrolyze milk proteins and thus to stimulate the growth of starter bacteria (4). We noticed also that MG1614 carrying plasmid pVS9 (the lactose-proteinase plasmid of *L. lactis* subsp. *lactis* SSL135) was unable to grow either in autoclaved or in pasteurized milk, although the strain degraded β-casein (unpublished observation). We have previously reported that plasmids pVS8 and pVS9 complement each other in growth experiments in autoclaved milk (15). This complementation also occurs in pasteurized milk.

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Because of these observations we wanted to investigate whether the cloned fragment on plasmid pVS8 was somehow involved in peptide utilization and to further characterize its gene products. In this study we describe the influence of plasmid pVS8 on the growth of the host strain on different casein breakdown products and characterize the proteins encoded in vitro by plasmid pVS8.

MATERIALS AND METHODS

Enzymes and chemicals. All restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were from Boehringer Mannheim GmbH (Penzberg, Federal Republic of Germany). *Bal*31 nuclease and T4 DNA polymerase were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Lysozyme and glycine were purchased from Sigma Chemical Co. (St. Louis, Mo.), and all other amino acids were purchased from E. Merck AG, (Darmstadt, Federal Republic of Germany).

Bacterial strains and growth conditions. The bacterial strains and plasmids are listed in Table 1. The strains were grown in M17 medium (13) supplemented with 0.5% glucose or lactose at 30°C. The ability to grow in milk was tested both in reconstituted autoclaved (120°C, 15 min) skim milk (Difco Laboratories, Detroit, Mich.) and in pasteurized (72°C, 15 s) nonfat milk.

Plasmid DNA isolation. Plasmid DNA was isolated as described previously (1). A Gene Clean kit (Bio 101, Inc., La Jolla, Calif.) was used in accordance with the manufacturer's instructions for restriction fragment isolation.

Transformation. Protoplasts of *L. lactis* subsp. *lactis* MG1614 were prepared and transformed as described previously (19).

Construction of deletion derivatives of plasmids. Deletion derivatives of plasmid pVS8 were constructed by *Bal*31 treatment as described previously (15). Plasmid pVS12 was linearized with *Bgl*II and treated with *Bal*31 nuclease and T4 DNA polymerase in the same way, except that the *Bal*31 treatment was for 5 min only.

Titratable acidity. Strains were grown in M17 medium to the mid-log phase, centrifuged, and washed with sterile

TABLE 1. *L. lactis* subsp. *lactis* strains and plasmids

Strain or plasmid	Phenotype[a]	Growth in autoclaved milk[b]	Description (reference)
Strains			
MG1614	Lac$^-$ Rif^r Str^r	−	Plasmid-free derivative of *L. lactis* subsp. *lactis* NCDO 712 (5)
VS230(pVS8)	Lac$^-$ Rif^r Str^r Cm^r	+	MG1614 transformed with pVS8 (20)
VS150(pVS9)	Lac$^+$ Rif^r Str^r Prt$^+$	−	MG1614 transformed with SSL135 plasmid pVS9 (20)
SSL135[c]	Lac$^+$ Prt$^+$	+	Isolated from a starter culture (18)
Plasmids (kilobases [kb])			
pVS2 (5)	Cm^r Em^r	−	Cloning vector constructed in this laboratory (20)
pVS7 (8.5)	Cm^r	−	Deletion derivative of pVS8 (20)
pVS8 (14.4)	Cm^r	+	Contains chromosomal fragment of SSL135 cloned on pVS2 (20)
pVS9 (>50)	Lac$^+$ Prt$^+$	−	Causes β-casein degradation (this study) but does not enable MG1614 to grow in milk (15)
pVS11 (6.5)	Cm^r	−	6.5-kb *Hin*dIII fragment of pVS8 (this study)
pVS12 (12.9)	Cm^r	−	Largest deletion derivative of pVS8 obtained by *Bal*31 treatment (15)
pVS19 (11)	Cm^r	−	pVS11 with 4.1-kb *Hin*dIII fragment of pVS8 (this study)
pVS32 (5.6)	Cm^r	−	pVS11 with 0.9-kb *Eco*RI fragment deleted (this study)
pVS35 (11.6)	Cm^r	−	Deletion derivative of pVS12 constructed by *Bal*31 treatment (from the *Bgl*II site) (this study)
pVS37 (12.6)	Cm^r	−	9.4-kb *Cla*I-*Bcl*I fragment of pVS8 ligated to the 3.2-kb *Cla*I-*Bcl*I fragment of pVS35 (this study)

[a] Lac$^+$, Lactose fermentation; Lac$^-$, no lactose fermentation; Prt$^+$, β-casein degradation; Cm^r, Rif^r, Str^r, and Em^r, resistance to chloramphenicol, rifampin, streptomycin, and erythromycin.

[b] Coagulation (+) or lack of coagulation (−) of milk in an overnight culture.

[c] Carries pVS9 and three cryptic plasmids.

water, and 5% inocula were made in autoclaved milk (supplemented with 0.5% glucose). The milk samples were incubated at 30°C. Samples of 10 ml were titrated after 20 and 40 h (DL20 compact titrator; Mettler Instrumente AG, Zürich, Switzerland).

Effect of preincubating milk with VS150 on the subsequent growth of VS230. Strains VS150 and MG1614 were grown to the mid-log phase in M17 medium. Cells were harvested by centrifugation and washed with Ringer solution (Ringer Tabletten; E. Merck AG), and 5% inocula were made in pasteurized milk (supplemented with 0.5% glucose). Inoculated milk samples were incubated for 6 h at 30°C. Rifampin (50 μg/ml) was used to prevent the growth of any contaminating bacteria. Strain VS230 was grown in M17 medium and washed, and 5% inocula were made in incubated milk. The growth of the strains was monitored by plating cells in parallel onto M17-glucose, M17-lactose-bromcresol purple (50 mg/liter), and M17-glucose-chloramphenicol (5 μg/ml) plates. The pH values of the milk samples were also determined.

Amino acid requirements. The amino acid requirements of the strains were tested with Otto synthetic medium (R. Otto, Ph.D. thesis, University of Groningen, Groningen, The Netherlands, 1981). The medium was prepared as follows. To 800 ml of deionized water was added 10 g of glucose, 2.5 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.6 g of triammonium citrate, 1.0 g of sodium acetate, and 0.5 g of L-(+)-ascorbic acid (the pH of this solution was kept at 6.4 with 1 N HCl); 10 ml of vitamin solution, 10 ml of trace element solution, 10 ml of nucleic acid base solution, relevant volumes of amino acid solutions, and 1 ml of $MnSO_4$ solution (2.8 g/100 ml) were added, and the volume was brought to 1 liter with deionized water. The vitamin solution (pH 7.0) contained (per liter of water) pyridoxol hydrochloride, 200 mg; nicotinic acid, 100 mg; thiamine hydrochloride, 100 mg; riboflavin, 100 mg; calcium pantothenate, 100 mg; sodium *p*-aminobenzoate, 1 g; D-biotin, 1 g; folic acid, 100 mg; vitamin B_{12}, 100 mg; orotic acid, 500 mg; 2-deoxythymidine, 500 mg; inosine, 500 mg; DL-6,8-thioctic acid, 250 mg; and pyridoxamine dihydrochloride, 500 mg. The trace element solution contained (per liter of deionized water) $MgCl_2 \cdot 6H_2O$, 20 g; $CaCl_2 \cdot 2H_2O$, 5 g; $FeCl_2 \cdot 4H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 0.5 g; $CoCl_2 \cdot 6H_2O$, 0.25 g; and $CuSO_4 \cdot 5H_2O$, 0.01 g. The nucleic acid base solution contained (per 10 ml of 0.1 N NaOH) adenine, 10 mg; uracil, 10 mg; xanthine, 10 mg; and guanine, 10 mg. The amino acid solutions were prepared separately and added to the chemically defined medium to final concentrations (milligrams per liter) as follows: L-alanine, 75; sodium L-glutamate monohydrate, 1,600; L-aspartic acid, 150; L-arginine monohydrochloride, 225; L-lysine monohydrochloride, 400; L-isoleucine, 50; L-methionine, 35; L-phenylalanine, 50; L-serine, 1,000; L-threonine, 50; L-tryptophan, 50; L-valine, 75; glycine, 100; L-histidine monohydrochloride, 275; L-tyrosine, 50; L-leucine, 100; L-ornithine monohydrochloride, 150; L-proline, 270; and L-citrulline, 150. All the solutions were sterilized by filtering. The amino acid solutions and vitamin stock solution were stored at −20°C. Strains were grown in M17 medium to the mid-log phase, centrifuged, and washed with sterile water. An inoculum with an A_{580} of 0.05 was made in the synthetic medium. One amino acid at a time was omitted from the medium, and the growth of the strains was measured spectrophotometrically (A_{580}) after incubation for 20 and 40 h at 30°C. Those amino acids without which the strain did not grow were considered to be necessary for the strain. The minimum concentrations of the necessary amino acids were also determined. The minimum concentration was defined as the lowest amino acid concentration that yielded an A_{580} of 0.6 in 20 h at 30°C. The minimum concentrations (in micrograms per milliliter) obtained for strains MG1614 and VS230 were as follows: methionine, 2.5; valine, 5; glutamic acid, 20; histidine, 2.5; leucine, 5; and isoleucine, 1. Glutamic acid and isoleucine were stimulatory; i.e., the strains grew without them in 40 h but not in 20 h.

Isolation of tryptic peptides, molecular size determination, and amino acid analysis. Tryptic peptides were isolated from

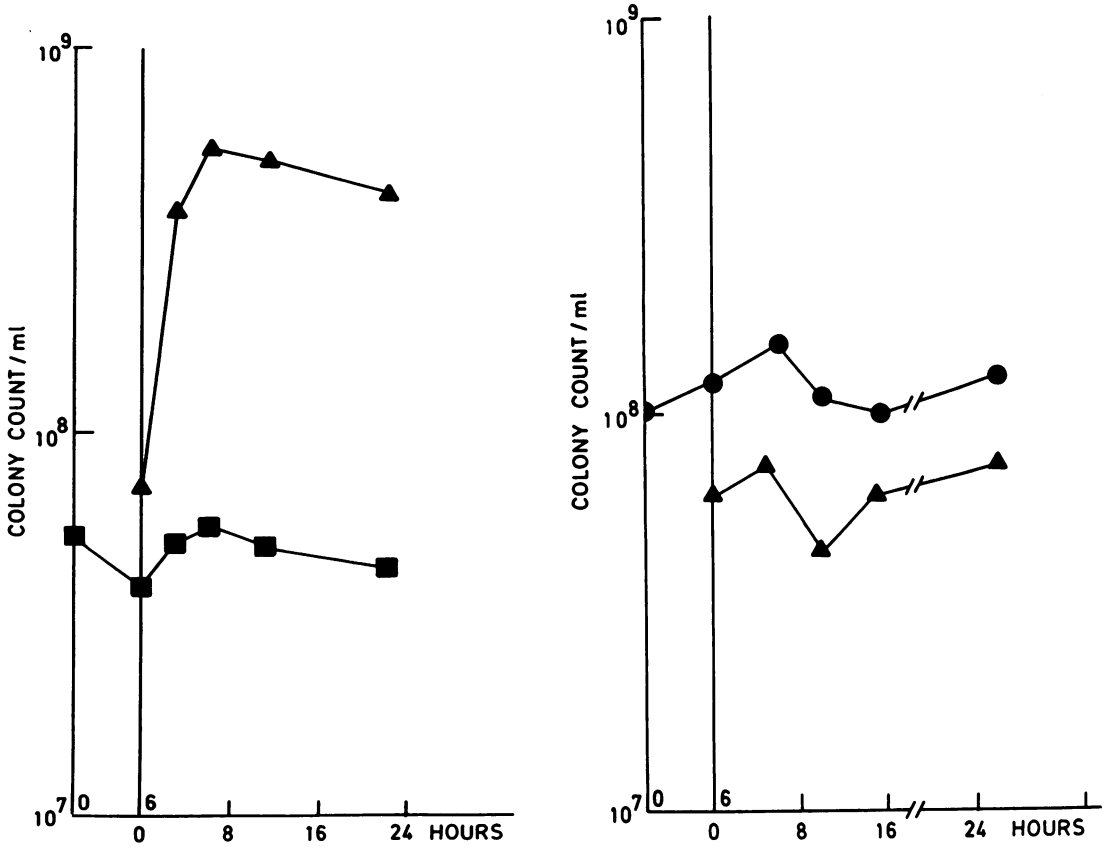

FIG. 1. Growth of strain VS230 in milk previously incubated with strain MG1614 or strain VS150. The first vertical axis represents the start of preincubation, and the second one represents the time at which the preincubated milk was inoculated with strain VS230. Symbols: ■, VS150; ●, MG1614; ▲, VS230.

tryptone (Difco) on a Bio-Gel P4 column (2 by 90 cm; 100/200 mesh; BioRad Laboratories, Richmond, Calif.). The eluent was 0.02 M sodium phosphate buffer (pH 7.6) containing 0.05 M NaCl. The flow rate was 1.8 ml/min. The peaks from three runs were collected, and individual peaks were pooled. The chromatography system consisted of fast protein liquid chromatography equipment (Pharmacia, Uppsala, Sweden) with a UV detector at 214 nm and a recorder. The molecular weights of the peptide fractions were determined with a Superose 12 column (Pharmacia) and the following standards (molecular weight): insulin B (3,495), bacitracin (1,450), actinomycin (1,280), bradykinin (1,060), and Pz-Pro-Leu-Gly-Pro-D-Arg (777). The peptide fractions collected from the Bio-Gel P4 column were hydrolyzed in 6 N HCl in Pierce vacuum hydrolysis tubes under nitrogen for 4 h at 107°C with a Reacti-Therm heating module (Pierce Chemical Co., Rockford, Ill.). The amino acid determinations were carried out with an LC 5001 amino acid analyzer (Biotronic, Pucheim, Federal Republic of Germany), the analog output of which was monitored with a C-R4A reporting integrator (Shimadzu). A lithium column and reagents were used as outlined in the Biotronic instruction manual. Amino acids were reacted with ninhydrin, and the instrument was calibrated with a type ANB standard (Benson Co., Reno, Nev.).

Growth on tryptic peptides. The growth medium was that of Otto but with all the necessary and stimulatory amino acids omitted and replaced by isolated tryptic peptides. The amount of peptide added was calculated to satisfy the minimum amino acid requirements of the strains. The utilization of the peptides was measured as an increase in the A_{580} after incubation for 20 h at 30°C.

In vitro protein synthesis. A DNA expression system kit (Dupont, NEN Research Products, Dreieich, Federal Republic of Germany) was used in accordance with the manufacturer's instructions. The label was [^{35}S]methionine. Labeled proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel, which was then fixed, dried, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Cross-feeding between strains carrying pVS9 and pVS8. The growth of the strains is shown in Fig. 1. Strain VS150 promoted the growth of strain VS230, the cell counts in pasteurized milk increasing sevenfold in 5 h and the pH decreasing to 4.5 in 10 h. Control strain MG1614 did not have this effect. VS150 itself did not grow in milk and did not support the growth of MG1614 (data not shown).

Growth of strains MG1614 and VS230 on tryptic peptides. The molecular masses of the isolated tryptic fractions were determined to be 12,300 and 8,700 daltons (Da) (fraction 1), 3,600 Da (fraction 2), 3,000 Da (fraction 3), 2,700 Da (fraction 4), and 2,100 Da (fraction 5). The growth of the strains on different tryptic fractions is shown in Fig. 2. Strain VS230 grew well on 3,600-Da tryptic peptides, while the growth of MG1614 was detectable only on 2,100-Da tryptic peptides.

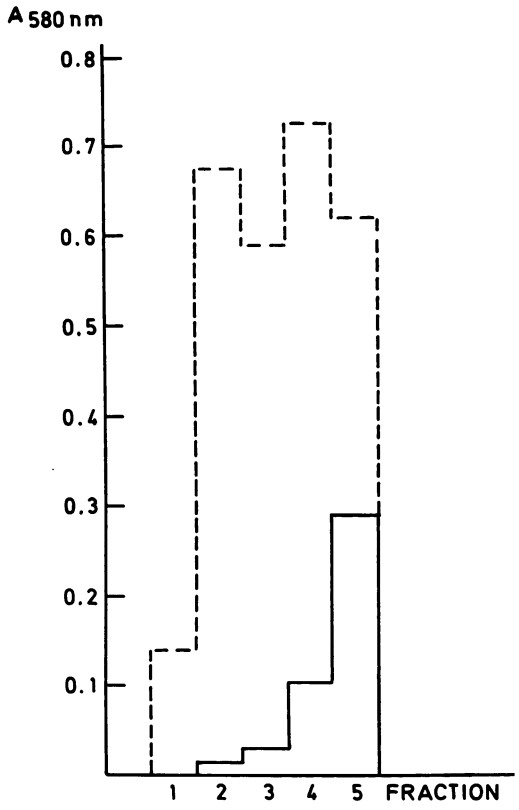

FIG. 2. Growth of strains MG1614 (——) and VS230 (– – –) in synthetic medium on tryptic peptide fractions of 12,300 and 8,700 Da (fraction 1), 3,600 Da (fraction 2), 3,000 Da (fraction 3), 2,700 Da (fraction 4), and 2,100 Da (fraction 5).

In vitro transcription-translation. Plasmid pVS8 encoded four proteins that were different from the protein products of the cloning vector pVS2. The sizes of the proteins were determined to be 69, 42, 38, and 36 kilodaltons (kDa), and the proteins were named A, B, C, and D, respectively. The autoradiographs of the proteins encoded by plasmid pVS8, its deletion derivatives, and subclones are shown in Fig. 3. The derivatives of plasmid pVS8 used in the in vitro transcription-translation experiments and the regions coding for different proteins (based on the autoradiography results) are shown in Fig. 4.

Effect of proteins encoded by pVS8 on titratable acidity. The ability of the strains to produce lactic acid in autoclaved milk at 30°C is shown in Table 2. The results showed that if the 69-kDa protein (A) was truncated (pVS19) or was missing (pVS12) the ability of the strain to produce acid slowed down. Neither 42- and 36-kDa proteins (B and D; pVS11) nor 69- and 38-kDa proteins (A and C; pVS37) alone supported the acid production phenotype. At least protein A, protein C, and either protein B or D or both are needed to achieve good growth and acid production in autoclaved milk.

DISCUSSION

Cooperation between proteolytic enzymes and peptidases of starter bacteria has a very important role not only for the cells themselves but also for the dairy industry. In this paper we describe the combined effects of a plasmid-encoded proteinase and an ability encoded by chromosomal DNA to utilize peptides.

Plasmid pVS9 in an MG1614 background encodes a proteinase that degrades β-casein (clearly seen in polyacrylamide gel electrophoresis; unpublished results), but the strain is unable to grow either in pasteurized or in autoclaved milk. Strain VS230 grows in hydrolyzed milk (either autoclaved or degraded by a pVS9-encoded proteinase) but is itself unable to hydrolyze α-, β-, or κ-casein, α-lactalbumin, or β-lactoglobulin (data not shown).

When strains harboring plasmid pVS9 or pVS8 are incubated together, it seems that the proteinase encoded by pVS9 supplements the growth of strain VS230. The ability of VS230 to use peptides does not, however, result in free amino acids or low-molecular-weight peptides in the medium, at least in amounts sufficient to support the growth of strain VS150. The poor growth of this strain suggests that it is deficient in amino acid and peptide transport in comparison with other known proteinase-positive strains. This hypothesis is supported by the results of growth experiments

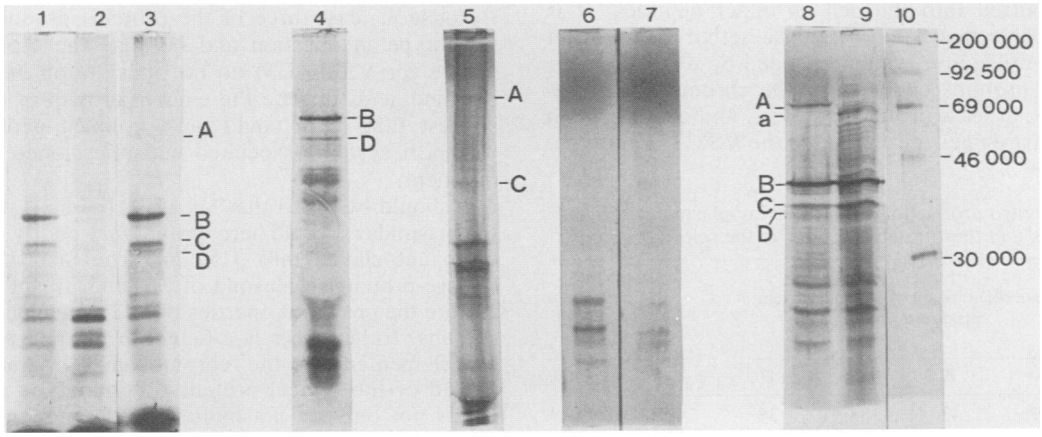

FIG. 3. Autoradiography of the proteins encoded by plasmid pVS8 and its derivatives. Lanes: 1, pVS12; 2, cloning vector pVS2; 3, pVS8; 4, pVS11; 5, pVS37; 6, pVS7; 7, pVS32; 8, pVS8; 9, pVS19; 10, ^{14}C-labeled standards with molecular weights of 200,000 (myosin), 92,500 (phosphorylase *b*), 69,000 (bovine serum albumin), 46,000 (ovalbumin), and 30,000 (carbonic anhydrase). Proteins A, a (the truncated form of protein A), B, C, and D, which are encoded by the cloned chromosomal fragment, are shown. Lanes 1 to 3, 4, 5, 6 and 7, and 8 to 10 were run separately under slightly different electrophoretic conditions. In each experiment proteins produced by pVS8 and molecular weight standards were used as references, although they are not included in the figure.

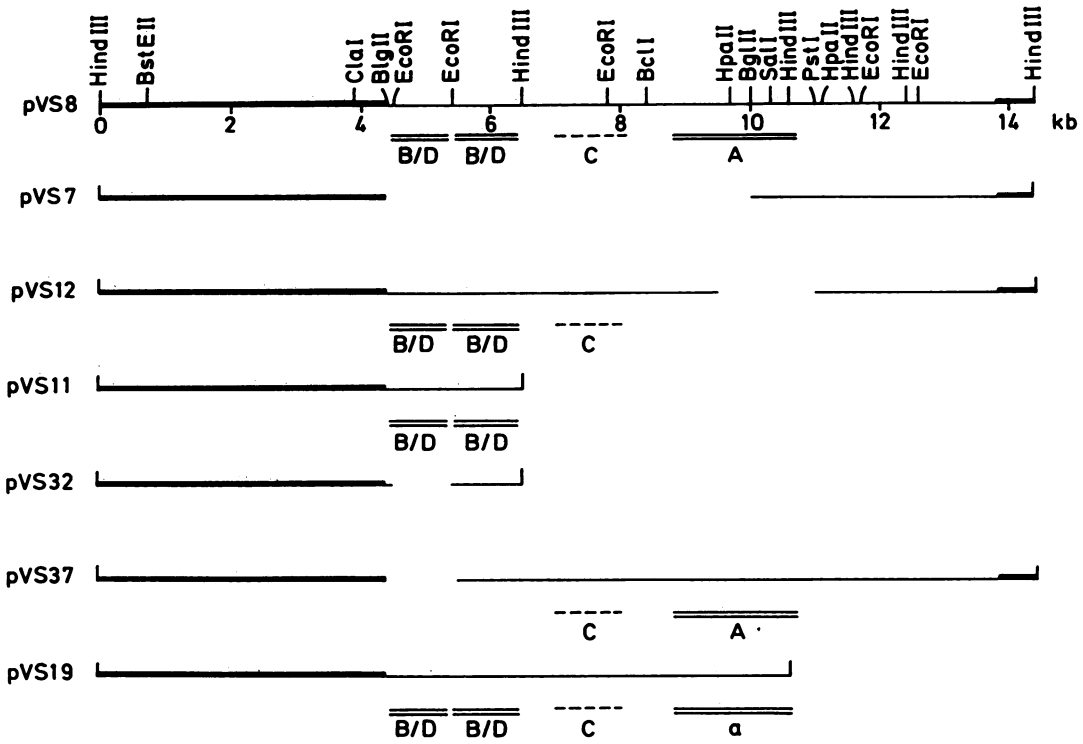

FIG. 4. Restriction maps and protein-coding regions of plasmid pVS8 and its derivatives. The thick lines represent the cloning vector pVS2. Protein-coding regions are shown as bars under the restriction maps. The dotted lines represents protein C, whose exact location in the *Hin*dIII-*Bcl*I fragment is not known. kb, Kilobases.

with tryptic peptides, in which it can be seen that the cloned chromosomal fragment on pVS8 is involved in peptide utilization. (Strain VS150 is not able to grow on tryptic peptides in synthetic medium [data not shown].)

In this study it was found that the largest peptide which could be used by VS230 was about 3,600 Da. The uptake limit for peptides in lactococci has been reported to be between three and seven amino acid residues (8, 12). Even though we do not know exactly how many amino acid residues 3,600-Da peptides have, it is doubtful whether they can be transported into the cell as intact peptides. It is therefore probable that some peptidase activity is involved in the peptide utilization system encoded by pVS8.

Of the four proteins encoded by the chromosomal fragment on pVS8, at least proteins A and C and either protein B or D or both are needed to produce the VS230 phenotype.

TABLE 2. In vitro production of proteins by plasmid pVS8 and its derivatives and the titratable acidity of the relevant strains

Plasmid	Size (kDa) of the following protein produced in vitro:				Titratable acidity of autoclaved milk (μmol/ml) at:	
	A	B	C	D	20 h	40 h
pVS8	69	42	38	36	42[a]	44[a]
pVS7					13	16
pVS12		42	38	36	17	34[a]
pVS11		42		36	11	16
pVS37	69		38		13	17
pVS19	65	42	38	36	13	32[a]

[a] Coagulates milk.

When protein A is absent (pVS12), the strain utilizes tryptic peptides in synthetic medium very poorly during the first 20 h. When incubation is continued for a further 20 h, the strain grows well on fractions 3 and 4 (data not shown). The corresponding phenomenon can also be seen in the titratable acidity of the strains in autoclaved milk (Table 2). Thus, the absence of protein A results in a "leaky" peptide utilization phenotype.

From the present results we are not able to deduce exactly the nature of the peptide utilization system we have cloned. Because at least three of the proteins produced affect the phenotype in question and because the *o*-phthalaldehyde results for VS230 (15) do not indicate an increase in free α-amino acids despite the evident growth of the strain, we suggest that there could be a coupled peptidase-peptide transport system associated with the cloned chromosomal fragment.

It should be noted that MG1614, the host strain for all of the plasmids reported here, grows very poorly in pasteurized or in autoclaved milk (15, 20). The fact that pVS9, the lactose-proteinase plasmid of SSL135, is not alone able to restore the growth properties of MG1614 may be due to two reasons. Either there is a defect in plasmid pVS9 which is complemented by the chromosomal fragment we have cloned or the typical proteolytic phenotype in general depends not only on plasmid-encoded functions but also on chromosomal genes, and a defect in either results in slow growth in milk. The latter hypothesis can be tested by introducing known proteinase plasmids into MG1614 and other proteinase-negative hosts (preferably with variable growth patterns in milk) and checking the resulting phenotypes. This work is in progress in our laboratory.

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