

Expression of the *Staphylococcus hyicus* Lipase in *Lactococcus lactis*

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Received 28 June 1999/Accepted 28 October 1999

The extracellular *Staphylococcus hyicus* lipase was expressed under the control of different promoters in *Lactococcus lactis* and *Bacillus subtilis*. Its expression at high and moderate levels is toxic for the former and the latter hosts, respectively. In *L. lactis*, the lipase was expressed at a high level, up to 30% of the total cellular proteins, under the control of the inducible promoter *PnisA*. About 80% of the lipase remained associated with the cells. Close to half of this amount remained associated with the inner side of the cytoplasmic membrane as unprocessed pre-pro-lipase. The other half was trapped by the cell wall and partially degraded at the N-terminal end. This result suggests that extracellular proteases degrade the lipase. Surprisingly, the kinetics and the pattern of lipase degradation were different in the two *L. lactis* subspecies, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*. The extracellular proteolytic systems that degrade lipase are thus different in these closely related subspecies. The incorrect export of the lipase is not due to an inappropriate leader peptide but may be due to an inefficiency of several steps of lipase secretion. We propose that (i) the *S. hyicus* lipase may require a special accessory system to be correctly exported or (ii) the kinetics of lipase synthesis may be a critical factor for proper folding.

Lactic acid bacteria are widely used in the production and preservation of foodstuffs. Since these bacteria are generally regarded as safe, their use as delivery vehicles for foreign proteins in the field of medicine can be envisaged. With recent advances in the field of molecular biology, efficient expression vectors have been developed and allow the expression of heterologous proteins in *Lactococcus lactis* (53, 57, 64, 68). The aim of this work was to express a bacterial lipase in *L. lactis* in order to use lactococci as a lipase delivery vehicle. This goal could alleviate lipase deficiency in the digestive tract during digestion (steatorrhea) or improve flavor development in some cheese-making processes (23, 35, 56).

Among the best-characterized bacterial lipases are those of several *Pseudomonas* and *Staphylococcus* species (24). The genes encoding these lipases have been tested for heterologous expression in a variety of potential industrial production hosts. It appears that the *Pseudomonas* lipase cannot be overexpressed in heterologous hosts to commercially acceptable levels because it requires a helper protein to fold properly (15, 25). Moreover, the high GC content of *Pseudomonas* genes may require resynthesis of a gene to fit with the codon bias of the new host. On the other hand, the lipase of *Staphylococcus hyicus* already has been expressed successfully in *Staphylococcus carnosus* (66), *Escherichia coli* (16), and *Lactobacillus curvatus* (67). No chaperone or specialized helper proteins were reported to be required for the proper folding and secretion of this lipase (24). The GC content of the *lip* gene, 37%, is similar to that of the lactococcal genes and is comparable to that of low-GC-content gram-positive bacterial genes (16). Moreover, general codon usage is not very different in staphylococci and lactococci. This lipase was therefore chosen for overexpression in lactic acid bacteria.

The *S. hyicus lip* gene encodes a pre-pro-protein composed of a signal peptide of 38 amino acids (aa), a pro-peptide of 207

aa, and a mature lipase of 396 aa (66). The pro-protein is secreted by the general secretion machinery. The pro-lipase (86 kDa) is further cleaved into the mature lipase (46 kDa) in the culture medium by the metalloprotease ShpII (3). The mature lipase is threefold more active than the pro-lipase. In heterologous hosts, such as *S. carnosus* and *E. coli*, which lack ShpII, the 86-kDa form is produced (16, 66). The biochemical properties of this lipase are well characterized (66). It has a broad substrate specificity and hydrolyzes first and second ester bonds. It also has high phospholipase activities (A1 and A2), a unique feature for bacterial lipases. These properties are compatible with its use as an enzyme in some food-making processes or as an additive to alleviate lipase deficiency during digestion. However, the amounts of the enzyme needed for these purposes vary greatly. Only a small amount of lipase activity is required in cheese making, while a large amount is needed in treating steatorrhea (35, 56).

In this paper, we describe the cloning and expression of the *lip* gene in *L. lactis* at different levels. Strains producing stable lipase production at a moderate level can be used in cheese-making processes. Moreover, we achieved the overproduction of the *S. hyicus* lipase in an amount that could allow the use of *L. lactis* as a lipase delivery vector for digestive enzymes in the digestive tract. However, the ability of *L. lactis* to correctly secrete this lipase is limited, and the limiting steps were investigated.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *L. lactis* strains were grown in M17 (59) with 0.5% glucose at 30°C. *E. coli*, *Bacillus subtilis*, and *S. carnosus* were grown in Luria-Bertani medium at 37°C. Chloramphenicol was used at a concentration of 10 µg/ml, and erythromycin was used at a concentration of 10 µg/ml for *L. lactis* and 1 mg/ml for *E. coli*. Nisin powder (ICN; 2.5% nisin content) was used at a concentration of 0.5 µg/ml.

Isolation of plasmid DNA and enzyme analysis. Plasmid DNA was isolated by the method of Holmes and Quigley (20) for *E. coli* and by the method of Anderson and McKay (2) for *L. lactis*, *B. subtilis*, and *S. carnosus*. *S. carnosus* was lysed after the addition of lysostaphin (4.5 U/ml). *E. coli* was transformed by the heat shock method (48). *L. lactis* was transformed by a procedure involving electroporation of cells grown in the presence of glycine to weaken the cell wall (21). *B. subtilis* was transformed by the standard method of Spizizen (55). Re-

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
Strains		
<i>B. subtilis</i>		
MTT19	<i>leuB6 trpC2 R⁻</i>	Bacillus Genetic Stock Center
JIM7381	MTT19 carrying pJIM2423	This work
<i>E. coli</i> TG1		
	<i>supE thiD (lac-proAB) hsdD5 F⁺ traD36 proAB lacIZΔM15</i>	10
<i>L. lactis</i>		
IL1403	<i>L. lactis</i> subsp. <i>lactis</i> , plasmid free	9
NZ9000	<i>L. lactis</i> subsp. <i>cremoris</i> , derivative of MG1363 <i>pepN::nisR nisK</i>	12
JIM7049	IL1403 <i>his::nisR nisK</i>	S. Calero
JIM5496	IL1403 carrying pJIM2429	This work
JIM5497	IL1403 carrying pJIM2430	This work
JIM5498	IL1403 carrying pJIM2431	This work
JIM5928	IL1403 carrying pJIM2098	This work
JIM7022	NZ9000 carrying pJIM2093	This work
JIM7048	JIM7049 carrying pJIM2093	This work
Plasmids		
pNEB193	Amp ^r	New England Biolabs
pLipPS1	Cm ^r , <i>lip</i> gene under the control of an <i>S. carnosus</i> promoter	38
pJIM2421	Amp ^r , <i>Pst</i> I fragment from pLipPS1 in pNEB193	This work
pJIM2279	Ery ^r , high- to low-copy-number vector for gram-positive bacteria	47
pBV502	Ery ^r Cm ^r , contains the P5- <i>cat-86</i> cassette	4
pGKV223	Ery ^r Cm ^r , contains the P23- <i>cat-86</i> cassette	65
pGKV232	Ery ^r , Cm ^r , contains the P32- <i>cat-86</i> cassette	65
pGKV244	Ery ^r , Cm ^r , contains the P44- <i>cat-86</i> cassette	65
pGKV259	Ery ^r , Cm ^r , contains the P59- <i>cat-86</i> cassette	65
pNuc11	Ery ^r , contains the <i>nuc</i> gene with the leader sequence for Usp45 under the control of P59	36
pNZ8008	pNZ273 derivative carrying the <i>gusA</i> gene under the control of <i>PnisA</i>	12
pJIM2422	Amp ^r Ery ^r , carrying the <i>lip</i> gene and obtained by fusion between pJIM2421 and pJIM2279	This work
pJIM2423	Ery ^r , carrying the <i>lip</i> gene under the control of <i>Pres</i>	This work
pJIM2424	Ery ^r , pJIM2423 carrying the P5- <i>cat-86</i> cassette upstream of the <i>lip</i> gene	This work
pJIM2425	Ery ^r , pJIM2423 carrying the P23- <i>cat-86</i> cassette upstream of the <i>lip</i> gene	This work
pJIM2426	Ery ^r , pJIM2423 carrying the P32- <i>cat-86</i> cassette upstream of the <i>lip</i> gene	This work
pJIM2427	Ery ^r , pJIM2423 carrying the P44- <i>cat-86</i> cassette upstream of the <i>lip</i> gene	This work
pJIM2428	Ery ^r , pJIM2423 carrying the P59- <i>cat-86</i> cassette upstream of the <i>lip</i> gene	This work
pJIM2429	Ery ^r , pJIM2423 carrying the <i>lip</i> gene under the control of P23	This work
pJIM2430	Ery ^r , pJIM2423 carrying the <i>lip</i> gene under the control of P44	This work
pJIM2431	Ery ^r , pJIM2423 carrying the <i>lip</i> gene under the control of P59	This work
pJIM2093	Cm ^r , pNZ8008 carrying the <i>lip</i> gene under the control of <i>PnisA</i>	This work
pJIM2098	pNuc11 containing the pro-lipase sequence instead of the mature <i>nuc</i> sequence	This work

striction and modification enzymes were purchased from Boehringer and used according to the instructions of the supplier.

Expression of the *lip* gene. (i) **Construction of a promoter-probe vector with the *lip* gene.** A 3.5-kb *Pst*I fragment of *S. carnosus* plasmid pLipPS1 (38) containing the *lip* gene, which encodes the pre-pro-lipase, was cloned into plasmid pNEB193 (New England Biolabs) to yield pJIM2421. This plasmid was digested with *Eco*RV and ligated into the general gram-positive cloning vector pJIM2279 (47) cleaved by *Pme*I, resulting in plasmid pJIM2422. A 3.7-kb fragment containing pNEB193 and a staphylococcal DNA fragment was then removed by *Sma*I-*Nru*I digestion to give pJIM2423 (Fig. 1). This plasmid should be able to replicate in most gram-positive hosts and carries the *lip* coding sequence under the transcriptional control of the weak constitutive plasmid promoter *Pres*.

(ii) **Insertion of lactococcal promoters upstream of the *lip* gene.** The strong and constitutive promoters P5 (4) and P23, P32, P44, and P59 (65) were cloned upstream of the *lip* gene as outlined in Fig. 1. *Xho*II fragments containing the promoter-*cat-86* cassette of pBV502 (4) and pGKV223, pGKV232, pGKV244, and pGKV259 (65) were inserted into the *Bam*HI site of pJIM2423 to yield, respectively, pJIM2424, pJIM2425, pJIM2426, pJIM2427, and pJIM2428. Then, the *cat-86* gene was deleted by *Sph*I restriction, followed by ligation. This procedure put the *lip* gene directly under the transcriptional control of these promoters.

(iii) **Expression of the *lip* gene under the control of the inducible promoter *PnisA*.** The *Xho*I-digested plasmid pNZ8008 (12) containing the inducible pro-

motor *PnisA* was introduced into the *Bam*HI site of pJIM2423 to yield pJIM2092. The initial vector carrying *PnisA* was then deleted by *Xho*I digestion, followed by ligation. The resulting plasmid, pJIM2093, was transformed in *L. lactis* strains containing *nisRK*, the genes necessary to induce *PnisA*. One strain is *L. lactis* subsp. *cremoris* NZ9000, a derivative of NZ3900 (12), and the other is *L. lactis* subsp. *lactis* JIM7049, a derivative of IL1403 (S. Calero, personal communication).

Substitution of the lipase signal peptide with the Usp45 signal peptide. The oligonucleotides used to amplify the *lip* gene without the region encoding the signal peptide were 5'-GGCGTGGCAGATGCATATGATTCG-3' and 5'-GTTTAAACCTGCGGCGCAATTTTGA-3'; the 2.55-kb PCR fragment obtained was cloned by use of *Nsi*I and *Not*I (italic letters), instead of the *nuc* gene on pNuc11 (36). In the resulting plasmid, pJIM2098, the pro-lipase is fused precisely with the leader peptide of Usp45 and is under the control of P59. The fusion was confirmed by sequence analysis. The product of the fused gene should be identical to that of the natural gene after secretion.

Preparation of cellular and supernatant fractions. The cells were recovered from the medium by centrifugation for 10 min at 6,000 × g and treated as previously described (37). The supernatants were passed through filters (0.25-μm pore size) to remove eventual cellular debris. The extracellular proteins were precipitated by the addition of solid ammonium sulfate to the supernatant to 70% (wt/vol) saturation (66). After agitation for 2 h at 4°C, the precipitate was

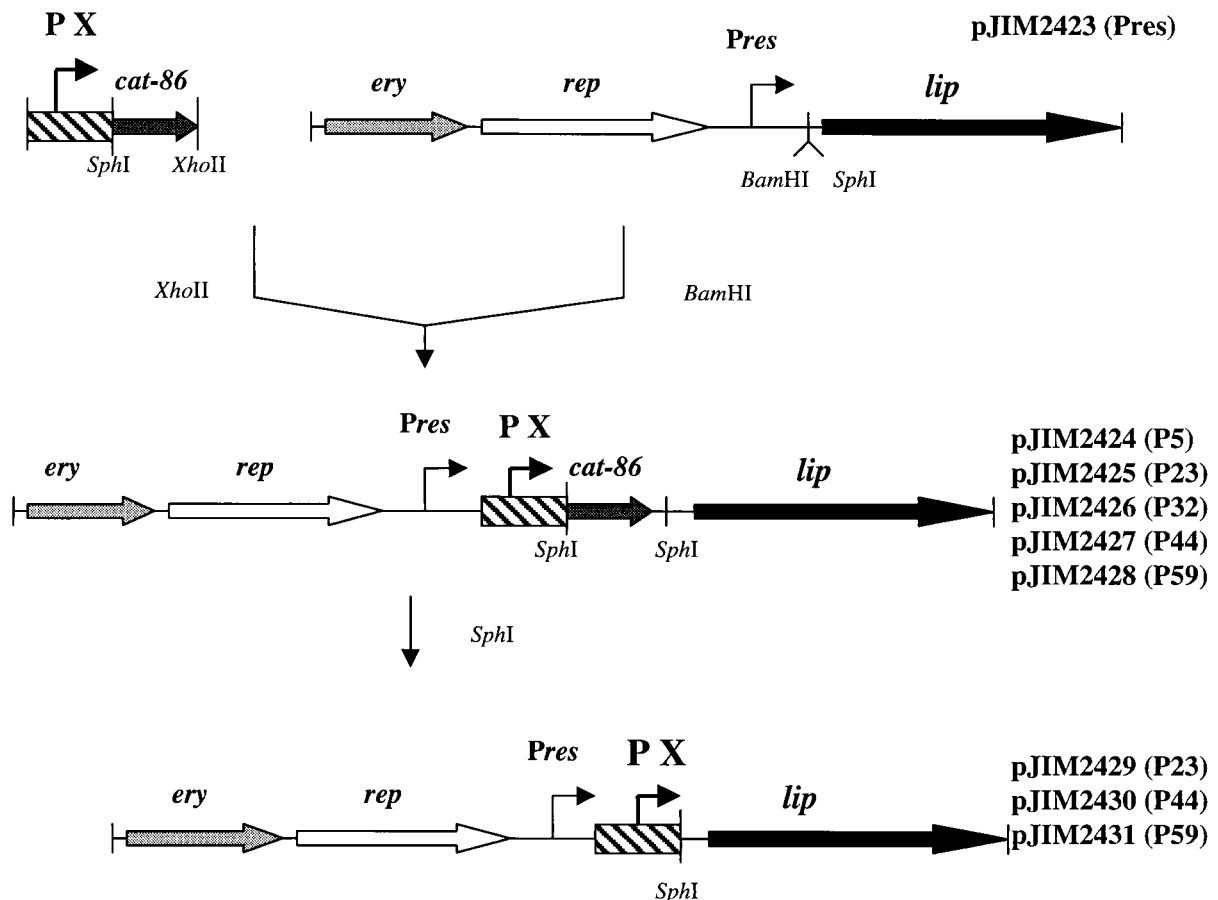


FIG. 1. Strategy used to place the *lip* gene under the control of a strong constitutive promoter. Chloramphenicol resistance is used to select the first cloning step in *L. lactis*. PX, P5, P23, P32, P44, or P59.

collected by centrifugation for 30 min at $10,000 \times g$ and 4°C . The pellets were resuspended in 20 mM Tris-HCl buffer (pH 8) and dialyzed for 14 h at 4°C .

Enzymatic assays. The lipase activity of lactococcal colonies was determined qualitatively by use of an agar plate assay medium containing 1% Tributyrin (Sigma) as a substrate and with nisin at $0.5 \mu\text{g/ml}$ for JIM7048 and JIM7022. Lipolysis is recognized as a zone of hydrolysis around the colonies. Lipase activity was determined quantitatively by monitoring the hydrolysis of tributyrin spectrophotometrically at 450 nm. The tributyrin was emulsified at 1% by sonication in a buffer consisting of 100 mM Tris-HCl (pH 8) and 25 mM CaCl_2 . The results obtained were converted to units per milliliter (1 U equals $1 \mu\text{mol}$ of liberated fatty acids/min). The lipase of *Rhizopus arrhizus* (Sigma; 50,000 U/ml) was used as a reference. Lactate dehydrogenase (LDH) activities were measured by monitoring the reduction of NAD in the presence of pyruvate spectrophotometrically at 340 nm (19).

Analysis of lipase and its cleavage products by Western blotting. To monitor the production and the degradation products of lipase in *L. lactis* and *B. subtilis*, antilipase antibodies directed against the C-terminal (from aa 222 to the end) and N-terminal (from aa 39 to aa 222) parts of the pro-lipase were produced. The C- and N-terminal parts of the protein were fused to the *E. coli* maltose binding protein in order to purify it. The C-terminal part was cloned from a 1.45-kb *Sal*I fragment of pJIM2423 and fused in frame with the maltose binding protein in the *Sal*I site of pMal-p2 (New England Biolabs). The N-terminal part corresponding to the beginning of the gene without the signal peptide was cloned from a 0.8-kb PCR fragment ending with *Bam*HI and *Sal*I. The oligonucleotides used were 5'-CGGCTTATGGATCCGCGTCGTCGGT-3' (*Bam*HI site in italic letters) and 5'-GGAACGTCGACTTGTTCGGT-3' (*Sal*I site in italic letters). The fusion proteins MBP-CtermLIP and MBP-NtermLIP were overproduced after induction by isopropyl- β -D-thiogalactopyranoside (IPTG) and purified on affinity columns according to the instructions of the supplier. The purified fusion proteins were inoculated into rabbits to produce polyclonal antisera. Anti-CtermLIP and anti-NtermLIP sera do not reveal proteins from the wild-type *L. lactis* strains in Western blotting and are specific for the *S. hyicus* lipase.

Anti-PepC antibodies used as a control for cell lysis were kindly provided by M.-P. Chapot-Chartier (Laboratoire de Recherches sur les Protéines, Institut National de la Recherche Agronomique, Jouy en Josas, France).

Proteins were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels

(34) and then either stained with Coomassie blue or immunoblotted with polyclonal rabbit antibodies (1/1,000), followed by ECL kit detection (Amersham).

Localization of the heterologous lipase. The culture was grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.5 and centrifuged for 10 min at $6,000 \times g$ and 4°C to separate the supernatant from the cells. Cells were washed and resuspended in a buffer containing 0.5 M sucrose, 0.04 M magnesium acetate, 1 mM ammonium acetate, 0.04 M CaCl_2 , and 2 mg of lysozyme (pH 7) per ml, incubated for 1 h at 37°C (50), and then divided into three aliquots. According to previously described protocols (1, 46), the first aliquot was kept for 30 min at 37°C as a control. The second aliquot was treated with trypsin (Sigma) (1 mg/ml) for 30 min at 37°C . In this sample, trypsin will digest only the proteins bound to the membrane and presented outside the cell. The third aliquot was treated with 1% Triton X-100 for 5 min and then with trypsin (1 mg/ml) for a further 30 min at 37°C . The Triton X-100 treatment will dissolve the membranes, and all proteins should be digested by trypsin. To terminate the trypsin digestion in the second and third aliquots, 4 mg of trypsin inhibitor (Sigma) per ml was added at the end of the incubation. The first two aliquots were then centrifuged for 10 min at $6,000 \times g$ to collect the protoplasts and separate them from the cell wall extract (supernatant of the lysozyme-treated cells). Finally, the protoplasts were suspended in a buffer provoking their osmotic lysis. The lysed samples were centrifuged for 20 min at $13,000 \times g$. The cytoplasmic fraction was in the supernatant, and the membrane-bound fraction was in the pellet.

Computer analysis. The search for the codon bias of highly expressed genes in staphylococci and lactococci was made with the help of the Nakamura database (41) and with 139,261 codons for *L. lactis* and 172,616 codons for *Staphylococcus aureus*. The bias for the highly expressed glycolytic genes previously reported in *L. lactis* (8) was confirmed in this analysis. *S. aureus* was selected as a representative of the genus *Staphylococcus* because the number of sequenced genes in *S. hyicus* is not sufficient for statistical analysis. The bias for highly expressed genes in *B. subtilis* was kindly communicated by Y. Moszer.

RESULTS

Constitutive expression of the lipase at a high level is toxic to *L. lactis* cells. Five strong promoters from *L. lactis* were

TABLE 2. Qualitative and quantitative lipolytic activities of different constructions carrying the *S. hyicus lip* gene in *L. lactis* IL1403^a

Plasmid	Cassette	Halos around colonies ^b	Maximal lipolytic activity (U/ml)	
			Intracellular	Extracellular
pJIM2422	No promoter	–	<0.3	<0.3
pJIM2423	<i>Pres</i>	+	<0.3	<0.3
pJIM2424	P5– <i>cat-86</i>	+	<0.3	<0.3
pJIM2425	P23– <i>cat-86</i>	–	<0.3	<0.3
pJIM2426	P32– <i>cat-86</i>	+	<0.3	<0.3
pJIM2427	P44– <i>cat-86</i>	–	<0.3	<0.3
pJIM2428	P59– <i>cat-86</i>	–	<0.3	<0.3
pJIM2429	P23	++	9.5	0.77
pJIM2430	P44	++	8.3	0.52
pJIM2431	P59	++	3.9	0.3
pJIM2093	<i>PnisA</i>	+++	110	7

^a The limit of detection is 0.3 U/ml. The maximal quantitative lipolytic activities were determined during exponential growth for the constitutive promoters and 3 h after induction by nisin for the inducible one.

^b –, no halo; +, small; ++, medium; +++, large.

cloned upstream of the *lip* gene, P5 (4) and P23, P32, P44, and P59 (65) (Fig. 1). P5 and P59 drive the expression of the rRNA operon, P23 drives the expression of a potential integral membrane protein, P32 drives the expression of the fructose 1-6 biphosphate aldolase, and P44 drives the expression of a cell division protein, FtsA. The procedure is represented in Fig. 1. In the first step, an *L. lactis* promoter–*cat-86* cassette was cloned upstream of the *lip* gene on a lactococcal replicative plasmid. The second step, removing the *cat-86* gene and its terminator and placing the *lip* gene directly under the control of the promoter, was carried out successfully for P23 (pJIM2429), P44 (pJIM2430), and P59 (pJIM2431), resulting in strains JIM5496, JIM5497, and JIM5498, respectively (Table 2). In these strains, lipase production significantly affects the growth of the cells, since the generation time is 80 min, instead of the 45 min for wild-type strain IL1403 (data not shown). Deletion of the *cat-86* gene failed for the other two promoters, P5 and P32.

The fact that repeated attempts to delete the *cat-86* gene from the two constructions containing P5 and P32 failed suggests that the *lip* gene under the control of these promoters is toxic for *L. lactis* cells. Indeed, the clones containing pJIM2424 or pJIM2426 gave halos, revealing lipase production despite the presence of the *cat-86* gene and its terminator between P5 or P32 and the *lip* gene, while no halo was detected with the other cassettes (Table 2). This result shows that these two promoters are stronger than the others in this plasmid context and confirms that lipase overexpression is toxic for the cells.

Overexpression of the lipase under the control of an inducible promoter. Since the expression of the *lip* gene under the control of constitutive promoters stronger than P23 was toxic for the cells (Table 2), we used an inducible promoter, *PnisA* (12) to increase the production of lipase. This promoter is tightly regulated, closed in the absence of nisin and opened in a dose-dependent manner in the presence of nisin in strains containing the *nisRK* regulators. Two such strains were used in this work, NZ9000, a derivative of *L. lactis* subsp. *cremoris* NZ3900 (12), and JIM7049, a derivative of *L. lactis* subsp. *lactis* IL1403 (S. Calero, personal communication). The lipase was induced by nisin as described in Materials and Methods at an OD₆₀₀ of 0.3 in strains JIM7022 and JIM7048 (respectively, NZ9000 and JIM7049 carrying pJIM2093). This condition was

determined previously as optimal for lipase overproduction (data not shown). Cell growth was severely affected after nisin induction in the presence of pJIM2093 (Fig. 2A), while it was not without nisin (generation time, 45 min); nisin had no significant effect on the parental strains lacking pJIM2093 (data not shown).

The lipolytic activities were determined in units per milliliter of cell extracts or supernatants (Table 2). The maximal lipolytic activity was reached 3 h after induction and was about 10-fold higher for JIM7022 and 5-fold higher for JIM7048 than the activity obtained with the constitutive promoter P23 in JIM5496 (Table 2 and Fig. 2). The activities in the cellular fractions were approximately 5- to 10-fold higher than the extracellular ones. This result suggests that secretion is inefficient or that a large amount of lipase remains bound to the cells. Cell-associated lipase production was also monitored by staining proteins with Coomassie blue. Scanning of the gel and measurement of the band density (of the pre-pro-lipase at 91 kDa, the pro-lipase at 86 kDa, and the shorter products of the lipase ranging between 52 and 83 kDa; see also below) indicated that the lipase represents about 30% of the protein in the cell (Fig. 3). Quantification by successive dilution of total cell extracts of JIM7022 on a Western blot showed that the lipase was synthesized in a quantity 60-fold higher in this strain than in JIM5496 (P23) (data not shown).

The lipase is degraded after membrane translocation. To study the secretion of lipase in *L. lactis*, we monitored its production on Western blots using anti-CtermLIP sera because the amount of lipase produced under the control of our constitutive promoters (P23, P44 or P59) was too low to be detected in protein gels by Coomassie blue staining. The lipase

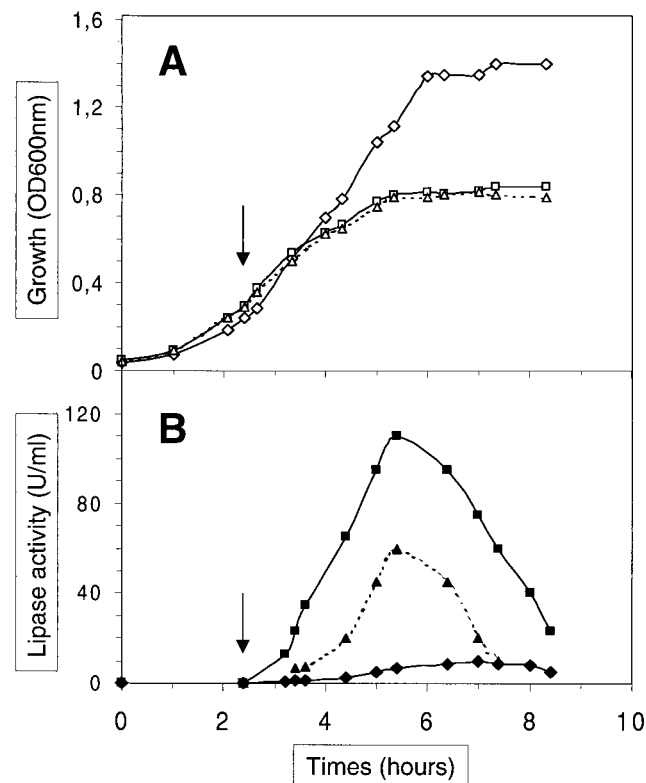


FIG. 2. Growth (A) and lipolytic activities (B) determined for cell extracts of strains JIM5496 (diamonds), JIM7022 (squares), and JIM7048 (triangles). Strains JIM7022 and JIM7048 were induced by nisin at an OD₆₀₀ of 0.3 (arrows).

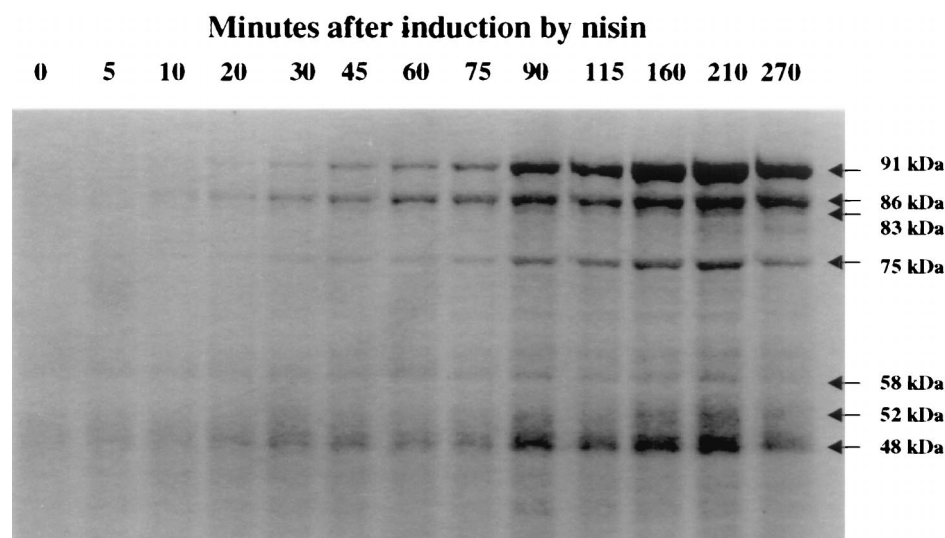


FIG. 3. Coomassie blue staining of JIM7022 total cell extracts. The samples (0.1 U, corresponding to the quantity of cells in 100 μ l of *L. lactis* culture at an OD₆₀₀ of 1) were loaded at different times after nisin induction (in minutes) as shown in Fig. 2.

was present in the total cell extracts in a quantity about 5-fold higher than that in the supernatants (Fig. 4A). A 91-kDa band, present only in the total cell extracts, could correspond to the pre-pro-lipase before it is translocated through the membrane. An 86-kDa band detected both in the supernatants and in the cells had the size expected for the pro-lipase after cleavage by the signal peptidase. Four additional truncated forms, at 81 kDa, 65 kDa, 54 kDa, and 52 kDa, larger than the mature lipase (46 kDa), were detected in significant amounts in the total cell extracts and in the supernatants.

The truncated lipase forms could result from the premature termination of synthesis or from degradation by protease(s). To discriminate between these hypotheses, we used anti-NtermLIP sera. In the first case, the truncated forms detected with anti-CtermLIP antibodies should be detected with anti-NtermLIP antibodies. Moreover, new, smaller bands corresponding to a premature stop upstream of the C-terminal part of the lipase could also be detected with anti-NtermLIP antibodies. In the case of degradation by protease(s), bands corresponding to degradation products truncated in the N-terminal part should disappear, while new bands corresponding to degradation products containing the first part of the lipase should appear. Western blots with specific anti-NtermLIP antibodies detected the potential 86-kDa pro-lipase and the 81-kDa form in the cell extracts and the supernatants, while the truncated 65-, 54-, and 52-kDa forms were not revealed (Fig. 4B). Thus, these do not contain the N-terminal part of the lipase and are most likely degraded forms of the lipase.

The degraded lipase forms were detected both associated with the cells and in the supernatants. They could be liberated either after cell lysis or after secretion. To test the possibility of lysis, we searched for the presence of the intracellular enzymes LDH (by an enzymatic assay) and PepC (with anti-PepC sera) in the supernatants. LDH activity (data not shown) and PepC activity (Fig. 4C) were detected in the cell extracts only. Lipase degradation thus probably takes place after translocation.

The extracellular proteolytic systems of the two *L. lactis* subspecies are different. We compared lipase expression in the cell extracts of two strains, *L. lactis* subsp. *cremoris* JIM7022 (*PnisA*) and *L. lactis* subsp. *lactis* JIM7048 (*PnisA*), using Western blots with anti-CtermLIP sera (Fig. 5). The pattern of the

degradation products seemed to be subspecies dependent. The bands observed with JIM7048 (*PnisA*) and JIM5496 (P23), both of which are *L. lactis* subsp. *lactis* but which produce different amounts of lipase, were the same: the pre-pro-lipase at 91 kDa, the pro-lipase at 86 kDa, and four degraded forms at 81, 65, 54, and 52 kDa. In *L. lactis* subsp. *cremoris* JIM7022 (*PnisA*), the degradation products of 81, 65, 54, and 52 kDa were not present, and four other bands appeared instead, at 83, 75, 58, and 48 kDa. We conclude that the proteases involved in lipase degradation in the two lactococcal strains have different cleavage specificities.

The kinetics of degradation of the lipase are different in the two strains, as deduced from the experiments shown in Fig. 6. In *L. lactis* subsp. *cremoris* JIM7022 (*PnisA*) (Fig. 6A), the bands corresponding to the lipase were visualized earlier after induction by nisin, leading to the production of a single band corresponding to the pro-lipase after 10 min. A second band corresponding to the pre-pro-lipase appeared after 20 min, and the intensity of these two bands increased progressively, to reach a maximum at about 150 min. The first degradation products were detected at very small amounts after 45 to 60 min, and the intensity of the corresponding bands increased progressively. In *L. lactis* subsp. *lactis* JIM7048 (*PnisA*) (Fig. 6B), the first bands appeared after 30 min. Since the early kinetics of induction by nisin are similar for the two strains with the luciferase gene as a reporter gene (data not shown), this result suggests that lipase is efficiently degraded at the early stage of production. Moreover, pre-pro-lipase, pro-lipase, and a degradation product are visualized simultaneously, confirming that degradation of the lipase occurs immediately upon its synthesis. Further degradation occurs later, as for the other strain.

Localization of the lipase and of its degradation products. The *S. hyicus* lipase and its degradation products were localized by separating the cell components into three fractions corresponding to the cytoplasm, the membrane, and the cell wall. Interestingly, the different bands observed previously were not present in the same cell fractions (Fig. 7). The 81-, 65-, 54-, and 52-kDa bands and the pro-lipase were found in the cell wall extract (Fig. 7, lane 2W), while only the 52-kDa product, a possible contaminant from the cell wall due to

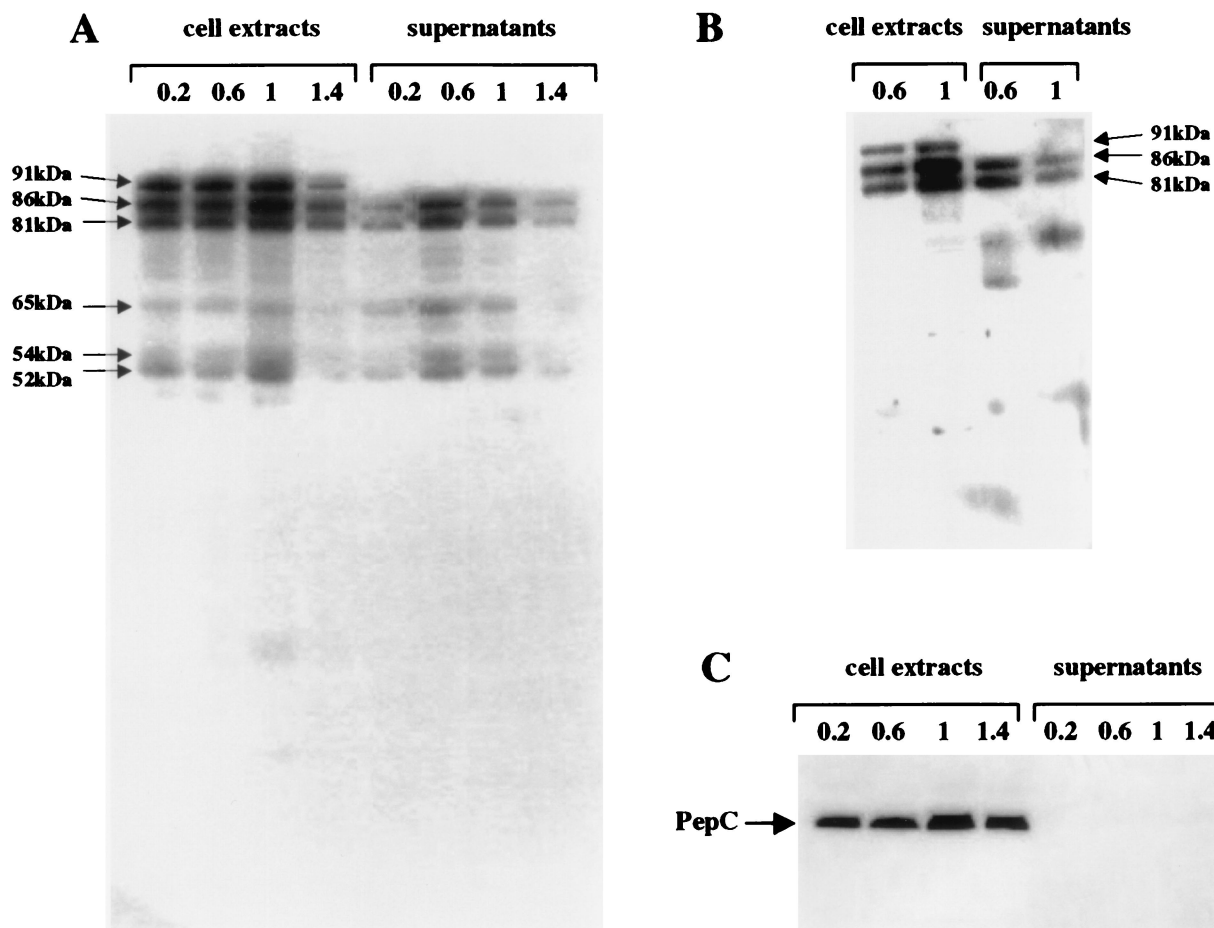


FIG. 4. Western blots of total cell extract (0.1 U) and supernatant (0.5 U) proteins from lactococcal strain JIM5496 with anti-CtermLIP (A), anti-NtermLIP (B), and anti-PepC sera (C). The samples were taken at different stages of growth (OD_{600s} of 0.2, 0.6, 1, and 1.4). See the legend to Fig. 3 for an explanation of U.

incomplete wall digestion, was present in small amounts in the cytoplasm and in the membrane (lanes 2C and 2M and lanes 3C and 3M). These results confirm that the lipase is degraded by protease(s) after export. Finally, the pre-pro-lipase was

found exclusively in the fraction containing the membrane (Fig. 7, lane 2M).

To determine the orientation of the lipase in the membrane, we performed trypsin digestion of protoplasts with or without Triton X-100 treatment. Trypsin will digest only the proteins displayed on the outside membrane surface of nontreated protoplasts. Triton X-100 treatment dissolves the membranes, and all the proteins should be digested by trypsin. The pre-pro-lipase was not digested by trypsin when it was added to intact protoplasts (Fig. 7, compare lanes 3M and 4M). Only one band smaller than 52 kDa could be detected, and it was resistant to trypsin degradation (Fig. 7, lane 5). This result shows that most of the lipase precursor is anchored to the membrane inside the cell.

The low efficiency of lipase translocation is probably not leader peptide dependent. To investigate the reason for the poor export of the lipase, we replaced its leader peptide with a lactococcal one. The *lip* leader has all the features known to be required for protein export in gram-positive bacteria. However, we assumed that the *S. hyicus* leader may not be well recognized or may have some other properties which render it not fully functional in *L. lactis*. The lipase was thus fused to the leader peptide of Usp45, the most abundant protein secreted by *L. lactis*. The fusion removed precisely the lipase leader and replaced it with the *L. lactis* one. A shorter pre-pro-lipase should thus be obtained, due to the length difference between

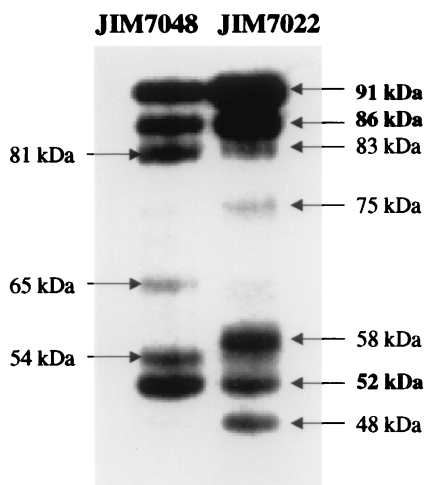


FIG. 5. Comparison of the degradation products of the *S. hyicus* lipase produced in JIM7048 (*L. lactis* subsp. *lactis*) and JIM7022 (*L. lactis* subsp. *cremoris*), as revealed by immunostaining with anti-CtermLIP sera.

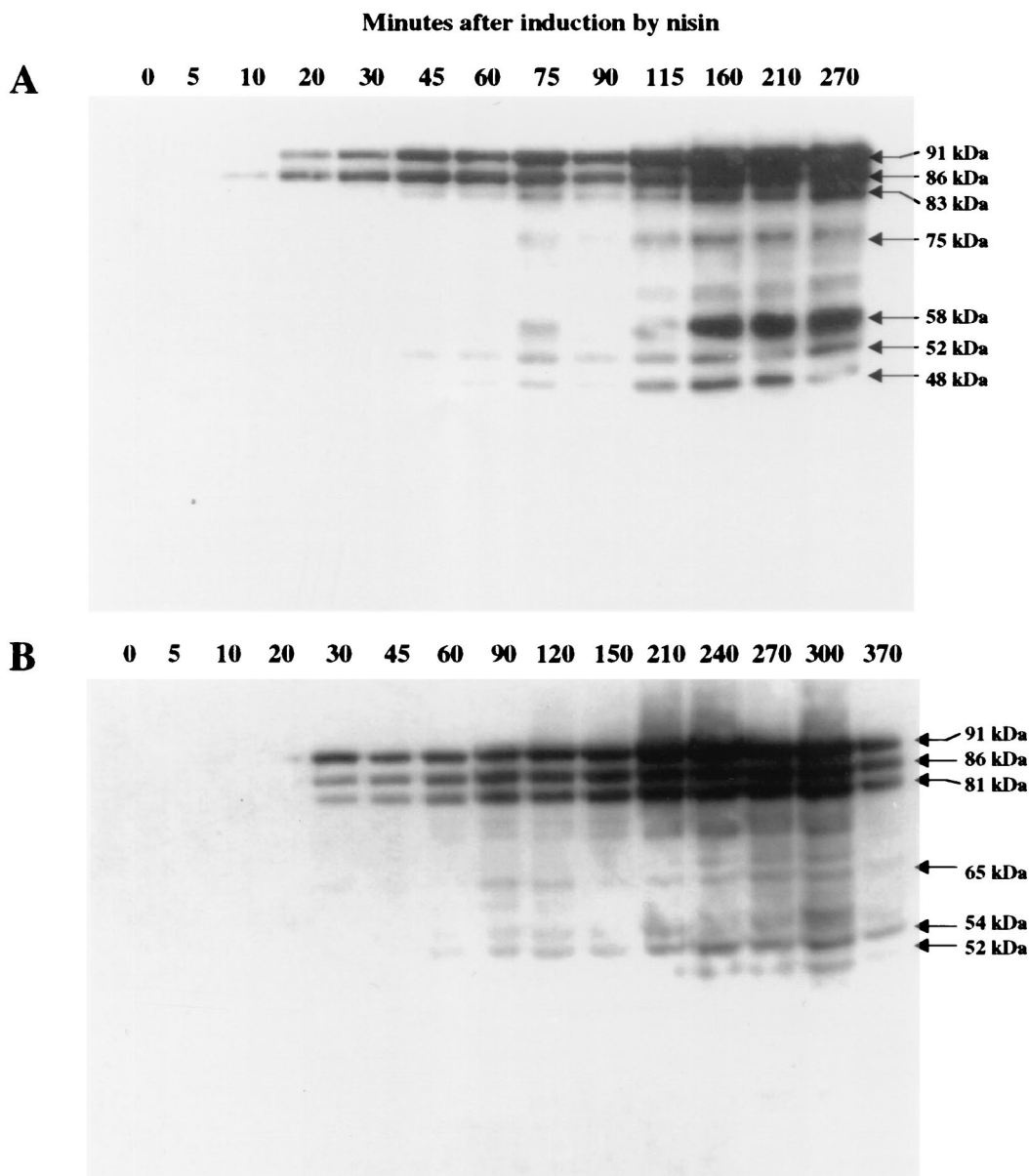


FIG. 6. Differences in the kinetics of lipase production and degradation of cell extracts (0.1 U) from JIM7022 (A) and JIM7048 (B), as revealed by immunostaining with anti-CtermLIP sera. Total cell extracts were loaded at different times after nisin induction (in minutes) as shown in Fig. 2. See the legend to Fig. 3 for an explanation of U.

the signal peptides of Usp45 (27 aa) and the lipase (38 aa). No significant improvement in lipase export was observed (Fig. 8). Moreover, the same degradation pattern was obtained with the native and the Usp45 leader peptides. A similar experiment with the synthetic pro-peptide LEISSTCDA (37) gave similar results (data not shown; P. Langella, personal communication). This result strongly suggests that the poor export of lipase is not due to its leader peptide.

Limitation of *S. hyicus* lipase secretion in *B. subtilis*. Lactococci do not naturally secrete many proteins and thus might lack a factor for efficient secretion of some heterologous proteins. We thus wanted to test if the low efficiency of lipase translocation that we encountered was specific for lactococci. Bacilli have a high capacity for secretion of proteins and are used industrially for the production of extracellular enzymes.

Moreover, some *Bacillus* species are able to naturally produce extracellular lipases sharing high homology with the staphylococcal lipases (29, 49). In order to study the expression of the *S. hyicus* lipase in *B. subtilis*, the model gram-positive bacterium for the secretion of proteins, pJIM2423 (*Pres*), pJIM2429 (P23), pJIM2430 (P44), and pJIM2431 (P59) were used to transform MTT19 cells. Despite the facts that MTT19 cells were highly competent and that the plasmids were purified by the same method, transformed clones were obtained only for pJIM2423 (*Pres*). This plasmid expresses the lipase from a low-copy-number constitutive promoter, *Pres*. The production and localization of lipase were monitored by Western blotting with anti-Cterm-LIP sera (data not shown). As in *L. lactis*, a form corresponding to the pre-pro-lipase was present in the cells. Moreover, significant amounts of smaller forms of the

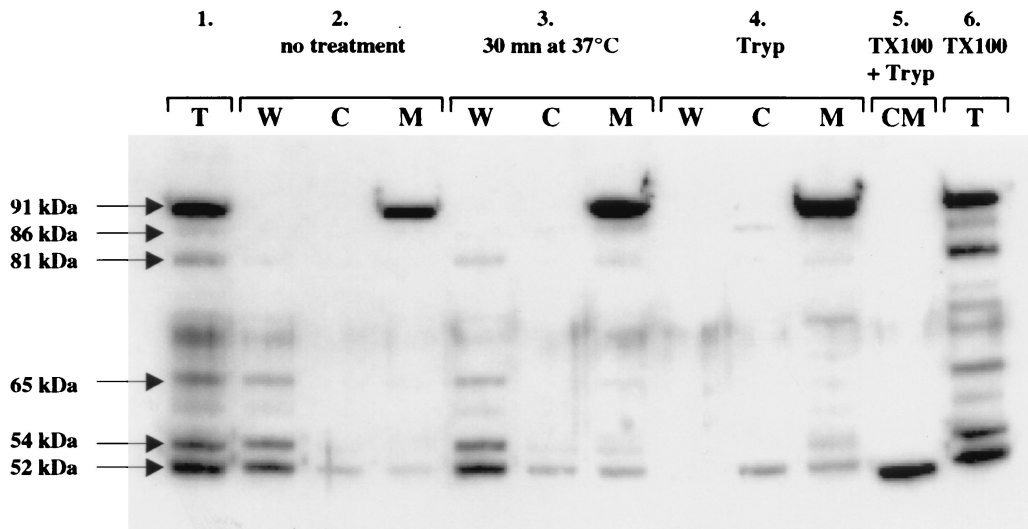


FIG. 7. Localization of the *S. hyicus* lipase in strain JIM5496 (P23) by Western blotting with anti-CtermLIP sera. Panel 1, total cell extract; panel 2, sample without treatment; panel 3, sample incubated for 30 min at 37°C; panel 4, cell wall and protoplast fractions incubated with trypsin for 30 min at 37°C; panel 5, protoplasts incubated with Triton X-100 and trypsin for 30 min at 37°C; panel 6, total cell extract incubated with Triton X-100 for 30 min at 37°C. Abbreviations: T, total cell extract; W, cell wall fraction; C, cytoplasm fraction; M, membrane fraction; CM, protoplasts; Tryp, trypsin; TX100, Triton X-100.

lipase were linked to the cells (data not shown). Finally, small amounts of fragments corresponding to degradation products of the lipase could be detected in the supernatants. These results show that *B. subtilis* does not express the lipase properly, although it has a high capacity to secrete heterologous proteins.

DISCUSSION

The *S. hyicus lip* gene was expressed under the control of strong constitutive promoters in *L. lactis*. The strongest promoters, such as P32, a promoter driving the expression of a glycolytic gene, the fructose 1-6 biphosphate aldolase gene, were toxic for the cells. This toxicity was even more pronounced in *B. subtilis*, although bacilli are naturally able to secrete large amounts of proteins, including lipases that share high homology with that of *S. hyicus* (29, 49). To overcome the

problem of toxicity and increase the production of lipase in *L. lactis*, the *lip* gene was expressed from an inducible promoter controlled by the addition of nisin in the medium. This strategy led to a 10-fold increase in lipolytic activity and allowed production of the lipase to about 30% of total cell protein. While the lipase activity was increased 10-fold, the bulk of lipase was increased 60-fold compared to the level obtained with constitutive promoters. The sixfold difference between the two measures suggests that an important fraction of the produced lipase is inactive. In order to understand the bottleneck for lipase production in *L. lactis*, the different forms of the enzyme were characterized.

The use of immunodetection techniques allowed us to show that in addition to the pre-pro-lipase and the pro-lipase, large amounts of smaller lipase forms were produced in *L. lactis* and *B. subtilis*. In *S. carnosus* overexpressing the lipase from pLipPS1, only the pro-lipase can be detected in the superna-

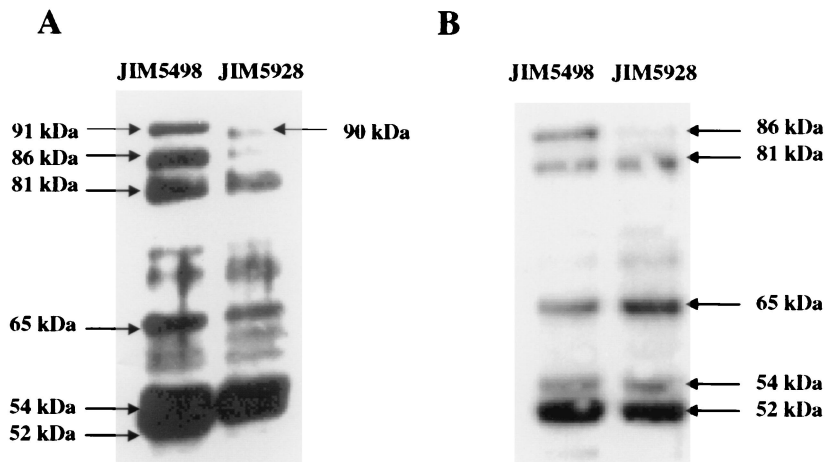


FIG. 8. Effect of the Usp45 leader peptide on the secretion of lipase. Shown is a Western blot of total cell extract (0.1 U) (A) and supernatant (0.5 U) (B) proteins from lactococcal strains JIM5498 (native leader) and JIM5928 (Usp45 leader). The samples were taken at an OD₆₀₀ of 0.8. See the legend to Fig. 3 for an explanation of U.

tant (data not shown); in *S. hyicus*, only the mature lipase is detected in the supernatant (data not shown). Most of the smaller forms were present in the cell wall extracts and in the supernatants. They include the C-terminal part of the lipase, suggesting that proteolytic cleavages occur at its N-terminal part by cell wall-associated proteases. This degradation occurs during or shortly after membrane translocation, especially in *L. lactis* subsp. *lactis*. The proteolysis of heterologous proteins is a factor that is often reported to limit the yield of a product. A good example is the degradation of *E. coli* OmpA upon secretion in *B. subtilis* and *S. carnosus* (40). The difference in the degradation patterns of the lipases of the two *L. lactis* subspecies (and also *B. subtilis*) suggests that the proteases involved have different specificities. However, the two *L. lactis* strains used in this work are devoid of the extracellular proteinase, PrtP, that was shown to be involved in AcmA autolysin degradation (6). In the recently sequenced *B. subtilis* and *L. lactis* subsp. *lactis* genomes, genes encoding proteins homologous to *E. coli* DegP, involved in the degradation of unfolded or misfolded proteins trapped in the cell wall (43, 58), were detected (A. Bolotin, personal communication).

Different factors might induce the degradation of the lipase by specialized proteases, such as DegP. The fact that most of the secreted lipase remains associated with the cell wall suggests that the lipase has some difficulties crossing the cell wall. It is thus trapped in a location favoring its degradation by the membrane and cell wall-bound proteases. We do not know why the lipase is not able to pass efficiently through the cell wall, since *L. lactis* and *B. subtilis* are able to secrete proteins larger than lipase, such as a plasmid-encoded protease (18). To our knowledge, the cell wall of *S. hyicus* is not thinner than or has no special properties compared to those of *L. lactis* and *B. subtilis*. The poor ability of the lipase to cross the cell wall could be due to its lack of proper folding during secretion.

In addition to its inability to cross the cell wall, only 20% of the produced lipase is secreted outside the cytoplasmic membrane. This finding indicates that another step in secretion is also ineffective. Indeed, most of the lipase is still bound to the inner side of the membrane. This finding is probably not due to an inefficient signal peptide. First, the lipase gene exhibits a consensus signal sequence for gram-positive bacteria, such as *L. lactis* and *B. subtilis*. This sequence is composed of an initial hydrophilic segment (aa 1 to 16), followed by a hydrophobic sequence (aa 17 to 34), and ends with Ala-Glu-Ala, a classical sequence of the cleavage site recognized by the general signal peptidase (16). Second, replacement with the lactococcal signal peptide of Usp45, the most abundant protein secreted by *L. lactis* (62), did not change the pattern of secretion of the lipase and did not improve lipase export. The factor affecting secretion should thus be encoded downstream of the peptide leader in the lipase gene or be an additional factor that interacts with the lipase.

It was reported that the region adjacent to the leader peptide has an effect on secretion (37). The synthetic pro-peptide LEISSTCDA, known to enhance the secretion of the *S. aureus* *nuc* and *Bacillus stearothermophilus* α -amylase genes, was introduced between the Usp45 leader peptide and the lipase pro-peptide (P. Langella, personal communication). However, it did not lead to better secretion of the *S. hyicus* lipase, showing that the leader peptide and the adjacent lipase sequence were not responsible for the lipase secretion-limiting step (data not shown). The presence of a large pro-peptide may improve secretion and prevent protein degradation, for example, by guiding the folding of the secreted protein (5, 11, 22). Interestingly, the pro-peptide is not present in *Bacillus* lipases (49), suggesting that it might be specific for staphylococci. However,

it improves the secretion of the *E. coli* OmpA protein in *B. subtilis* (40), suggesting a more subtle role during protein secretion.

From the structure of the *lip* leader peptide and current knowledge, it is expected that the lipase is secreted through the general pathway already described for *L. lactis* (37, 44, 45, 52). The factors known to be involved in the export and in the folding of the protein during secretion (33, 39) are present in the *L. lactis* genome (13, 30, 63; A. Bolotin, personal communication), although *L. lactis* secretes small amounts of proteins. A single secreted protein, Usp45, of unknown function, can be systematically identified in sodium dodecyl sulfate-polyacrylamide gels, while only traces of other proteins are detectable in *L. lactis* (62). These results suggest that *L. lactis* does not have a large potential for secreting proteins; thus, some components of the secretion machinery could be present in limiting amounts. An example of such a limiting factor has been documented for *B. subtilis*, the model organism for the secretion of protein in gram-positive bacteria. In this organism, the overproduction of PrsA allows an increase in the secretion of a single-chain antibody fragment (69). PrsA, a membrane-associated lipoprotein, plays the role of an extracellular chaperone, allowing the correct folding of secretory proteins after their translocation across the cytoplasmic membrane (31, 32). A homologue of this protein is present in *L. lactis* (45), and it could be of interest to test if it might be a limiting factor in the secretion of the lipase. However, the patterns of lipase production are similar when the lipase is expressed at different levels, in particular at a low level close to the limit of detection in Western blotting. Moreover, *L. lactis* is able to secrete large amounts of certain heterologous proteins, such as staphylococcal nuclease (36). These results do not support the hypothesis of a general factor limiting secretion.

Another possibility is that the lipase requires a specialized factor for its proper secretion. This is the case for *Pseudomonas* and *Vibrio cholerae*, where the secretion of the lipase encoded by *lipA* requires the expression of the linked *lipB* gene (15, 25, 42). Indeed, the *S. hyicus* lipase is well secreted in *S. hyicus* and *S. carnosus* but not in *E. coli* (16), *L. lactis*, and *B. subtilis*. Secretion efficiency in *L. curvatus*, a lactic acid bacterium, is not known, because the lipolytic activity was determined only by qualitative analysis (67). It is thus possible that staphylococci possess a specific foldase, not yet characterized but required for efficient lipase secretion.

Last, the rate of protein synthesis and the occurrence of pauses may have a determinant effect on the folding of the product (17, 26, 28, 61). Rate and pauses may be affected by codon bias (26, 54, 60) and codon context (7, 14). Highly expressed genes have a strong bias, allowing optimal synthesis (27, 51). The codon biases for highly expressed genes in *L. lactis*, *B. subtilis*, and *S. aureus* differ significantly (unpublished data; see Materials and Methods). In particular, UUA is the preferred leucine codon in *S. aureus* (80 to 90%) but is almost absent in *L. lactis* (less than 1%), while UUG and CUU are rare in *S. aureus* (1 and 5%, respectively) but frequent in *L. lactis* (38 and 53%, respectively). Notwithstanding the absence of a cluster of rare codons in the lipase gene, it is possible that pauses or slowing down in the translation of the *lip* message takes place in different regions in *L. lactis*, *B. subtilis*, and *S. hyicus*. This process could lead to incorrect folding of the nascent peptide in the heterologous hosts and thus to an inactive and inefficiently exported lipase protein.

ACKNOWLEDGMENTS

We thank F. Götz for providing plasmid pLipPS1, O. P. Kuipers for providing plasmid pNZ8008 and strain NZ9000, and Y. Le Loir and P.

Langella for providing plasmid pNuc11. We thank J. Anba, S. Bonneau, S. Calero, P. Langella, and I. Poquet for advice during this work and A. Bolotin for communication of the *L. lactis* genome sequence.

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