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Secretion of TEM β-Lactamase with Signal Sequences Isolated from the Chromosome of *Lactococcus lactis* subsp. *lactis*

MERVI SIBAKOV,†* TEIJA KOIVULA, ATTE VON WRIGHT,‡ AND ILKKA PALVA

Genesit Oy, Valimotie 7D, SF-00380 Helsinki, Finland

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With TEM β-lactamase as a reporter gene, a set of expression-secretion-promoting fragments were isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. The fact that only translocated β-lactamase renders cells resistant to ampicillin allowed direct ampicillin selection with an *Escherichia coli* vector (pKTH33). The clones showing the greatest ampicillin resistance were subcloned onto a replicon capable of replication in lactic acid bacteria (pVS2), and the nucleotide sequences of the relevant fragments were determined. The structure of the secretion-promoting fragments in general resembled that of gram-positive true signal sequences, with a strongly positively charged N terminus, a long hydrophobic core, and a putative signal peptidase recognition site. The promoterlike sequences preceding the signal sequences matched well with those of previously published lactococcal promoters. In addition to *E. coli*, the functioning of these expression-secretion cassettes was studied in three gram-positive hosts: *Bacillus subtilis*, *L. lactis*, and *Lactobacillus plantarum*. Efficient expression and secretion of TEM β-lactamase into the culture medium of each gram-positive host was obtained. Furthermore, when a strain of *L. lactis* subsp. *lactis* showing increased sensitivity to lysozyme was compared with a standard laboratory strain, threefold-higher secreted enzyme activities were detected.

In recent years, considerable knowledge about the molecular biology of lactic acid bacteria has accumulated. However, so far the area of protein secretion, at the molecular level, has been almost totally unexplored. Along with other gram-positive organisms, lactic acid bacteria are most likely able to translocate their proteins to the outside of the cell. In fact, this has been shown to be the case with *Lactococcus lactis* subsp. *cremoris* protease, to our knowledge the only secreted protein of the species studied at the molecular level. The genes coding for the protease from two different. *L. lactis* subsp. *cremoris* species have been isolated and sequenced (15, 37). Examination of their DNA sequences reveals a typical gram-positive signal peptide. Other than this information, no secretion-promoting fragments from the *Lactococcus* chromosome have been described in detail.

The purpose of the present study was to look for secretion-promoting fragments from the *L. lactis* subsp. *lactis* chromosome with the aid of a "signal sequence" probe vector. Studying the structure and function of such fragments is important for basic molecular biology, since there is little information about protein secretion from the gram-positive bacteria for species other than bacilli and to some extent streptomycetes. Lactic acid bacteria are also commercially important. For instance, their use as starters in various processes is crucial to the dairy industry. Improvement of the strains' properties to meet the demands of present and future processes would make lactic acid bacteria attractive candidates for genetic modification. Another line of application could be the production of heterologous proteins. For that purpose, e.g., construction of efficient secretion vectors, characterization of secretion-promoting fragments is also needed.

* Corresponding author.

† Present address: Valio Finnish Co-operative Dairies' Association, Research and Development Centre, P.O. Box 176, SF-00181 Helsinki, Finland.

‡ Present address: University of Kuopio, Department of Pharmaceutical Chemistry, P.O. Box 6, SF-70211 Kuopio, Finland.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Growth media. For propagating *Escherichia coli* and *Bacillus subtilis* strains, Luria broth (17) was used; for *L. lactis*, M17G or M17GS broth (29), and for *L. plantarum*, MRS broth (5), was used. The antibiotics used for selection and maintenance are listed in Table 2.

DNA isolation and modifications. Rapid isolation of plasmid DNA from *E. coli* for screening of the clones was done by the method of Holmes and Quigley (10). DNA for restriction enzyme digests was prepared by the method of Birnboim and Doly (3) from either 1 or 10 ml of liquid culture. RNase (Boehringer) was added prior to restriction enzyme treatments.

Isolation of plasmid DNA from *B. subtilis* was carried out as described by Gryczan et al. (7). Isolation of plasmid DNA from *L. lactis* subsp. *lactis* was carried out according to Anderson and McKay (1) for both small-scale and large-scale isolations. Further purification of DNA, if needed, was done by CsCl-ethidium bromide density gradient centrifugation, regardless of the source of DNA.

Chromosomal DNA from *L. lactis* subsp. *lactis* was isolated by the above-described method; only the chromosomal band was collected from CsCl runs.

Restriction enzyme digestions were performed according to the manufacturers' recommendations (Boehringer, Bethesda Research Laboratories, and Promega). Selected restriction fragments were obtained by separation of the digested DNA by electrophoresis on a 0.8% agarose gel, after which DNA extraction and purification were performed by the phenol-liquid nitrogen freezing method as follows. A slice of agarose containing the desired fragment was transferred to a siliconized Eppendorf tube and mashed with a glass rod. About 250 μl of TE buffer (1) was added together with an equal volume of phenol. After thorough mixing in a Vortex shaker, the tube was immersed in liquid nitrogen until frozen. The phases were separated by centrifugation at 12,000 × *g* for 15 min, after which phenol extraction was

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Origin or reference
E. coli TG1	K-12 Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	European Molecular Biology Laboratory
B. subtilis IH6064	*metB5 sacA321*	23
L. lactis subsp. *lactis*		
MG1614		6
V207		Valio[a]
L. plantarum V755		Valio[a]
Plasmids		
pBR322		4
pVS2	Plasmid capable of replication in *E. coli*, *B. subtilis*, *L. lactis*, and *L. plantarum*, 5 kb, Em^r Cm^r	36
pKTH33	Signal sequence probe vector (Fig. 1), 4.6 kb, Cm^r	Genesit Oy
Plasmids bearing *L. lactis* chromosomal DNA[b]		
pKTH1795	Clone ss1	This work
pKTH1796	Clone ss11	This work
pKTH1797	Clone ss30	This work
pKTH1798	Clone ss38	This work
pKTH1799	Clone ss45	This work
pKTH1801	Clone ss80	This work
pSV2-based plasmids[c]		
pKTH1803	ss1	This work
pKTH1804	ss11	This work
pKTH1805	ss30	This work
pKTH1806	ss38	This work
pKTH1807	ss45	This work
pKTH1809	ss80	This work

[a] Valio Finnish Co-operative Dairies' Association.
[b] Derived from pKTH33.
[c] pSV2 bearing *Cla*I-*Pvu*II fragments of the indicated clones.

repeated and the resulting aqueous layer was treated with ether and ethanol precipitated.

Modification of the ends of DNA fragments. For the generation of blunt-ended fragments, the Klenow fragment of DNA polymerase I (Promega) was used. As alternative methods, T4 DNA polymerase (Promega) or mung bean nuclease (Promega) was used. For dephosphorylation of 5′ phosphorylated ends, calf intestinal phosphatase (Boehringer) was used, and T4 polynucleotide kinase (Promega) was used for phosphorylation of the 5′ hydroxyl ends.

The ends of the DNA fragments were joined by T4 DNA ligase (Promega). All modifying enzymes were used according to the manufacturers' recommendations.

Preparation of chromosomal DNA. *L. lactis* subsp. *lactis* chromosomal DNA was sonicated to get random fragments of 500 to 600 bp. (Restriction enzymes tested gave fragments of 2 kb or larger.) The extent of sonication was checked by running a small aliquot of the treated sample in a 0.8% agarose gel. The total sonicated DNA was then applied to a 0.8% agarose gel and electrophoresed. A pool of 500- to 600-bp fragments were extracted by the phenol-liquid nitrogen method, and the ends of the purified DNA fragments were treated with the Klenow fragment of DNA polymerase I for blunt-end ligation.

TABLE 2. Media and markers used for various hosts

Host	Growth medium	Antibiotic[a] and concn (μg/ml)
E. coli TG1	Luria	Ap (50), Cm (11), Tc (12.5)
B. subtilis IH6064	Luria/2× Luria	Cm (5), Km (10)
L. lactis MG1614	M17G, M17GS	Cm (4–5)
L. plantarum V755	MRS	Cm (4–5)

[a] Ap, Ampicillin; Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin.

DNA transformations. Transformation of *E. coli* cells was accomplished by the method of Hanahan and Meselson (8). *B. subtilis* cells were transformed by the method of Gryczan et al. (7). *L. lactis* protoplast transformation was carried out according to von Wright et al. (35). *L. plantarum* transformation by electroporation was performed by the method described below. For electroporation experiments, cells were grown to an A_{600} of 0.5 to 0.6, chilled in ice, harvested by centrifugation, washed twice in sucrose-magnesium electroporation buffer (SMEB; 272 mM sucrose, 1 mM $MgCl_2$), and resuspended in 1/50 of a volume of the same buffer. An aliquot (0.8 ml) of the ice-cold cell suspension was mixed with 0.5 to 1.0 μg of plasmid DNA. Cells were kept on ice for 2 to 3 min before and after electroporation. Electroporation was performed with a GenePulser apparatus (Bio-Rad Laboratories, Richmond, Va.) at a constant capacitance of 25 μF, a field strength of 7,500 V/cm, and a pulse controller setting of 800 Ω. Expression was done in MRS broth at 30°C for 1 h, and the transformants were plated on appropriate selection plates.

Enzymatic assays. β-Lactamase (derived from plasmid pBR322) was assayed by the method of O'Callaghan and Morris (21). Cell and supernatant fractions were separated by centrifugation after growth in appropriate liquid medium. Cells were incubated in lysozyme (4 mg/ml) for 30 min at 37°C and disrupted by sonication (four times for 15 s each time; Bransonic sonicator), and the cell debris was pelleted by centrifugation, after which the supernatant was assayed for enzymatic activity. For the rapid analysis of β-lactamase-positive status, bacterial colonies were suspended in 250 μl of 0.1% Nitrocefin (Glaxo) in 100 mM potassium phosphate buffer (pH 7.0) in microtiter wells. A 1% stock solution of Nitrocefin was made in dimethyl sulfoxide. Positive colonies turned red after 1 to 30 min of incubation at room temperature.

DNA sequencing. All DNA sequencing was based on the method of Sanger et al. (26). For plasmid sequencing, a Sequenase (United States Biochemical Corporation) system was used, as described by Hattori and Sakaki (9).

In vitro protein synthesis. In vitro translation of pre-β-lactamase was performed with a DNA expression system (in vitro DNA Directed, Prokaryotic by NEN Products, DuPont), after which the products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) and subjected to autoradiography.

MIC determination for ampicillin. An overnight colony was suspended in 1 ml of 50 mM phosphate buffer (pH 7.0). From the suspension, a drop was spread with a glass rod on a set of Luria plates containing different concentrations of ampicillin. The MIC was defined as the lowest concentration that inhibited growth.

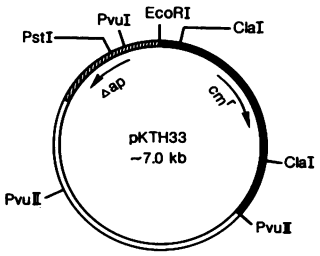

FIG. 1. Restriction map of pKTH33. Symbols: solid box, pC194 DNA; open box pBR322 DNA; hatched box, ΔAp^r gene within pBR322 DNA.

RESULTS

Screening for the promoter-signal sequence fragments with plasmid pKTH33. Plasmid pKTH33 (Fig. 1) contains the part of the *E. coli* TEM β-lactamase gene coding for the mature protein, preceded by an *Eco*RI linker (23a). Part of the plasmid originates from pBR322, allowing its replication in *E. coli*.

If a sequence bearing an expression-secretion signal is inserted in frame with the marker gene for β-lactamase, active enzyme is produced if the protein is translocated through the cytoplasmic membrane. This in turn renders the transformants resistant to ampicillin. By plating the transformants directly on ampicillin plates, positive selection for signal sequence fragments is obtained. Plasmid pKTH33 was cleaved with *Eco*RI, treated with the Klenow fragment of DNA polymerase I to obtain blunt-ended molecules, and purified by phenol extraction and ethanol precipitation.

The pool of size-selected *Lactococcus* chromosomal DNA fragments was ligated to the vector in a molar ratio of 2:1 (insert-vector), and the ligation mixture was transformed into *E. coli* TG1 cells and plated on Luria-ampicillin (50 μg/ml) plates. Several transformants were screened for β-lactamase activity by the Nitrocefin assay in microtiter wells. Clones were judged good producers if they turned red after 1 to 30 min of incubation at room temperature. In this way, almost 100 ampicillin-resistant clones were obtained. The transformants were purified by single colony streaks, and the MICs of ampicillin for them were determined. For further analysis, clones with ampicillin MICs from 500 μg/ml upwards were chosen. At the same time, rapid isolations of plasmid DNA were performed to verify the sizes of the signal sequence inserts.

Subcloning of promoter-secretion signal fragments into a replicon that functions in lactic acid bacteria. Although the use of pKTH33 allowed direct selection of the desired fragments, the clones could not be propagated as such in lactic acid bacteria. It was therefore necessary to change the replicon by subcloning the promoter-signal sequence fragments into the plasmid pVS2.

The insert and the entire β-lactamase gene were cleaved off from the β-lactamase-positive *E. coli* clones by *Cla*I-*Pvu*II double digestion, and the desired fragments were extracted from a 0.8% agarose gel as described above and treated with the Klenow fragment of DNA polymerase I to generate blunt ends. The vector pVS2 was cleaved with *Hin*dIII and treated with the Klenow enzyme as described above.

Ligation was performed at an insert/plasmid molar ratio of 2:1 under standard conditions, and the mixture was transformed into *E. coli* TG1 cells and plated on Luria-chloramphenicol (11 μg/ml) plates. The production of β-lactamase was checked by the Nitrocefin microtiter well assay as described above. Rapid isolation of plasmid DNA was done for positive clones, and the size of the insert was verified by restriction enzyme digests.

After the initial screening in *E. coli* TG1, *B. subtilis* IH6064, *L. lactis* MG1614, and *L. plantarum* V755 hosts were each transformed with the correct DNA constructs.

During the subcloning procedures, several original constructs were lost because of instability. For constructional reasons, the subclones harbored two replicons, both pBR322 and pVS2, which may have been one cause of instability. The approach of using a shuttle vector in the initial screening had to be abandoned for the same reason. For the final closer characterization in the three gram-positive hosts, only six clones which had retained their β-lactamase activity and correct restriction enzyme pattern were used (ss1, ss11, ss30, ss38, ss45, and ss80).

Nucleotide sequences. The nucleotide sequences and the deduced amino acid sequences of the six selected secretion-proficient clones are presented in Fig. 2. In each case, a translation initiation codon and a ribosome-binding-sequencelike sequence were found upstream from the reporter gene. In the clones ss30, ss38, and ss45, the distance from the reporter gene to the translation initiation codon was over 200 nucleotides, corresponding to about 70 amino acids, so it is very likely that only part of the fragment acts as a signal sequence. In the clones ss1 and ss11, the distance was 44 and 43 codons, respectively, which may fall within the limits of gram-positive signal sequences. In the clone ss80, the distance was very short, only 22 or 31 codons (29). The initiation codon appeared to be ATG in five out of six clones. The only likely start codon in the clone ss45 was GTG.

These secretion-promoting fragments had features in common with the known gram-positive signal sequences. They encoded one or more positively charged amino acid residues at the N terminus, followed by a long (10 to 20 residues) hydrophobic core and in most cases a putative signal peptide processing site.

The secretion-promoting sequences were preceded by putative promoter regions. Conserved areas around −35 and −10 were seen, which closely matched the previously published *L. lactis* subsp. *cremoris* promoter sequences (32) and, in general, *E. coli* and *B. subtilis* promoters transcribed by the vegetative σ factors. A more detailed characterization of the promoter sequences is given in the accompanying paper (14).

In vitro synthesis of β-lactamase precursors. To find out the correct assignment of the translation initiation site in the isolated promoter-signal sequence cassettes, four of the best producers were subjected to an in vitro transcription-translation assay for the synthesis of pre-β-lactamase. Figure 3 shows the proteins synthesized from the clones ss30, ss38, ss45, and ss80. For confirmation, the protein products were immunoprecipitated with β-lactamase antiserum (data not shown). The sizes of the precursor proteins closely matched the translation start points deduced from the nucleotide sequence data. In the clones ss30, ss38, and ss45, there was no other translation initiation codon close to the predicted one. Clone ss80 differed from the others in that there were two ATG codons, in frame, only eight codons apart. Starting from the ATG, which lies 32 codons upstream from the N terminus of the reporter gene, a very typical looking gram-positive signal peptide with three positively charged amino acids at the N terminus was found. However, mapping of the 5′ end of the mRNA (14) suggested that translation starts

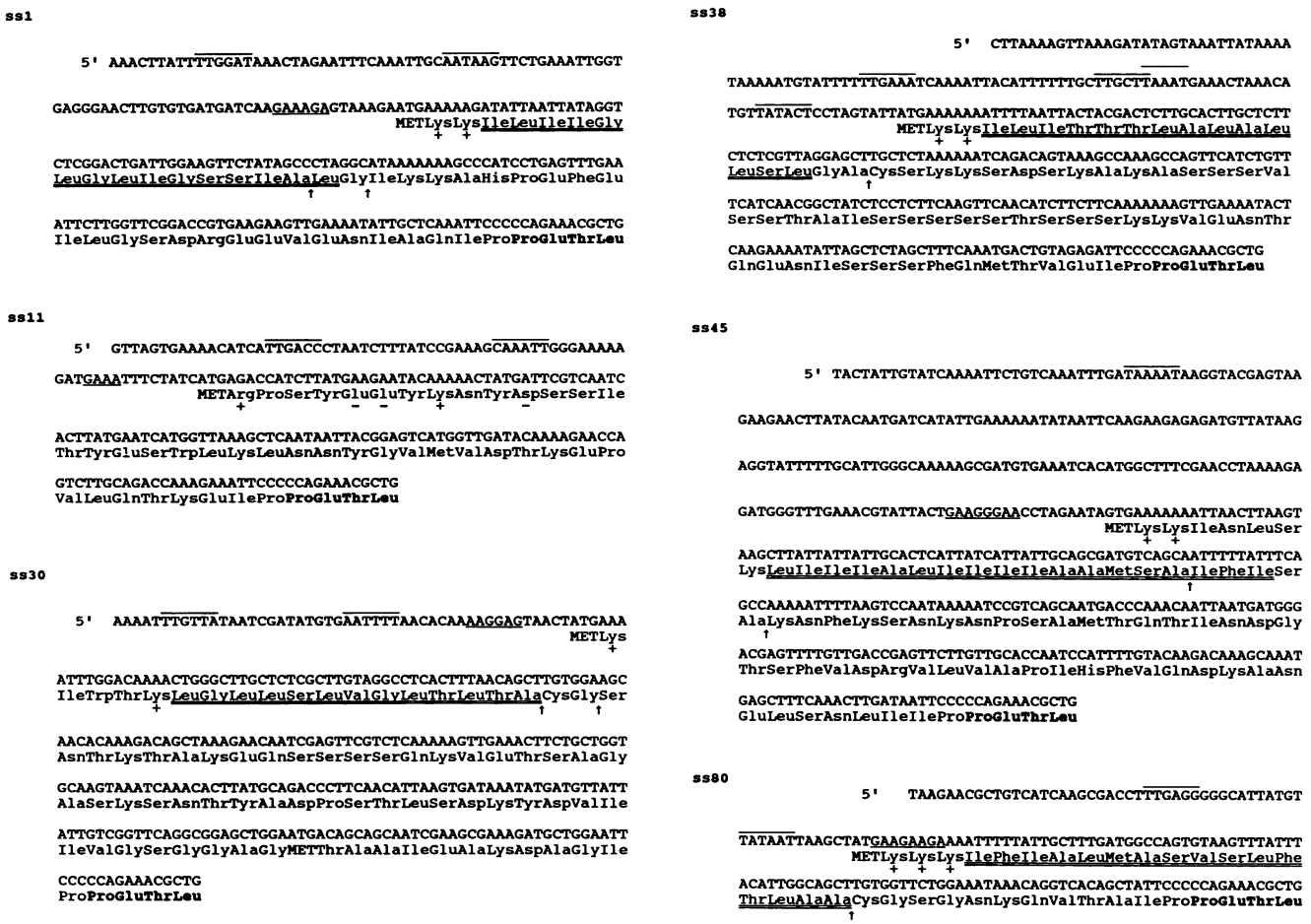

FIG. 2. Nucleotide sequences and deduced amino acid sequences of the six chromosomal fragments promoting expression and secretion of β-lactamase. Putative −35 and −10 sequences are indicated by overlining above the nucleotides, and the ribosome-binding sites are underlined. With the signal sequences, positive charges (+), hydrophobic core (double underlining), and putative processing sites (↑) are indicated where possible. The reporter gene is printed in boldface.

from the latter ATG, yielding a signal peptide devoid of its N-terminal charges.

Functioning of the promoter-signal sequence fragments in *E. coli*. For closer analysis in *E. coli*, six clones were chosen. These clones had high MICs for ampicillin, which should correlate with efficient export. Clones ss1, ss11, ss30, ss38, ss45, and ss80, all carried by the original probe vector pKTH33, were grown in Luria broth supplemented with ampicillin to 100 Klett units (no. 66 filter). The supernatant and cells were separated by centrifugation. The cell fraction was osmotically shocked to release the periplasmic β-lactamase. Table 3 shows the β-lactamase activities found in different cellular fractions of *E. coli* TG1.

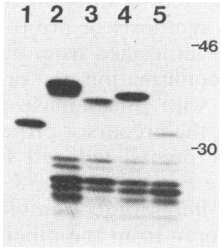

FIG. 3. Sizes of in vitro-synthesized β-lactamase precursor proteins. The plasmid-coded proteins were translated in vitro with [^{35}S]methionine label. Samples were run on SDS–12% polyacrylamide gels and autoradiographed. Lanes: 1, β-lactamase control; 2, ss30; 3, ss38; 4, ss45; 5, ss80. Sizes of reference proteins are given in kilodaltons.

With the β-lactamase fusion proteins, rather low levels of enzymatic activities corresponded to high ampicillin resistance. The lower export efficiency could be due to the fact that the hybrid proteins do not make the right conformation for proper release from the membrane.

Expression and secretion of TEM β-lactamase in gram-positive hosts. To study the functioning of the lactococcal expression-secretion fragments or cassettes in *B. subtilis*, *L. lactis* subsp. *lactis*, and *L. plantarum*, each host transformed with a hybrid plasmid bearing β-lactamase was assayed for enzymatic activity. The strains were grown in appropriate growth media as described in Materials and Methods, from an overnight 1:20 dilution until the mid-exponential phase of growth (100 Klett units). Samples were then withdrawn at intervals, and the supernatant and cell fractions were analyzed separately.

TABLE 3. Functioning of signal sequences in *E. coli*

Plasmid[a]	β-Lactamase activity (U/ml)		Ampicillin MIC (μg/ml)
	Periplasm	Cell fraction	
ss1	19	15	2,500
ss11	4	19	600
ss30	37	300	3,500
ss38	663	62	5,500
ss45	4	91	2,200
ss80	4	58	3,500
pBR322	1,009	4	7,500

[a] pKTH33 carrying the indicated clone or pBR322.

The most important findings for the three host bacteria were the efficient recognition of the lactococcal expression signals and the secretion of β-lactamase into the culture medium. The production kinetics and secretion efficiencies of the four clones in each host are presented in Fig. 4. Clones ss30, ss38, and ss45 behaved in a similar way in that they secreted over 95% of the β-lactamase activity into the culture medium at any time point (cell fractions not shown). Clone ss80 was clearly different from the others; a large proportion of the enzymatic activity remained cell attached and was only slowly released into the supernatant.

Secretion of TEM β-lactamases from two different *L. lactis* subsp. *lactis* strains. Secretion of TEM β-lactamase was also tested in two different *L. lactis* subsp. *lactis* strains. One, MG1614, is a standard laboratory strain cured of any resident plasmids; the other, V207, harbors several cryptic plasmids and has increased sensitivity to lysozyme. Over threefold-higher activities were obtained with the strain V207 than with MG1614. The production curves of the two best producers, ss38 and ss45, in MG1614 and V207 are shown in Fig. 5. The β-lactamase activity which remained in the cellular fractions (<5%) is not shown.

DISCUSSION

During this study, a set of expression-secretion-promoting fragments were isolated from the *L. lactis* subsp. *lactis* chromosome by direct ampicillin selection in *E. coli*.

The approach of using plasmid pKTH33 as the probe vector gave nearly 100 clones resistant to ampicillin at over 50 μg/ml from a ligation mixture of 1 μg of DNA. During repeated single-colony isolations, several clones lost their β-lactamase-positive status. For the closer characterization, only clones with high ampicillin resistance (>500 μg/ml) were chosen, since this was supposed to correlate with efficient β-lactamase secretion. In the subcloning procedures onto the replicon for lactic acid bacteria, additional clones were lost because of structural instability, so that finally only six clones fulfilled the criteria of β-lactamase production and expected restriction enzyme patterns. One reason for the instability problems could be that the subclones in lactococci harbored two replicons. This would be in accord with the previously reported results (22, 25) concerning true shuttle vectors.

Attempts have also been made to look for translocation-proficient fragments from the chromosome of *Saccharomyces cerevisiae* (12), *E. coli* (38), and *B. subtilis* (27, 28). In the first two cases, very similar approaches were used. Probe

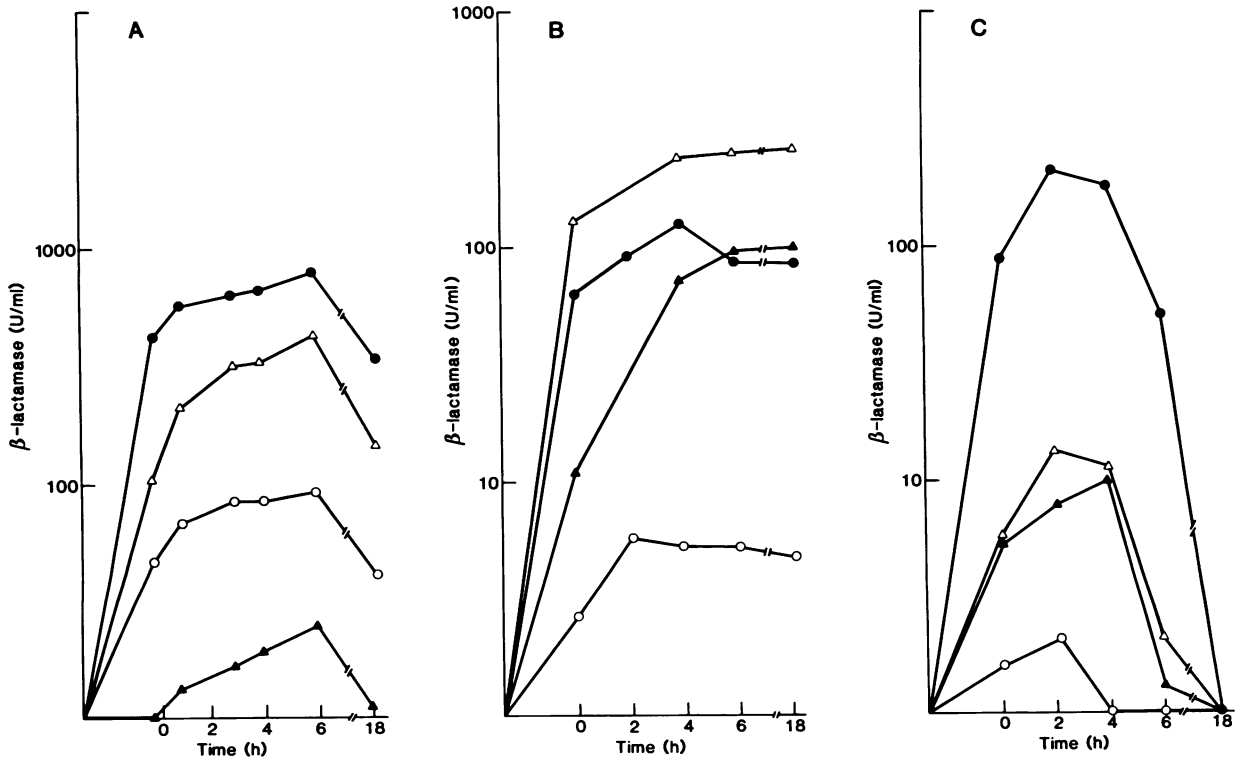

FIG. 4. Secretion of β-lactamase from three gram-positive hosts. (A) *B. subtilis*, (B) *L. lactis* subsp. *lactis*, (C) *L. plantarum*. Symbols: ○, ss30; ●, ss38; △, ss45; ▲, ss80. Cells were grown in appropriate media to 100 Klett units (time 0 in the graphs), after which samples were withdrawn. Only values for the supernatant fractions are presented. See text for details.

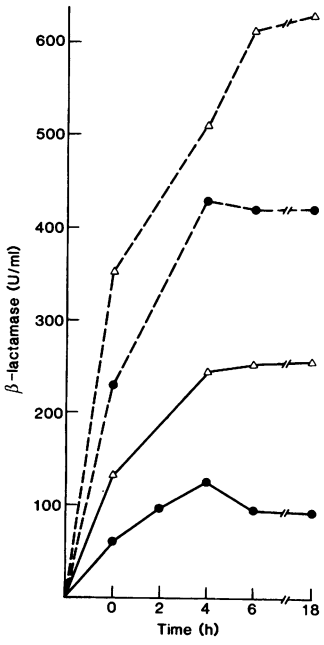

FIG. 5. Secretion of β-lactamase with the best constructs from two different *L. lactis* subsp. *lactis* strains. Symbols: ●, ss38; △, ss45; ——, strain MG1614; – – –, strain V207. Only values for the supernatant fractions are presented. See text for details.

vectors contained fixed promoters and reporter genes. The authentic signal sequences were deleted and replaced by a pool of quasi-random short (<200 bp) DNA sequences. Among these fragments, sequences restoring some translocation of invertase in *S. cerevisiae* or β-lactamase in *E.coli* were found at high frequency (up to 20%). The only common feature of the sequences was their hydrophobicity, and the translocation efficiencies were generally below 10% of the wild-type level. These sequences do not possess the characteristics needed for true signal sequences. The stringency of the experimental set-up apparently lowers the ability to find true signal sequences.

The only reported study concerning gram-positive bacteria (27, 28) describes a set of secretion-promoting fragments isolated from the chromosome of *B. subtilis*. These fragments better resembled true signal sequences, although none of them was identical to the previously described ones. In most of them, the typical tripartite structure, with a positively charged N terminus, a long hydrophobic core, and a putative signal sequence processing site, was seen. The initial screening was done either in *E. coli* or in *B. subtilis*. Both approaches yielded structurally and functionally similar signal sequences.

The structure of the secretion-promoting fragments isolated in this study is very analogous to that of those reported by Smith et al. (28), which are true signal sequences. In both cases, chromosomal fragments were looked for by direct ampicillin selection in *E. coli*. The probe vectors used allowed selection of large fragments, which were likely to contain coding regions for natural genes. This might have raised the possibility that true signal sequences rather than short hydrophobic sequences, as described for *S. cerevisiae* and *E. coli*, would be chosen.

All our fragments except ss80 encoded two or more positively charged amino acids at the very N terminus. The hydrophobic core was about 10 amino acids long in each case, and a processing site fitting the description of von Heijne (33) could be deduced from the sequence in most of the fragments.

Secretion of active TEM β-lactamase should be indicative of the processing of the signal peptide. In our case, the exact cleavage site was not determined. It is surprising that in three (ss30, ss38, and ss80) of the six sequences, a conserved stretch resembling the lipoprotein signal peptidase recognition site was found. According to von Heijne (34), the signal peptidase II (SPase II) consensus is Leu-Ala(Gly/Ala) ↓ Cys, where Cys in position +1 is the only absolutely required residue. In the clones ss30 and ss38, the conserved sequences were Leu-Thr-Ala-Cys and Leu-Gly-Ala-Cys, respectively. In the clone ss80, a perfect consensus, Leu-Ala-Ala-Cys, was found. Until now, only three lipoprotein signal sequences from gram-positive bacteria have been characterized (11, 18, 19). In our sequences, the Cys residue was in amino acid position 20 or 21, depending on the clone, which fits well with the average length of lipoprotein signal peptides (34), but the possible presence of a fatty acid modification has not been verified. The secretion kinetics obtained with *B. subtilis* were, however, not typical of lipoproteins, since no membrane-bound intermediates were detected.

In the clones ss30 and ss38, in addition to SPase II-like sequences, there were also putative recognition sequences for the SPase I-like signal peptidase recognition in the vicinity of residues 30 to 40, whereas the rest of the clones, ss1, ss11, and ss45, had only SPase I-like recognition sites.

Comparison of secretion efficiencies between the secretion-promoting fragments was difficult, since each fragment was preceded by a different promoter. This applies to the comparison of the different fragments in one host and also when one fragment was tested in different hosts, where differences in, for example, promoter functioning, namely its strength or the growth phase-dependent activity, may confuse the picture.

The secretion efficiency of TEM β-lactamase in *B. subtilis* with a heterologous *Bacillus* expression-secretion signal is known to be over 95% (24). It was not previously known how lactococcal secretion signals promote secretion of foreign proteins. Therefore, the efficient secretion into the culture medium of TEM β-lactamase from *L. lactis* subsp. *lactis*, *B. subtilis*, and *L. plantarum* was an important finding.

The production kinetics of the four expression-secretion cassettes in *B. subtilis* were very similar. The maximum activity was reached late in the stationary phase of growth, after which the activity fell gradually. Our previous results have shown that exoproteases secreted by *B. subtilis* rapidly degrade TEM β-lactamase (26a). An addition of 5% glucose was needed here to obtain the reported enzymatic activities, since the strain used is a wild-type protease producer. Without the repression of proteases, the activities fell to zero at the onset of stationary phase.

In *L. plantarum*, peak activity occurred at the end of logarithmic phase, followed by a fast lowering of activity in the stationary phase. This resembles the kinetics obtained with foreign proteins in *B. subtilis* if the exoproteases are not repressed (24, 30).

In *L. lactis*, more heterogeneity in the production kinetics between the fragments was seen. The timing of the peak activity was somewhere between that of *B. subtilis* and *L. plantarum*. *L. lactis* differed from the other two hosts in that the level of TEM β-lactamase activity remained high even in

overnight cultures, indicating a lower level of host protease activity.

The secretion kinetics obtained with ss80 were clearly different from the others. There was a block in efficient secretion, in the logarithmic phase of growth, since 50% (*B. subtilis*) or 90% (*L. lactis*) of the TEM β-lactamase activity remained cell bound, and in *L. plantarum* the peak activity was also reached later. Some clarification of the retarded kinetics comes from inspection of the nucleotide sequence of this clone. First, no obvious signal sequence cleavage site can be deduced from the sequence, which may indicate inefficient processing of the TEM β-lactamase precursor. Second, the location of a putative TATA box and a Shine-Dalgarno sequence, together with the mapping of the 5′ end of the β-lactamase mRNA, suggest that the correct translation initiation codon is the latter of the two in-frame ATG codons. This would yield a signal peptide lacking its positive N terminus instead of the strong positive charge, which could have been deduced from the nucleotide sequence.

A rather unexpected finding was that the overall level of enzymatic activity was highest in *B. subtilis*, although the expression-secretion cassettes originated from *L. lactis*. mRNA quantitation (14) confirmed the similar functioning of the promoters in the two hosts. When the units of TEM β-lactamase per cell over time secreted by each host were calculated, it looked as if *L. lactis* had a lower ability than *B. subtilis* to secrete foreign proteins. The secretion kinetics of *L. lactis* could be further improved nearly 10-fold by replacing the ss45 promoter with another lactococcal promoter (14). The level of β-lactamase rose nearly to the amount previously obtained with the efficient α-amylase promoter in *B. subtilis* (24).

On the basis of our earlier findings (unpublished), another way to yield improvement might be modification of the host's cell wall. Here it was shown that when a more lysozyme sensitive *L. lactis* subsp. *lactis* was used, a threefold increase in the amount of secreted enzyme was obtained.

Very little is known about signal sequences or protein secretion in general in lactic acid bacteria. In lactococci, the only well-characterized signal sequence is that of *L. lactis* subsp. *cremoris* protease (15, 37). None of the signal sequences isolated during our study was identical to the protease signal. There are also only a few reports on the expression and secretion of foreign proteins in lactococci (13, 20, 31). In these cases, the production levels have been very low, which has prevented further analysis of the secretion kinetics. There is one study on *L. plantarum* describing a high expression and over 94% secretion of *Clostridium thermocellum* endoglucanase into the culture medium (2). Our results prove that lactic acid bacteria do possess secretion machinery analogous to that of other gram-positive bacteria. They also appear to have the potential for efficient expression and secretion of foreign proteins, which may find interesting applications both in the improvement of starter cultures and in the production of heterologous proteins.

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REFERENCES

1. **Andersson, D. J., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Bates, E. E. M., H. J. Gilbert, G. P. Hazlewood, J. Huckle, J. I. Laurie, and S. P. Mann.** 1989. Expression of a *Clostridium thermocellum* endoglucanase gene in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **55**:2095–2097.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bolivar, F., R. L. Rodrigues, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. Gene **2**:95–113.
5. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130–135.
6. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
7. **Gryczan, T., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
8. **Hanahan, D., and M. Meselson.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
9. **Hattori, M., and Y. Sakaki.** 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152**:232–238.
10. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
11. **Hussain, M., F. I. J. Pastor, and J. O. Lampen.** 1987. Cloning and sequencing of the *blaZ* gene encoding β-lactamase III, a lipoprotein of *Bacillus cereus* 5691H. J. Bacteriol. **169**:579–586.
12. **Kaiser, C. A., D. Preuss, P. Grisati, and D. Botstein.** 1987. Many random sequences functionally replace the secretion signal sequence for *Saccharomyces cerevisiae* invertase. Science **235**: 312–337.
13. **Klessen, C., K. H. Schmidt, J. J. Ferretti, and H. Malke.** 1988. Tripartite streptokinase gene fusion vectors from gram-positive and gram-negative procaryotes. Mol. Gen. Genet. **212**:295–300.
14. **Koivula, T., M. Sibakov, and I. Palva.** 1991. Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. Appl. Environ. Microbiol. **57**:333–340.
15. **Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Lederboer, and G. Venema.** 1988. Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54**:231–238.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
18. **McLaughlin, J., C. L. Murray, and J. Rabinowitz.** 1981. Unique features in the ribosome-binding sequence of the gram-positive *Staphylococcus aureus* β-lactamase gene. J. Biol. Chem. **256**: 11283–11291.
19. **Nielsen, J. B. K., and J. O. Lampen.** 1982. Membrane-bound penicillinases in gram-positive bacteria. J. Biol. Chem. **257**: 4490–4495.
20. **Novak, S. R., and C. A. Batt.** 1987. Expression of the intensely sweet protein thaumatin in *Streptococcus lactis*. FEMS Microbiol. Rev. **46**:P15.
21. **O'Callaghan, C. H., and A. Morris.** 1972. Inhibition of β-lactamases by β-lactamase antibiotics. Antimicrob. Agents Chemother. **2**:442–448.
22. **Ostroff, G. R., and J. J. Pene.** 1984. Molecular cloning with bifunctional plasmid vectors in *Bacillus subtilis*. I. Construction and analysis of *B. subtilis* clone banks in *E. coli*. Mol. Gen. Genet. **193**:299–305.
23. **Palva, I.** 1982. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. Gene **19**:81–87.

23a. **Palva, I.** 1983. Ph.D. thesis. University of Helsinki, Helsinki, Finland.

24. **Palva, I., M. Sarvas, P. Lehtovaara, M. Sibakov, and L. Kääriäinen.** 1982. Secretion of *Escherichia coli* β-lactamase from

Bacillus subtilis by the aid of α-amylase signal sequence. Proc. Natl. Acad. Sci. USA **79**:5582–5586.
25. **Primrose, S. B., and S. D. Ehrlich.** 1981. Isolation of plasmid deletion mutants and study of their instability. Plasmid **6**:193–201.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26a. **Sibakov, M.** 1986. Ph.D. thesis. University of Helsinki, Helsinki, Finland.
27. **Smith, H., S. Bron, J. van Ee, and G. Venema.** 1987. The construction and use of signal sequence selection vectors in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **169**:3321–3328.
28. **Smith, H., A. de Jong, S. Bron, and G. Venema.** 1988. Characterization of signal-sequence-coding regions selected from the *Bacillus subtilis* chromosome. Gene **70**:351–361.
29. **Terzaghi, P. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Environ. Microbiol. **29**:807–813.
30. **Ulmanen, I., K. Lundström, P. Lehtovaara, M. Sarvas, M. Ruohonen, and I. Palva.** 1985. Transcription and translation of foreign genes in *Bacillus subtilis* by the aid of a secretion vector. J. Bacteriol. **162**:176–182.
31. **van de Guchte, M., J. M. B. M. van der Vossen, J. Kok, and G. Venema.** 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55**:224–228.
32. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53**:2452–2457.
33. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
34. **von Heijne, G.** 1989. The structure of signal peptides from bacterial lipoproteins. Prot. Eng. **2**:531–534.
35. **von Wright, A., A.-M. Taimisto, and S. Sivelä.** 1985. Effect of Ca^{2+} ions on plasmid transformation of *Streptococcus lactis* protoplasts. Appl. Environ. Microbiol. **50**:1100–1102.
36. **von Wright, A., S. Tynkkynen, and M. Suominen.** 1987. Cloning of a *Streptococcus lactis* subsp. *lactis* chromosomal fragment associated with the ability to grow in milk. Appl. Environ. Microbiol. **53**:1584–1588.
37. **Vos, P., G. Simons, and W. M. de Vos.** 1987. Primary structure and organisation of the *S. cremoris* SK11 proteinase gene. FEMS Microbiol. Rev. **46**:P33.
38. **Zhang, Y., and J. K. Broome-Smith.** 1989. Identification of amino acid sequences that can function as translocators of β-lactamase in *Escherichia coli*. Mol. Microbiol. **3**:1361–1369.