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A Model System for the Investigation of Heterologous Protein Secretion Pathways in *Lactococcus lactis*

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The capacity of recombinant strains of *Lactococcus lactis* to secrete a heterologous protein was investigated by constructing two expression-secretion vectors (pLET2 and pLET3) for use with a lactococcal gene expression system driven by the highly active T7 RNA polymerase. The vectors incorporated different lactococcal secretion leaders and translation initiation sequences. When tetanus toxin fragment C (TTFC) was used as a test protein, the quantities of TTFC produced by the pLET2-TTFC strain exceeded the rate of secretion of TTFC into the growth medium. However, nearly all of the soluble TTFC associated with the cell (3.4%) was translocated through the cell membrane. The pLET3-TTFC strain did not accumulate TTFC intracellularly and exhibited growth characteristics and viability identical to the growth characteristics and viability of the control strain. This strain secreted approximately 2.9 mg of TTFC per liter into the growth medium after 6 h of growth under test tube conditions. Our results indicate that *L. lactis* is capable of secreting substantial amounts of heterologous protein and also confirm the findings of other workers that the cell wall may serve as a functional barrier to the diffusion of some secreted proteins into the growth medium.

The development of efficient gene expression and protein secretion systems suitable for use in innocuous lactic acid bacteria could enable these microorganisms to be used for the production by secretion of a number of heterologous proteins. Provided that suitable methods for protein recovery are available, higher initial levels of purity can be obtained when a product is secreted, and the protein is less likely to be subject to intracellular proteolysis or aggregation. Correct folding of proteins may occur more readily in the secretory pathway, and this may reduce or abolish the need for complex renaturation processes (7).

Despite these potential advantages there have been few studies of heterologous protein secretion in *Lactococcus lactis* (1, 3, 4, 10), presumably because of the lack of well-developed expression systems for this species. The *Bacillus subtilis* neutral protease was correctly processed and secreted in *L. lactis* when transcription was initiated on a lactococcal plasmid. However, the amounts of neutral protease formed by *L. lactis* were only 1 to 2% of the amounts produced by the same construct in *B. subtilis* (10). Several lactococcal signal sequences which secrete TEM β-lactamase in *L. lactis* have been isolated by using an *Escherichia coli* signal sequence probe vector (4). More recently, a variety of lactococcal protein secretion signals have been identified by using β-lactamase or α-amylase reporter plasmids (3). A comparison of the expression-secretion efficiencies of these export elements in *E. coli*, *B. subtilis*, and *L. lactis* revealed that in addition to the secretion elements themselves, the host organism and the reporter gene influenced the level of protein secretion. The secretion of bovine prochymosin in *L. lactis* has been obtained by constructing transcriptional gene fusions in which prochymosin secretion is directed by the N-terminal leader sequence of the major *L. lactis* secreted proteinase (1). Prochymosin could be detected in cell supernatants by immunoblotting, but the amounts of protein produced intracellularly and secreted into the medium were not determined.

We have described elsewhere the development of a highly active gene expression system for use in *L. lactis* (13), and in this study we investigated some of the characteristics of this system when gene expression is coupled to protein secretion. In this paper we describe the construction of two novel expression-secretion vectors which can be used for protein production and secretion in *L. lactis* in conjunction with this lactococcal expression system. We used the 52-kDa tetanus toxin fragment C (TTFC) as a test protein in this study.

In this expression system (13) the T7 RNA polymerase gene has been placed under the control of the lactococcal *lac* promoter and cloned into a low-copy-number pIL277 vector (5) derived from the enterococcal pAMβ1 replicon. Expression of the T7 RNA polymerase in suitably transformed cells of *L. lactis* can be induced by substituting lactose for glucose in the growth medium.

We have also described previously (13) the construction of a high-copy-number expression shuttle (*E. coli-L. lactis*) vector designated pLET1, which includes the T7 RNA polymerase promoter, the T7 bacteriophage gene 10 translation initiation sequences, and the T7 terminator sequence. In order to derive expression-secretion vectors from pLET1, the T7 expression cassette in pLET1 (Fig. 1C) was modified by separately incorporating DNA fragments encoding two different lactococcal secretion leader sequences. In one case (pLET3) the highly efficient gene 10 translation initiation sequences were replaced with the sequences that lie directly upstream of the lactococcal *prt* signal leader.

The signal leader sequence of an abundant lactococcal secreted protein (*usp45*) of unknown function (9) was synthesized by using long overlapping oligonucleotides which were annealed and extended by using T7 DNA polymerase. The double-stranded product was cut with *Nde*I and *Bam*HI

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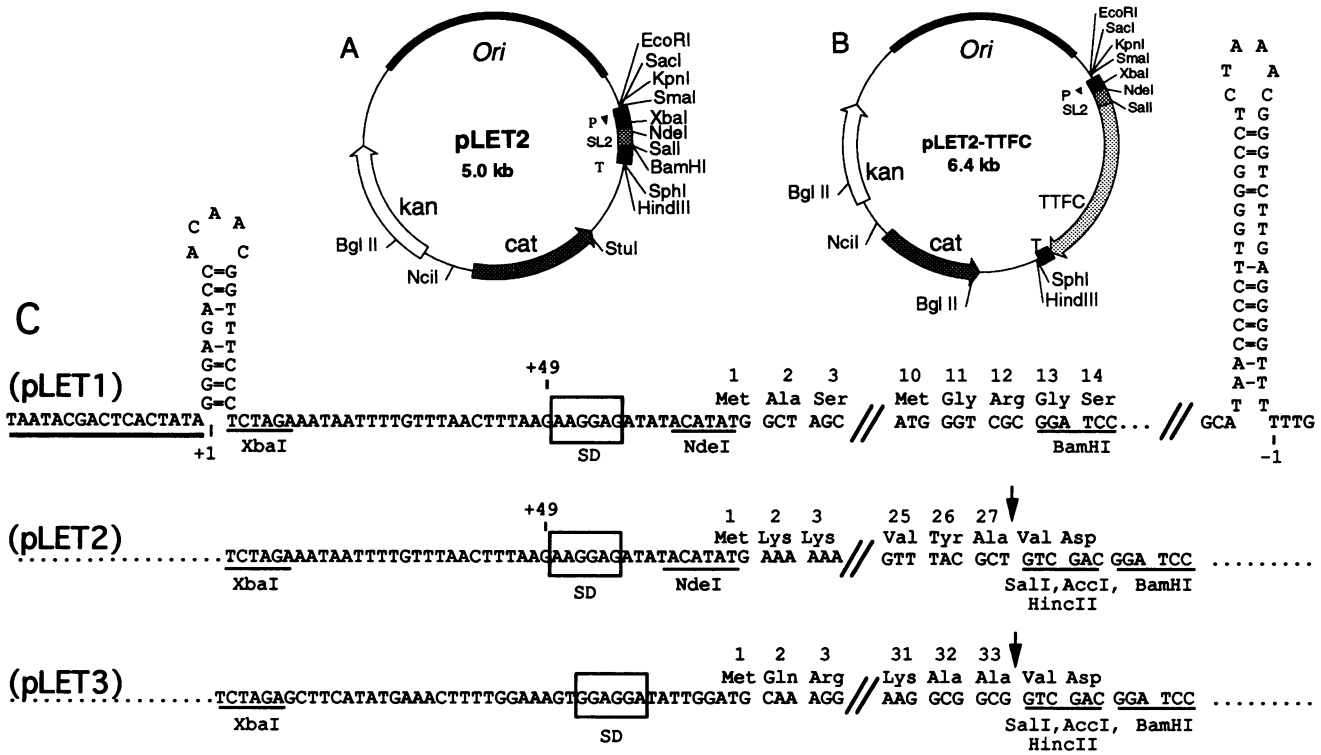

FIG. 1. (A) Plasmid pLET2. (B) Plasmid pLET2-TTFC. The coding sequences of the TTFC gene and the chloramphenicol (cat) and kanamycin (kan) resistance genes are shown as arrows; the direction of transcription is indicated. *Ori*, origins of replication; T, terminator; P, T7 promoter; SL2, *usp45* signal secretion leader. (C) Sequences of the T7 expression cassettes constructed for protein secretion in *L. lactis*. The cognate promoter of the T7 RNA polymerase is underlined. The predicted transcription start site and the last nucleotide of the terminated transcript are indicated by +1 and −1, respectively, as determined by Studier et al. (8). The protein sequences (numbered and with three-letter amino acid codes) are shown above the nucleotide sequence. The potential stem-loop structures at the 5′ and 3′ ends of the RNA are shown, and the Shine-Dalgarno (SD) sequence in each construct is enclosed in a box. The predicted signal peptide cleavage site is indicated by an arrow. The *Sal*I fusion cloning site and other relevant sites are underlined. The *Sal*I, *Xba*I, and *Bam*HI sites are unique in all vectors. Discontinuities in the sequence shown are indicated by angled parallel lines.

and ligated between the *Nde*I and *Bam*HI sites in the T7 cassette cloned in pLET1 to generate pLET2 (Fig. 1C). The *usp45* signal leader replaced the T7 bacteriophage gene 10 coding sequence without altering the nucleotide spacing between the bacteriophage gene 10 ATG start codon and the Shine-Dalgarno sequence as shown in Fig. 1. A *Sal*I restriction endonuclease site was included at the 3′ end of the signal peptide to allow gene fusions to the *usp45* leader to be made.

The second signal secretion leader was obtained by polymerase chain reaction amplification of a DNA fragment of the *L. lactis prt* gene containing both the translation initiation sequences and the signal leader (11). The polymerase chain reaction-amplified *prt* gene leader was cloned between the *Xba*I and *Bam*HI sites in the pLET1 expression cassette to generate pLET3 (Fig. 1C). As described above for pLET2, a *Sal*I restriction site was included at the 3′ end of the signal sequence.

A DNA fragment of pSS1261 (2) comprising the TTFC gene (amino acids 856 to 1316) was cloned between the *Sal*I and blunt-ended *Bam*HI sites in pLET2 and pLET3 to give pLET2-TTFC and pLET3-TTFC, respectively. The pLET2 and pLET2-TTFC vector constructs are illustrated in Fig. 1A and B. The pLET expression vectors containing the TTFC gene in frame with either the *usp45* signal leader or the *prt* signal leader were transferred to the *L. lactis* host strain for T7 expression (13) (MG1820 harboring pILPol) by electroporation (12). To provide a nonexpressor control strain for the ensuing experiments, the host expression strain was transformed with vector pMIG1 (12), which lacks both the T7 expression cassette and the TTFC gene.

The expression strains were induced by replacing glucose with lactose in the growth medium, and the expression and secretion of TTFC in *L. lactis* were monitored by immunoblotting. Preparation of total cell protein extracts and the immunoblotting procedure were carried out as previously described (13). Proteins from the cell-free supernatant were precipitated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by adding trichloroacetic acid to a final concentration of 10% at 0°C. The protein pellet was recovered by centrifugation and was resuspended in SDS-PAGE sample buffer.

The results of the immunoblotting experiment showed that TTFC was present in the cell-free supernatants of both expression-secretion strains (Fig. 2A and B). However, the two strains differed with respect to the amounts of TTFC detected in the total cell extracts. The results obtained with the *prt* signal leader fusion construct (pLET3-TTFC) suggest that TTFC is selectively secreted from these cells, since it was only present in low amounts (and at only one time point)

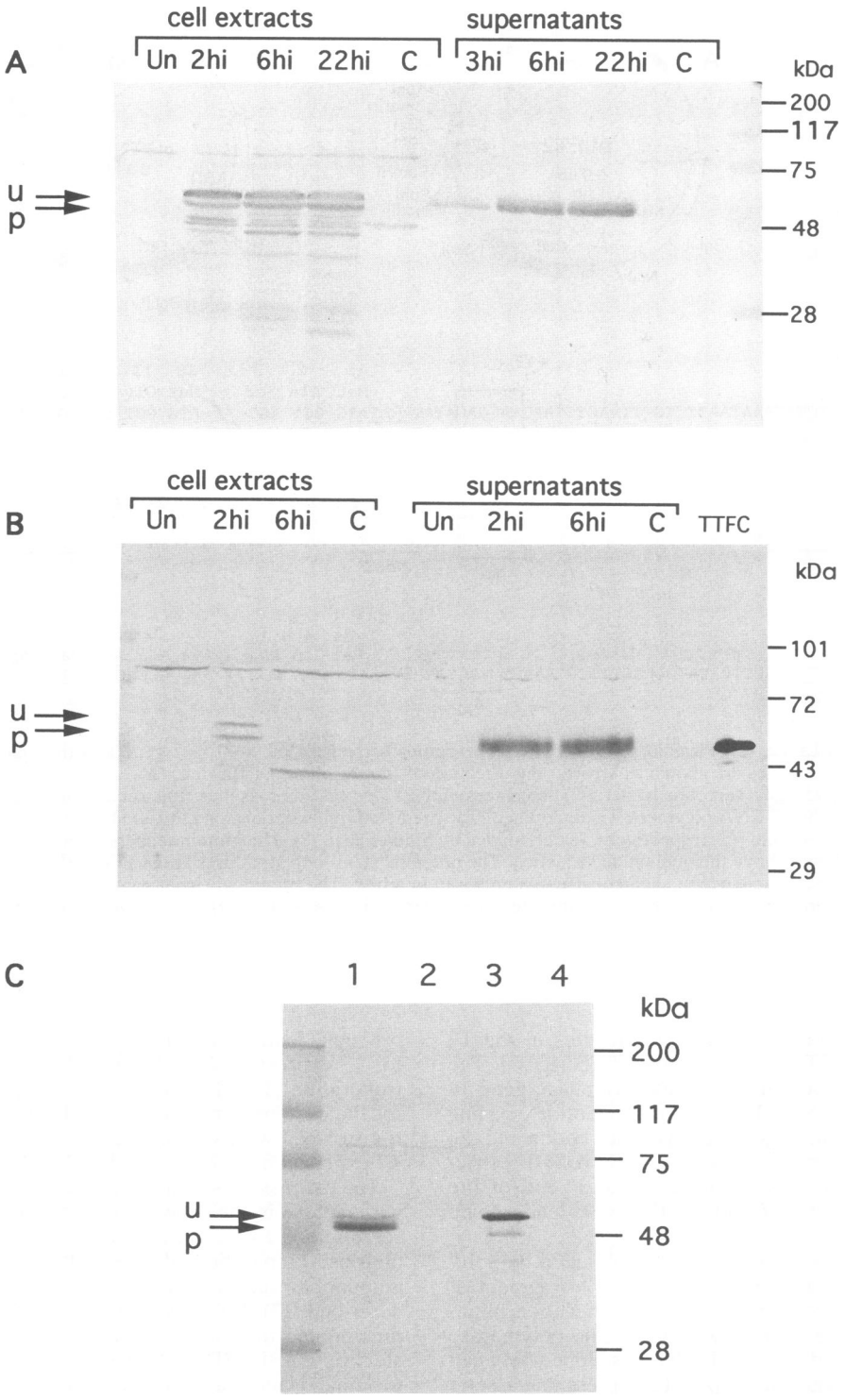

FIG. 2. Immunoblots of total cell extracts and supernatant proteins from the lactococcal expression strain harboring pLET2-TTFC (A) and pLET3-TTFC (B). Total cell protein extracts obtained from approximately 2×10^8 uninduced cells (tracks Un) or from the same number of cells at 2 h (track 2hi), 6 h (track 6hi), or 22 h (track 22hi) after induction were loaded into the tracks. Proteins obtained from cell-free supernatants were precipitated from 200-μl portions of culture supernatants and loaded into the tracks; tracks 2hi, 3hi, 6hi, and 22hi, contained supernatants obtained 2, 3, 6, and 22 h after induction, respectively. Total cell extracts or supernatant proteins from the pMIG1 control strain were loaded into tracks C. A 500-ng portion of recombinant TTFC purified from *E. coli* was loaded into track TTFC. The approximate molecular masses of the prestained marker proteins are indicated on the right. The positions of the putative unprocessed (u) and processed (p) forms of TTFC, as deduced from a comparison with the mobility of rTTFC on these and other Western blots (immunoblots), are indicated by arrows. (C) Immunoblot of proportional amounts of soluble and insoluble extracts of the *L. lactis* expression strain harboring pLET2-TTFC or the vector control pMIG1. Tracks 1 and 3, contained pLET2-TTFC soluble and insoluble extracts, respectively; tracks 2 and 4 contained pMIG1 soluble and insoluble extracts, respectively.

in total protein extracts obtained from approximately 2×10^8 expressing cells (approximately 12 μg of total protein), while TTFC was easily detected among the proteins precipitated from 200-μl portions of cell-free supernatants obtained from induced cultures having cell densities of 2.5×10^8 to 5×10^8 cells per ml (Fig. 2B). In contrast, substantial amounts of TTFC were detected in the total cell extracts prepared from the pLET2-TTFC strain containing the *usp45* signal leader (Fig. 2A), suggesting that the level of TTFC expression exceeded the rate of TTFC secretion into the growth medium. The high-molecular-weight species of TTFC detected in the total cell extracts from this strain was most likely the unprocessed form (signal sequence plus TTFC) of the protein (Fig. 2, arrow) as it was consistently found to be larger than recombinant TTFC (rTTFC) purified from *E. coli*, which lacks a signal leader (data not shown). No TTFC was detected in the total cell extracts or in culture supernatants of the control strain which harbored the pMIG1 vector lacking the T7 expression sequences and the TTFC gene (Fig. 2). The protein recovered from the supernatants of these expression-secretion strains was the same size as purified rTTFC, suggesting that it had been properly processed and translocated through the cell membrane.

In order to obtain further evidence that the signal secretion leaders contributed to the secretion of TTFC and that TTFC was not just released from damaged or lysed cells, we examined the numbers of cells in our cultures which became permeable to the DNA chelating dye propidium iodide and also assayed for the presence of lactic dehydrogenase (a strictly cytoplasmic enzyme) in the cell-free supernatants of induced cultures. Both of these assays provide quantitative indicators of cell death. At different times after induction propidium iodide (10 μg/ml) was added to dilutions of the cultures in phosphate-buffered saline, and the cells were examined by phase-contrast microscopy and fluorescence microscopy. The results indicated that less than 0.5% of the cells of both the pLET3-TTFC strain and the control strain were permeable to propidium iodide 6 h after induction. Since 0.5% of the cells in the expression cultures could not have released the quantity of TTFC detected in the supernatants (Fig. 2B), the propidium iodide procedure provided further evidence that TTFC was actively secreted into the supernatant by the pLET3-TTFC strain. In the pLET2-TTFC culture approximately 5% of the bacteria became permeable to propidium iodide after 2 h, and the proportion increased to approximately 10% at 6 h. Again, this degree of cell permeability cannot account for the total amount of processed TTFC found in the cell-free supernatant. It is, however, possible that a portion of the TTFC detected had been released from damaged or dying cells. Assays of the lactate dehydrogenase activities of the culture supernatants (data not shown) indicated that barely detectable levels of lactate dehydrogenase activity were present in the supernatants of all strains and that there were no differences among the lactate dehydrogenase activities of supernatants derived from cultures of the control strain and the two expression-secretion strains.

The expression strain harboring pLET2-TTFC was also fractionated by mechanical homogenization (13) 2 h after induction, and extracts of the soluble and insoluble proteins were immunoblotted with polyclonal antiserum to TTFC. Most of the TTFC recovered in the soluble protein fraction had a lower molecular weight than the TTFC associated with the insoluble fraction (Fig. 2C). This implied that most of the soluble cellular protein had been processed to its secreted form, while the intracellular insoluble protein remained unprocessed. It was determined by an enzyme-linked immunosorbent assay that TTFC accounted for approximately 3.4% of the total soluble protein recovered from the pLET2-TTFC expression strain. Hence, nearly all of the TTFC recovered as soluble protein from the cells had been translocated across the cell membrane and was probably present in the periplasmic space between the cell membrane and the cell wall. This indicates that for the pLET2-TTFC expression strain the rate of diffusion of TTFC through the cell wall must be a limiting step in the secretion of TTFC, at least under the growth conditions used by us. The TTFC detected in the insoluble fraction by SDS-PAGE and immunoblotting might have arisen from the aggregation and precipitation of protein in the cells, as is commonly observed for other recombinant proteins which have been overexpressed in *E. coli*. Alternatively, the unprocessed form of TTFC might be associated with cell membranes which were pelleted with the cell walls during fractionation.

The TTFC detected in the soluble and insoluble fractions of mechanically homogenized cells was apparently undegraded, while some degradation was evident in total cell extracts prepared by a slow extraction procedure involving the incubation of cells with lysozyme and mutanolysin at 37°C (Fig. 2A and C). It is likely that enzyme digestion of the walls and the subsequent washing steps in the absence of any protease inhibitors activated degradative processes in dying cells. In contrast to the observed partial degradation of intracellular TTFC, the TTFC secreted into the growth medium of cultured cells by the expression-secretion strains remained undegraded even after incubation for 22 h. Similarly, Sibakov et al. (4) and Perez-Martinez et al. (3) have shown that the activity of TEM β-lactamase is stable when this enzyme is secreted by *L. lactis*. However, in other hosts, such as *B. subtilis* or *Lactobacillus plantarum*, proteolytic degradation of secreted β-lactamase has been observed (4, 6). Our results support the view that protease degradation should not impede the use of *L. lactis* for the production of extracellular heterologous proteins.

In order to determine the amounts of TTFC secreted by the *L. lactis* expression strains harboring pLET3-TTFC and pLET2-TTFC, cultures of these strains were induced in the exponential growth phase (optical density at 600 nm, 0.5), and samples of the culture supernatants (taken at different times after induction) were assayed for TTFC by an enzyme-linked immunosorbent assay (13). Figure 3A shows that these strains reached the stationary phase of growth approximately 4 h after induction and that the pLET2-TTFC strain grew more slowly than cells which carried pLET3-TTFC or the pMIG1 control and did not reach the same final cell density. When TTFC secretion was assayed over a 6-h time period following induction (Fig. 3B), the amounts of TTFC secreted into the growth medium by the strain which carried pLET3-TTFC reached a plateau (approximately 1 μg/ml) when the cells entered the stationary phase. The quantities of TTFC detected in the growth medium of the pLET2-TTFC expression strain were higher (approximately 2 μg/ml at 6 h postinduction). TTFC was not detected in the supernatants of the pMIG1 control strain.

Since the growth and viability of the pLET3-TTFC strain were not affected by the induction of TTFC production, this strain was grown from a low cell density in the presence of the inducer, and its rates of growth and TTFC secretion were monitored for 6 h. The amount of TTFC secreted into the growth medium was proportional to the cell density (Fig. 3C). After 6 h approximately 2.9 mg of TTFC per liter had been secreted into the growth medium.

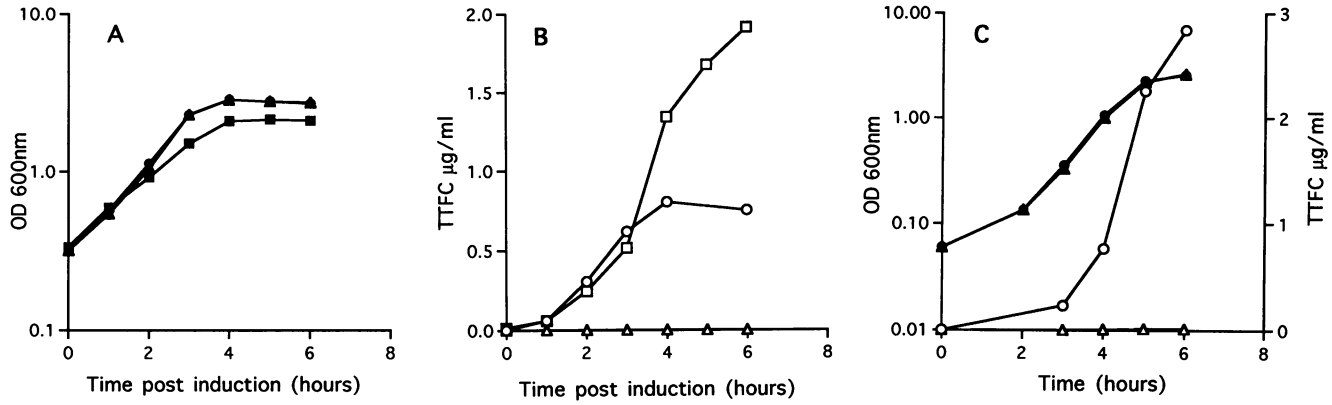

FIG. 3. (A) Growth curves for different strains after induction of TTFC expression in the exponential phase of growth. (B) Amounts of TTFC secreted into the culture supernatant following induction in the exponential phase of growth. (C) Growth curve and amounts of TTFC secreted into the culture supernatant by the pLET3-TTFC and pMIG1 control strains when the cultures were grown from low cell densities in the presence of lactose. All values for optical density at 600 nm (OD 600 nm) were plotted on a logarithmic scale. The symbols used to identify the different strains are the same in all graphs. Open symbols, secreted TTFC concentration; solid symbols, optical density measurements. Symbols: ■ and □, pLET2-TTFC; ● and ○, pLET3-TTFC; ▲ and △, pMIG1 control.

As both expression-secretion vectors were able to direct the secretion of TTFC into the growth medium, the intracellular accumulation of TTFC in the pLET2-TTFC strain (3.4% of the soluble protein) was probably a consequence of higher levels of expression. The only difference between the two constructs was that in pLET3 all of the sequences downstream of the T7 promoter and the RNA stabilizing sequence, including the ribosome binding site, were of lactococcal origin. One possible reason for the lower levels of expression obtained with the pLET3-TTFC construct is that the 5′ secondary structure sequence and the Shine-Dalgarno sequences of the two mRNAs promote protein translation initiation at different rates. We are currently investigating these issues.

In separate studies we found (data not shown) that similar amounts of TTFC are produced by our host strains when the TTFC gene is present on either a low-copy-number expression vector or a high-copy-number expression vector. This implies that a high copy number of the target gene is not required for the T7 RNA polymerase-based expression system to yield substantial quantities of the target gene product. Chromosomal integration of genes encoding expression products should therefore not diminish product yield. It is possible that the limitation which we have observed on the quantities of TTFC that can be secreted may not be typical of all proteins and that higher levels (>3 mg/liter) of secreted protein could be obtained by testing other translation control and signal leader sequences in the types of vectors described here. It was also evident from the results of the fractionation and immunoblotting experiments performed with the pLET2-TTFC expression strain that the cell wall may serve as a functional barrier to the diffusion of some secreted proteins into the growth medium. Other workers have also demonstrated that this might be the case for TEM β-lactamase as threefold-higher levels of activity were obtained with a strain showing increased sensitivity to lysozyme (4).

The results reported here indicate that the vectors described provide powerful experimental systems with which to probe the potential productivity of the protein secretion pathways in *L. lactis*, since levels of gene expression which outpace protein secretion can now be obtained. Moreover, the prolonged stability of the secreted protein which we investigated is in marked contrast to the proteolysis encountered in some other gram-positive systems and indicates that *L. lactis* is worthy of further investigation as a production organism for the secretion of recombinant proteins in soluble forms.

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