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Leaky *Lactococcus* Cultures That Externalize Enzymes and Antigens Independently of Culture Lysis and Secretion and Export Pathways†

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A novel system that leaks β-galactosidase (β-gal) without a requirement for secretion or export signals was developed in *Lactococcus lactis* by controlled expression of integrated phage holin and lysin cassettes. The late promoter of the lytic lactococcal bacteriophage φ31 is an 888-bp fragment (P_{15A10}) encoding the transcriptional activator. When a high-copy-number P_{15A10}::*lacZ.st* fusion was introduced into *L. lactis* strains C10, ML8, NCK203, and R1/r1t, high levels of the resultant β-gal activity were detected in the supernatant (approximately 85% of the total β-gal activity for C10, ML8, and NCK203 and 45% for R1/r1t). Studies showed that the phenotype resulted from expression of Tac31A from the P_{15A10} fragment, which activated a homologous late promoter in prophages harbored by the lactococcal strains. Despite the high levels of β-gal obtained in the supernatant, the growth of the strains was not significantly affected, nor was there any evidence of severe membrane damage as determined by using propidium iodide or transmission electron microscopy. Integration of the holin-lysin cassette of phage r1t, under the control of the phage φ31 late promoter, into the host genome of MG1363 yielded a similar "leaky" phenotype, indicating that holin and lysin might play a critical role in the release of β-gal into the medium. In addition to β-gal, tetanus toxin fragment C was successfully delivered into the growth medium by this system. Interestingly, the X-prolyl dipeptidyl aminopeptidase PepXP (a dimer with a molecular mass of 176 kDa) was not delivered at significant levels outside the cell. These findings point toward the development of bacterial strains able to efficiently release relevant proteins and enzymes outside the cell in the absence of known secretion and export signals.

Lactococcus lactis is best known for its role in mesophilic dairy fermentations, including those used in production of cheddar cheese, buttermilk, and sour cream. Its long history of safe use in the food industry and generally recognized as safe (GRAS) status provide new opportunities for using *L. lactis* in important roles in food biotechnology, particularly in the presentation of vaccines, antimicrobial agents, or intracellular peptidases involved in cheese ripening, outside of the cell. Three major mechanisms have been exploited in these studies. First, signal sequences of the lactococcal secreted protein Usp45 (43), the lactococcal proteinase (46), and the S-layer protein (encoded by *slpA*) (45) of *Lactobacillus brevis* have been employed to secrete heterologous proteins from *L. lactis* via the secretory pathway. ATP-binding cassette–transporter export systems have also been used to export heterologous bacteriocins from *L. lactis* (for a review, see reference 1). Lastly, induction of cell autolysis can result in efficient release of homologous and heterologous proteins from the cell. Interest in naturally occurring autolytic strains has focused on the release of intracellular enzymes, namely peptidases, into the cheese medium to enhance flavor development and accelerate cheese ripening. Strains of *L. lactis* differ widely in their ability to undergo autolysis (25). Recent studies have implicated both the major lactococcal autolysin AcmA (5) and "leaky" low-level expression of a prophage-encoded lysin (21, 27) as causes of cell autolysis.

Advances in the molecular techniques available for studying *L. lactis* have increased efforts to genetically engineer autolytic strains to control cell lysis. For instance, the lactococcal autolysin AcmA was cloned under the control of two regulated promoters, the chloride-inducible promoter (39) and the promoter-operator region of the temperate phage r1t (5, 32), generating strains which, upon induction of the promoter, lyse to release intracellular enzymes into the supernatant. Another major advance in this area has been the cloning and characterization of several lactococcal bacteriophage lysins and holins (reviewed in reference 15). In contrast to the lactococcal autolysin AcmA, the bacteriophage lysins do not possess a signal sequence (4). Externalization occurs via a small, transmembrane holin which oligomerizes to form nonspecific pores in the host membrane (for reviews, see references 3, 15, 52, and 53). The genes encoding several bacteriophage lysins and/or holins have been exploited to design strains which lyse under controlled conditions. For example, the holin and lysin genes of phages r1t and US3 were placed under the control of P_{nisA} (6) and the chloride-inducible promoter (39), generating strains which released intracellular peptidases into the medium upon induction of the promoter. Overproduction of only the holin from phage φUS3, via P_{nisA}, resulted in an immediate inhibition of cell growth and partial lysis of the host cell (6).

The use of bacteriophage lytic cassettes is an exciting ad-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, phage, plasmid, or promoter fragment	Description[a]	Reference or source
Strains		
E. coli		
JM110	*E. coli* cloning host	51
XL1-Blue	*E. coli* cloning host	Gibco-BRL
L. lactis		
NCK203	Subsp. *lactis*; KP1 derivative	18
ML8	Subsp. *lactis*	U. of Laval[b]
C10	Subsp. *lactis*	12
R1/r1t	Subsp. *cremoris*; carries r1t prophage	22
R1C	Subsp. *cremoris*; r1t-cured derivative	22
MG1363	Subsp. *cremoris*	14
MM210	Subsp. *cremoris*; industrial strain	NCSU[c]
Bacteriophage ϕ31.9	Phage ϕ31 derivative with F142L mutation in Tac31A	10
Plasmids		
pTRKH2	Em^r; high-copy-number cloning vector	34
pNZ18	Cm^r; high-copy-number shuttle vector	7
pGhost8	Tet^r; lactococcal integration vector	INRA[d]
pTRK390	Em^r; lactococcal promoter screening vector	36
pTRK391	Em^r; pTRK390 (P_{15A10}::*lacZ.st*)	36
pTRK406	Cm^r; pNZ18::*abiA*, $AbiA^+$	8
pTRK483	Em^r; pTRK390 ($P_{566–888}$::*lacZ.st*)	47
pTRK609	Em^r; pTRK390 (P6::*lacZ.st*)	This study
pTRK610	Em^r; pTRK390 ($P_{15A10ϕ31.9}$::*lacZ.st*)	This study
pTRK611	Em^r; pTRK390 ($P_{15A102X}$::*lacZ.st*)	This study
pTRK617	Em^r; $P_{15A102X}$::TTFC on pTRKH2	This study
pTRK618	Em^r; P6::TTFC on pTRKH2	This study
pTRK619	Em^r; $P_{15A102x}$::*pepXP* on pTRKH2	This study
Promoter fragments		
P6 promoter	Strong, constitutive *Lactobacillus* promoter	9
P_{15A10}	888-bp ϕ31 late promoter encoding *tac31A* and two phage-inducible transcription start sites	36
$P_{15A10ϕ31.9}$	P_{15A10} derivative encoding mutant *tac31A* (F142L mutation), resulting in a 50% reduction in promoter activity	10, this study
$P_{15A102x}$	P_{15A10} derivative with mutation in inverted repeat downstream of transcription start sites, resulting in a 20 to 3-fold increase in promoter activity	47, this study
$P_{566–888}$	Tightly regulated version of ϕ31 late promoter which does not encode *tac31A*	47

[a] Abbreviations: Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.
[b] University of Laval, Laval, Quebec, Canada.
[c] NCK culture collection at North Carolina State University, Raleigh.
[d] Institut National de la Recherche Agronomique, Paris, France.

vance in the continued development of expression systems allowing the delivery of proteins and enzymes to the outside environment. However, these systems often cause rapid cessation of growth and, eventually, cell lysis. Moreover, since lysin works extracellularly, the growth of other lactococcal strains used in combination would also be affected. In this study, we describe a novel expression system which allows the release of significant levels of a *Streptococcus thermophilus* β-galactosidase (β-gal) (40) in *L. lactis* without the use of signal sequences and without causing severe losses of cell viability or integrity. The system utilizes the phage transcriptional activator Tac31A (formerly open reading frame 2 [ORF2] and *tac* [36, 47]) to activate a late promoter residing on a prophage integrated in the genome of *L. lactis*. Low-level activation of the late promoter, driving downstream expression of holin and lysin, resulted in a leaky behavior that efficiently externalized an enzyme and a vaccine, with minimal losses of other cytoplasmic enzymes.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were propagated at 30°C in M17 medium (Difco) (42) supplemented with 0.5% glucose (GM17). Where necessary, erythromycin, tetracycline, and/or chloramphenicol was added at 5, 2, and 7.5 μg/ml, respectively. *Escherichia coli* strains were grown in Luria-Bertani broth at 37°C with shaking or on Luria-Bertani medium supplemented with 1.5% agar. Erythromycin resistance in *E. coli* was selected by plating on brain heart infusion agar (Difco) supplemented with 120 μg of erythromycin/ml (34).

Bacteriophage propagation and phage DNA isolation. When necessary, the resident prophages of the lactococcal strains used in this study were induced by using mitomycin C at a level of 10 μg/ml (for strains NCK203, C10, and ML8) or 2 μg/ml (for R1/r1t). Prophage DNA was isolated and purified as described by Raya et al. (37) as modified by Walker et al. (48). In some cases, *L. lactis* genomic DNA preparations were prepared 1 h after mitomycin C induction, using the procedure of Hill et al. (17). To determine the number of r1t phages present in the culture supernatant, culture samples were filtered (0.45 μm pore size) and serial dilutions were spotted onto a lawn of R1Cs cells (prophage cured). $CaCl_2$ (10 mM) was added to the medium, and soft agar was used to prepare the lawn of the sensitive host.

DNA isolation. Small-scale *E. coli* plasmid preparations were performed by the alkaline-sodium dodecyl sulfate method (38). Large-scale *E. coli* plasmid preparations were performed using a Qiagen plasmid kit (Qiagen Inc., Chatsworth, Calif.) according to manufacturer's directions. Small-scale isolation of plasmids from *L. lactis* was performed as previously described (35), except that ethidium bromide was not used prior to phenol-chloroform extraction.

DNA manipulations and transformations. Standard procedures were used for the DNA manipulations (38). Restriction enzymes and T4 DNA ligase were provided by Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the manufacturer's instructions. Southern hybridizations were performed at 65°C in a Robbins (Robbins Scientific, Inc., Sunnyvale, Calif.) hybridization oven per the manufacturer's instructions. DNA probes were ^{32}P labeled

by using the Multiprime DNA labeling system (Amersham, Piscataway, N.J.). The r1t *attP* fragment used to probe genomic DNA preparations for the induction of prophages contained the 3′ portion of integrase, *attP*, ORF50, and the 3′ portion of lysin (nucleotides 31728 to 185 of the published sequence [44]). The fragment was amplified from R1/r1t genomic DNA by PCR using the forward primer 5′-GGCTATCACACAGCAAACCTATATC-3′ and the reverse primer 5′-CGTTCCTACTCGGCACAGGTCAAG-3′. Ligations were transformed into RbCl-competent *E. coli* strains. RbCl-competent *E. coli* cells were prepared by the procedure of Hanahan (16). Cell preparations were frozen at −70°C in 100-μl aliquots and transformed by a procedure described for $CaCl_2$-competent cells (38). After being screened to verify their proper insertion in *E. coli*, plasmids were electroporated into *L. lactis* by the procedure of Holo and Nes (19), modified as described by Walker and Klaenhammer (47). Electroporations were carried out in a 0.2-cm-path-length cuvette with a Bio-Rad (Richmond, Calif.) Gene Pulser, using 100 μl of cell preparation and the following conditions: 25 μF, 2.45 kV, and 200 Ω. Cell recovery was achieved by incubation in GM17 supplemented with 10 mM $MgCl_2$ and 1 mM $CaCl_2$ for 2 h at 30°C prior to plating on media with appropriate antibiotics.

PCR. PCR was performed with *Taq* DNA polymerase (Boehringer Mannheim) according to the manufacturer's instructions. In each case, 40 cycles were used to amplify the regions of interest. Annealing temperatures were 5 to 10°C below the lowest melting temperature of each primer pair. To facilitate cloning of PCR products, restriction enzyme sites (indicated throughout the manuscript by underlining) were designed for the 5′ ends of the primers.

RNA manipulations. RNA was isolated from *L. lactis* strains by using TRIzol reagent (Gibco-BRL, Gaithersburg, Md.) according to the procedure of Dinsmore and Klaenhammer (8). Slot blot Northern hybridizations using equivalent amounts of RNA from each sample (approximately 10 μg) were performed on a Bio-Rad slot blot apparatus (Bio-Rad, Richmond) according to the manufacturer's protocol. The r1t lysin probe used to measure the induction of lysin mRNA was amplified from a genomic DNA preparation of R1/r1t, using the forward primer 5′-CGTTCCTACTCGGCACAGGTCAAG-3′ and the reverse primer 5′-CCAAACTCTTTATCGACTTC-3′, and consisted of nucleotides 32065 to 32815 of the published r1t sequence (44). The r1t holin probe used to measure the induction of holin mRNA was amplified using the forward primer 5′-GCA CAAGCAATGATTGGCGCTTTGG-3′ and the reverse primer 5′-TTGACTA GGCTTGCTGTATTATCG-3′ and consisted of nucleotides 31866 to 32023 of the published r1t sequence (44). The DNA probe used to measure induction of mRNA from the late region of the uncharacterized prophage of *L. lactis* strains C10, ML8, and NCK203 was designed using sequence data from the late region of the lytic phage φ31 and contained the 3′ portion of ORF3, *cos*, ORF4, and the 5′ portion of ORF5 (48). The fragment was amplified from phage φ31 genomic DNA by using the forward primer 5′-CGTGATTGGTCTTCTTATG-3′ and the reverse primer 5′-AGAAATGAGCTTCAAGAACAA-3′.

Enzyme assays. β-gal determinations were performed using the *o*-nitrophenyl-β-D galactopyranoside (ONPG) assay described by Miller (30), as modified by O'Sullivan et al. (36). To determine the level of β-gal in the supernatant of samples, 100-μl samples of the whole culture (cells plus growth medium) or just the filter-sterilized supernatant were tested. Both reactions (whole culture and supernatant) were stopped upon development of a yellow color in the reaction containing the whole culture. Absorbances at 420 nm were read after centrifugation to remove any cell debris. Percentages were determined with the formula (A_{420} supernatant/A_{420} whole culture) × 100.

Intracellular X-prolyl dipeptidyl aminopeptidase (PepXP) activity present in the whole culture versus that in the supernatant was measured in 100-μl samples of the whole culture or filter-sterilized supernatant as described by Nivens and Mulholland (33). Whole cultures were permeabilized by using chloroform. Both reactions were stopped by addition of 30% acetic acid when color development was detected in the whole-culture sample. Absorbances were read at 410 nm, and percentages were determined as described above.

Determination of the proportion of cells with damaged membranes. The integrity of *Lactococcus* cell membranes was measured by a fluorescence procedure described by Niven and Mulholland (33). Basically, *L. lactis* strains C10, ML8, R1/r1t, and NCK203, with and without P_{15A10} (containing *tac31A*), were propagated to an initial optical density at 600 nm (OD_{600}) of 0.65 and centrifuged to remove the medium. Cell pellets were resuspended in phosphate-buffered saline (PBS) to an OD_{600} of 0.9. Viable-cell counts were performed. The samples were divided into four tubes, each of which received one of the following treatments: no treatment, treatment with propidium iodide (PI) alone (30 μmol/liter), treatment with the permeabilizing agent cetyltrimethylammonium bromide (CTAB) alone (0.2 mmol/liter), or treatment with a combination of PI and CTAB. After a 30-min incubation at room temperature, fluorescence was measured using an excitation wavelength of 500 nm and an emission wavelength of 600 nm. Readings were also taken for PBS containing PI, CTAB, or PI plus CTAB. The ratio of cells with damaged membranes was determined with the formula $(cells_{PI} - cells_{alone} - PBS_{PI})/(cells_{PI+CTAB} - cells_{alone} - PBS_{PI+CTAB})$.

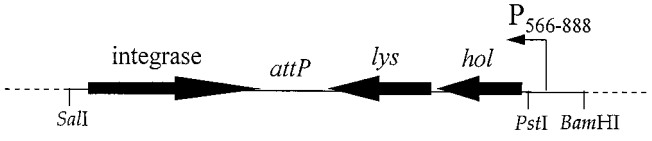

FIG. 1. Organization of construct used for integration of the $P_{566–888}$::holin-lysin cassette into *L. lactis* MG1363.

Electron microscopy. The cells were prepared for transmission electron microscopy as described by Dykstra (11).

Integration of the r1t holin-lysin cassette in MG1363. An integration cassette containing the r1t holin and lysin genes under the control of the tightly regulated phage φ31 late promoter $P_{566–888}$ was cloned into pGhost8, a temperature-sensitive vector (29). PCR primers (P1 [5′-GATCGTCGACTGTCTGACGGC TGGGTAATGT-3′] and P2 [5′-GTTTCTGCAGTCGGTTCAGCCAGTGATT GTTC-3′]) were used to amplify a single fragment (*Sal*I-*Pst*I) (see Fig. 1) containing the integrase, the *attP* region, and the holin-lysin cassette from a genomic DNA preparation of R1/r1t (nucleotides 31738 to 1391 of the published r1t sequence [44]). This fragment also contained the putative ORF50 coding region, which is located just downstream of the lysin gene (44). PCR primers P3 (5′-ATAGGATCCGTGTCACATAACTGAGCGCC-3′) and P4 (5′-GATGC TGCAGTATTGGCTTGCCACATATTC-3′) were used to amplify $P_{566–888}$ (*Bam*HI-*Pst*I) (Fig. 1) (47). The two PCR products were restricted, gel purified, and ligated into pGhost8 which had been restricted with *Bam*HI and *Xho*I. Ligation reaction products were transformed directly into MG1363 competent cells. After outgrowth at 30°C, the transformants were selected on GM17 with tetracycline (2 μg/ml) at the nonpermissive temperature of 37°C.

Construction of $P_{15A102X}$::TTFC and $P_{15A102X}$::PepXP fusions. The coding region for tetanus toxin fragment C (TTFC) was amplified from pLET1-TTFC (50) using the forward primer 5′-GATCCTGCAGTTGTTTAACTTTAAGAA GGAG-3′ (*Pst*I site underlined) and the reverse primer 5′-ATATCTCGAGTA GTTCCTCCTTTCAGCA-3′ (*Xho*I site underlined). The fragment, which contained the translation initiation region utilized on pLET1-TTFC, was cloned into pTRKH2 which had been restricted with *Pst*I and *Xho*I. To achieve adequate expression levels, a stronger, mutated version of the phage φ31 late promoter, $P_{15A102X}$, containing *tac31A* and the phage-inducible transcription start sites (36, 47) was cloned upstream of the TTFC coding region to yield pTRK617. In the mutated $P_{15A102x}$, a small inverted repeat downstream of the transcription start sites was eliminated by site-directed mutagenesis, resulting in a two- to threefold increase in expression levels (47). The forward primer 5′-ATATGGATCCGCA GAGCATTTGTAAGGTTGG-3′ (*Bam*HI site underlined) and the reverse primer 5′-GATGCTGCAGGATTGGCTTGCCACATATTC-3′ (*Pst*I site underlined) were used to amplify the 888-bp $P_{15A102X}$, which was cloned into the *Bam*HI-*Pst*I sites upstream of the TTFC coding region. Since unintentional promoter activity from the vector allowed low-level expression of the transcriptional activator (unpublished data), this cloning strategy, and all others to follow, retained the phage promoter in the same orientation as that in pTRK391. As another control, the strong *Lactobacillus* P6 promoter (9) was cloned upstream of the TTFC coding region to yield pTRK618.

The PepXP coding region was amplified with its own translation initiation region from *L. lactis*, using forward primer 5′-TAAGCTAAAAGTATTATCA TGTTTATTACGGAGG-3′ and reverse primer 5′-GTCGAGCAACTGTGTT GTAAGGAG-3′ (*Sal*I site underlined). Primers were designed using sequence information from Nardi et al. (31). The *pepXP* fragment was initially cloned into pT7Blue (Novagen, Madison, Wis.) to introduce a *Bam*HI site at the 5′ end of the coding region. The *pepXP* fragment was removed by using *Bam*HI and *Sal*I and cloned into pTRKH2 restricted with *Bam*HI and *Sal*I. $P_{15A102x}$ was cloned upstream as a *Bam*HI fragment.

Western blotting to detect TTFC. Cell extracts were prepared from strains propagated to mid-log phase (OD_{600} = 0.5) in GM17 supplemented with erythromycin. The cells were centrifuged, washed in 100 mM Tris-HCl (pH 8.0), and resuspended at a 40× concentration in 100 mM Tris-HCl. The cells were broken by bead beating two times for 1 min each, with a 30-s intermission, on ice. The cell extract was collected by removing the supernatant after centrifugation at 4°C. Concentrated (50-fold) supernatant samples were prepared by dialyzing filter-sterilized supernatants of the cultures against water (overnight), freeze drying, and resuspending in 100 mM Tris-HCl. Dialysis was necessary to remove con-

TABLE 2. Levels of β-gal detected in supernatants of Tac31A$^+$ (encoded by P_{15A10} or $P_{15A10\phi31.9}$) and Tac31A$^-$ cultures

Strain	Prophage with homologous $P_{\phi31}$[a]	% of β-gal detected for construct:		
		P6::*lacZ.st*	P_{15A10}::*lacZ.st*	$P_{15A10\phi31.9}$::*lacZ.st*
MM210	No	—[b]	<10	—
MG1363	No	—	<10	—
NCK203	Yes	10	88.3	—
C10	Yes	4.3	84.0	—
ML8	Yes	6.1	88.0	—
R1/r1t	Yes	<10	Unstable	45.3
R1C	No	—	—	<10

[a] $P_{\phi31}$ refers to the phage φ31 late promoter, designated P_{15A10} in this paper.
[b] —, not tested.

centrated solutes which interfered with electrophoresis. Denatured cell extracts and concentrated supernatants were electrophoresed on a 10% polyacrylamide gel, using a Bio-Rad mini-PROTEAN II electrophoresis unit, by standard procedures (38). The gels were transferred overnight to polyvinylidene difluoride membranes (0.2 μm pore size) by using a Bio-Rad Mini Trans-blot cell at 25 V. TTFC was detected with a rabbit anti-TTFC antibody (1:1,000 dilution; Calbiochem, La Jolla, Calif.) followed by an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Boehringer Mannheim). Color development was accomplished by using the colorimetric substrates 4-nitroblue tetrazolium chloride (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS

Leakage of β-gal into *Lactococcus* culture supernatants. The 888-bp late-promoter fragment, P_{15A10}, from the lytic bacteriophage φ31 (36) encodes its own transcriptional activator, Tac31A (formerly ORF2 and *tac* [47]). It was observed that when *L. lactis* NCK203 carried a P_{15A10}::*lacZ.st* fusion (pTRK391), β-gal activity was detected largely in the culture supernatant and not in the cells. A total of five *L. lactis* strains were electroporated with pTRK391(P_{15A10}::*lacZ.st*), and the level of β-gal activity in each supernatant was measured (Table 2). Three of the strains (C10, ML8, and NCK203) contained prophages harboring a late-promoter region homologous to P_{15A10} in φ31 (48), while two strains (MM210 and MG1363) did not. In the three prophage-bearing strains C10, ML8, and NCK203, 84 to 88% of the β-gal activity was detected in the culture supernatant. Less than 10% of the β-gal activity was detected in the supernatants of *L. lactis* strains that did not harbor prophages. Introduction of pTRK609(p6::*lacZ.st*), in which β-gal expression is driven by the strong, constitutive P6 promoter (9), into strains C10, ML8, and NCK203 failed to yield comparable levels of β-gal in the supernatants. The results showed that significant levels of β-gal activity were located in the supernatants of the lysogenic strains when the promoter P_{15A10} was used to drive *lacZ* expression from the high-copy-number replicon.

These results indicated that the leaky behavior resulted from expression of Tac31A, in trans, in combination with prophages encoding a homologous $P_{\phi31}$ promoter. Prophage-cured derivatives of C10, ML8, and NCK203 were not available for use as negative controls to confirm this result. However, phage r1t, of *L. lactis* R1, also harbors a $P_{\phi31}$-homologous promoter (48), and a prophage-cured derivative, designated R1C, is available (22). Therefore, *L. lactis* R1/r1t and R1C (prophage cured) were transformed with plasmid constructs encoding P_{15A10}::*lacZ.st* or P6::*lacZ.st*. Several transformants of each were propagated in GM17 plus erythromycin overnight, and the percentage of β-gal activity in each of the supernatants was determined. Very little β-gal was detected in the supernatants when P6::*lacZ.st* was present in R1/r1t or R1C (Table 2) or when P_{15A10}::*lacZ.st* was present in prophage-cured R1C. When P_{15A10}::*lacZ.st* was present in R1/r1t, various levels of β-gal were detected in the supernatants of the transformants, ranging from very little (no more than that obtained with P6::*lacZ.st*) to about 40 to 50% of the total β-gal activity. After successive passage of the R1/r1t(P_{15A10}::*lacZ.st*) transformants, none exhibited the leaky β-gal phenotype. Further investigation revealed a loss of the r1t prophage from the transformants. It appeared that the presence of *tac31A* on P_{15A10} was sufficiently lethal to select for r1t-cured derivatives in the population, explaining the variability and instability of the leaky behavior in this phage r1t lysogen background. This instability was resolved by reconstructing the *lacZ.st* fusion with the promoter $P_{15A10\phi31.9}$, which encodes a mutated version of *tac31A* and yields a 50% reduction in promoter activity compared to P_{15A10} (10). $P_{15A10\phi31.9}$ was directionally cloned into *Bam*HI- and *Pst*I-restricted pTRK390 upstream of *lacZ.st* to generate pTRK610 and transformed into R1/r1t and R1C. Table 2 shows that β-gal levels were low (10% or less) in the supernatants of R1C transformants, whereas an average of 45% of the total β-gal activity was detected in the supernatants of the R1/r1t transformants. This leaky phenotype was evident after multiple passages. These results clearly indicated that the presence of the r1t prophage was responsible for the increased levels of β-gal detected in the supernatants when Tac31A was expressed in trans.

Leaky behavior is independent of prophage induction. It was important to determine whether Tac31A induced replication of a prophage or simply activated expression over a late region. This question could not be answered by using *L. lactis* strains NCK203, ML8, and C10, since no sensitive lactococcal hosts were available to determine the titers of phages that might appear from Tac31A$^+$ derivatives of these cultures. Two alternative approaches were therefore used to address this question. The first approach utilized the AbiA abortive phage defense mechanism, which targets phage replication (17, 18) and can reduce the burst size of phage r1t 10-fold after induction with mitomycin C (unpublished observations). Since C10, ML8, and NCK203 contain prophages with homology to r1t, there existed the possibility that AbiA would severely inhibit their replication as well. To test this, pTRK483 ($P_{566–888}$::*lacZ.st*) was combined with pTRK406 (pNZ18::*abiA* [8]), or the control plasmid pNZ18, in *L. lactis* NCK203. The tightly regulated $P_{566–888}$ does not encode Tac31A (47); therefore, no β-gal will be detected from the $P_{566–888}$::*lacZ.st* fusion unless Tac31A is provided in trans via induction of the resident prophage with mitomycin C (47, 48). When pTRK483 ($P_{566–888}$::*lacZ.st*) was combined with pNZ18, addition of mitomycin C to the lysogen led to efficient induction of β-gal from the phage promoter, as described previously by Walker et al. (48). However, when AbiA was introduced on pTRK406, β-gal expression was eliminated after mitomycin C induction, indicating that replication of the prophage was inhibited (data not shown). The final experiment was conducted by combining pTRK406 (AbiA$^+$) with pTRK391 (P_{15A10}::*lacZ.st*) in *L. lactis* NCK203. Efficiency of plaquing (EOP) assays using phage φ31

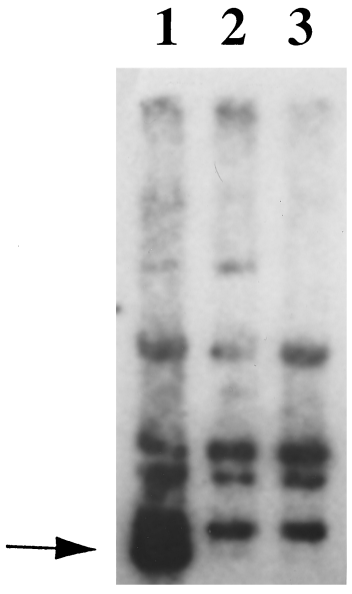

FIG. 2. Southern hybridization of genomic DNA restricted with *Pvu*II and probed with a ^{32}P-labeled fragment containing the r1t *attP* region. Lane 1, NCK203 genomic DNA isolated after induction with mitomycin C; lane 2, NCK203(P_{15A10}::*lacZ.st*) genomic DNA; lane 3, NCK203 (control) genomic DNA. The arrow indicates the extra band appearing after excision of the prophage from the host genome.

confirmed that AbiA was functional in the transformants. The levels of β-gal in the supernatants of chosen colonies were comparable to those obtained with P_{15A10}::*lacZ.st* alone (Table 2) or with P_{15A10}::*lacZ.st* plus pNZ18, demonstrating that prophage induction and replication were not responsible for the leaky behavior.

A second approach to evaluate whether prophage induction was occurring used Southern hybridization to assess any changes in the conformation of the *attP* site in leaky cells. *Pvu*II-digested genomic DNAs isolated from *L. lactis* NCK203, NCK203 treated with mitomycin C (positive control for prophage induction), and NCK203(pTRK391; P_{15A10}::*lacZ.st*) were electrophoresed on an agarose gel and transferred to a Magnacharge nylon membrane (38). The membrane was probed with a ^{32}P-labeled fragment containing the r1t *attP* region, since this region is conserved among several other temperate members of the P335 lactococcal phage species (e.g., TUC2009 and LC3). Figure 2 shows that hybridization of the *attP* region to restriction digests of the NCK203 genomic DNA, after induction of the resident prophage, yielded an extra band representing the annealing of *attP* after prophage excision from the host chromosome. This extra band was not detected when P_{15A10} was present (Fig. 2, lane 2). These results, confirmed by using ML8 and C10 (data not shown), provided further evidence that the resident prophages of NCK203, C10, and ML8 were not induced to detectable levels by Tac31A. The results are speculative, however, since linkage of the homologous r1t *attP* region in NCK203 to the resident prophage encoding the homologous P_{15A10} region has not been established.

Tac31A causes a slight activation of the late region of the resident prophages. The results suggested that the leaky phenotype was directed by Tac31A activation of the late region of resident prophages, which typically include holin-lysin cassettes. The genes encoding the holin and lysin of the C10, ML8, and NCK203 resident prophages are uncharacterized and different from r1t (see above). Therefore, to assess holin-lysin expression, a fragment consisting of the late region of phage ϕ31 (48) just downstream of the late promoter and the right *cos* site was used to probe RNAs isolated from mitomycin C-treated NCK203, NCK203(P6::*lacZ.st*), and NCK203(P_{15A10}::*lacZ.st*). RNAs isolated from mitomycin C-treated ML8, ML8 (P6::*lacZ.st*), and ML8(P_{15A10}::*lacZ.st*) were probed in the same manner. The results (Fig. 3) showed that the presence of P_{15A10} encoding Tac31A resulted in approximately a twofold

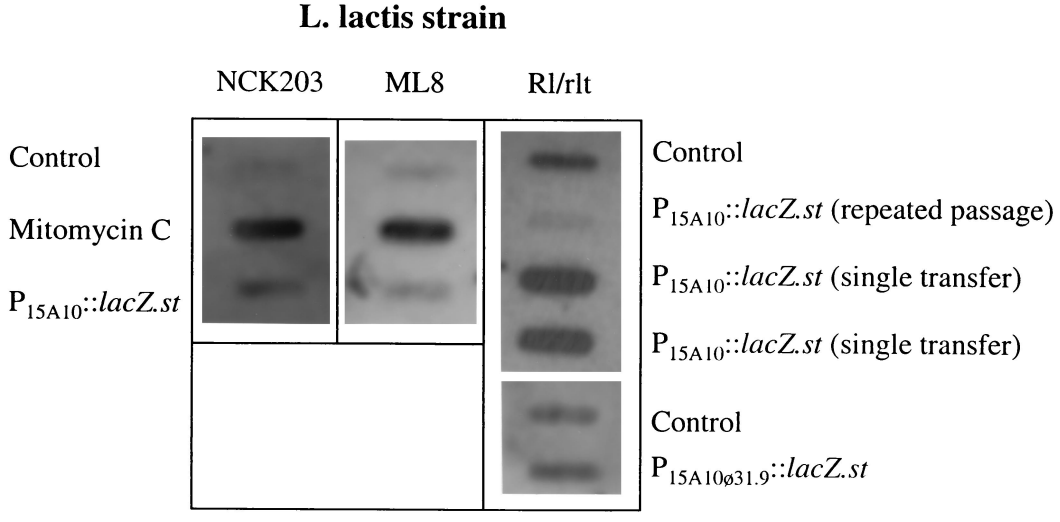

FIG. 3. RNA slot blots measuring induction of the late regions of strains NCK203, ML8, and R1/r1t, with and without Tac31A (encoded on P_{15A10}::*lacZ.st*) or mutant Tac31A (encoded on $P_{15A10\phi31.9}$::*lacZ.st*). RNA isolated from strains NCK203 and ML8 was probed with the beginning of the late region of phage ϕ31 (see Materials and Methods). RNA isolated from strain R1/r1t was probed with both an r1t lysin probe and an r1t holin probe. The results were identical, and thus only data for the r1t lysin probe are shown.

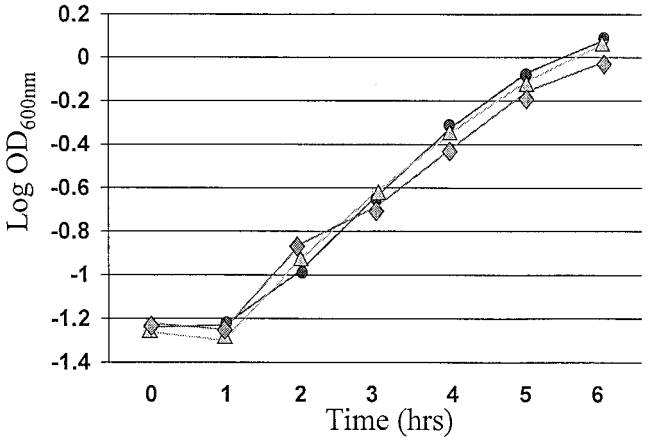

FIG. 4. Growth curve for *L. lactis* NCK203 (circles), compared to its derivatives harboring pTRK609 (P6::*lacZ.st*) (triangles) and pTRK391 (P_{15A10}::*lacZ.st*) (diamonds).

increase in the level of RNA from the late region over that of the controls. This level of expression was significantly lower than that obtained after induction of the resident prophage with mitomycin C. The same results were obtained with RNA slot blots of C10 RNA (data not shown).

This experiment was repeated for the R1/r1t and R1C transformants. The RNA was probed with either a ^{32}P-labeled r1t lysin probe or a ^{32}P-labeled r1t holin probe, and identical results were achieved (Fig. 3). RNA isolated from R1/r1t(P_{15A10}::*lacZ.st*) transformants propagated once showed a marked induction of both holin and lysin compared to RNA from R1/r1t. If R1/r1t(P_{15A10}::*lacZ.st*) transformants were subjected to repeated passages, the levels of lysin and holin mRNAs began to decrease until very little could be detected, indicating that the r1t prophage was being cured from the population. As expected, lysin and holin mRNAs were not detected in any R1C transformants (data not shown). Therefore, the presence of low levels of Tac31A in R1/r1t resulted in significant expression of holin and lysin mRNAs, much higher levels than the expression of the late region achieved with the C10, ML8, and NCK203 prophages (see above). This high level of expression may explain why the leaky phenotype is so unstable in R1/r1t. RNA isolated from the R1/r1t($P_{15A10\phi31.9}$::*lacZ.st*) transformants and probed with a ^{32}P-labeled r1t lysin fragment (Fig. 3) showed little or no induction over that seen with the control R1 cells, which is more consistent with results obtained with the C10, ML8, and NCK203 prophages.

Effect of leaky expression on the growth, viability, and appearance of *L. lactis*. Growth curves were constructed for *L. lactis* NCK203 and R1 parents and their counterparts harboring plasmids encoding P6::*lacZ.st* or P_{15A10}::*lacZ.st*. The presence of Tac31A on P_{15A10} had no significant effect on the growth of these strains, as measured by changes in both OD_{600} and CFU per milliliter (Fig. 4; data not shown for CFU per milliliter or R1). The fluorescent dye PI was used to determine the proportion of dead or membrane-compromised cells in Tac31A$^+$ populations (33). In this experiment, the cells were washed before being tested, thereby eliminating any contribution made by lysed cells. With *L. lactis* C10 and NCK203, the presence of Tac31A on P_{15A10} did not significantly increase the proportion of dead or compromised cells in the population. In all cases, the percentage of dead or damaged cells was less than 10% of the total (see Table 3 for NCK203 data). The same was true for R1/r1t harboring the $P_{15A10\phi31.9}$::*lacZ.st* construct (Table 3). These results were confirmed by viable-cell counts. Similar CFU-per-milliliter levels were obtained at the same OD regardless of whether P_{15A10} was present (data not shown). In addition, transmission electron microscopy showed that the presence of Tac31A did not significantly alter the appearance of NCK203(pTRK391) compared to the control strain NCK203 (pTRKH2) (data not shown). No ghost cells or cellular debris were observed, providing further evidence that cell lysis was not occurring.

Integration of the r1t holin-lysin cassette in MG1363 results in leaky expression of β-gal. To establish that expression of holin and lysin was responsible for the leaky phenotype, the r1t holin-lysin cassette was integrated into the prophage-free strain MG1363 by using the r1t integrase and *attP* region. One stable integrant was obtained, and PCR analysis (data not shown) confirmed that the holin and lysin genes were in the proper orientation with respect to the phage ϕ31 late promoter (illustrated in Fig. 1). The integrant was then transformed with the P_{15A10}::*lacZ.st*, $P_{15A10\phi31.9}$::*lacZ.st*, and P6::*lacZ.st* constructs independently, and the resulting derivatives were compared. Both β-gal and PepXP levels were measured in the whole cultures and in the supernatants (Table 3). Although the presence of wild-type Tac31A in MG1363/*hol-lys* resulted in 66% of the β-gal activity being found in the supernatant, the phenotype was very different from that obtained with C10, ML8, and NCK203 and was more similar to results obtained with R1/r1t, in which it was lethal. Analysis of cell damage with PI showed that 40% of the MG1363/*hol-lys*(P_{15A10}::*lacZ.st*) cells took up the dye compared to the control. In addition, these cells grew extremely slowly and exhibited considerable decreases in OD_{600} after overnight storage at 4°C (data not shown). When the mutant Tac31A was present on $P_{15A10\phi31.9}$, an average of 46% of the β-gal activity was detected in the supernatant and only a slight increase in the percentage of damaged cells could be detected with the PI test. These results strongly suggest that activation of the integrated cassette including the holin and lysin genes by Tac31A was largely responsible for the leaky phenotype. Moreover, relatively undamaged (as determined via PI analysis) and highly leaky cells did not release comparable amounts of an intracellular peptidase (PepXP) into the supernatant, providing further evidence for an externalization process independent of cell lysis.

Leaky expression of enzymes and other proteins. It was of considerable interest to determine if other heterologous proteins or enzymes could leak into the culture supernatant by this mechanism. The first protein evaluated was TTFC, a 47-kDa model antigen in vaccine delivery systems designed for *L. lactis* (49, 50). $P_{15A102X}$::TTFC constructs were transformed separately into NCK203, C10, and MG1363 (prophage-cured control). Western blot analysis was used to detect TTFC in the supernatants and cell extracts of each strain (Fig. 5). High levels of TTFC were detected in the cell extracts of MG1363 ($P_{15A102x}$::TTFC), whereas no TTFC was detected in the supernatant. In contrast, in the leaky constructs of NCK203 and C10 (harboring $P_{15A102x}$::TTFC), TTFC was detected mostly in

TABLE 3. Direct comparison of levels of β-gal and PepXP detected in supernatants and proportion of membrane-damaged cells in strains carrying various constructs

Strain	Construct carried	% of enzyme in supernatant		% of cells damaged[a]
		β-gal	PepXP	
NCK203	P6::*lacZ.st*	10	5.9	5.5
	P_{15A10}::*lacZ.st*	88.3	9.2	4.8
	$P_{15A102X}$::TTFC	—[b]	—	—
	$P_{15A102X}$::PepXP	—	6.4	—
R1/r1t	P6::*lacZ.st*	<10	10.5	—
	$P_{15A10\phi31.9}$::*lacZ.st*	45.3	5.0	4.3
R1C	$P_{15A10\phi31.9}$::*lacZ.st*	<10	—	3.0
MG1363/hol-lys	None	—	—	8.1
	P6::*lacZ.st*	6.0	—	4.3
	P_{15A10}::*lacZ.st*	66	40–90[c]	40.0
	$P_{15A10\phi31.9}$::*lacZ.st*	46	5.0	15.0

[a] Determined with PI.
[b] —, not tested.
[c] Due to the large deviation in these readings, this value is given as a range rather than an average.

the supernatants, and little was found associated with the cell extracts.

Since the leaky behavior described herein could be useful for the externalization of flavor enzymes from *Lactococcus* cultures during cheese manufacture, secretion of the intracellular enzyme PepXP was also studied. The proportions of PepXP activity present in the supernatants of log-phase cultures of NCK203, with and without Tac31A, and R1/r1t, with and without the mutant Tac31A, were measured (Table 3). Interestingly, while PepXP activity was detected in the whole-culture samples, it was not detected at significant levels (<10%) in the supernatants of NCK203 or R1/r1t, even when Tac31A or its mutant was present. Attempts were made to increase the background cytoplasmic levels of PepXP levels by creating a $P_{15A102x}$::*pepXP* fusion in pTRKH2 to yield pTRK619. This fusion construct was transformed into NCK203, and levels of PepXP activity in the supernatant were measured. Again, significant levels of PepXP activity were not detected in the supernatant (Table 3). Therefore, Tac31A does not promote leakage of PepXP to the supernatant. Moreover, these data provide additional evidence that leaky *L. lactis* cells are not lysing to release β-gal or TTFC into the supernatant.

DISCUSSION

This paper describes a novel expression system that allows release of certain proteins and enzymes into the growth medium without the use of export or secretion pathways and without significant effects on cell viability or cell membrane integrity. The leaky behavior, first observed for a heterologous β-gal enzyme expressed from the P_{15A10} phage φ31 late promoter (P_{15A10}::*lacZ.st*), depends on two features: the Tac31A transcriptional activator of the phage φ31 promoter, and a resident prophage containing a promoter homologous to P_{15A10} which directs low-level expression of a downstream holin-lysin cassette.

Several lines of evidence confirm the importance of Tac31A to the leaky phenotype. Tac31A expression from pTRK391 resulted from low-level promoter activity associated with vector sequences. Earlier studies had suggested that higher Tac31A expression levels were lethal in *L. lactis* NCK203 (47), possibly due to induction of lethal gene products from an uncharacterized prophage harboring sequences identical to the φ31 late promoter (48). The lethality of Tac31A was confirmed in *L. lactis* R1/r1t, in which even low-level expression from pTRK391 resulted in an unstable leaky phenotype and, ultimately, selection of an r1t-cured derivative of the strain. The use of a mutated version of Tac31A established a stable leaky phenotype in R1/r1t. Expression of equivalent or higher levels of β-gal from a strong *Lactobacillus* promoter (P6::*lacZ.st*) in these strains did not result in high levels of β-gal in the growth medium, proving that the strain by itself did not allow the release of significant levels of β-gal.

One interesting question arising from this study was why *L. lactis* R1/r1t was more sensitive to the wild-type Tac31A than were C10, ML8, and NCK203. There are several possible explanations for this difference. First, *tac31A* mRNA levels were not measured, and the possibility that Tac31A was expressed more efficiently in R1/r1t cannot be ruled out. Second, R1/r1t undergoes spontaneous prophage induction (20), which can lead to approximately 10^3 to 10^4 phages/ml in the culture supernatant. This level of spontaneous induction may lead to an increased number of phage genomes replicating in many of the cells and, therefore, to an increased induction of lysin and holin by Tac31A. Third, although the holin- and lysin-encoding regions of the responsible resident prophages of C10, ML8, and NCK203 have not been identified, we have obtained evidence that they are different from those encoded by r1t. Different activity levels of the lysin and/or holin, or even different transcription and/or translation efficiencies of these gene products, may explain the differences. For example, on the phage r1t genome, the holin-lysin cassette lies at the end of a very long, late transcript. Lastly, the difference in sensitivity simply may be due to strain differences.

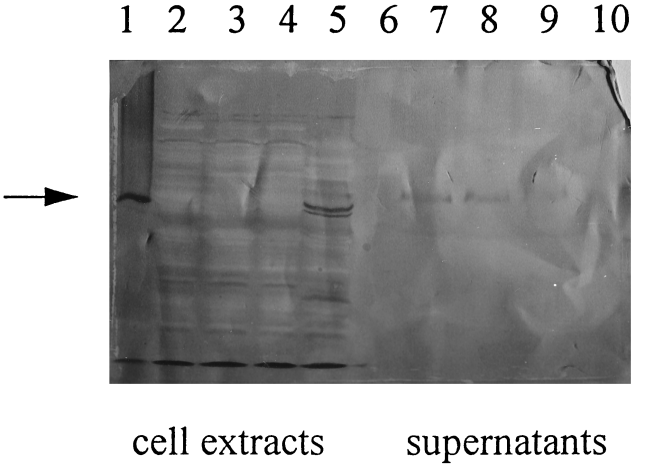

FIG. 5. Western blot of the cell extracts and supernatants of lactococcal strains carrying $P_{15A102X}$::TTFC constructs. The arrow marks the position of the TTFC standard. Cell extracts (lanes 2 to 5) and supernatants (lanes 7 to 10) of lactococcal strains carrying $P_{15A102x}$::TTFC constructs were examined for expression of TTFC. Lane 1, TTFC standard; lanes 2 and 7, C10; lanes 3 and 8, NCK203a; lanes 4 and 9, NCK203b; lanes 5 and 10, MG1363 (prophage-negative control); lane 6, Rainbow high-molecular-weight markers (Amersham). NCK203a and NCK203b are two clones carrying the same construct.

Initially, it was considered highly probable that the leaky phenotype resulted from Tac31A induction of a prophage from a small proportion of the population. The phage infection cycle would increase β-gal levels in that proportion of cells by activation of the P_{15A10}::*lacZ.st* cassette, and lysis would result in higher levels of β-gal in the supernatant. However, none of the experiments described in this paper support this theory. First, no differences were observed in culture growth, OD, or CFU per milliliter even though a clear majority of the β-gal activity was found in the supernatant. Second, Southern hybridization with an r1t *attP* probe failed to show any significant induction of the resident prophage in C10, ML8, or NCK203 when Tac31A was present. Third, the presence of AbiA, an abortive-infection protein which inhibits phage replication, did not affect the leaky phenotype in NCK203; hence, replication of the prophage does not appear to be a requirement for release of β-gal activity. Fourth, the presence of the mutant Tac31A in R1/r1t did not significantly increase the number of spontaneously induced phages present in the culture supernatant (data not shown). Taken together, these data suggest that the leaky phenotype is due not to induction and replication of the resident prophage but rather to induction of the late region of the integrated prophage. RNA slot blot analyses measuring induction of the late region of the resident prophages of C10, ML8, and NCK203 support this conclusion. The RNA data showed that only a low-level activation of the late region is required for the release of β-gal (Fig. 3).

An MG1363 derivative containing an integrated r1t holin-lysin cassette under the control of the tightly regulated $P_{566–888}$ late promoter confirmed the importance of holin and lysin to the leaky phenotype. The phenotype of MG1363/*hol-lys* was very similar to that observed in R1/r1t containing P_{15A10}::*lacZ.st* or $P_{15A10\phi31.9}$::*lacZ.st*, strongly suggesting that activation of lysin and holin at a very low level is largely responsible for the release of β-gal into the growth medium. In almost all reported circumstances, induction of holin and lysin results in cell death as well as lysis (6; for reviews, see references 15 and 39). Also, induction of holin alone usually results in cessation of growth due to increased cell membrane permeability and collapse of the membrane potential (6; reviewed in reference 53), sometimes followed by partial loss of turbidity. In most of the studies, however, induction was achieved by utilizing expression vectors which would efficiently express the gene(s), thus allowing for fairly high-level production of the holin and lysin products (6; reviewed in references 15 and 39). In contrast, a recent report by Husson-Kao et al. (21) suggested that leaky low-level expression of a prophage lytic cassette due to incomplete prophage repression was not lethal to the cell until environmental forces (lactose depletion or solvent addition) intervened.

Certain bacterial strains have been found to utilize phage-encoded holins and/or lysins to release cellular products. Expression and release of *Serratia marcescens* extracellular nuclease (2, 23) and bacteriocin 28b (13) were found to be due to putative prophage-encoded operons containing genes for a transcriptional activator, holin, and lysin. In both cases, however, evidence suggests that release may be mediated by cell lysis. In other studies, low-level expression of holin led to the leakage of intracellular enzymes or compounds without substantially affecting cell growth. Kyogoku and Sekiguchi (24) found that expression of a *Bacillus licheniformis* holin in *E. coli* resulted in β-gal leakage into the supernatant without loss of cell viability. However, the leakage of β-gal was considerably less than that observed in this study and was possibly due to low levels of cell lysis (24). In addition, low-level expression of a *Streptococcus thermophilus* holin gene, *lyt50*, in *E. coli* (before induction of the expression vector) resulted in a high background of isocitrate dehydrogenase activity in the culture medium, with no effects on cell viability (41). Lastly, low-level expression of small *B. licheniformis* and *Bacillus subtilis* proteins possessing the characteristics of a holin was found to complement certain alkaline phosphatase-deficient mutants of *E. coli*, presumably by altering the cell membrane permeability so that the XP substrate entered more readily and was hydrolyzed by cytoplasmic phosphatases (26, 28). The proteins were not lethal unless induced in *E. coli*, but no measurement of the degree of leakiness was given. These studies are interesting because they suggest that low-level expression of certain holins may allow for release of intracellular enzymes into the growth medium while allowing continued growth of the culture.

Work with other proteins and enzymes suggested that this system could be utilized to release other relevant products into the growth medium. TTFC was detected at higher levels in the supernatants than in the cell extracts. Interestingly, another enzyme was not released efficiently, if at all; PepXP activity was not detected to any significant extent in the supernatant, regardless of whether Tac31A was present, even when PepXP was expressed from the P_{15A10} promoter. The only exception was with MG1363/*hol-lys*, for which expression of the wild-type Tac31A resulted in high levels of PepXP in the medium and concomitant cell lysis. Work by Wells et al. (49) has suggested that the cell wall can act as a barrier to the diffusion of some proteins or enzymes into the medium. Perhaps PepXP is similarly affected, but this seems unlikely since one would expect at least a slight increase of PepXP in the supernatant even if diffusion was somewhat limited. At this point, we do not understand why some proteins and enzymes (e.g., β-gal and TTFC) are externalized while others (PepXP) are retained. All previous complementation studies have indicated that holins are nonspecific, allowing for the release of heterologous lysin products outside the cell (reviewed in reference 53). Data from this study suggest that holins allow export of other proteins while restricting others. Further investigation is needed to elucidate the mechanism through which leaky cells externalize select proteins and enzymes.

To our knowledge, this is the first report describing the release of significant levels of β-gal and other, heterologous proteins into the growth medium without the use of export signals and without seriously compromising the viability of the cells by inducing autolysis or prophage induction. Leaky lactic acid bacteria are expected to find valuable applications as delivery vehicles in bioprocessing, food, and the gastrointestinal tract.

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