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Production of Human Papillomavirus Type 16 E7 Protein in *Lactococcus lactis*

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The E7 protein of human papillomavirus type 16 was produced in *Lactococcus lactis*. Secretion allowed higher production yields than cytoplasmic production. In stationary phase, amounts of cytoplasmic E7 were reduced, while amounts of secreted E7 increased, suggesting a phase-dependent intracellular proteolysis. Fusion of E7 to the staphylococcal nuclease, a stable protein, resulted in a highly stable cytoplasmic protein. This work provides new candidates for development of viral screening systems and for oral vaccine against cervical cancer.

Infection with human papillomavirus type 16 (HPV-16) is the main factor associated with development of cervical cancer (42). The HPV-16 E6 and E7 proteins are constitutively produced in cervical carcinomas, and E7 was shown to interact with several cell compounds, causing deregulation of the cell cycle and cell transformation (43). E7 is a 98-amino-acid nuclear phosphoprotein that is devoid of any known enzymatic activity (36). In eukaryotic cells, E7's half-life is short (30 to 40 min); its degradation is mediated by the ubiquitin-proteasome pathway (31). E7 protein is widely studied because of its implication in carcinoma onset. It is also considered to be a good antigen candidate for the development of new vaccines against cervical cancer.

E7 production systems have been developed in both eukaryotes (1, 39) and prokaryotes (27, 34). Since the 1990s, several workers have investigated the use of bacteria as E7 antigen delivery vehicles to elicit an immune response against HPV-16 (14, 22). The gram-positive and generally regarded as safe (GRAS) commensal bacterium *Streptococcus gordonii* was used for this purpose to display E7 protein at the cell surface in fusion with export signals (30). These recombinant *S. gordonii* strains could elicit an immune response in mice and monkeys (23, 26). Although encouraging, these results rely on a commensal, GRAS but non-food-grade bacterium. One risk of commensal, and hence persistent, microorganisms is the induction of immunotolerance. Thus, a transient presentation of the antigen to the immune system by a noncommensal bacterium may be needed to avoid this risk.

None of the systems mentioned above seems to provide the combination of safety, sufficient yields, and simplified methods that would allow both purification and eventual oral immunization using E7. We therefore considered an alternative system for native E7 production based on a food-grade lactic acid bacterium. The best-known lactic acid bacterium, *Lactococcus lactis*, has been extensively engineered for the production of heterologous proteins (5, 6, 10, 18, 20, 21, 28, 29, 35, 37). Protein production in *L. lactis* offers advantages: *L. lactis* is a food-grade gram-positive bacterium that produces very low amounts of native exoproteins. It is therefore a good candidate for heterologous protein secretion in different applications ranging from industrial production of high-added-value proteins to in vivo use as a live vaccine. As *L. lactis* is a noncommensal and transient bacterium in the digestive tract (12), the risk of eliciting a tolerance response to a given antigen is diminished; furthermore *L. lactis* has already been used to produce a viral epitope (18) and a viral protein (7).

In this work, we used the nisin-inducible system (4, 16) to express the *E7* gene in *L. lactis*. E7 synthesis was directed to cytoplasmic or extracellular locations. Both native and Nuc-fused E7 proteins were successfully expressed in both locations. Expression levels and stability of these proteins are reported under different growth conditions and in different genetic backgrounds. These studies show that E7 can be stably produced in either native or hybrid form under different growth conditions when exported from *L. lactis*.

Bacterial strains and plasmids and methods used. The bacterial strains and plasmids used in this work are listed in Table 1. *L. lactis* strains were grown in M17 medium (Difco) (38) supplemented with 1% glucose (GM17) or brain heart infusion (Difco) at 30°C without agitation. *Escherichia coli* was grown in Luria-Bertani (33) at 37°C. Unless otherwise indicated, plasmid constructions were first established in *E. coli* and then transferred to *L. lactis* by electrotransformation (17). Plasmids were selected by addition of antibiotics as follows (concentrations in micrograms per milliliter): for *L. lactis*, streptomycin (1,500), rifampin (50), erythromycin (5), chloramphenicol (10), and erythromycin and chloramphenicol together (2.5 and 5, respectively); for *E. coli*, ampicillin (100) and chloramphenicol (10). Plasmid DNA isolation and general procedures for DNA

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Replicon	Characteristic(s)[a]	Reference or source
Strains			
E. coli TG1		*supE hsd* Δ*5 thi* Δ(*lac-proAB*) F′(*traD36 proAB-lacZ*Δ*M15*)	13
L. lactis MG1363		Wild type, plasmid free	11
L. lactis MG1363 *clpP*		Ery^r, *clpP* disrupted by single-crossover recombination, plasmid free	8
L. lactis MG1363 *dnaK*		Ery^r, *dnaK* disrupted by single-crossover recombination, plasmid free	15
L. lactis NZ9000		MG1363 (*nisRK* genes into chromosome), plasmid free	16
L. lactis NZ9000 *clpP*		Str^r Rif^r Ery^r, MG1363 transconjugant carrying *nisRK* genes and *clpP* disruption, plasmid free	This work
L. lactis NZ9000 *dnaK*		Str^r Rif^r Ery^r, MG1363 transconjugant carrying *nisRK* genes and *dnaK* disruption, plasmid free	This work
Plasmids			
pBS SK II+	(ColE1)	Ap^r	Stratagene
pVE8001	(ColE1)	Ap^r, PCR fragment encoding *trpA* transcription terminator	I. Poquet[b]
pBS:*Nsi*I:*nuc*	(ColE1)	Ap^r, PCR fragment, with *Nsi*I sites at both extremities, encoding Nuc mature moiety	S. Nouaille[b]
pGEM-T	(ColE1)	Ap^r	Promega
pGEM-E7	(ColE1)	Ap^r; PCR fragment encoding E7	This work
pBS-E7	(ColE1)	Ap^r; PCR fragment encoding E7	This work
pCYT-Nuc	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes NucB mature moiety	7
pSEC-Nuc	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes SP_{Usp}-NucB precursor	7
pCYT-E7	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes the native E7 protein	This work
pSEC-E7	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes SP_{Usp}-E7 precursor	This work
pCYT-Nuc-E7	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes a NucB-E7 fusion	This work
pSEC-Nuc-E7	(pWV01)	Cm^r; gene, expressed under P_{nisA} encodes SP_{Usp}-NucB-E7 precursor	This work

[a] For strains, genotypic and phenotypic characteristics are given; for plasmids, plasmid and cloned-cassette characteristics are given.
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manipulations were essentially performed as described previously (33). PCR (apparatus from Perkin-Elmer Cetus, Norwalk, Conn.) was performed using Vent DNA Polymerase (Promega) and PCR sequences were confirmed using the Dye terminator sequencing kit (ABI PRISM BigDye Terminators; Applied Biosystems).

Induction for E7 expression was performed using nisin (1 ng/ml; Sigma) for a 1- or 3-h period as previously described (7). As E7 is labile (31), the protein sample preparation from *L. lactis* cultures was adapted to include protease inhibitors and mild precipitation procedures. Briefly, protein samples were prepared from 2 ml of cultures. Cell pellet and supernatant were treated separately, essentially as described (20). To inhibit proteolysis in supernatant samples, 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol were added. Proteins were then precipitated by addition of 100 μl of 100% trichloroacetic acid, incubated 10 min on ice, and centrifuged 10 min at 17,500 × *g* at 4°C. For the cell fraction, TES-Lys buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0], lysozyme [10 mg/ml]) was complemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and immunorevelation with anti-Nuc or anti-E7 antibodies were performed as described (20).

Cloning and inducible expression of *E7* in *L. lactis*. To achieve E7 production in different locations in *L. lactis*, several plasmids were constructed. The *E7* gene was PCR amplified from vector pCDNA3-E7 (kindly provided by J. Alcocer, Laboratorio de Inmunología y Virología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Nuevo Léon, Mexico). Primers used (and their sequences) were E7-HPV1 (5′-GATGCATCACAACATGGAGATACACCTAC ATTGCAT-3′)for the coding strand and E7-HPV2 (5′-GGA GCTGTTATGGTTTCTGAGAACAGATGG-3′) for the complementary strand. The PCR product (313 bp) was cloned into pGEM-T Easy Vector (Promega), resulting in pGEM-E7 plasmid (Table 1). The *rho*-independent transcription terminator *trpA* (3) was fused just downstream of the *E7* gene by insertion of a *Sal*I-*Apa*I fragment isolated from pGEM-E7 into the *Sal*I-*Apa*I-cut pVE8001 vector (29). The resulting plasmid pBS-E7 was used for further constructions (Table 1). Two cassettes were constructed to produce HPV-16 E7 protein in cytoplasmic and secreted forms. An *E7-trpA* cassette was isolated from an *Eco*RV-*Nsi*I-cut pBS-E7 and cloned into purified backbones isolated from *Eco*RV-*Nsi*I-cut pCYT-Nuc and pSEC-Nuc (7, 32) resulting in pCYT-E7 and pSEC-E7 (Table 1; Fig. 1). In pSEC-E7, the *E7* gene is fused in frame with a DNA fragment containing the ribosome binding site and the signal peptide of *usp45* (SP_{Usp45}), the gene encoding Usp45, the predominant *L. lactis*-secreted protein (Fig. 1) (40). In pCYT-E7, the fragment encoding SP_{Usp45} is absent. In both pSEC-E7 and pCYT-E7, expression of the *E7* cassettes is controlled by the P_{nisA} promoter (4). These plasmids were introduced into *L. lactis* strain NZ9000, which carries chromosomal copies of regulatory genes *nisR* and *nisK* (Table 1) (16).

The secreted form of E7 is stably produced in *L. lactis*. The capacity of *L. lactis* to accumulate E7 in either the cytoplasm or the extracellular medium was examined using strains NZ9000 containing pCYT-E7 and pSEC-E7, respectively (Table 1). Noninduced and induced culture samples were examined by Western blotting using anti-E7 antibodies. In the absence of nisin, no E7 signal was detected in either strain under different growth conditions. In late-exponential-phase (optical density at 600 nm [OD_{600}] = 0.9 to 1.0) cells, induced NZ9000(pCYT-E7) cultures contained a distinct band in the cell fraction at the expected size for native E7 (19 kDa) (27), whereas no signal was detected in the supernatant (Fig. 2A). Similar analysis of NZ9000(pSEC-E7) resulted in two bands: (i) a weak band in the cell fraction corresponds to SP_{Usp}-E7 precursor (preE7) (approximately 21 kDa) and (ii) a band in the supernatant

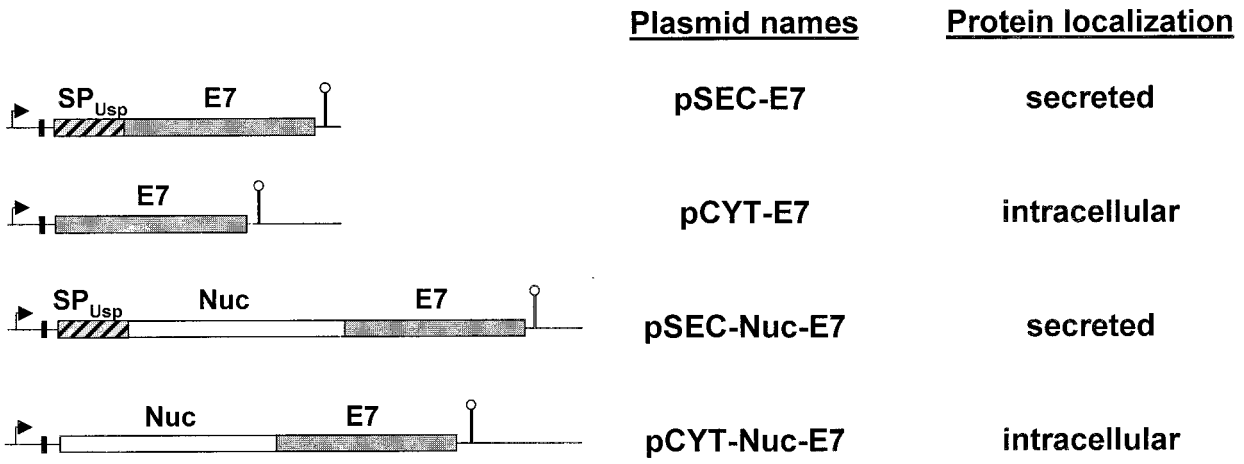

FIG. 1. Expression cassettes for E7 production and export in *L. lactis*. Schematic structures of native E7 or Nuc-E7 fusions (at left) expressed under control of the lactococcal P_{nisA} promoter and carried by the indicated plasmids. For details of plasmid constructions, see the text and Table 1. Arrows indicate *L. lactis* promoter sequences of the nisin-inducible promoter (P_{nisA}), solid vertical bars indicate the ribosome binding sites of the *usp45* gene, striped bars indicate signal peptides of the *usp45* gene; shaded bars indicate E7 coding region, open bars indicate NucB coding sequences, and stems topped with circles indicate transcription terminators (not to scale).

fraction corresponds to the secreted mature E7 (Fig. 2A). E7 secretion appears to be efficient, as about 95% of the protein is detected in the supernatant. We estimate that E7 yield is about threefold higher for secreted protein than for cytoplasmic protein. We already observed this phenomenon in *L. lactis* for different proteins such as Nuc, rotavirus nonstructural protein 4 (NSP4) (7), *Brucella abortus* immunodominant antigen L7/L12 (32), or bovine β-lactoglobulin (2). Thus, in *L. lactis*, secretion seems the best strategy to achieve high production yields for eukaryotic (β-lactoglobulin), viral (E7, NSP4), or prokaryotic (L7/L12, Nuc) proteins regardless of their native locations. The above results suggest that proteins that are exported may avoid intracellular proteolysis.

Interestingly, analysis of protein samples extracted from stationary-phase ($OD_{600} > 1$) cultures of the above strains reveals a striking difference in E7 production: amounts of cytoplasmic E7 are markedly decreased, while amounts of secreted E7 are increased (Fig. 2B). We suggest that intracellular proteolytic degradation is greater in stationary growth phase and that the secreted protein can escape outside the cell via translocation.

We asked whether *clpP* and/or *dnaK*, factors known to be involved in intracellular protein degradation, also affect E7 turnover. ClpP is an ATP-dependent protease and the major cytoplasmic housekeeping protease in *L. lactis* (8), and the DnaK chaperone may promote proteolysis by maintaining misfolded proteins in a disaggregated state (41). To test whether ClpP and DnaK are involved in E7 degradation, we constructed an *L. lactis* NZ9000 strain harboring the *clpP* mutation (NZ9000 *clpP*) or *dnaK* mutation (NZ9000 *dnaK*) by conjugation using the following strategy: the donor strain was an erythromycin-resistant (Ery^r) MG1363 *clpP* or MG1363 *dnaK* (kindly provided by H. Ingmer or K. Hammer, respectively) (8, 15). A spontaneous streptomycin-resistant (Str^r) and rifampin-resistant (Rif^r) strain was selected from NZ9000 and was used as the conjugation recipient. Conjugation was performed as described (17), and transconjugants were selected as triply Str^r, Rif^r, and Ery^r. Chromosomal structure of the NZ9000 *clpP* or NZ9000 *dnaK* transconjugants was confirmed by PCR or Southern hybridization (data not shown). E7 production in NZ9000 *clpP* or NZ9000 *dnaK* containing plasmids pCYT-E7 or pSEC-E7 was analyzed by Western blot and compared to that of a wild-type (wt) strain. In exponential- and stationary-phase cultures, no significant differences in E7 patterns were observed between wt and *clpP* or *dnaK* strains: cytoplasmic E7 was equally degraded and secreted E7 yields were unchanged (data not shown).

Together, these results indicate that E7 intracellular proteolysis is ClpP and DnaK independent. Until recently, only two cytoplasmic proteases, ClpP and FtsH (8, 24), were identified in *L. lactis*. The existence of a third, as yet unidentified, protease was postulated by studies of a *clpP* mutant suppressor (9). E7 may thus be a useful screening target for identifying a putative *L. lactis* protease that, as suggested by our data, is activated in stationary phase.

Fusion of Nuc to the E7 N terminus stabilizes E7 production in *L. lactis*. Recent studies in our laboratory suggest that fusion of a protein of interest to Nuc could rescue and/or enhance production of a secreted heterologous protein in *L. lactis*, particularly when low yields are initially observed (32). Nuc is a well-characterized and stable protein that is resistant to denaturation and has readily detectable activity (19, 29). Furthermore, in a eukaryotic system, E7 is reportedly protected from proteolysis when epitope-tagged (Myc-tagged) at the N terminus (but not at the C terminus [31]). To test the effect of fusing Nuc to the N terminus of E7 (Nuc-E7), for both cytoplasmic and secreted forms, a *nuc* cassette harboring an *Nsi*I restriction site at both extremities was purified from *Nsi*I-cut pBS:*Nsi*I:*nuc* (Table 1) (kindly provided by S. Nouaille, URLGA, INRA, Jouy en Josas, France) and cloned into *Nsi*I-cut pSEC-E7 and pCYT-E7 backbones. The resulting plasmids, pCYT-Nuc-E7 and pSEC-Nuc-E7, were introduced into *L. lactis* NZ9000, and cytoplasmic and secreted Nuc-E7 production was then examined using these strains. Nuc-E7 production from induced exponential- and stationary-phase cultures was analyzed by Western blot experiments using anti-Nuc (data not shown) or anti-E7 antibodies (Fig. 3A).

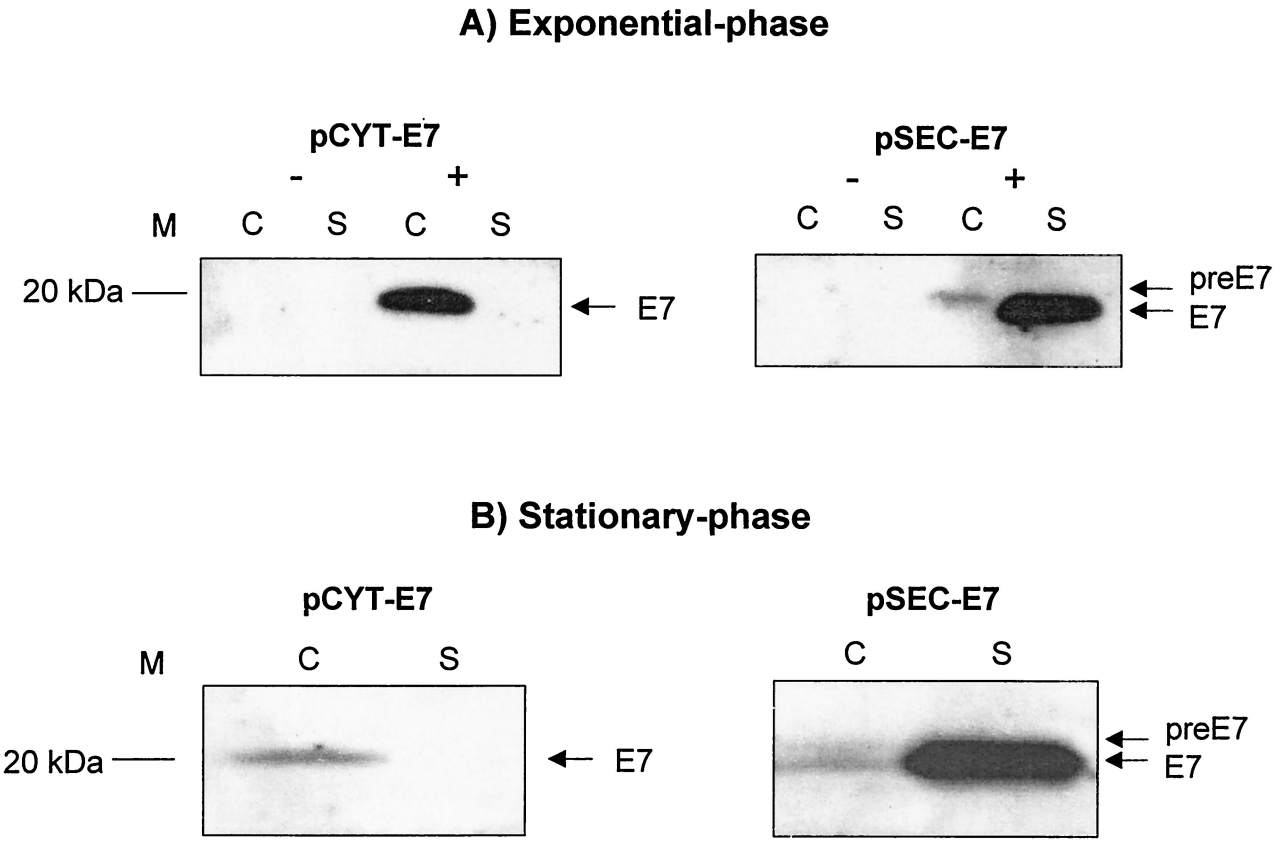

FIG. 2. Native E7 production in wt *L. lactis* depends on growth phase. E7 production and secretion were analyzed by Western blotting from cultures induced at different times such that 1 h after induction, the samples were harvested at exponential ($OD_{600} \approx 0.5$ to 0.6) or stationary ($OD_{600} \approx 1.5$) phase. Strains contain plasmid pCYT-E7 (encoding native E7, cytoplasmic form) or pSEC-E7 (encoding the precursor preE7, i.e., SP_{Usp45} fused to E7). (A) Exponential-phase cultures. Noninduced (−) and induced (+) cultures of wt *L. lactis* containing plasmids are as indicated. (B) Stationary-phase cultures. Arrows indicate positions of E7 mature and precursor forms. There is only a slight difference in migration positions of the preE7 (in cell lysates) and the secreted E7 mature form (in supernatant fraction). Abbreviations: C, cell lysates; S, supernatant fraction; M, positions and sizes of molecular mass markers.

For induced exponential- and stationary-phase cultures of NZ9000(pCYT-Nuc-E7), one major band, present in the cell fraction, is detected with anti-E7 antibodies. This band migrates at the expected size for a Nuc-E7 fusion (35 kDa). In the stationary-phase culture, a weak additional band is also present (Fig. 3A), which most likely corresponds to a Nuc-E7 degradation product. Thus, in contrast to results with the cytoplasmic form of native E7, the Nuc-E7 fusion accumulates in stationary-phase samples, suggesting that the Nuc moiety protects the E7 moiety from degradation.

For induced cultures of NZ9000(pSEC-Nuc-E7), Western blotting revealed three bands in the cell fraction in both exponential- and stationary-phase samples (Fig. 3A). The major upper band migrates at the expected size for the Nuc-E7 precursor (38 kDa). The two other bands comigrate with the intracellular forms found in NZ9000(pCYT-Nuc-E7) and correspond to mature Nuc-E7 and a putative cleavage product. In the supernatant, a single weak band is detected with anti-E7 antibodies, corresponding to the secreted Nuc-E7 fusion (Fig. 3A). In this case, secretion efficiency was only ~10% (compared to ~95% for native E7). Thus, exported Nuc-E7 protein remains cell associated, while exported E7 alone is released into the medium. Nuc activity plate assays performed on NZ9000(pSEC-Nuc-E7) showed a clear activity halo around colonies (data not shown), suggesting that Nuc-E7 is displayed on the cell surface (it was previously demonstrated that cytoplasmic Nuc forms give only a faint halo [19, 29]). To estimate amounts of Nuc-E7 versus E7 product, the same protein samples were analyzed using both anti-E7 and anti-Nuc antibodies (Fig. 3B). Nuc-E7 protein patterns were similar using both antibodies. To get an idea of the order of magnitude of E7 production yield in *L. lactis*, we estimated Nuc-E7 and E7 concentrations as follows. By comparison of Nuc-E7 signals with a Nuc standard loaded on the same gel (ImageQuant) (20), we estimated the quantity of total Nuc-E7 to be about 15 μg/ml (data not shown) (20). Using anti-E7 antibodies, total native E7 production was estimated to be about threefold lower than that of Nuc-E7. As purified E7 protein is not available, we used the Nuc-E7 protein concentration as determined by anti-Nuc antibodies as the standard; known amounts of Nuc-E7 were then used to estimate native E7 concentration (Fig. 3B). Using anti-E7 antibodies, we could then make an indirect estimation of native E7 concentration to be around 5 μg/ml (data not shown). These results show that despite greater amounts of total protein in the strain expressing pSEC-Nuc-E7, more E7 was secreted from the pSEC-E7 construc-

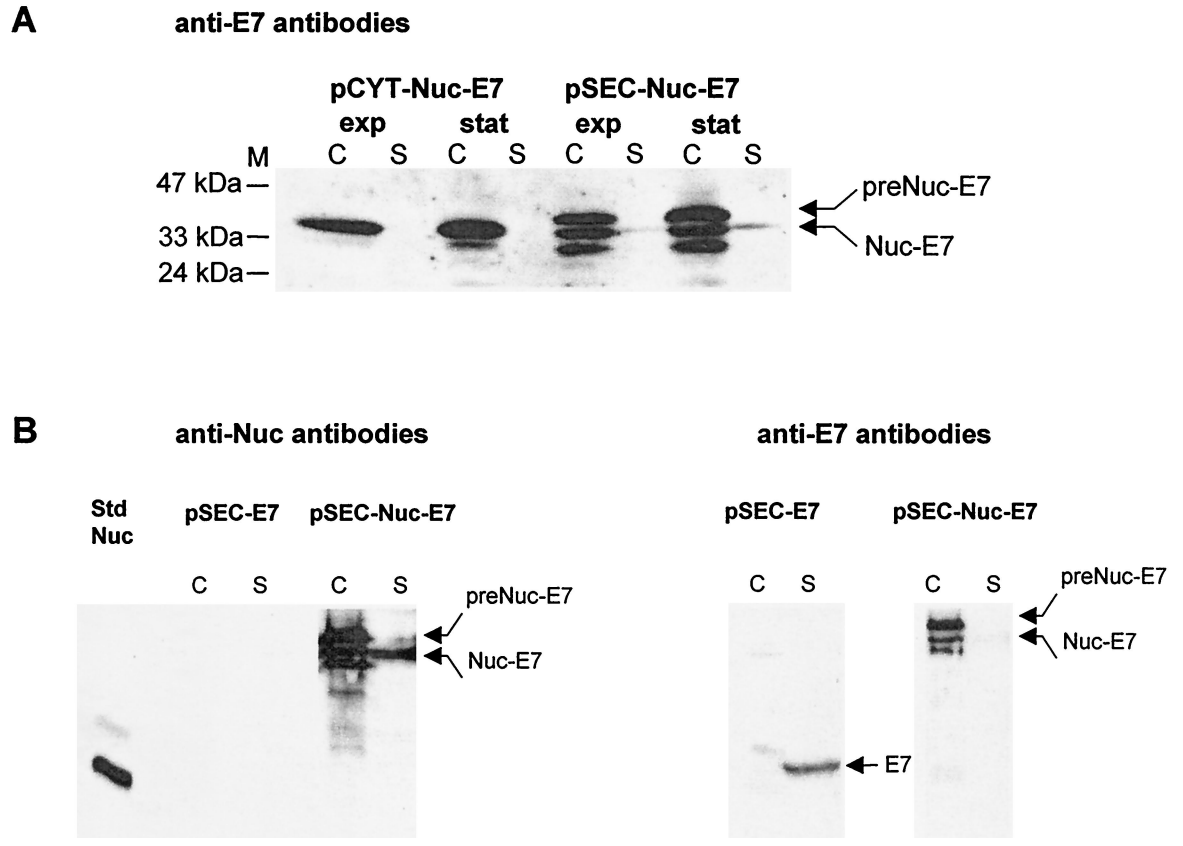

FIG. 3. (A) Production of Nuc-E7 fusion is stable in *L. lactis*. Nuc-E7 production was analyzed by Western blot analysis using anti-E7 antibodies on exponential- and stationary-phase cultures of *L. lactis* strains containing pCYT-Nuc-E7 (encoding Nuc-E7) or pSEC-Nuc-E7 (encoding preNuc-E7). Arrows indicate positions of Nuc-E7 mature and precursor forms. Other bands in the supernatant fraction of *L. lactis* (pSEC-Nuc-E7) probably correspond to putative products resulting from secondary proteolytic cleavage of Nuc-E7. (B) Estimation of Nuc-E7 (pSEC-Nuc-E7) and native E7 (pSEC-E7) yields in *L. lactis*. Production yields were estimated for the secreted forms of both Nuc-E7 (pSEC-Nuc-E7) and native E7 (pSEC-E7). The left panel shows quantification of Nuc-E7 production by Western blot analysis using anti-Nuc antibodies. Nuc-E7 signal was compared to the signal of known amounts of commercial Nuc by quantitative scanning of blots after immunodetection (ImageQuant) (19). Total Nuc-E7 concentration is estimated to be around 15 μg/ml, of which about 3 to 5 μg/ml is in the supernatant. The right panel shows the analysis, after dehybridization, of the same membrane using anti-E7 antibodies. Signal intensities of native E7 forms were compared to those of Nuc-E7 forms in the panel at left. Total native E7 concentration is estimated to be about three times less than that of Nuc-E7. Abbreviations: C, cell lysates; S, supernatant fraction (note that culture supernatant samples were concentrated about fivefold prior to loading); M, positions and sizes of molecular weight markers; exp, exponential phase; stat, stationary phase; Std Nuc, NucA standard (50 μg/ml for std Nuc, i.e., 1 μg loaded on the gel; note that the faint higher band corresponds to NucB form enzyme).

tion. Thus, secretion may be the system of choice to obtain stable native E7 production.

Interestingly, although pCYT-Nuc-E7 and pSEC-Nuc-E7 have an essentially identical design, the yield of exported Nuc-E7 protein is significantly greater (two- to threefold) than of the cytoplasmic form. These results are similar to those observed for native E7. We suggest that the secretion machinery may protect proteins from degradation by cytoplasmic proteases and thus account for the higher observed yields. Our results further show that the fusion of Nuc at the N terminus of E7 can stabilize E7 production in both cytoplasmic and secreted forms.

In summary, we successfully used *L. lactis* to produce HPV-16 E7, known as an extremely labile protein (31), in either the cytoplasm or the extracellular medium. Both these forms will be valuable in vaccine development trails. The amount of native E7 produced (estimated at 5 μg/ml for the secreted form) offers a promising starting point for E7 protein purification for physical and biochemical characterization, development of HPV screening assays and eventual production of purified vaccines, with essentially no risk of contamination with a toxic by-product.

The system developed in this study to produce HPV-16 E7 in *L. lactis* is interesting for the development of a new live vaccine against cervical cancer. Note that the use of the complete protein reportedly gives better interactions with the immune system components than that obtained with synthetic peptides (25). E7 was previously expressed at the *S. gordonii* surface and found to elicit an immune response in mice (23). While these results are promising, alternative E7 presentation systems will be useful in determining whether a totally innocuous and nonpersistent bacterium, *L. lactis*, can lead to development of a totally safe vaccine, with reduced risk of colonization or spread. In vivo immunogenicity tests using secreted and cytoplasmic forms of E7 for vaccination are now being developed in our laboratories.

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Yves Le Loir and Roberto Montes de Oca-Luna share credit in this work for senior authorship.

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