JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 5135–5139
0021-9193/94/$04.00+0

Vol. 176, No. 16

# Direct Screening of Recombinants in Gram-Positive Bacteria Using the Secreted Staphylococcal Nuclease as a Reporter

Y. LE LOIR, A. GRUSS, S. D. EHRLICH, AND P. LANGELLA*

*Laboratoire de Génétique Microbienne, Institut de Biotechnologie, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France*

Received 2 March 1994/Accepted 16 May 1994

**A system for direct screening of recombinant clones in *Lactococcus lactis*, based on secretion of the staphylococcal nuclease (SNase) in the organism, was developed. The *nuc* gene (encoding SNase) was cloned on both rolling-circle and theta-replicating plasmids. *L. lactis* strains containing these *nuc*$^+$ plasmids secrete SNase and are readily detectable by a simple plate test. A multicloning site (MCS) was introduced just after the cleavage site between leader peptide and the mature SNase, without affecting nuclease activity. Cloning foreign DNA fragments into any site of the MCS interrupts *nuc* and thus results in *nuc* mutant clones which are easily distinguished from *nuc*$^+$ clones on plates. The utility of this system for *L. lactis* was demonstrated by cloning an antibiotic resistance marker and *Escherichia coli* chromosomal DNA fragments into the MCS of the *nuc*MCS cassette. Both cloning vectors containing the *nuc*MCS cassette were also introduced into *Streptococcus salivarius* subsp. *thermophilus*, in which direct screening of *nuc* mutant recombinant clones was also achieved. The potential uses of *nuc* as a secretion reporter system are discussed.**

The food-grade bacterium *Lactococcus lactis* is of considerable economic importance because of its wide use in starter cultures for the manufacture of dairy products. The genetic characterization of *L. lactis* is limited by the lack of availability of molecular cloning systems. Although the development of cloning vectors and electrotransformation procedures has allowed considerable progress with *L. lactis* (4), the selection of recombinant clones remains a problem because of poor transformation efficiency with in vitro-ligated DNA molecules and the lack of a screening system for identification of recombinant clones. No equivalent of the β-galactosidase α-complementation (18) system of *Escherichia coli* is presently available for *L. lactis*.

In order to develop an efficient system to screen clones of *L. lactis*, we used the *nuc* gene encoding the staphylococcal nuclease (also called heat-stable nuclease, thermonuclease, and micrococcal nuclease and hereafter referred to as SNase) as the reporter gene (14). This *nuc* gene, first isolated from *Staphylococcus aureus* Foggie (14), has already been cloned and expressed in *E. coli* (14), *Bacillus subtilis* (12), and *Corynebacterium glutamicum* (11). SNase is a small, stable, biochemically well-characterized enzyme secreted by *S. aureus*, present either as a polypeptide of 168 amino acids (SNase B) or as a 149-amino-acid derivative (SNase A) which is missing the 19 N-terminal amino acids present in SNase B (14). SNase is synthesized as a precursor with a signal sequence of 60 amino acids (14).

**Bacterial strains and plasmids and methods used.** Mesophilic strains *Lactococcus lactis* subsp. *lactis* IL1403 (2) and *Lactococcus lactis* subsp. *cremoris* MG1363 (3) and thermophilic strain *Streptococcus salivarius* subsp. *thermophilus* IL73 were grown on M17 medium (16) in which lactose was replaced by glucose and were incubated at 30 and 37°C, respectively. *E. coli* TG1 (5) was grown on Luria-Bertani medium (12) and incubated at 37°C. Plasmids (Table 1) were established in *E. coli* by the competent-cell procedure (13) and in *L. lactis* and *S. thermophilus* by electrotransformation (10), carried out with a gene pulser and a pulse controller apparatus (Bio-Rad Laboratories, Richmond, Calif.). Unless otherwise indicated, plasmid constructions were first established in *E. coli* and then transferred to *L. lactis*. Plasmids were selected by the addition of antibiotics as follows (concentrations in micrograms per milliliter): for *L. lactis* and *S. thermophilus*, erythromycin (10), chloramphenicol (10), and tetracycline (2); and for *E. coli*, erythromycin (150) and ampicillin (100). Plasmid DNA was isolated as previously described (1). For *L. lactis* cells, TES buffer (50 mM Tris, 10 mM $Na_2EDTA$, 25% sucrose, pH 8) containing 10 mg of lysozyme per ml was used for 10 min at 37°C to prepare protoplasts. Nuclease activity assays are described below. General procedures for DNA manipulations were essentially performed as described previously (13). PCRs (1 min at 94°C, 2 min 55°C, and 2 min 72°C) were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus. *Thermophilus aquaticus* DNA polymerase (Promega) was used as recommended by the manufacturer.

**Cloning and expression of the *nuc* gene in *L. lactis*.** A 1.6-kb *Bam*HI-*Pst*I fragment of the *E. coli-C. glutamicum* shuttle plasmid pWNuc5 (11) containing *nuc* was cloned onto the broad-host-range rolling-circle plasmid pVE3509 to construct pGK::*nuc*1.6. This plasmid was first established in *E. coli* TG1. For identification of *nuc*$^+$ clones, a nuclease plate assay was used: plates were overlaid with Toluidine Blue-DNA agar (TB-D agar; 0.05 M Tris [pH 9], 10 g of agar per liter, 10 g of NaCl per liter, 0.1 mM $CaCl_2$, 0.3% [wt/vol] salmon sperm DNA, 90 mg of Toluidine Blue O dye per liter [9]). *E. coli* TG1(pGK::*nuc*1.6) developed an easily detectable pink halo (corresponding to the expression of the SNase) after 1 h at 37°C as previously observed (14), whereas the *E. coli* TG1 control had no halo on TB-D agar. pGK::*nuc*1.6 was introduced by electrotransformation into *L. lactis* IL1403. *L. lactis* *nuc*$^+$ clones on TB-D agar were detected on brain heart infusion medium (Difco) (Fig. 1) and checked for the presence of pGK::*nuc*1.6. The presence of SNase in *L. lactis* culture supernatants is attributed to its secretion and not to cell lysis. The intracellular marker enzyme (lactate dehydrogenase) was detected in cellular fractions but not in supernatants (data not shown). These observations show that staphylococcal transcription and secretion signals are recognized in *L. lactis*.

To remove the nonsequenced DNA part of the 1.6-kb *nuc*

* Corresponding author. Phone: (1) 34 65 25 26. Fax: (1) 34 65 25 21.

TABLE 1. Plasmids used in this study

| Plasmid | Description | Reference or source |
|---|---|---|
| pWNuc5 | Ap$^r$ Km$^r$ *nuc*$^+$; 12.3 kb | 11 |
| pUC18::Cm | Ap$^r$ Cm$^r$; 3.8 kb; pUC18 carrying the 901-bp *Taq*I-*Sau*3A fragment containing the Cm$^r$ determinant of pIP501 | 17 |
| pGK12 | Em$^r$ Cm$^r$; 5.2 kb | 8 |
| pVE3501 | Em$^r$; 3.4 kb; *Msp*I-*Bsp*XI digestion of pGK12 to delete the Cm$^r$ gene | This work |
| pVE3503 | Em$^r$; 3.9 kb; 445-bp *Pvu*II fragment containing MCS of pBS[a] inserted into *Nsi*I-cut and blunted pVE3501 | E. Maguin[b] |
| pVE3509 | Em$^r$ Cm$^r$; 4.8 kb; 900-bp *Sma*I Cm$^r$ fragment of pUC18::Cm inserted into *Sma*I-cut pVE3501 | This work |
| pGK::*nuc*1.6 | Em$^r$ *nuc*$^+$; 5.5 kb; 1.6-kb *Bam*HI-*Pst*I *nuc* fragment of pWNuc5 inserted into *Bam*HI-*Pst*I-cut pVE3509 | This work |
| pGK::*nuc* | Em$^r$ *nuc*$^+$; 4.8 kb; 870-bp *Eco*RI *nuc* fragment of pBS::*nuc* inserted into the 3.9-kb *Eco*RI fragment of pVE3509 | This work |
| pGK::*nuc*MCS | Em$^r$ *nuc*$^+$; 4.3 kb; 900-bp *Eco*RI-blunted fragment of pBS::*nuc*MCS inserted into *Nsi*I-cut and blunted fragment of pVE3501 | This work |
| pVE3530 | Em$^r$ *nuc* Tc$^r$; 6.6 kb; 2.3-kb Tc$^r$ *Sal*I-*Sma*I-cut fragment of pVE1015 inserted into *Sal*I-*Eco*RV-cut pGK::*nuc*MCS | This work |
| pIL253 | Em$^r$; 5 kb | 14 |
| pIL253::*nuc* | Em$^r$ *nuc*$^+$; 5.8 kb; 870-bp *Eco*RI *nuc* fragment inserted into *Eco*RI-cut pIL253 | This work |
| pVE3533 (pIL) | Em$^r$; 4.9 kb; *Sst*I-cut and religated pIL253 | This work |
| pIL::*nuc*MCS | Em$^r$ *nuc*$^+$; 5.8 kb; 900-bp *Eco*RI fragment of pBS::*nuc*MCS inserted into *Eco*RI-cut pIL | This work |
| pVE3542 | Em$^r$ *nuc* Tc$^r$; 2.3-kb Tc$^r$ *Sal*I-*Sma*I-cut fragment of pVE1015 inserted into *Sal*I-*Eco*RV-cut pIL::*nuc*MCS | This work |
| pBS | Ap$^r$; 2.9 kb | Stratagene |
| pVE1015 | Ap$^r$ Tc$^r$; 5.2 kb; 2.3-kb *Hin*dIII Tc$^r$ fragment of pT181 inserted into *Hin*dIII-cut pBS | P. Duwat[b] |
| pBS::*nuc* | Ap$^r$ *nuc*$^+$; 3.8 kb; 858-bp PCR *nuc* product cut by *Eco*RI inserted into *Eco*RI-cut pBS | This work |
| pBS::*nuc*MCS | Ap$^r$ *nuc*$^+$; 3.8 kb; synthetic oligonucleotide cut by *Nsi*I inserted into *Nsi*I-cut pBS::*nuc* | This work |

[a] pBS, pBluescript KS.
[b] Laboratoire de Génétique Microbienne, Institut de Biotechnologie, Institut National de la Recherche Agronomique, Jouy en Josas Cedex, France.

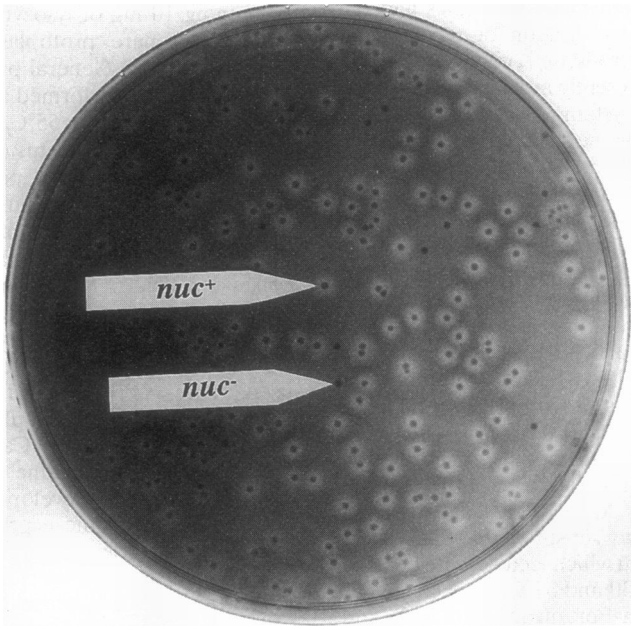


FIG. 1. Nuclease plate test for detection in *L. lactis* of *nuc* mutant clones among *nuc*$^+$ clones. A protocol for optimal detection in *L. lactis* of *nuc* mutant clones among *nuc*$^+$ clones on petri plates was developed. A low-phosphate medium, in this case, brain heart infusion, was used for plating cells, as phosphate interferes with the pink-blue *nuc* test by interacting with the Toluidine Blue stain of TB-D agar. The plating efficiency of *L. lactis* on brain heart infusion is as good as that on the high-phosphate M17 medium. Every strain of *E. coli*, *L. lactis*, and *S. salivarius* tested was first checked for a potential *nuc* activity: after 1 h at 37°C on TB-D agar, no nuclease activity was detected on any strain. They were all considered *nuc* mutant strains and consequently as good hosts for cloning of the *nuc* gene. Twenty-four hours after plating and incubation, transformant plates were overlaid with TB-D agar and incubated for 1 h at 37°C to detect *nuc* mutant clones. On this plate, 26 *nuc* mutant clones were detected among a total of around 240 *nuc*$^+$ clones which developed a pink halo corresponding to the secretion of SNase as described previously (14). Longer incubation of plates with TB-D agar leads to diffusion of pink halos and prevents good detection of *nuc* mutant clones.

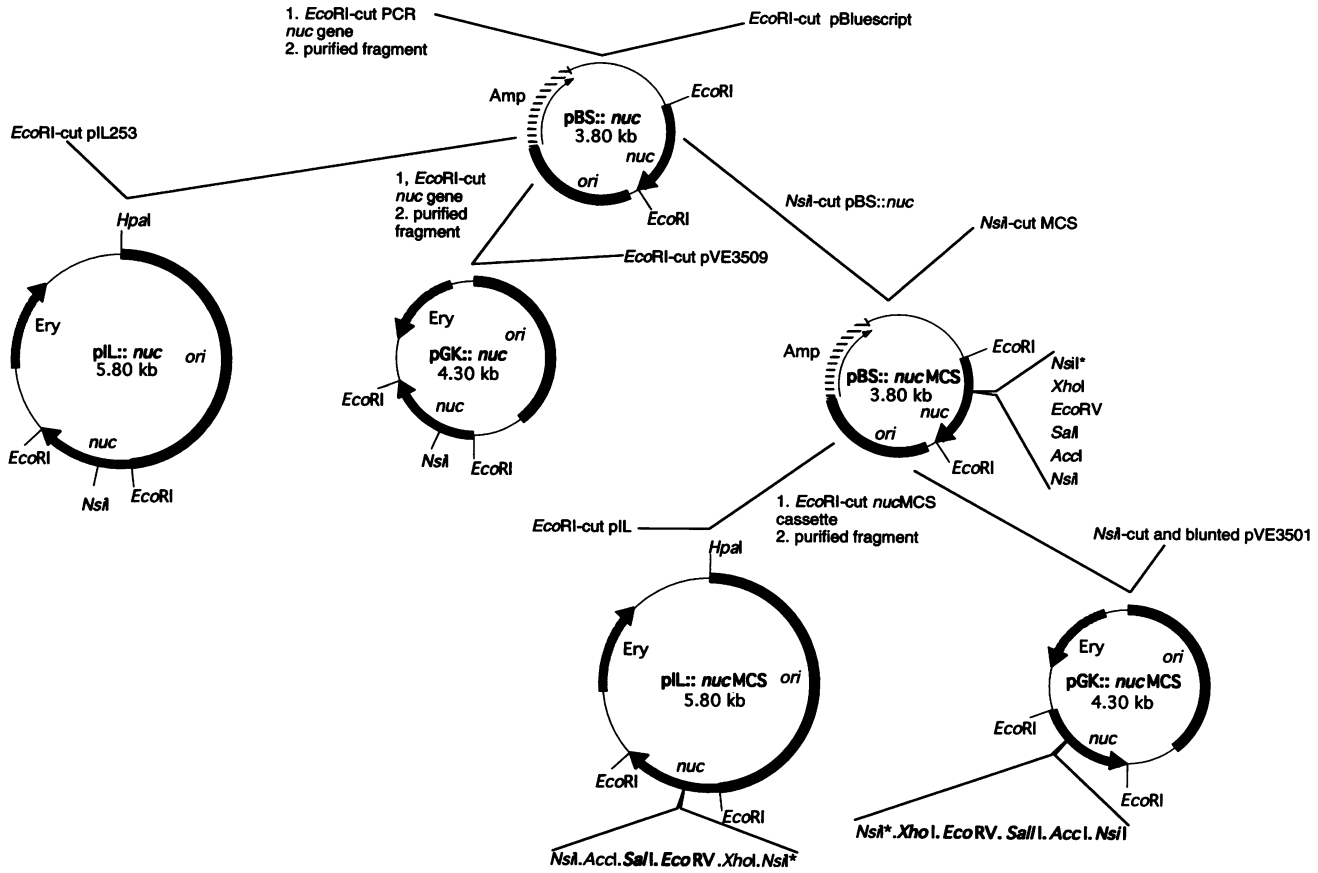


FIG. 2. Construction of *nuc* vectors and *nuc*MCS vectors. pGK::*nuc*1.6 was used as the matrix in one PCR to amplify one 858-bp *nuc* fragment that we first cloned in *E. coli* TG1 by using pBluescript SK$^+$. Single-stranded DNA oligonucleotide primers directed to the *nuc* gene were synthesized by Appligene (Strasbourg, France). The coding strand primer is 5′-CAGAATTACCAGAAGTTGAAACCG-3′, and the complementary strand primer is 5′-TGTAGTTTCTTCTTCAGTTTCTGC-3′. This fragment still carries staphylococcal promoter region, ribosome binding site, staphylococcal signal peptide, and structural gene of SNase. *E. coli* clones containing the expected plasmid, pBluescript SK$^+$::*nuc*, were still *nuc*$^+$. The arrows indicate the direction of transcription. Amp, resistance to ampicillin; Ery, resistance to erythromycin; *ori*, origin of replication; *nuc*, 858-bp PCR nuclease gene. The synthetic MCS was obtained by annealing after phosphorylation of the single-stranded DNA (5′-ATGCATCA CAAGTCGACGATGATATCTCGAGTGCA-3′) and the complementary strand (5′-TGCACTCGAGATATCGTCGACTTGTGATGCCAT-3′) with kinase. This synthetic MCS contains unique restriction sites for *Nsi*I, *Sal*I, *Acc*I, *Eco*RV, and *Xho*I. *Nsi*I*, mutated site for *Nsi*I. Unique restriction sites for pIL::*nuc*MCS and for pGK::*nuc*MCS are in boldface type.

fragment, the *nuc* gene was recovered by PCR of an 858-bp fragment containing its native expression signal. Derivatives were constructed to examine *nuc* expression and to facilitate the use of these *nuc* plasmids as cloning vectors (Fig. 2). The *nuc* fragment obtained by PCR was cloned onto rolling-circle plasmid pVE3509 (present at 20 to 30 copies per chromosome in *L. lactis*; data not shown) and pIL253 (15), a theta-replicating plasmid (present at 80 to 100 copies per chromosome in *L. lactis* [15]), resulting in pGK::*nuc* and pIL253::*nuc*, respectively (Fig. 2). Both plasmids conferred a *nuc*$^+$ phenotype on *L. lactis*. SNase production appears to be gene dosage dependent in *L. lactis*, as halos around colonies of *L. lactis* IL1403(pIL253::*nuc*) were significantly larger and more intense than those obtained with *L. lactis* IL1403(pGK::*nuc*) (data not shown). This effect was confirmed by comparing *nuc* activities in filtered supernatants from overnight cultures of the two strains (data not shown).

**Construction of *nuc*MCS vectors.** Our goal was the construction of new vectors for gram-positive bacteria which allow the direct screening of recombinant clones based on the expression of the SNase. A multicloning site (MCS) containing *Nsi*I, *Sal*I, *Acc*I, *Eco*RV, and *Xho*I restriction sites was inserted in the *Nsi*I site of the *nuc* gene, which corresponds to the cleavage site between the leader peptide and the mature SNase (Fig. 3). Sequencing confirmed its orientation and presence in monocopy. The insertion of the MCS in frame with the SNase open reading frame did not affect SNase activity on plate assays using pBluescript SK$^+$::*nuc*MCS in *E. coli* TG1 as the test system. However, cloning into any of the restriction sites of the MCS will interrupt the *nuc* gene and result in a *nuc* mutant phenotype. The *nuc*MCS cassette was transferred to vectors pGK and pVE3533 (pIL), a derivative of pIL253 (Fig. 2), and established in *L. lactis*; it exhibited SNase activity in all contexts.

**Use of *nuc*MCS vectors to clone foreign DNA in *L. lactis*.** The potential application of the *nuc* detection system was first examined by cloning directly in *L. lactis* the *tet* gene of *S. aureus* plasmid pT181 (7). A 2.3-kb *Sal*I-*Sma*I fragment containing the Tc$^r$ marker was cloned into *Sal*I-*Eco*RV-cut pIL::*nuc*MCS. Transformants of MG1363 were screened for inserts by the *nuc* plate test, and *nuc* mutant colonies were further checked for DNA inserts by screening for Tc$^r$ (Table 2). Of 27 *nuc* mutant

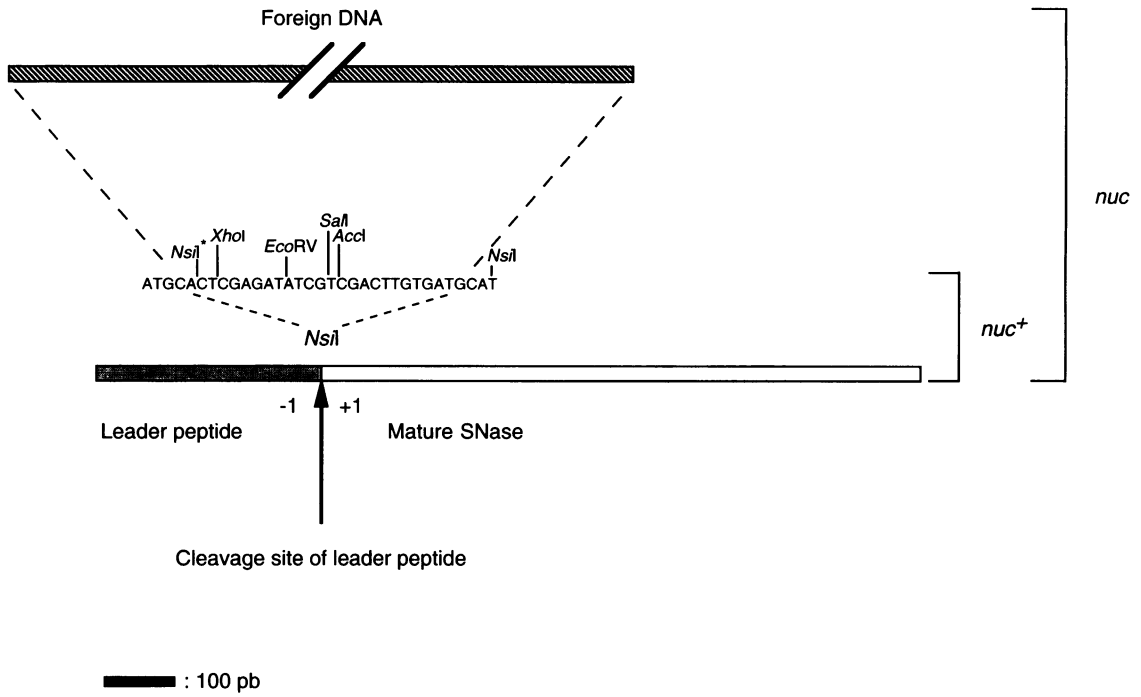


FIG. 3. Insertion of the MCS in the cleavage site of the SNase leader peptide. The MCS containing five restriction sites was inserted into the *Nsi*I site of the *nuc* gene which corresponds to the cleavage site between leader peptide and the mature SNase. This insertion does not hamper the secretion of the SNase (*nuc*$^+$). Cloning foreign DNA in the MCS will block the translation of *nuc*, and clones carrying *nuc*MCS plasmids with an insert will not secrete SNase (*nuc* mutants).

clones, 17 contained the Tc$^r$ marker, thus demonstrating the efficiency of the screening test. False-positive clones are likely to be due either to overdigestion of the backbone or to an illegitimate (in vivo) religation of the backbone. The *nuc* mutant Em$^r$ Tc$^r$ MG1363 clones contained the expected plasmid construction. The same experiment was performed with IL1403 by using pGK::*nuc*MCS as the test plasmid. In this experiment, 10 of 34 *nuc* mutant clones contained the insert (Table 2).

In a second test, we cloned *E. coli* chromosomal DNA fragments onto both *nuc*MCS plasmids in *L. lactis*. Vectors pGK::*nuc*MCS and pIL::*nuc*MCS linearized by *Eco*RV were ligated with *E. coli* TG1 *Eco*RV-digested total chromosomal DNA. The majority of fragments had sizes between 0.1 and 7 kb as estimated by agarose gel migration. A 1:9 ratio (wt/wt; plasmid/chromosomal DNA) was used for ligation. These DNA mixtures were then used to transform IL1403. Routinely, $10^3$ transformants per μg of religated DNA, which is about 100-fold fewer than with intact vectors, were obtained. The nuclease plate test was used to screen for plasmids carrying inserts. When pGK::*nuc*MCS was used as a vector, 263 *nuc* mutant clones were present among 1,475 transformants, which corresponds to an 18% foreign-DNA insertion rate. Eighty percent of 24 *nuc* mutant clones analyzed carried an insert with a size range of 0.25 to 2.5 kb (Table 2). In similar experiments performed with pIL::*nuc*MCS, 297 *nuc* mutant clones were found among 1,533 transformants (19% foreign-DNA insertion rate); 39 of 55 *nuc* mutant clones analyzed carried inserts with sizes of 0.25 to 2.3 kb (Table 2).

**Introduction of *nuc*MCS vectors in *S. salivarius* subsp. *thermophilus*.** The applicability of the *nuc*MCS vectors to other gram-positive species was examined. *S. salivarius* subsp. *thermophilus* IL73 does not secrete nuclease activity, as verified by the nuclease plate assay. Transformation of this strain with pGK::*nuc*MCS and pIL::*nuc*MCS gives rise exclusively to Em$^r$ *nuc*$^+$ colonies, confirming that SNase was expressed in *S. thermophilus* IL73. Plasmids in which the *nuc* gene was interrupted by a Tc$^r$ marker (pVE3530 and pVE3542) were also introduced in *S. thermophilus* IL73 and, as in *L. lactis*, were Em$^r$ Tc$^r$ and carried the *nuc* mutation.

TABLE 2. Use of *nuc*MCS vectors to clone foreign DNA in *L. lactis*.

| Plasmid | Tc$^r$ gene | | *E. coli* DNA fragments[a] | | |
|---|---|---|---|---|---|
| | % *nuc* mutant clones among transformants (no. of mutant clones/total no. of transformants) | % Tc$^r$ *nuc* mutant clones among *nuc* mutant clones (no. of Tc$^r$ clones/total no. of mutant clones) | % *nuc* mutant clones among transformants[b] | % Insert-containing *nuc* mutant clones among *nuc* mutant clones | Size (kb) of insert |
| pGK::*nuc*MCS | 57 (24/42) | 41 (10/24) | 18 | 80 | 0.25–2.5 |
| pIL::*nuc*MCS | 31 (27/87) | 63 (17/27) | 19 | 71 | 0.25–2.3 |

[a] Size distribution of *Eco*RV-generated fragments, 0.1 to 7 kb.
[b] Approximately 1,500 transformants per experiment.

***nuc*MCS cloning vector system.** The *nuc*MCS cloning vectors allow a rapid direct screening of recombinant clones. Expression of the *nuc* gene by its natural promoter in many gram-positive bacteria (including *B. subtilis*, *C. glutamicum*, *L. lactis*, and *S. thermophilus*), as well as the use of two broad-host-range vectors, allows a wide application of this system. In *E. coli*, cloning is most often detected by interruption of a plasmid-carried *lacZ* truncated gene which requires that the host strain contain the complementary part of the gene (18). The *lacZ* system was also adapted for cloning in *B. subtilis*, but its use is limited to a single strain (6). In contrast, the *nuc*MCS cloning vector has no host requirement other than carrying the *nuc* mutation. It can therefore be used in a wide range of gram-positive strains, as well as those *E. coli* strains which are not adapted for the use of the *lacZ* detection system. To our knowledge, no such system has been available for gram-positive bacteria until now.

***nuc* gene as a new reporter gene in *L. lactis*.** The properties of the SNase make it an attractive candidate as a reporter of gene expression and secretion in *L. lactis*. SNase is a small protein with a compact structure. Its activity does not seem to be altered by the addition of an N-terminal peptide, as the B form of SNase contains 19 extra amino acids at the N terminus and is as active as the smaller A form. Since nuclease activity does not seem to be diminished by its N-terminal tail, this raises the possibility that fusion proteins (fused at the SNase N terminus) could also be active. We are currently testing the use of SNase as a reporter of secretion of fusion proteins. In addition, SNase retains activity even after treatment with denaturing agents such as sodium dodecyl sulfate (SDS) or trichloroacetic acid (data not shown), thus allowing us to localize SNase in cellular fractions and identify it in complex protein SDS-polyacrylamide gel electrophoresis gel patterns. In conclusion, SNase promises to be a new powerful tool for genetic analysis of gram-positive bacteria.

We thank P. Duwat, E. Maguin, P. Morel, and J. C. Piard for helpful discussions during this work. We thank W. Liebl for providing us the plasmid pWNuc5 and our photographer, M. Nezonde, for her contribution.

This work was supported in part by BRIDGE contract BIOT-CT91-0263 of the Commission of the European Communities.

## REFERENCES

1. **Birnboim, H., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11**:260–263.
3. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
4. **Gasson, M. J.** 1993. Progress and potential in the biotechnology of lactic acid bacteria. FEMS Microbiol. Rev. **12**(1–3):3–19.
5. **Gibson, T. J.** 1984. Ph.D. thesis. University of Cambridge, Cambridge, England.
6. **Haima, P., D. van Sinderen, S. Bron, and G. Venema.** 1990. An improved β-galactosidase α-complementation system for molecular cloning in *Bacillus subtilis*. Gene **93**:41–47.
7. **Khan, S., and R. Novick.** 1983. Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from *Staphylococcus aureus*. Plasmid **10**:251–259.
8. **Kok, J., J. M. B. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48**:726–731.
9. **Lachica, R. V. F., C. Genigeorgis, and P. D. Hoeprich.** 1971. Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl. Microbiol. **21**:585–587.
10. **Langella, P., Y. Le Loir, S. D. Ehrlich, and A. Gruss.** 1993. Efficient plasmid mobilization by pIP501 in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **175**:5806–5813.
11. **Liebl, W., A. J. Sinskey, and K. H. Schleifer.** 1992. Expression, secretion, and processing of staphylococcal nuclease by *Corynebacterium glutamicum*. J. Bacteriol. **174**:1854–1861.
12. **Miller, J. R., S. Kovacevic, and L. E. Veal.** 1987. Secretion and processing of staphylococcal nuclease by *Bacillus subtilis*. J. Bacteriol. **169**:3508–3514.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Shortle, D.** 1983. A genetic system for analysis of staphylococcal nuclease. Gene **22**:181–189.
15. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70**:559–566.
16. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Environ. Microbiol. **29**:807–813.
17. **Trieu-Cuot, P., G. de Cespedes, and T. Horaud.** 1992. Nucleotide sequence of the chloramphenicol resistance determinant of the streptococcal plasmid pIP501. Plasmid **28**:272–276.
18. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.