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Isolation of a Replication Region of a Large Lactococcal Plasmid and Use in Cloning of a Nisin Resistance Determinant

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The replication region of a 28-kilobase-pair (kbp) cryptic plasmid from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis SSD207 was cloned in *L. lactis* subsp. *lactis* MG1614 by using the chloramphenicol resistance gene from the streptococcal plasmid pGB301 as a selectable marker. The resulting 8.1-kbp plasmid, designated pVS34, was characterized further with respect to host range, potential cloning sites, and location of replication gene(s). In addition to lactococci, pVS34 transformed *Lactobacillus plantarum* and, at a very low frequency, *Staphylococcus aureus* but not *Escherichia coli* or *Bacillus subtilis*. The 4.1-kbp *Cla*I fragment representing lactococcal DNA in pVS34 contained unique restriction sites for *Hin*dIII, *Eco*RI, *Xho*II, and *Hpa*II, of which the last three could be used for molecular cloning. A region necessary for replication was located within a 2.5-kbp fragment flanked by the *Eco*RI and *Cla*I restriction sites. A 3.8-kbp *Eco*RI fragment derived from a nisin resistance plasmid, pSF01, was cloned into the *Eco*RI site of pVS34 to obtain a nisin-chloramphenicol double-resistance plasmid, pVS39. From this plasmid, the streptococcal chloramphenicol resistance region was subsequently eliminated. The resulting plasmid, pVS40, contains only lactococcal DNA. Potential uses for this type of a nisin resistance plasmid are discussed.

Lactococcal dairy starter strains contain a wide variety of plasmids with sizes ranging from about 2 kilobase pairs (kbp) to more than 80 kbp. Large plasmids are often associated with such phenotypes as lactose fermentation, proteolysis, conjugation, slime formation, bacteriophage resistance, etc. (for a review, see reference 24). The majority of the lactococcal plasmids, however, are cryptic. Small cryptic plasmids, such as pWV01 and pSH71, have been used by several research groups for vector construction by marking them with antibiotic resistance genes derived from plasmids of other gram-positive bacteria (7, 12, 19, 28, 32).

The lactococcal cloning vectors constructed so far share an ability to replicate in a wide range of bacterial hosts, such as *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and some lactobacilli (8, 12, 19, 28). Although this property is very useful for research purposes, it may be unacceptable for food-grade vectors aimed at genetic manipulation of actual dairy starter strains. It would be undesirable if a recombinant plasmid were to spread into other genera of bacteria in the dairy or farm environment or in the digestive tracts of humans or domestic animals.

Lactococcus lactis subsp. *lactis* biovar diacetylactis SSD207, isolated from a dairy starter culture, shows in gel electrophoresis at least 12 plasmid bands ranging in size from 2 to more than 30 kbp (29). No attempt has been made to identify the covalently closed, relaxed circular, and multimeric forms of different plasmids. In hybridization experiments (unpublished observation), none of the SSD207 plasmids hybridized with pVS2, a typical broad-host-range vector based on a 2.1-kbp lactococcal plasmid, pSH71 (11). We therefore screened this strain for plasmid replication functions with a limited capacity to function outside the lactic acid bacteria. In this report, we describe the isolation and properties of one such replication determinant and its use for cloning nisin resistance.

Nisin resistance was chosen for cloning experiments because of its potential use as a primary selection marker in lactococcal transformations. Nisin is a polypeptide antibiotic of 34 amino acids and is produced by several strains of *L. lactis* subsp. *lactis* (16, 17). Several groups have studied the genetics of both nisin resistance and production and have attempted selection for nisin resistance in experiments in lactococci. Nisin resistance, nisin production, and sucrose fermentation appear often to be linked, and genetic evidence suggests involvement of a plasmid or plasmids in these phenotypes. However, the physical isolation of a single sucrose fermentation and nisin production and resistance plasmid has not so far succeeded (13, 26). On the other hand, a 64-kbp plasmid was isolated which coded for both lactose fermentation and nisin resistance (18), but transconjugants could not be selected with the nisin resistance marker (18). Linkage between nisin resistance and bacteriophage resistance has been firmly established by McKay and Baldwin (25), who demonstrated that a 64-kbp plasmid, pNP40, was responsible for the observed phenotypes. Subsequently, Froseth et al. (10) located the replication region and nisin resistance determinant of pNP40 within a 7.6-kbp *Eco*RI fragment which could be established as an independent replicon. It was concluded that this nisin resistance determinant could be useful as a secondary selection marker.

In the present study, the nisin resistance marker was derived from the nisin-producing *L. lactis* subsp. *lactis* 10.084 obtained from the Danish Government Research Institute for Dairy Industry. The strain was known because of its high resistance to nisin, but no genetic studies on this phenotype had so far been performed.

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TABLE 1. Plasmids and recipient strains used in this work

Plasmid or strain	Phenotype conferred[a]	Plasmid size (kbp)	Relevant genotype or phenotype	Description, source, or reference
Plasmid				
pBR322	Amp^r Tet^r	4.3		4
pVC5	Amp^r Cam^r Tet^r	8.4		4.1-kbp *Cla*I fragment containing the chloramphenicol resistance gene of pGB301 (2) cloned to the single *Cla*I site of pBR322 (S. Tynkkynen, M.Sc. thesis)
pVS1	Cam^r	4.5		2.8-kbp *Cla*I-*Hpa*II fragment of pVC5 cloned on the 1.7-kbp *Cla*I fragment of pSH71 (11) (S. Tynkkynen, M.Sc. thesis)
pVS2	Cam^r Ery^r	5.1		32
pGKV10	Ery^r	4.6		28
pSW211	Cam^r Nis^r Ery^r	12.8		This work
pSW221	Ery^r Nis^r	8.4		This work
pVS34	Cam^r	8.1		This work
pVS39	Cam^r Nis^r	11.8		This work
pVS40	Nis^r	7.8		This work
Strain				
L. lactis subsp. *lactis* MG1614			Str^r Rif^r	11
L. lactis subsp. *lactis* LM0230				9
E. coli AB259			*thi*	23
B. subtilis 3G18			Ade Met Trp	Gerard Venema, University of Groningen, The Netherlands
S. aureus RN451				Richard Novick, Public Health Research Institute of the City of New York, Inc.
L. plantarum 755			Contains a cryptic plasmid of about 37 kbp	Strain collection, Research and Development Centre, Valio Finnish Co-operative Dairies' Association, Helsinki

[a] Nis, Nisin.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *L. lactis* subsp. *lactis* biovar diacetylactis SSD207 is described in the introduction. *L. lactis* subsp. *lactis* 10.084 is a nisin-producing strain able to ferment both lactose and sucrose and containing at least five uncharacterized plasmids (see Fig. 4). Recipient strains and different plasmids used in the transformations and cloning experiments are detailed in Table 1. M17 medium (27) supplemented with either 0.5% lactose (for SSD207 and 10.084) or glucose (for the rest of strains) was used throughout this work with *L. lactis* subsp. *lactis* and *S. aureus*. *E. coli* and *B. subtilis* were grown in L broth or L agar as defined by Maniatis et al. (22). MRS medium (6) was used with *Lactobacillus plantarum*. The incubation temperature was 32°C for the lactococci, 30°C for *L. plantarum*, and 37°C for the others.

DNA isolation. The method of Anderson and McKay (1) was used both for plasmid screening and (followed by cesium chloride-ethidium bromide ultracentrifugation) for preparative purposes with the lactococci. With *E. coli*, the method of Birnboim (3) was applied with minor modifications. DNA was extracted from agarose gels by using the GeneClean kit (Bio 101, Inc., La Jolla, Calif.) according to the instructions of the manufacturer.

Nisin solutions. Nisin was contained in the preparation called Nisaplin (Aplin & Barret, Trowbridge, Wiltshire, England). According to the manufacturer, Nisaplin contains 1,026 IU of nisin per mg. In the present report, the nisin concentrations were calculated according to this information. Stock solutions of nisin were prepared by dissolving Nisaplin at 10 mg/ml in 0.02 N HCl without further sterilization. Only freshly prepared solutions were used for selective purposes.

Transformation. *L. lactis* subsp. *lactis* MG1614 and LM0230 protoplasts were transformed as described previously (30). The method of Mandel and Higa (21) was used to transform *E. coli* AB259. Chloramphenicol (5 μg/ml) and erythromycin (2.5 μg/ml) were used as the selection agents with the lactococci, and chloramphenicol, either alone or together with ampicillin (at concentrations of 5 and 50 μg/ml, respectively), was used for *E. coli*. The nisin resistance phenotype of chloramphenicol-resistant MG1614 transformants was checked on agar containing 500 IU of nisin per ml. The use of nisin for primary selection is described in a separate section.

Competent cells of *B. subtilis* were transformed by the method of Boylan et al. (5). Chloramphenicol (5 μg/ml) was used for selection. To check the effects of multimeric forms of plasmids on the transformation frequencies, plasmids were linearized with suitable restriction enzymes (*Hin*dIII for pVS2 and *Eco*RI for pVS34) and religated before transformation in some experiments, using untreated plasmids as controls.

L. plantarum and *S. aureus* were transformed by electroporation. A fresh inoculum of cells grown overnight was made in the appropriate broth to give an absorbance of 0.15 to 0.18 at 600 nm. The cultures were grown until the absorbance reached 0.5 to 0.7, and the cells were harvested by centrifugation at 4°C, washed twice and finally suspended at 0.05 culture volume in electroporation buffer (272 mM sucrose, 15% glycerol). The electroporation mixture (200 μl of cell suspension held in ice for 2 min in an electroporation cuvette with a 2-mm electrode gap, to which was added up to 20 μl of purified plasmid DNA in 10 mM Tris hydrochloride–1 mM EDTA [pH 7.5]) was given a single electric pulse in a Genepulser apparatus (Bio-Rad Laboratories, Richmond,

Calif.). The capacitance was 25 μF and the voltage was either 2 kV (*L. plantarum*) or 2.5 kV (*S. aureus*). The cuvette was connected in parallel to a 1,000-Ω (*L. plantarum*) or 600-Ω (*S. aureus*) resistor (Bio-Rad Pulse Controller). The cells were then kept in ice for 2 min, suspended in 10 ml of prewarmed culture broth, incubated for 1 to 2 h, and harvested by centrifugation of 9 ml at room temperature, the pellet being resuspended in the remaining 1 ml. The suspension was spread on selection plates containing either 10 μg (for *L. plantarum*) or 5 μg (for *S. aureus*) of chloramphenicol per ml. *L. plantarum* plates were incubated anaerobically at 30°C for 48 h, while aerobic conditions and 37°C for 24 to 48 h were applied to *S. aureus*.

In some experiments, electroporation was also applied to *L. lactis* subsp. *lactis* MG1614. The procedure was the same as described above with the following modifications. The growth medium contained 0.5 M sucrose and 1% glycine. The final cell suspension was 0.02 culture volume, and 100 μl was used for the electroporation mixture. The voltage was 2 kV, and the resistor setting was 400 Ω. The selection media were the same as for protoplast transformation.

Use of nisin for primary selection. Nisin was used for direct selection of lactococcal transformants in both protoplast transformations and electroporation experiments. Early experiments employed protoplast transformation, for which selection was on plates containing 100 IU of nisin per ml. Later on, electroporation was used, and here selection was on 7 IU/ml. In all cases, nisin solutions were added to the melted agar media immediately before the plates were poured. Practically no spontaneous nisin-resistant colonies were observed on control plates.

Molecular cloning. Restriction enzymes and T4 DNA ligase were purchased from New England Bio-Labs, Inc. (Beverly, Mass.) and used according to the instructions of the supplier. The general procedures for DNA manipulation were essentially those recommended by Maniatis et al. (22).

Plasmid elimination. Novobiocin gradient plates were used for plasmid elimination as described previously (31).

Southern hybridization. Two nonradioactive DNA-labeling methods were applied. The DNA Labeling and Detection Kit (Boehringer Mannheim GmbH, Penzberg, Federal Republic of Germany) based on random-primed DNA synthesis and digoxigenin-labeled dUTP was used according to the instructions of the manufacturer. DNA transfer was performed as described by Maniatis et al. (22) with 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and a Gene Screen nylon membrane (Dupont, NEN Research Products, Boston, Mass.). Alternatively, the DNA labeling was done with biotinylated dUTP (Boehringer Mannheim GmbH) by the method of Leary et al. (20). The transfer was done to nitrocellulose filter paper (Schleicher & Schuell, Dassel, Federal Republic of Germany). The biotin-labeled probe was prepared with the Nick Translation Reagent Kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and staining was performed with the Vectastain Alkaline Phosphatase Kit (Vector Laborato-

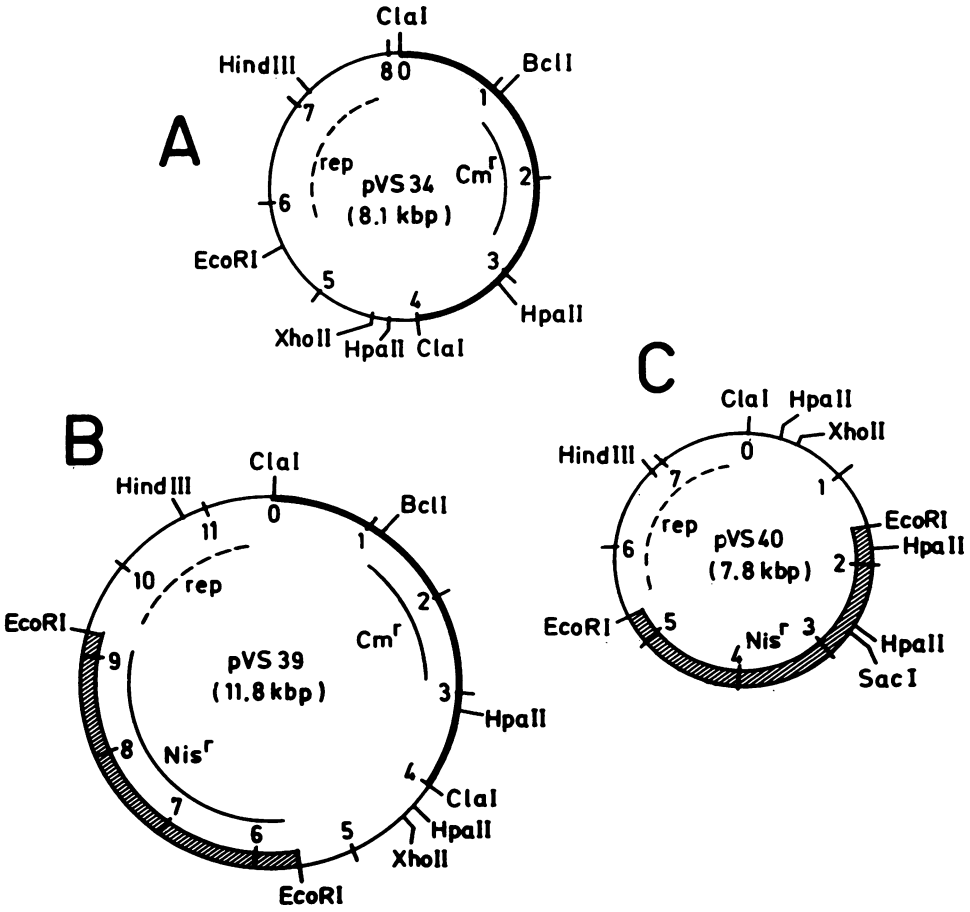

FIG. 1. Restriction maps of plasmids pVS34 (A), pVS39 (B), and pVS40 (C). For further information about the restriction sites within the nisin resistance region of pVS39 and pVS40, see Fig. 3. ——, SSD207 plasmid DNA; ▬, pGB301 DNA; ▨, pSW211 DNA.

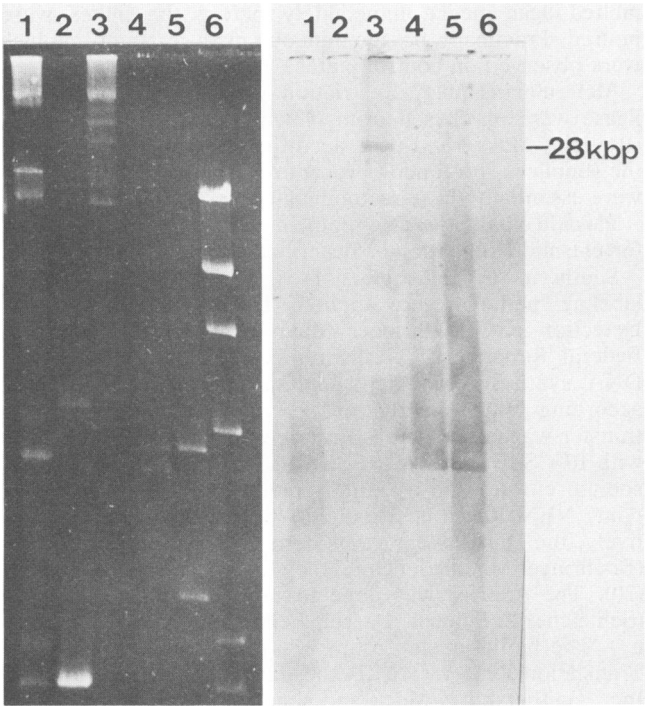

FIG. 2. Hybridization of the 4.1-kbp *Cla*I fragment of pVS34 (containing the replication functions) with the 28-kbp cryptic plasmid of *L. lactis* subsp. *lactis* biovar diacetylactis SSD207. The size of the plasmid was determined, using in addition to the 2.1-, 3.6-, 7.5-, and 55-kbp plasmids of NCDO 712 (lane 1) (9) other plasmids of known molecular weight not included in the picture. Lanes: 1, plasmids of NCDO 712; 2, pSH71; 3, SSD207; 4, *Eco*RI digestion of pVS40; 5, *Cla*I-*Bcl*I digestion of pVS34; 6, *Hin*dIII-digested λ DNA.

ries Inc., Burlingame, Calif.) according to the instructions of the manufacturer.

RESULTS

Screening of SSD207 plasmid replication regions. The 4.0-kbp *Cla*I fragment from plasmid pVC5 (Soile Tynkkynen, M.Sc. thesis, University of Helsinki, Helsinki, Finland, 1985) containing the chloramphenicol resistance gene of streptococcal plasmid pGB301 (2) was mixed with and ligated to the total plasmid DNA of SSD207 digested with *Cla*I. The ligation mixture was used to transform MG1614 protoplasts, with selection for chloramphenicol resistance.

Thirty transformants were obtained from the ligation mixture, which contained about 1 μg of DNA. The majority of the transformants carried plasmids of about 8 kbp, while the rest apparently contained randomly inserted extra *Cla*I-generated fragments (data not shown). One clone was arbitrarily chosen for further studies and was designated pVS34. The restriction map of this plasmid is presented in Fig. 1A. In DNA-DNA hybridization experiments, the 4.1-kbp *Cla*I fragment of pVS34 (containing the replication functions) hybridized with the approximately 28-kbp plasmid of SSD207 (Fig. 2).

Location of pVS34 replication functions. To test whether any of the known restriction sites of pVS34 interrupt the replication region of the plasmid, fusion plasmids with pBR322 were generated by linearizing pBR322 with *Hin*dIII, *Cla*I, or *Bam*HI and ligating to pVS34 digested with *Hin*dIII, *Hpa*II, or *Xho*II, respectively. *E. coli* was transformed with these ligation mixtures by using ampicillin selection. Transformants were tested on chloramphenicol (5 μg/ml) plates. Plasmid DNA from chloramphenicol-resistant transformants was used to retransform MG1614 protoplasts. Only with the construction in which the *Hin*dIII ligation formed the hybrid plasmid were no transformants produced. Normal transformation frequencies (about 10^4 chloramphenicol-resistant clones per μg of DNA) were obtained with the rest, and the plasmids extracted from the transformants had the expected sizes and restriction patterns. This, together with the successful use of the *Eco*RI site for cloning (see below), led to the conclusion that at least part of the replication region of pVS34 is located within the 2.5-kbp *Eco*RI-*Cla*I restriction fragment.

Transformation of different bacterial hosts with pVS34. Purified pVS34 DNA (from lactococcal preparative isolations) was used to transform *E. coli* AB259, *B. subtilis* 3G18, *L. plantarum* 755, and *S. aureus* RN451. As positive controls, parallel experiments were done with equal amounts of pVS2 (which contains the same chloramphenicol resistance gene as pVS34), and the transformation frequencies were compared. The results (Table 2) indicated that pVS34 was able to transform only *L. plantarum* and, at a very low frequency, *S. aureus*. Subsequently, the presence of pVS34 DNA was demonstrated in *L. plantarum* and *S. aureus* transformants (data not shown).

Isolation of nisin resistance determinant. Total plasmid DNA from the nisin-resistant strain *L. lactis* subsp. *lactis* 10.084 was isolated and used to transform MG1614 with selection for nisin resistance (100 IU of nisin per ml of selection medium). Plasmid DNA from the transformants was used in subsequent rounds of transformation. A clone

TABLE 2. Transformation of different recipients by plasmids pVS2 and pVS34[a]

Plasmid	Transformant frequency/μg of plasmid DNA				
	L. lactis MG1614	*E. coli* AB259	*B. subtilis* 3G18	*S. aureus* RN451	*L. plantarum* 755
pVS2	1.0×10^4	1.0×10^5	2.6×10^3	3.2×10^2	1.4×10^2
pVS2 cut and religated[b]	—[c]	—	2.0×10^5	—	—
pVS34	1.0×10^4	ND[d]	ND	3.3×10^1	5.7×10^2
pVS34 cut and religated[b]	—	—	ND	—	—

[a] Each transformation was performed at least three times except for the experiments with cut and religated DNAs, which were done once. The numbers represent the highest transformant numbers obtained. The amount of DNA varied from 5 to 100 ng per transformation depending on the transformation method and recipient (optimization was done with pVS2 as a model plasmid).
[b] Cutting and religation were performed to create plasmid multimers for *B. subtilis* transformation (see Materials and Methods).
[c] —, Experiment not done.
[d] ND, No transformants detected.

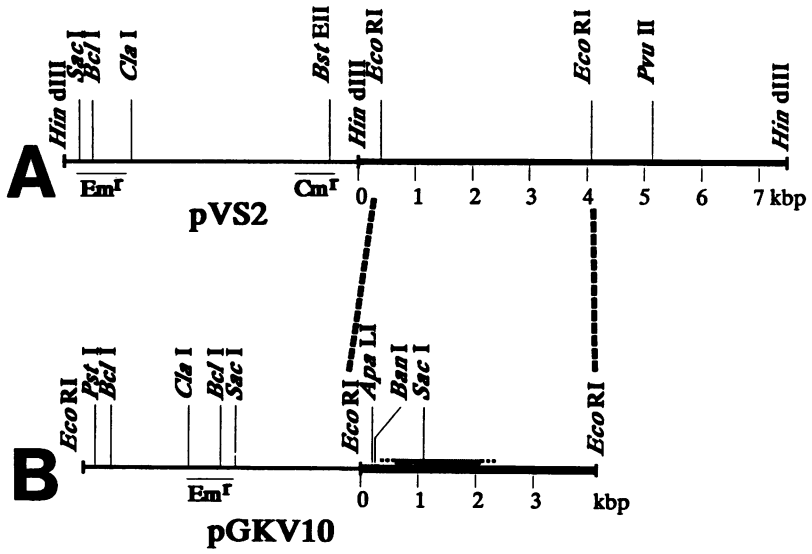

FIG. 3. Restriction map of two plasmids encoding nisin resistance. (A) pSW211. (B) pSW221. The thick lines represent the DNA cloned from *L. lactis* subsp. *lactis* 10.084. The location of the nisin resistance determinant on pSW211 (crosshatched area) was based solely on the observation that deletion of the smaller *Eco*RI-*Sac*I fragment of the insert results in inactivation of the resistance.

containing a single 46-kbp plasmid was obtained. This plasmid, designated pSF01, was associated with nisin resistance, lactose fermentation, and proteinase activity but not with nisin production or sucrose fermentation. To subclone the nisin resistance determinant, pSF01 was digested with *Hin*dIII and the resulting fragments were mixed and ligated with *Hin*dIII-digested pVS2 DNA. Transformation of MG1614 with this ligation mixture produced 28 nisin-resistant transformants per 0.4 μg of pSF01 DNA in the ligation mixture. Twenty-six of the transformants were found to be also resistant to chloramphenicol and erythromycin. Plasmid DNA was isolated from eight transformants showing the expected phenotype, all of them containing, among others, a 7.5-kbp insert in pVS2. From one clone with this insert only, the plasmid was transformed into LM0230 and designated pSW211 (Fig. 3A). To reduce the size of the insert containing the nisin resistance determinant, the 3.8-kbp *Eco*RI fragment of pSW211 was isolated and subcloned with pGKV10 as a vector. The resulting construct, designated pSW221 (Fig. 3B), conveyed to both MG1614 and LM0230 a nisin resistance phenotype identical to that coded by pSW211.

The origin of the 3.8-kbp *Eco*RI fragment was confirmed by hybridization experiments. A probe prepared from this fragment hybridized both with the 7.5-kbp *Hin*dIII fragment and with the original pSF01 (Fig. 4).

Cloning of nisin resistance on pVS34. The 3.8-kbp *Eco*RI fragment of pSW211 was ligated to the single *Eco*RI site of pVS34. MG1614 protoplasts were transformed with this ligation mixture with selection for chloramphenicol resistance. Transformants were tested for nisin resistance and analyzed for plasmid content. The restriction map of one of the nisin-chloramphenicol double-resistance plasmids, denoted pVS39, is shown in Fig. 1B.

Excision of streptococcal chloramphenicol resistance gene from pVS39. To construct a nisin resistance plasmid containing only lactococcal DNA, pVS39 was digested with *Cla*I,

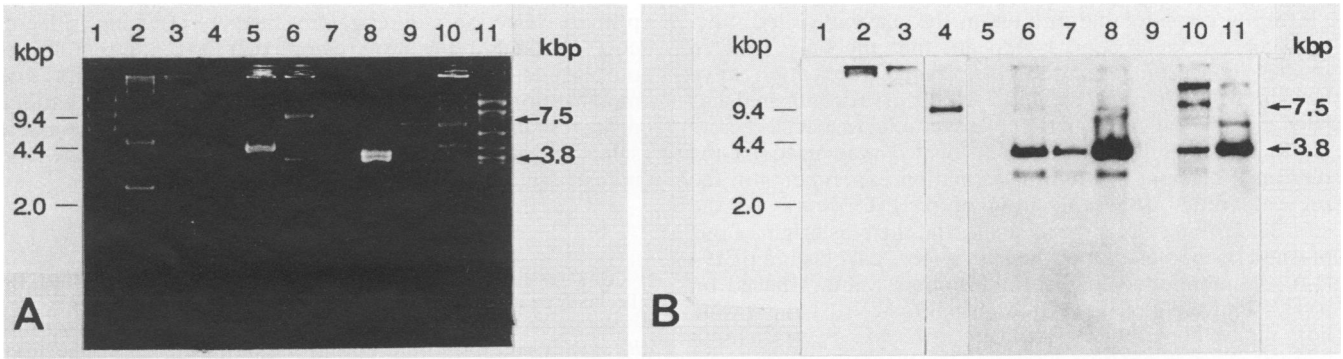

FIG. 4. Southern hybridization of nisin resistance determinant to various plasmids described in this study. (A) Agarose gel (0.8%). (B) Hybridization membrane. Probe consisted of the 3.8-kbp *Eco*RI fragment of pSW221. Lanes: 1, lambda DNA digested with *Hin*dIII (molecular size marker); 2, total plasmid DNA of strain 10.084; 3, pSF01; 4, pSF01 digested with *Hin*dIII; 5, pGKV10 digested with *Eco*RI; 6, pSW211 digested with *Eco*RI; 7, pSW221 digested with *Eco*RI; 8, pvS40 digested with *Eco*RI; 9, pVS34 doubly digested with *Eco*RI and *Hin*dIII; 10, total plasmid DNA of strain 10.084 digested with *Eco*RI; 11, pSF01 digested with *Eco*RI. The molecular size markers are indicated on the left. The size of the *Hin*dIII Nis^r fragment and its *Eco*RI subfragment are indicated on the right. The Boehringer-Mannheim Nonradioactive DNA Labeling and Detection Kit was used.

TABLE 3. Electroporation of MG1614 with plasmids pSW211 and pVS40[a]

Plasmid	Primary selection agent[b] and its concn	Transformants/μg of DNA
pSW211	Cm (5 μg/ml)	6×10^5
	Nis (7 IU/ml)	2.4×10^5
pVS40	Nis (7 IU/ml)	1.7×10^3

[a] The numbers are results of representative experiments.
[b] Nis, Nisin; Cm, chloramphenicol.

and the 7.8-kbp *Cla*I fragment containing both the nisin resistance and replication regions of pVS39 was isolated from an agarose gel and self-ligated. This DNA was used together with similarly topologically relaxed plasmid DNA (coding for chloramphenicol resistance) to transform MG1614 protoplasts. The plasmid used in the cotransformation, pVS1, had been first linearized with *Cla*I and self-ligated again. The ratio of the 7.8-kbp *Cla*I fragment of pVS39 to pVS1 DNA in the cotransformation mixture was about 3 to 1. Chloramphenicol-resistant transformants were selected and then tested for resistance to nisin. Three doubly resistant clones were found among the 20 tested, and all contained two plasmids corresponding in size to intact pVS1 and covalently closed supercoiled 7.8-kbp DNA. One of the clones was arbitrarily chosen for novobiocin curing of pVS1. Nisin (500 IU/ml) was used in both agar layers of novobiocin gradient plates. One of 100 purified single colonies from the novobiocin plates was nisin resistant and chloramphenicol sensitive. It contained a single plasmid representing the self-ligated 7.8-kbp *Cla*I fragment of pVS39. This plasmid was designated pVS40 (Fig. 1C).

Nisin resistance as the primary selection marker. A series of electroporation experiments were conducted to evaluate the usefulness of nisin resistance as a primary selection marker for pVS40 in the lactococci. As a control, parallel experiments were performed with pSW211, plating half of the electroporation mixture on selection plates containing nisin and the other half on chloramphenicol plates. With pSW211, the nisin selection and the chloramphenicol selection give equal transformation frequencies (Table 3). The efficiency of transformation with pVS40 is 2 magnitudes lower than with pSW211.

DISCUSSION

Despite considerable interest in the plasmid-coded functions of the lactococci, it appears that the only detailed studies concerning lactococcal plasmid replication have been done with pSH71. This 2.1-kbp cryptic plasmid has been used in the construction of several lactococcal vectors (7, 8, 12), including pVS2 (32), which was used as the reference plasmid in the transformation experiments in the present study. The replication of pSH71 occurs via the so-called rolling circle mechanism, and the plus origin of this plasmid resembles that of the staphylococcal plasmid pE194 (14). As mentioned in the Introduction, vectors based on pSH71 and the closely related plasmid pWV01 have a wide host range, being able to replicate in *E. coli* as well as in a variety of gram-positive bacteria. On the other hand, very little is known about the replication mechanisms and host range of the large lactococcal plasmids, although most of the plasmids coding for technologically interesting metabolic functions have a size of more than 20 kbp. Recently Hayes et al. (15) have published the minimal replicon of a cryptic 8.7-kbp lactococcal plasmid, pCI305. The replication region of this plasmid spans 1.6 kbp and contains both a gene for a *trans*-acting replication protein and a separate site for an origin of replication. The replication machinery of pCI305 seems to have a limited ability to function outside the lactococci. The present report describes isolating another replication region from a large, cryptic lactococcal plasmid and marking it with an antibiotic resistance gene to obtain an easily selectable replicon with a size practical for genetic manipulations.

This study describes the family of plasmids pVS34, pVS39, and pVS40, all of which have a replication region derived from *L. lactis* subsp. *lactis* biovar diacetylactis SSD207. At least some function essential for replication is located within a 2.5-kbp fragment. In contrast to lactococcal vectors based on pSH71 or pWV01, the replication region described here derives from a large plasmid and does not allow transformation of *E. coli* or *B. subtilis* (Table 2). These differences in size and host range may reflect a mechanism of replication other than the rolling circle pathway characteristic of many of the small plasmids in gram-positive bacteria.

Plasmid pVS40 differs from the rest of the constructions described here in that it contains only lactococcal plasmid DNA. As the selectable marker it carries the nisin resistance determinant of *L. lactis* subsp. *lactis* 10.084. Although the transformant yields are lower than those obtained with pSW211, primary selection of pVS40 with nisin is fully possible (Table 3). The reason for the difference in transformant numbers between these two plasmids is, at present, not known. This phenomenon might reflect differences in plasmid copy numbers, but in the absence of actual copy number determinations, this remains speculative. (Similar transformation results have been obtained with LM0230 [data not shown].) On the basis of these results, pVS40 could be acceptable as a first-generation food-grade cloning vector or marker plasmid for cotransformation of useful plasmids into starter strains. Previously, nisin resistance has been considered as a useful secondary marker in genetic experiments (10). Further research is needed to determine whether the successful primary selection for nisin resistance reported here depends on the particular nisin resistance determinant isolated or on the plasmid vehicle and selection conditions used. The unique *Xho*II site in pVS40 has been used successfully in cloning the lactose fermentation genes on the *Bcl*I B fragment of a derivative of the lactococcal plasmid pLP712 (unpublished data). These first experiments with a narrow-host-range lactococcal replicon and a lactococcal primary selection marker justify further screening of lactococcal plasmid replication regions that have different effects on plasmid copy number, host range, incompatibility, and mobilization. By subsequently joining such fragments to the nisin resistance marker or some other ethically acceptable selectable gene, a series of cloning vectors useful in the food industry may be generated.

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