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Single-Crossover Integration in the *Lactobacillus sake* Chromosome and Insertional Inactivation of the *ptsI* and *lacL* Genes

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Single-crossover homologous integration in *Lactobacillus sake* was studied. Integration was conducted with nonreplicative delivery vector pRV300. This vector is composed of a pBluescript SK$^-$ replicon for propagation in *Escherichia coli* and an erythromycin resistance marker. Random chromosomal DNA fragments of *L. sake* 23K ranging between 0.3 and 3.4 kb were inserted into pRV300. The resulting plasmids were able to integrate into the chromosome by homologous recombination as single copies and were maintained stably. The single cross-over integration frequency was logarithmically proportional to the extent of homology between 0.3 and 1.2 kb and reached a maximum value of 1.4×10^3 integrants/μg of DNA. We used this integration strategy to inactivate the *ptsI* gene, encoding enzyme I of the phosphoenolpyruvate:carbohydrate phosphotransferase system, and the *lacL* gene, which is one of the two genes required for the synthesis of a functional β-galactosidase. The results indicated that our method facilitates genetic analysis of *L. sake*.

Lactobacillus sake is the predominant species in the microflora of fermented meat. Its use as a starter culture for the preparation of dry sausages and other raw fermented meat products may help in the development of quality traits, such as texture, color, taste, aroma, and safety (2, 8, 31). In addition, this bacterium is thought to have a beneficial effect on the extension of the storage life of vacuum- or modified gas atmosphere-packed raw meat (21, 26). To best use the biotechnological potential of this organism in practical applications and to carry out a strain improvement program, detailed knowledge of its cellular metabolism and gene expression and regulation is required.

However, only five *L. sake* chromosomal gene clusters have been cloned and sequenced (10, 19, 25, 28, 30), and it is clear that a number of important tools for genetic manipulation of *L. sake*, including chromosomal integration systems, remain to be developed in order to engineer strains for agroindustrial use. In lactic acid bacteria (LAB), the most straightforward approach of integration relies on a nonreplicating plasmid (usually a conventional *Escherichia coli* ColE1-based vector) which carries a cloned region of DNA homologous to a particular target within the recipient strain chromosome and an antibiotic resistance marker (5, 16). The introduction of such a plasmid yields transformants when a single crossover (SCO) between the plasmid and the recipient chromosome occurs at the region of homology, which results in integration of the entire plasmid flanked by copies of the homologous DNA. This Campbell-like integration event was demonstrated first in *Lactococcus lactis* by Leenhouts et al. (13) and subsequently has been exploited in many LAB species for gene disruption and heterologous gene expression (5, 16) (for reviews, see references 6 and 17).

The introduction of a selectable marker by SCO recombination often appears to be unstable, and as a consequence, antibiotics are still needed to maintain the integrities of integrated structures in resulting strains. In *Lactococcus lactis*, use of the erythromycin resistance gene as a selective marker facilitates the stable maintenance of single copies of some integrated plasmids (5, 14). SCO integration after transformation generally occurs at a low frequency (6, 17). In practice, it is limited to bacteria that can be transformed at a high frequency. We used these observations to devise a strategy by which single copies of a selectable marker can be introduced into the *L. sake* chromosome by homologous recombination. Recently, we reported an optimized electrotransformation procedure for *L. sake* 23K which yields up to 10^6 transformants per μg of DNA with selection for erythromycin resistance (3). Here, we employed this high level of transformability and the nonreplicating plasmid pRV300 to inactivate genes in the *L. sake* chromosome by homologous recombination. This plasmid is essentially a pBluescript SK$^-$ replicon (Stratagene) carrying the erythromycin resistance marker of plasmid pAMβ1 from *Streptococcus faecalis*, which is expressed in gram-positive bacteria. Replication of this plasmid is not functional in *L. sake*. We used this delivery vector to examine SCO integration at several random locations on the *L. sake* chromosome and at a defined locus. Stable single-copy integration was obtained. Integration frequencies ranged from 10^{-5} to 10^{-3} and were affected by the lengths of homologous sequences. We observed a log-linear relationship between the extent of homology and integration frequency for DNA inserts of between 0.3 and 1.2 kb and almost constant recombination frequencies for larger inserts, corresponding to approximately 10^3 integrants/μg of DNA. We also observed that besides its length, the region of homology and its location influenced integration frequency.

We also disrupted the *L. sake* 23K *ptsI* gene, which codes for enzyme I of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), and constructed a *lacL* mutant in which β-galactosidase activity was suppressed. The effects of the *ptsI* gene disruption on the growth rate and carbohydrate transport systems in *L. sake* have been discussed elsewhere (25).

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TABLE 1. List of strains and plasmids

Strain or plasmid	Genetic marker(s) and/or description	Source or reference
Strains		
L. sake		
23K	Plasmid free	3
160x1K	Plasmid free	3
DSM 20017	Natural isolate	Deutsche Sammlung von Mikroorganismen
RV1000	23K *ptsI*::pRV10	This work
RV3018	23K *lacL*::pRV3016	This work
E. coli		
TG1	*supE* Δ*hsd-5 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	20
KA 796	*ara thi* Δ(*lac-proAB*)	R. Schaaper
Plasmids		
pBluescript SK$^-$	Cloning vector; Ap^r	Stratagene
pGK12	Cloning vector; Cm^r Em^r	11
pIL253	Cloning vector; Em^r	23
pRB473	*E. coli* and *B. subtilis* shuttle vector; Ap^r Bm^r Cm^r	R. Bruckner
pMOB1000	8.65-kb *Hin*dIII *lac* fragment of the DSM 20017 chromosome in pRB473	19
pVE6023	1,113-bp *Hha*I-blunt-ended pIL253 fragment joined to the *Hin*cII-restricted pUC1318; Ap^r Em^r	E. Maguin
pRV300	*Ssp*I-restricted pBluescript SK$^-$ joined to a 1,130-bp *Sma*I fragment of pVE6023; Ap^r Em^r	This work
pRV3001 to pRV3014	*Hin*dIII-, *Eco*RV-, or *Eco*RI-restricted pRV300 joined to a *Hin*dIII, *Eco*RV, or *Eco*RI random chromosomal fragment of 23K; Ap^r Em^r	This work
pRV3015	*Eco*RV-*Hin*dIII deletion of pRV3001A, leaving a 1.2-kb fragment	This work
pRV3016	*Eco*RI-*Hin*dIII and *Eco*RV-*Hin*dIII deletion of pRV3001A, leaving a 0.7-kb fragment	This work
pRV3017	*Eco*RV deletion of pRV3001A, leaving a 0.5-kb fragment	This work
pRV3018	*Cla*I-*Eco*RI 1,563-bp *lacL* fragment from pMOB1000 joined to *Cla*I-*Eco*RI-restricted pRV300	This work
pRV5	*Eco*RV-*Hin*dIII-restricted pBluescript SK$^+$ joined to a 3.2-kb *Eco*RV-*Hin*dIII *ptsI* chromosomal fragment of 160x1K	25
pRV10	*Eco*RV-*Hin*dIII-restricted pRV300 joined to the 716-bp *Hin*dIII-*Hpa*I internal *ptsI* fragment from pRV5	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. From a phylogenetic point of view, *L. sake* 160x1K, 23K, and DSM 20017 are closely related, as deduced from random amplified polymorphic DNA grouping (2a). *E. coli* strains were grown in Luria-Bertani broth at 37°C with vigorous agitation. *L. sake* strains were grown at 30°C in DeMan-Rogosa-Sharpe (MRS) medium with 0.5% glucose, unless otherwise indicated. Solidified growth media contained 1.5% agar. Erythromycin was added at 5 μg/ml for *L. sake* and 150 μg/ml for *E. coli*, and ampicillin was used at 100 μg/ml for *E. coli.*

Molecular cloning techniques. The molecular cloning techniques used were essentially those of Sambrook et al. (20). DNA was introduced into *E. coli* by electrotransformation (7). Electroporation of *L. sake* was performed by the method of Berthier et al. (3) and yielded approximately 10^6 transformants per μg of plasmid DNA for 23K.

Total DNA from *L. sake* was prepared by the method of Anderson and McKay (1). To detect integration by homologous recombination, purified DNA was treated with restriction endonucleases and separated by agarose gel electrophoresis (20). Gels were analyzed by Southern hybridizations (24) with nick-translated (20) DNA probes.

Construction of the nonreplicative delivery vector. A 1,130-bp *Sma*I fragment carrying the pAMβ1 Em^r gene from plasmid pVE6023 was ligated into the *Ssp*I restriction sites of pBluescript SK$^-$ (Stratagene). This ligation resulted in two 3,539-bp plasmids, depending on the Em^r gene orientation. The pRV300 derivative, which harbors the Em^r gene in the same orientation as the direction of pBluescript replication, was retained for further experiments.

Cloning of random *L. sake* chromosomal fragments into pRV300. Chromosomal DNA of *L. sake* 23K was digested to completion with *Eco*RI, *Eco*RV, or *Hin*dIII. Chromosomal fragments of between 0.3 and 3.4 kb were purified from agarose gels with Geneclean (Bio 101, Inc., La Jolla, Calif.) and ligated to pRV300 digested with *Eco*RI, *Eco*RV, or *Hin*dIII enzymes. Recombinant plasmids were established in *E. coli* TG1 cells. DNA was isolated from independent recombinant clones to verify the plasmid structures and insert sizes. The plasmids used for SCO integration into the *L. sake* chromosome were designated pRV3001 to pRV3014. Suffix letters (A, B, and C) designate plasmids with inserts of the same size (see Table 2). Plasmids were purified by CsCl density gradient centrifugation.

Deletion of a chromosomal fragment of unknown function. Recombinant plasmid pRV3001A, which harbors a 3.4-kb *Eco*RI fragment of the *L. sake* 23K chromosome, was used to generate a set of deletion derivatives in the DNA insert by restriction digestion. For plasmid constructions and insert sizes, see Fig. 4.

SCO integration into the *L. sake* chromosome. Recombinant plasmids were used to electrotransform *L. sake* 23K cells. Transformants were selected at 30°C on MRS agar containing 5 μg of erythromycin per ml. Plasmid pGK12 (11), which replicates in *L. sake*, was used as a control for transformation efficiency. Integration frequencies were estimated as ratios of the numbers of Em^r clones obtained with the test plasmids to the numbers of Em^r transformants obtained with the replicating pGK12 plasmid. To analyze integrants, restriction enzymes *Sna*BI and *Ssp*I, which cut only the vector moiety, were used.

Construction of a *ptsI* mutant. An internal 716-bp *Hin*dIII/*Hpa*I fragment of the *ptsI* gene from plasmid pRV5 (25) was cloned in pRV300 digested with *Eco*RV and *Hin*dIII by using *E. coli* TG1. This plasmid, pRV10, was used to disrupt the *ptsI* gene in *L. sake* 23K. The resulting *ptsI* mutant strain was named RV1000.

Construction of a *lacL* mutant. An internal 1,563-bp fragment of the *lacL* gene, originating from *L. sake* DSM 20017 and further cloned on plasmid pMOB1000 (19), was amplified by PCR with two oligonucleotides of 21 bp, corresponding to amino acids 24 to 27 and 537 to 541. To facilitate further cloning, an additional tail of nine nucleotides containing a *Cla*I restriction site was added at the 5′ end of the coding-strand primer. The complementary-strand primer carried an *Eco*RI restriction site at its 5′ end. The amplified *Cla*I-*Eco*RI fragment was cloned in integration insertion vector pRV300 by using a Δ*lac E. coli* strain, KA 796. This plasmid, pRV3018, was used to disrupt the *lacL* gene in *L. sake* 23K. The resulting *lacL* mutant strain was named RV3018.

Determination of the stability of integrants. To assess the stability of the Em^r phenotype of transformants, cells were grown in the absence of this antibiotic. In the case of *ptsI* mutant RV1000, MRS supplemented with gluconate was used, whereas for the other strains tested, glucose was used as the carbohydrate source. After approximately 100 generations, the number of cells in each culture and the

TABLE 2. Integration frequencies of nonreplicating plasmids in *L. sake*[a]

Plasmid(s)	Insert (kb)	Mean integration frequency ± SD[b]
pRV3001(A, B)	3.4	$(1.5 \pm 0.1) \times 10^{-3}$
pRV3002	2.7	1.0×10^{-3}
pRV3003(A, B)	2.6	$(4.7 \pm 1.4) \times 10^{-5}$
pRV3004	2.3	6.7×10^{-5}
pRV3005(A, B)	2.0	$(7.6 \pm 1.4) \times 10^{-4}$
pRV3006	1.8	7.2×10^{-4}
pRV3007(A, B)	1.2	$(1.4 \pm 0.3) \times 10^{-3}$
pRV3008(A–C)	1.0	$(8.5 \pm 0.7) \times 10^{-4}$
pRV3009(A, B)	0.9	$(6.2 \pm 1.3) \times 10^{-4}$
pRV3009C	0.9	4.2×10^{-5}
pRV3010	0.8	2.0×10^{-4}
pRV3011	0.6	1.1×10^{-4}
pRV3012	0.5	1.0×10^{-4}
pRV3013(A–E)	0.4	$(6.9 \pm 2.0) \times 10^{-5}$
pRV3014	0.3	6.4×10^{-5}

[a] Random *L. sake* 23K chromosomal fragments were generated with different enzymes as described in Materials and Methods. The fragments were cloned into nonreplicating vector pRV300. Plasmid names and insert sizes are indicated.

[b] For each insert size, one to five measurements of integration frequency were performed.

proportion of Em^r cells were determined by plating appropriate dilutions on a nonselective medium and replica plating about 3,000 colonies on selective and nonselective media.

Recombination between the repeated *lacL* fragment in strain RV3018 restores a functional *lacL* gene. Therefore, the recombination frequency was calculated from the proportion of $LacZ^+$ cells from the population of blue colonies formed on MRS agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Cells were grown at 30°C in MRS medium for at least 100 generations. The number of cells in the culture and the proportion of parental and recombinant cells were determined by spreading appropriate dilutions on nonselective MRS agar plates supplemented with 80 μl of X-Gal (2% [wt/vol]).

RESULTS

Integration into the *L. sake* chromosome by a Campbell-like mechanism. Plasmid pRV300 was constructed; it carries an origin of replication for *E. coli* (pBluescript SK^-) not for *L. sake* and the constitutive Em^r gene from pAMβ1. We examined whether pRV300 could be used as a vehicle to insert DNA sequences into the chromosome of *L. sake*. Random *L. sake* chromosomal fragments were cloned into pRV300 in *E. coli* TG1. These fragments were of 14 sizes, ranging from 0.3 to 3.4 kb, and the resulting plasmids were designated pRV3001 to pRV3014 (Table 1). Some plasmids carried different inserts, indistinguishable in size, and were labelled by a suffix, A to E (Table 2). A total of 26 plasmids were constructed and used to measure integration frequencies along the *L. sake* chromosome. For this purpose, *L. sake* 23K was transformed by electroporation with 500 ng of each recombinant plasmid, with selection for Em^r (see Materials and Methods). As a control, the *Lactococcus lactis* 4,377-bp cloning vector pGK12 (11), which replicates in *L. sake*, was used to transform competent cells to Em^r. The transformation frequency of pGK12 was constant (2×10^6 transformants per μg of pGK12 DNA). Since comparable frequencies were obtained with various pGK12 derivatives, ranging from 3.2 to 5 kb (data not shown), strain 23K competence was used as an internal standard. Thus, plasmid integration frequencies, relative to the transformation frequency of replicating plasmid pGK12, were deduced from the proportion of transformants in two experiments. They ranged between 10^{-3} and 10^{-5} (Table 2). No transformant was obtained with vector pRV300 without a chromosomal insert (frequency, $<2 \times 10^{-7}$).

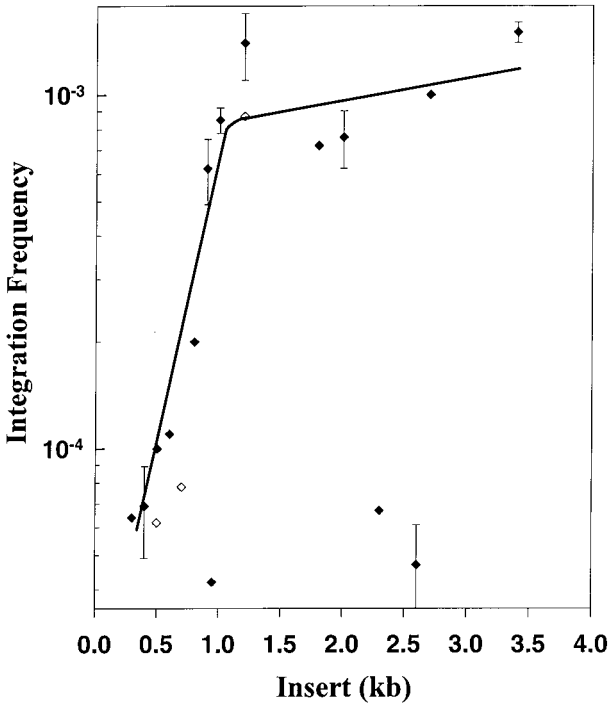


FIG. 1. Relationship between insert size and integration frequency. For each insert size (Table 2), one to five measurements of integration frequency were performed; the means and standard deviations are indicated when available. A set of three deletion derivatives were made from pRV3001 with different enzymes (Fig. 4). The integration frequencies obtained with these plasmid derivatives are indicated by open symbols.

A log-linear relationship between the integration frequency and extent of homology was observed for most inserts of between 0.3 and 1.2 kb, with a slope of 1.46 per 100 bp (Fig. 1). For most fragments of between 1.2 and 3.4 kb, integration frequencies appeared to reach a plateau of about 10^{-3}, which corresponds on average to an efficiency of 1.4×10^3 integrants/μg of DNA. No transformants were obtained with three 290-bp internal fragments of nonessential genes (integration frequency, $<10^{-6}$ [7a]).

Some plasmids integrated at very low frequencies that did not fit the log-linear curve (around 5×10^{-5} for pRV3003, pRV3004, and pRV3009C), indicating that factors other than insert length are implicated (see below).

Two transformants obtained with each of the recombinant plasmids were analyzed by Southern hybridization. Total DNA was extracted from each transformant and from strain 23K and then was digested with several restriction enzymes for which unique sites were present on the vector part of the plasmids but not on the chromosomal DNA insert. The results of one hybridization experiment, using plasmid vector pRV300 as a probe, are presented in Fig. 2. Southern hybridization of undigested total DNA revealed the absence of free plasmid, since the only signal detected was located at the chromosomal level (Fig. 2A). We digested total DNA with *Sna*BI, which recognizes a single site within the vector. Figure 2B shows that two junction segments were generated for each sample; all the electrophoretic patterns were different. These results indicate that each plasmid integrated at a distinct position. Data supporting Campbell-like integration were obtained by treating integrant DNAs with either *Hin*dIII, *Eco*RI, or *Eco*RV. These restriction sites are at the vector-insert junction, and digestion should release a single linear plasmid vector band. Indeed, Fig.

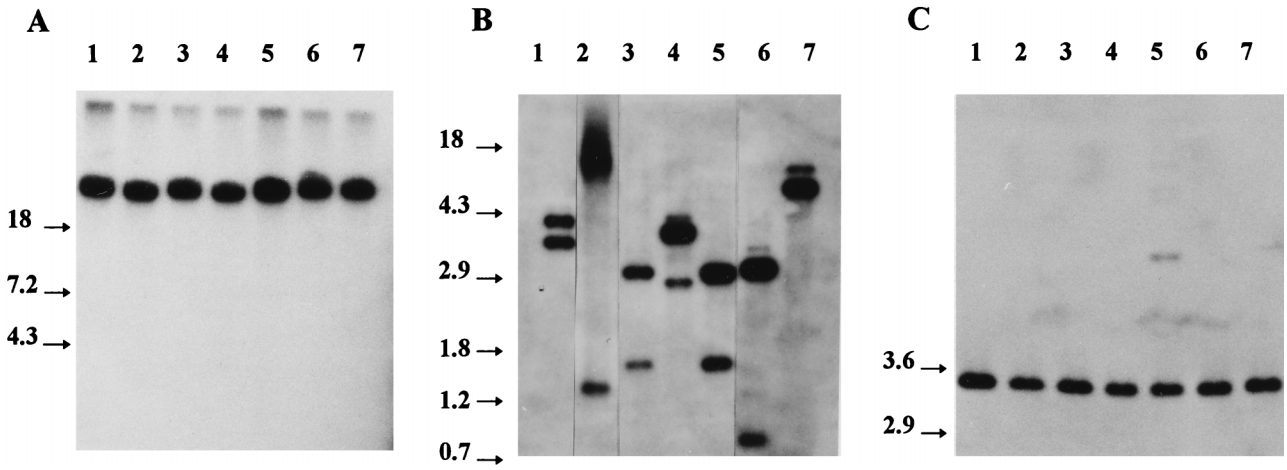


FIG. 2. Campbell-like integration of pRV300 derivative plasmids along the chromosome of *L. sake* 23K. Chromosomal DNAs of integrants were analyzed by agarose gel electrophoresis and Southern hybridization with pRV300 as a probe. The integrants analyzed were from the following integration plasmids: pRV3011 (lanes 1), pRV3009A (lanes 2), pRV300B (lanes 3), pRV3007B (lanes 4), pRV3006 (lanes 5), pRV3002 (lanes 6), and pRV3001B (lanes 7). The supercoiled DNA ladder (A) and Raoul marker (Appligene) (B and C) were used as DNA standards. DNA sizes (in kilobases) are indicated on the left. (A) Undigested chromosomal DNA was used. (B) Chromosomal DNA digested with *Sna*BI, which recognizes a single site within the pRV300 vector, was used. (C) Chromosomal DNA digested with either *Hin*dIII, *Eco*RI, or *Eco*RV (depending on the insert fragment), with sites located at the vector-insert junction, was used.

2C shows a hybridizing fragment of 3.4 kb which was present in each transformant DNA but not in the digest of the total DNA of strain 23K.

To prove SCO integration, we used the chromosomal inserts as probes. Probing chromosomal DNA from the nontransformed host strain with inserts should reveal the corresponding DNA fragments. After Campbell-like integration, these hybridizing bands should be absent in the DNA digests of transformants (Fig. 3B). To verify this prediction, we treated total DNA from transformants obtained with plasmid pRV3007A and from nontransformed strain 23K with *Ssp*I. *Ssp*I has a unique restriction site in the vector part. The results are presented in Fig. 3. Probing with the 1.2-kb insert showed a 9-kb band hybridizing with the 23K host DNA (Fig. 3A, lane 1). This band disappeared in the DNAs of transformants (Fig. 3A, lanes 2 and 3). Instead, two hybridizing bands of the expected sizes were detected. These signals were of equal intensity and corresponded to duplication of the insert. A similar analysis of several recombinants obtained after transformation with plasmids pRV3001A and pRV3005A revealed that the plasmids were also integrated by SCO (data not shown).

Integration frequencies at one chromosomal locus. Deletions in the 3.4-kb chromosomal insert from plasmid pRV3001 were generated and cloned into pRV300 (Fig. 4). While plasmid pRV3001, carrying the entire 3.4-kb insert, integrated into the chromosome at a frequency of 2.9×10^{-3} (in the plateau of the curve), the three deletion derivatives (0.5, 0.7, and 1.2 kb) showed integration frequencies that fit the log-linear portion of the curve in Fig. 1.

Insertional inactivation of the *L. sake* 23K *ptsI* gene. The *ptsH* and *ptsI* genes of *L. sake* 160x1K, encoding the general enzymes of the PTS system, have previously been cloned and sequenced (25). The *ptsI* gene of *L. sake* 23K was inactivated by gene disruption with a pRV300-based integration plasmid harboring an internal 716-bp *Hin*dIII/*Hpa*I fragment of the *ptsI* gene. This plasmid, pRV10, was transferred to *L. sake* 23K by electroporation and plating on MRS-erythromycin supplemented with gluconate as a non-PTS carbohydrate source. Em^r clones were obtained with a frequency of 1.4×10^{-4}, which was expected from the extent of homology. Five randomly chosen Em^r colonies were propagated on a chemically defined medium, MCD (12), supplemented with various PTS and non-PTS carbohydrates. As expected, the five clones did not grow on sucrose, fructose, mannose, or *N*-acetylglucosamine, all of which were demonstrated to be PTS carbohydrates in *L. sake* (12). In contrast, they all grew on ribose and arabinose, two non-PTS carbohydrates, with improved rates compared to the

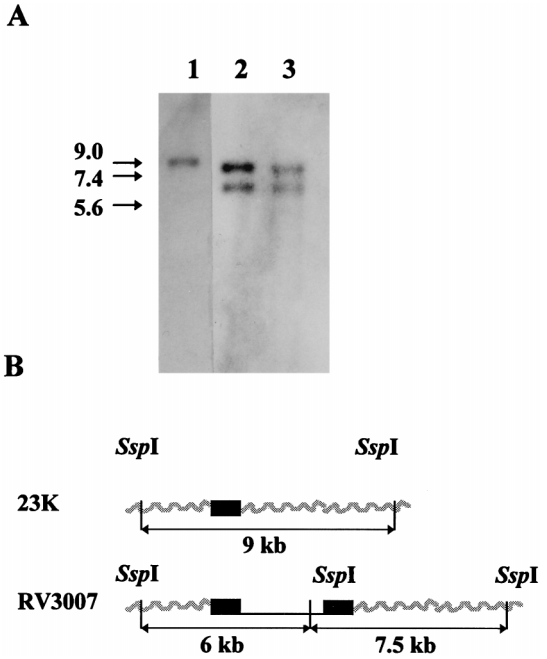
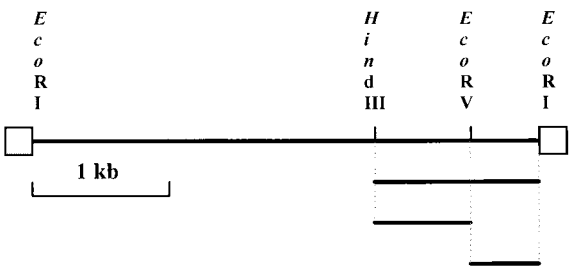


FIG. 3. (A) Southern hybridization analysis of *L. sake* 23K and a strain containing pRV3007B integrated into its chromosome. Chromosomal DNA was digested with *Ssp*I, separated by agarose gel electrophoresis, and hybridized with the 1.2-kb insert fragment as a probe. Lane 1, strain 23K; lanes 2 and 3, two independent integrants obtained with plasmid pRV3007B. The Raoul marker was used as a reference, and DNA sizes (in kilobases) are indicated on the left. (B) Schematic representations of the relevant parts of the chromosomes of strain 23K and a transformant strain after Campbell-like integration of plasmid pRV3007B. A solid box indicates the cloned 1.2-kb insert fragment.



Plasmid	Insert size (kb)	Integration frequency (mean ± SD)
pRV3001	3.4	$(2.2 \pm 0.7) \times 10^{-3}$
pRV3015	1.2	$(8.7 \pm 1.2) \times 10^{-4}$
pRV3016	0.7	$(7.8 \pm 0.6) \times 10^{-5}$
pRV3017	0.5	$(6.2 \pm 0.1) \times 10^{-5}$

FIG. 4. Frequencies of integration of plasmid pRV3001 deletion derivatives. A 3.4-kb *Eco*RI fragment of the *L. sake* 23K chromosome of unknown content was cloned into pRV300, generating pRV3001. Three deletion derivatives were constructed from pRV3001 with different enzymes, as shown in the schematic representation. Solid boxes indicate pRV300 sequences. Plasmid names and insert sizes are shown. For each plasmid, at least two independant measurements were done; the means and standard deviations (SD) are also indicated.

growth of the wild-type strain, whereas the growth on glucose was slightly impaired (25).

To demonstrate that disruption of the *ptsI* gene was the result of integration of pRV10, Southern hybridizations were conducted with chromosomal DNAs from one mutant strain, RV1000, and wild-type strain 23K, with the 716-bp *Hin*dIII/*Hpa*I fragment of the *ptsI* gene as a probe (Fig. 5). The *ptsI* probe hybridized with an ~20-kb *Bam*HI fragment of *L. sake* 23K. This signal disappeared in *ptsI* mutant RV1000, and

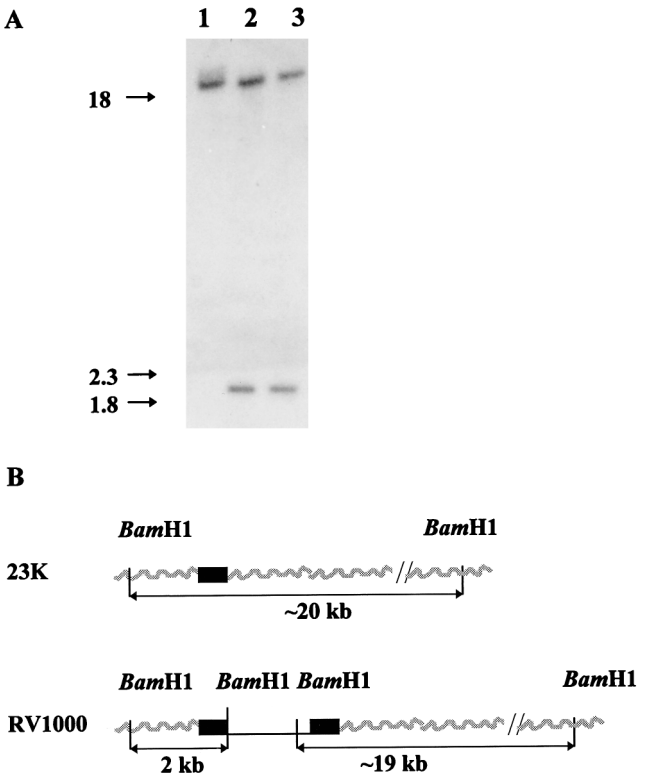


FIG. 5. (A) Southern hybridization analysis of *L. sake* 23K and *ptsI* mutant RV1000. Chromosomal DNA was digested with *Bam*HI, separated by agarose gel electrophoresis, and hybridized with the 716-bp *Hin*dIII/*Hpa*I fragment of the *ptsI* gene as a probe. Lane 1, strain 23K; lanes 2 and 3, two independent integrants obtained with plasmid pRV1000. The Raoul marker was used as a reference, and DNA sizes (in kilobases) are indicated on the left. (B) Schematic representations of the relevant parts of the chromosomes of strain 23K and *ptsI* mutant RV1000 after Campbell-like integration of plasmid pRV1000. A solid box indicates the cloned 716-bp *Hin*dIII/*Hpa*I insert fragment.

two fragments of 2 and 19 kb hybridized with the probe. Similar results were obtained with three other mutant clones (data not shown).

The effects of loss of PTS enzyme I activity, growth, and involvement in non-PTS carbohydrate utilization have been discussed elsewhere (25).

Inactivation of the *L. sake* 23K *lacL* gene encoding β-galactosidase. Sequence analysis revealed that the β-galactosidase of *L. sake* DSM 20017 is encoded by two overlapping genes, *lacL* and *lacM* (19). *E. coli* clones containing *lacL* and *lacM* genes were kindly provided by M. Obst, Stuttgart, Germany. The results of hybridization experiments revealed a similar gene organization in our *L. sake* laboratory strain, 23K (data not shown). Therefore, we disrupted the *lacL* gene of strain 23K by the insertion of integration vector pRV3018, which contained a 1,563-bp internal *lacL* segment from strain DSM 20017 (for construction of plasmid pRV3018, see Materials and Methods). Em^r clones were recovered at a frequency of $6 \times 10^{-4}/\mu g$ of DNA, which is close to the value expected for the length of the homologous insert. The absence of β-galactosidase expression in the disruption mutants was indicated by the formation of white colonies on MRS agar containing X-Gal (*L. sake* 23K harboring the pAMβ1 derivative pIL253 [23] was used as the $LacZ^+$ control strain). The $LacZ^-$ phenotype of disruption mutants was further confirmed by API ZYM test (Biomerieux). The Southern hybridization pattern obtained with a clone chosen randomly indicated that there was Campbell-like integration of pRV3016 into the *lacL* gene on the chromosome of strain 23K (data not shown).

Stability of the Em^r phenotype. To estimate the stability of the integrated plasmid, different strains were grown in MRS broth in the absence of erythromycin for 100 generations and plated on media with and without this antibiotic. Wild-type strain 23K, in which replicative plasmid pIL253 is stably maintained, was used as a control. No significant differences in colony counts on the two media were observed for strains RV3006 and RV3009. Replica plating of colonies formed on nonselective media revealed Em^s cells, arising from the loss of the integrated plasmid, at frequencies of 9.2×10^{-5} and 2.5×10^{-4}, respectively (Table 3). This indicates rather stable maintenance of the integrated plasmid flanked by homologous repeats of 1.8 and 0.9 kb, respectively. A similar stability was observed with *lacL* strain RV3018, where the loss of plasmid was assessed by restoration of the $LacZ^+$ phenotype, yielding blue colonies on plates supplemented with X-Gal (Table 3). In contrast, the stability of the integrated plasmid in *ptsI* strain RV1000 was much lower, since only 13% of cells remained Em^r after 30 generations of growth without this antibiotic

TABLE 3. Stabilities of nonreplicating plasmids integrated into the *L. sake* chromosome

Strain	Em^r CFU (%) after:		Recombination frequency[c]
	Plating[a]	Replica plating[b]	
23K(pIL253)	100	100	$<1.1 \times 10^{-5}$
RV1000	13[d]	ND[e]	ND
RV3006	98	99	9.2×10^{-5}
RV3009	92	97	2.6×10^{-4}
RV3018	99	ND	5.9×10^{-5}

[a] Values were determined by direct plating onto selective and nonselective media or by plating onto MRS agar supplemented with X-Gal after growth for 100 generations.

[b] Values were determined by plating onto nonselective medium and replica plating an average of 3,000 colonies onto selective medium.

[c] The recombination frequency is the proportion of culture which lost the integrated plasmid divided by the number of generations of nonselective growth (~100 generations).

[d] Values were determined after 30 generations of growth under nonselective conditions.

[e] ND, not done.

(Table 3). Analysis of the Em^s progeny showed reversion to the Pts$^+$ phenotype and the absence of sequences homologous to the integration vector (data not shown).

DISCUSSION

Here, we have described the construction and use of an integration vector (pRV300) for *L. sake* that consists of a nonreplicative plasmid (pBluescript SK$^-$) and a functional erythromycin resistance gene from *S. faecalis* plasmid pAMβ1. When it carries a homologous chromosomal DNA segment, this plasmid is able to integrate into the chromosome of *L. sake* 23K. This is achieved by the highly efficient transformation procedure we previously developed for this host (3). The integration of DNA into the chromosome occurs through SCO homologous recombination, taking place along the chromosome and with as little as 0.3 kb of homology. The relative integration frequencies for randomly cloned segments (0.3 to 3.4 kb) vary by a factor of about 25 (Fig. 1) and are logarithmically proportional to the extent of homology for inserts of between 0.3 and 1.2 kb. Previous studies of integration by recombination in *Bacillus subtilis* (9, 18) and *E. coli* (18, 27, 32) revealed either a linear or an exponential shape for the yield-versus-insert-size curve, depending on the experimental system used, which were all different from that presented here (interplasmid recombination [18], suicide [27, 32], and thermosensitive [Ts] plasmids [9]). Interestingly, a previous study of SCO integration in *Lactococcus lactis*, with a Ts plasmid derivative of pWV01 (pG$^+$ host), showed a similar log-linear correspondence, although the experimental system used (Ts plasmid delivery vector) was different (4). The reasons for the nonlinear relationship are not known at present. In both *Lactococcus lactis* and *L. sake* chromosomes, the integration frequencies observed with short inserts (0.35 to 1 kb) are similar. However, in *Lactococcus lactis*, they vary by 3 orders of magnitude within the total length range (0.35 to 2.5 kb) and the optimal values are observed with inserts larger than those used in the present study (2.5 instead of 1.2 kb). The difference could be related to the use of nonreplicative and autonomously replicating plasmids in the two studies.

The integration frequencies for randomly cloned inserts of 0.9 kb varied by a factor of about 10 (Fig. 1). Very low integration frequencies were also observed for inserts of 2.3 and 2.6 kb (at the background detection level). This variation could be due to the presence of specific signals on the homologous segment or to the target location, creating mutations and new phenotypes or making important modifications in the local conformation surrounding the segment. Such location-specific effects are expected from random insertions and were previously reported for *Lactococcus lactis* (4) as well as for other bacteria (18, 29).

Campbell-like integration in the *L. sake* chromosome was observed with an approximately 0.3-kb homologous fragment, but not with shorter fragments (290 bp), suggesting that the minimal length for efficient homologous integration is between these two values. Using the pG$^+$ host Ts vector, Biswas and coworkers (4) also noticed that the integration frequencies in *Lactococcus lactis* dropped off dramatically for lengths of between 330 and 356 bp. They proposed that the minimal length for homologous recombination had been reached. The values for the two LAB species are higher than those reported for *B. subtilis* (70 bp) (9) and *E. coli* (23 to 27 bp) (22). The fact that *L. sake* and *Lactococcus lactis* have similar length requirements and a logarithmic relationship for integration frequency versus extent of homology suggests that the cellular factors which are implicated in homologous recombination are similar in these two species.

Interestingly, hybridization analysis showed that the chromosomes of the various strains constructed in this study carry a unique integrated plasmid copy. Amplification concomitant to nonreplicative-plasmid integration was reported for *Lactococcus lactis*. This was probably caused by integration of plasmid multimers (13). No amplification was observed in the *L. sake* chromosome, although some plasmid preparations of pRV300 integration derivatives contained substantial amounts of multimers (data not shown). In addition, higher levels of erythromycin (up to 80 μg/ml) did not yield amplification subsequent to integration of plasmids pRV3015 and pRV3016 (not shown), attesting that a single copy of the Em^r gene is sufficient to confer resistance in *L. sake.*

Using integration vector pRV300, containing an internal coding region of the *L. sake ptsI* gene, we disrupted the chromosomal *ptsI* gene copy. We demonstrated that integration into the chromosome occurred through SCO homologous recombination and that integration took place at the *ptsI* locus. The integrants obtained and characterized by the Em^r phenotype were, as expected, unable to grow on PTS carbohydrates (25). Furthermore, we used gene disruption to inactivate the *L. sake lacL* gene. As expected, no β-galactosidase activity was detected in isolates containing an integrated pRV300. To our knowledge, this is the first report of a phenotypic property of *L. sake* being eliminated by genetic engineering. The use of PtsI$^-$ *L. sake* helped to elucidate the metabolic significance of the PTS system in this LAB species (25), and LacZ$^-$ derivatives should contribute to a better understanding of the physiological role of lactose metabolism in LAB prevailing in meat fermentation (7b).

Maintenance of the integration plasmid copy in the *L. sake* chromosome was variable. For example, after nonselective growth of strains RV3006, RV3009, and RV3018, Em^s cells were generated with very low frequencies, ranging between 5.9×10^{-5} and 2.6×10^{-4} per generation. For strain RV30018, Em^s cells were most probably the result of the precise excision of pRV300 by homologous recombination between the 1.5-kb repeats, which would restore a functional *lacL* gene, since they displayed β-galactosidase activity on X-Gal–MRS plates. In contrast, strain RV1000, which contained a 0.7-kb *ptsI* segment, was highly unstable. Again, the integration plasmid was apparently lost under nonselective growth conditions by a mechanism of precise excision. Variations in the

stability of plasmids integrated in the chromosome of LAB have previously been described. They were attributed either to the residual replicative activity of integration plasmids, which led to their loss, or to recombination events in the directly repeated plasmid DNA when amplified (5, 14, 15). None of these explanations could apply to the present results since the pRV300 vector cannot replicate in the *L. sake* host and no amplification (concomitant or subsequent to integration) was observed in the strains constructed in this study. Therefore, we propose that the stability of each integrated sequence is influenced by the nature and/or location of the insert or that selection leads to the enrichment of recombinant cells. Previous reports have mentioned that the location on the chromosome affected the frequency of recombination in *Lactococcus lactis* (15) and *B. subtilis* (29).

In conclusion, the present study demonstrates that Campbell-like integration is a valuable technique for gaining access to the *L. sake* chromosome. Further work is now required for the development of the gene replacement technique in *L. sake.*

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