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K. Leenhouts · A. Bolhuis · G. Venema · J. Kok

Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*

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Abstract A food-grade vector system was developed that allows stable integration of multiple plasmid copies in the chromosome of *Lactococcus lactis*. The vector consists of the plus origin of replication (Ori^+) of the lactococcal plasmid pWV01, the sucrose genes of the lactic acid bacterium *Pediococcus pentosaceus* PPE1.0 as selectable marker, a multiple-cloning site, and a lactococcal DNA fragment of a well-characterized chromosomal region. The system includes two *L. lactis* strains, LL108 and LL302, which produce the pWV01 RepA protein essential for replication of the Ori^+ vectors. These helper strains allow the construction and isolation of the replicating form of the integration plasmids from a homologous background. Single-cross-over integration of the plasmids in *L. lactis* MG1363 resulted in amplifications to a level of approximately 20 copies/chromosome after selection of the transformants on medium containing sucrose as the only fermentable sugar. The amplifications were stable under selective growth conditions. In glucose-containing medium a limited loss of integrated plasmid copies was detected at a rate of $(7.5–15) \times 10^{-2}$ copies per generation. One strain, MG124, was isolated that had retained 11 integrated copies after a period of 120 generations of non-selective growth. These results show that the single-cross-over integration system described here represents a simple procedure for the engineering of stable food-grade strains carrying multiple copies of a gene of interest.

K. Leenhouts · A. Bolhuis · G. Venema · J. Kok (✉)
Biomolecular Sciences and Biotechnology Institute,
Department of Genetics, University of Groningen,
Kerklaan 30, 9751 NN Haren,
The Netherlands
Tel.: +31 50 632111
Fax: +31 50 632348
e-mail: kokj@biol.rug.nl

Introduction

Lactic acid bacteria are of great economic importance because of their widespread use in industrial food fermentation processes. The rapid development of genetic engineering technology for this group of GRAS organisms (generally regarded as safe) and the increased knowledge of gene structure and expression, and of protein secretion, render them potentially useful for new biotechnological applications, including new or improved food products and medicines (Gasson 1993).

The safe use of recombinant lactic acid bacteria requires the development of food-grade vector systems, that are entirely composed of DNA from the homologous host or from closely related (GRAS) organisms and do not rely on antibiotic-resistance genes for their selection. A few potentially useful food-grade plasmid systems have been developed for the genus *Lactococcus lactis*. Hughes and McKay (1991) used a derivative of the lactococcal plasmid pNP40 to construct pFK012, a plasmid that confers resistance to nisin. The pivotal role of the lactose-specific phosphocarrier factor III (LacF) in the lactococcal lactose metabolic pathway was exploited by de Vos et al. (1989), to construct pSH71 derivatives that allow direct selection of lactose-proficient transformants by complementation of a *lacF*-deficient host. However, plasmid systems such as these may suffer from segregational instability during non-selective growth (de Vos et al. 1989).

Integration of the genes of interest into the chromosome of the target organism could provide more stability. Gene-replacement strategies have been developed that are suitable for the chromosomal insertion of genes in a food-grade manner (Leenhouts et al. 1991b, 1993; Biswas et al. 1993), but have the disadvantage that the resulting strains have a low gene dosage. Single-cross-over (Campbell-type) integration offers the possibility of amplifying the integrated plasmid (Leenhouts et al. 1989, 1991a; Chopin et al. 1989) and, consequently, the gene of interest. Temperature-sensitive derivatives of the

lactococcal plasmids pSK11L and pWV01 are potentially useful for these purposes (Polzin and McKay 1992; Biswas et al. 1993), but a drawback may be that continued growth of the recombinant strains at the non-permissive temperature is required to secure stability of the integrated structures.

In previous work we have described derivatives of the lactococcal plasmid pWV01 (Ori^+ vectors) that can be used as integration vectors for *L. lactis*. Specially designed *Bacillus subtilis* and *Escherichia coli* strains have been engineered to enable the construction and isolation of the integration vectors (Leenhouts et al. 1991c, 1996; Law et al. 1995). Here we describe the construction of *L. lactis* strains that provide the pWV01 RepA protein *in trans* to enable replication of Ori^+ vectors. These strains allow the isolation of the integration vectors from a food-grade homologous host. Campbell-type Ori^+ integration vectors were constructed carrying, as food-grade selectable marker, the sucrose genes of the lactic acid bacterium *Pediococcus pentosaceus* PPE1.0 (Gonzalez and Kunka 1986). This host/vector system may contribute considerably to food science and health applications.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in TY medium (Rottlander and Trautner 1970) and *L. lactis* was grown in M17 medium (Terzaghi and Sandine 1975) containing 0.5% glucose (GM17). After electrotransformation, *L. lactis* cells were plated onto GM17 agar plates containing 0.3 M sucrose. Glucose was omitted when colonies were selected for their ability to ferment sucrose. Kanamycin (Km) was used at a final concentration of 30 μg/ml for *E. coli*. Erythromycin (Em) was used at final concentrations of 100 μg/ml and 5 μg/ml for *E. coli* and *L. lactis* respectively. Chloramphenicol (Cm) was used at a final concentration of 5 μg/ml for *L. lactis*.

Transformation

E. coli and *L. lactis* were transformed by electrotransformation as described before (Holo and Nes 1989; Zabarovsky and Winberg 1990).

Construction of integration plasmids

Two integration plasmids carrying pWV01 *repA*, pKL15A and pUK30, were constructed. Plasmid pKL15A is a derivative of the pBR322-based Campbell-type integration plasmid pHV60A (Leenhouts et al. 1989), in which *repA* from plasmid pUC23rep3 (Leenhouts et al. 1991c) was inserted. The replacement-type integration vector pUK30 was obtained by cloning the *repA* fragment of pUC23rep3 in the multiple-cloning site of pUK29. The latter plasmid is a derivative of pUK21 (Vieira and Messing 1991) carrying the Em-resistance gene of pUC19E in the *Xho*I site, the 3′ end of the *L. lactis pepX* gene (Mayo et al. 1991) as a 1.5-kb *Xba*I fragment in the *Xba*I site, and the 5′ end of *pepX* as a 1.5-kb *Spe*I-*Mlu*I fragment in the *Bgl*II and *Sph*I sites.

To construct the pWV01 Ori^+-based integration vectors, the Em-resistance gene of pUC19E was cloned as a *Bam*HI fragment into the *Bam*HI site of pUK21, resulting in pUK21E. The *Spe*I fragment carrying the Em-resistance gene of pUK21E was treated with Klenow enzyme to create blunt ends. A 601-bp *Taq*I fragment of pWV01 (Leenhouts et al. 1991d), which carries the plus origin of replication (Ori^+) but lacks the gene encoding the replication-initiation protein (*repA*), was also treated with Klenow enzyme and was subsequently ligated to the blunt-ended *Spe*I fragment of pUK21E, resulting in pORI22. The integration vectors pINT23, 25, and 42 were constructed by inserting fragments of the *pepX* gene region (Mayo et al. 1991) into the multiple-cloning site of pORI22 (see also Fig. 1). The 0.8-kb *Xho*II-*Pvu*II internal fragment of *pepX* was inserted into the *Bgl*II and *Stu*I sites to obtain pINT23. Plasmid pINT25 was constructed by cloning the 3′ end of *pepX* as a 1.5-kb *Xba*I fragment into the *Xba*I site. The 5′ end of the *pepX* gene was taken as a 1.5-kb *Spe*I-*Mlu*I fragment and inserted into the *Bgl*II and *Sph*I sites of pORI22, after the modification of the recessed ends, to obtain pINT42. Plasmid pINT29 was obtained by ligation of the *Spe*I fragment of pUK29, carrying the Em-resistance gene and two parts of *pepX*, to the 601-bp *Taq*I fragment of pWV01. All Ori^+ vectors were constructed using the *E. coli* $RepA^+$ helper strain EC101 (Law et al. 1995).

Bioassays

The ability of *L. lactis* to ferment sucrose (Suc^+) was tested by direct plating of cells or by streaking colonies onto M17 agar plates containing 0.5% sucrose (SM17) and 0.005% bromocresol purple as a pH indicator. Suc^+ colonies stain yellow on these plates whereas Suc^- colonies remain white. PepX-deficient strains were identified by a PepX plate assay as described before (Leenhouts et al. 1991b).

Stability assay

Overnight cultures of strains MG124, 125 and 142 cultured in SM17 ($t = 0$), were diluted to 10^3 colony-forming units (cfu)/ml in GM17 and were grown to the stationary phase (approximately 20 generations). Six identical transfers in GM17 medium were carried out to reach 120 generations of non-selective growth ($t = 120$). At $t = 0$, $t = 60$ and $t = 120$, dilutions of the cultures were plated onto SM17 medium containing 0.005% bromocresol purple to determine the Suc phenotype of over 1000 cfu for each strain. Similar dilutions were plated at these intervals onto GM17 agar plates and three colonies of each strain were randomly selected at each assay time and cultured in GM17. Chromosomal DNA for Southern hybridization was isolated from these cultures and from the cultures of strains MG124, 125 and 142 at $t = 0$, $t = 60$ and $t = 120$.

DNA isolation and analysis

Chromosomal DNA was isolated as previously described (Leenhouts et al. 1990). Plasmid DNA was extracted according to the method of Birnboim and Doly (1979) with the following modifications. *L. lactis* cells were incubated with lysozyme for 15 min at 55°C to obtain optimal lysis. Large-scale plasmid isolation was followed by CsCl-gradient purification (Maniatis et al. 1982). Plasmid and chromosomal DNA treated with restriction enzymes were analysed using 0.8% agarose gels. DNA transfer to Qiabrane Nylon Plus membranes (Qiagen, Düsseldorf, Germany) was according to the protocol of Chomczynsky and Qasba (1984). Probes were labelled using the ECL labelling kit and hybridization conditions and probe detection were according to the instructions of the supplier (Amersham International, Amersham, United Kingdom).

Determination of integrated plasmid copy numbers

The number of integrated plasmid copies in strains MG124, MG125 and MG142 was determined using Southern blots with membranes containing dilutions of the chromosomal DNA of

Table 1 Bacterial strains and plasmids. *dco* double cross-over, *sco* single cross-over

Strains or plasmid	Relevant properties	Source or reference
E. coli		
NM522	*supE, thi,* Δ*(lac-proAB), hsd5(r⁻, m⁻)*[F′, *proAB, lacI^qZ*ΔM15]	Stratagene (La Jolla,Calif.)
EC101	RepA$^+$ JM101 (Yanisch et al. 1985), Km^r, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	Law et al. 1995
L. lactis		
MG1363	Plasmid free NCDO712, Suc$^-$, RepA$^-$	Gasson 1983
SH4352	Suc$^+$ derivative of NCDO712	Gasson 1984
LL108	RepA$^+$ MG1363, Cm^r, carrying multiple copies of pWV01 *repA* in chromosomal fragment A (Leenhouts et al. 1990)	This work
LL302	RepA$^+$ MG1363, carrying a single copy of the pWV01 *repA* in *pepX*	This work
MG124	Suc$^+$ MG1363, carrying multiple copies of pINT124 in *pepX*	This work
MG125	Suc$^+$ MG1363, carrying multiple copies of pINT125 in the 3′-end of *pepX*	This work
MG129	MG1363, carrying one copy of the sucrose genes *scrA* and *scrB* in *pepX*	This work
MG142	Suc$^+$ MG1363, carrying multiple copies of pINT142 in the 5′ end of *pepX*	This work
Plasmids		
pKL15A	Cm^r, pHV60A (Leenhouts et al. 1989) derivative carrying *repA* of pWV01	This work
pUC19E	Ap^r, Em^r, pUC19 (Yanisch-Perron et al. 1985) carrying the Em^r gene of pE194 (Horinouchi and Weisblum 1982) in the *Sma*I restriction site	Laboratory collection
pUK21	Km^r, α*lacZ*	Vieira and Messing 1991
pUK21E	Km^r, Em^r, pUK21 carrying the Em^r gene of pUC19E in the *Bam*HI restriction site	This work
pUK29	Km^r, Em^r, pUK21 derivative specific for integration in *pepX*	This work
pUK30	Km^r, Em^r, pUK29 derivative specific for dco integration of pWV01 *repA* in *pepX*	This work
pWV01	cryptic plasmid of *L. lactis* Wg2	Leenhouts et al. 1991d
pORI22	Em^r, *ori*$^+$ of pWV01, replicates only in strains providing *repA in trans*	This work
pINT23	Em^r, derivative of pORI22 specific for sco integration in *pepX*	This work
pINT25	Em^r, derivative of pORI22 specific for sco integration in *pepX*	This work
pINT29	Em^r, *ori*$^+$ derivative specific for dco integration in *pepX*	This work
pINT42	Em^r, derivative of pORI22 specific for sco integration in *pepX*	This work
pINT124	Suc$^+$, derivative of pINT23	This work
pINT125	Suc$^+$, derivative of pINT25	This work
pINT129	Em^r, Suc$^+$, derivative of pINT29	This work
pINT142	Suc$^+$, derivative of pINT42	This work

these strains and of strain MG129, containing a single copy of the sucrose genes. Dilution factors were determined by using chromosomal fragment A (Leenhouts et al. 1990), which is present in a single copy in the chromosome of each strain, as a probe. A *Pvu*II fragment containing *scrA* and *scrB* was used as a probe in these experiments. A Pharmacia LKB2222-020 UltraScan XL laser densitometer (LKB Produkter AB, Pharmacia, Bromma, Sweden) was used to measure the intensity of the hybridizing bands.

Determination of growth rates

Strains were grown at 30 °C in a 96-well microtitre plate in 200-μl samples (eight times for each strain) of 1 in 100 dilutions of overnight cultures. The absorbance at 600 nm was determined at 15-min intervals by using a Thermomax microtitre plate reader (Molecular Devices Corporation, Menlo Oaks, Calif.). The growth rate (μ_{max}) was determined by calculating the maximum value of the first derivative of the growth curve.

Results

Construction of RepA$^+$ *L. lactis* strains

Plasmid pUK30 was used in a two-step gene-replacement strategy (Leenhouts et al. 1991b), to obtain strain LL302. This strain contains one copy of pWV01 *repA* inserted in *pepX* (data not shown).

Integration of pKL15A into the chromosome of strain MG1363 by selection for chloramphenicol resistance resulted in strain LL108 carrying approximately 15 tandem copies of the integration plasmid, as has been described earlier for similar plasmids (Leenhouts et al. 1989). Strains LL302 and LL108 both allow the replication of pWV01-based vectors that lack *repA*. The copy number of such vectors in strain LL302 is low and comparable to that of pWV01-derived vectors in strain MG1363. A significantly higher copy number was observed in strain LL108. As visualized on agarose gels, approximately ten times more plasmid DNA was pres-

ent in minipreparations from LL108 than in those from strain LL302 (data not shown).

Construction of food-grade integration plasmids

The sucrose genes *scrA* and *scrB*, encoding the sucrose-specific enzyme II and the sucrose-6-phosphate hydrolase, respectively, of the lactic acid bacterium *P. pentosaceus* PPE1.0, have been cloned previously (to be described elsewhere) and were used in this study as food-grade selectable genes for the construction of the integration plasmids. An *Aat*II-*Sal*I fragment, corresponding to nucleotides 13 837–17 897 of the *P. pentosaceus* sucrose operon (accession number L32093 of the Genome Sequence database and accession number Z32771 of the EMBL Sequence database), encompassing *scrA* and *scrB*, was inserted in the *Aat*II-*Sal*I sites of pINT42, 23, 25 and 29. The Em^r genes of pINT42, 23 and 25 were removed by *Bam*HI digestion and self-ligation. The final constructs were designated pINT142, 124, 125 and 129. A schematic representation of pINT125 is shown in Fig. 1A. The location on the MG1363 chromosome of the chromosomal fragments used in the construction of the integration plasmids is shown in Fig. 1B. Plasmids pINT142, 124, 125 and 129 were selected in the $RepA^+$ helper strains LL108 and LL302 using a growth medium with sucrose as the selectable substrate.

Single-copy integration of the sucrose genes

Plasmid pINT129 allows single-copy integration of the sucrose genes by gene replacement using a two-step procedure (Leenhouts et al. 1991b). The sucrose genes in pINT129 are positioned between the two chromosomal fragments (Fig. 1B). An Em^r gene is present in another part of the vector. In the first step there is no selection for the sucrose genes. The integration of pINT129 in the MG1363 chromosome is accomplished by selection for Em resistance. The second step involves the resolution of part of the cointegrate structure in non-selective M17 medium with glucose as the only carbon source. Transformants with the sucrose genes inserted into *pepX* by gene replacement were identified using the PepX colorimetric assay and by Southern hybridization, and one of these was designated MG129. Strain MG129 was unable to grow in M17 medium with sucrose as the sole carbon source. Although small colonies were visible on SM17 agar plates, no acidification was observed when the pH indicator bromocresol purple had been added, and therefore no sucrose was fermented (Suc^-).

Suc^+ transformants were obtained when the integration of pINT129 was directly selected for on SM17 agar plates. However, all these transformants appeared to be Em^r as well and Southern blot analysis showed that, in all cases investigated, chromosomal amplification of the entire integration plasmid had occurred.

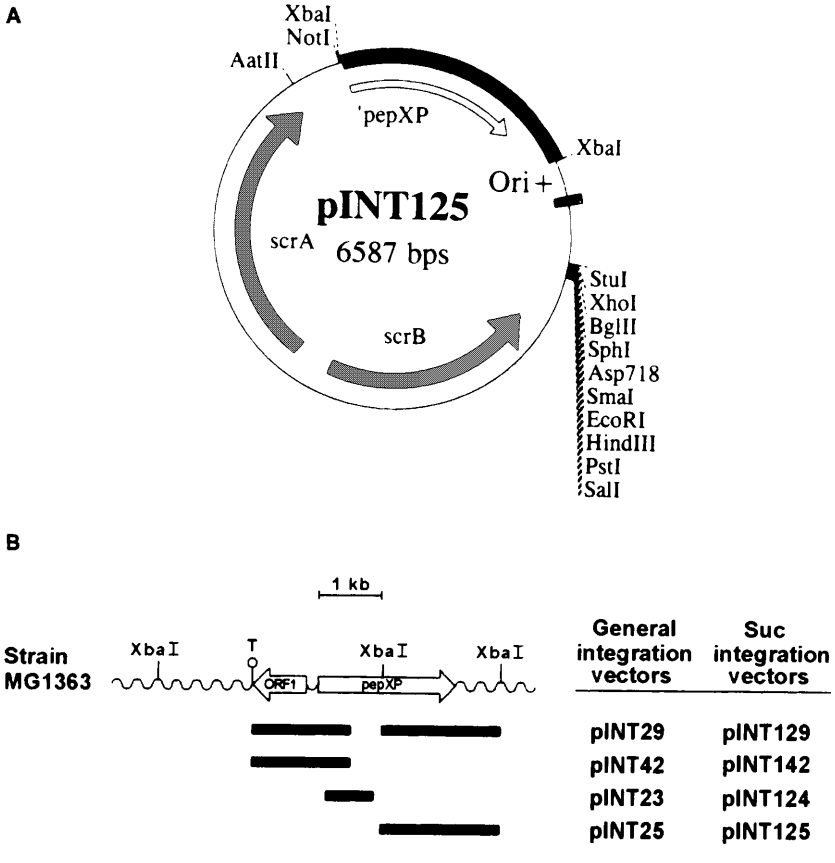

Fig. 1 **A** Plasmid map of the food-grade integration plasmid pINT125. *Black bar* chromosomal DNA of strain MG1363, *′pepXP 3′* end of *pepX*, *scrA* gene encoding the sucrose-specific enzyme II, *scrB* gene encoding sucrose-6-phosphate hydrolase, Ori^+ plus origin of replication of pWV01. **B** Schematic representation of the chromosomal location of the DNA fragments present in various integration vectors

From these data we concluded that the sucrose genes of *P. pentosaceus* PPE1.0, expressed from their native promoters, are not suitable to select for single-copy integrations in *L. lactis*.

Multi-copy integrations using sucrose genes as selectable marker

Single-cross-over integrations into the chromosome of strain MG1363 were obtained using one of the plasmids pINT124, 125 or 142, by direct selection of transformants on SM17 agar plates. None of the transformants analysed contained free replicating plasmid DNA, and Southern blot analysis of their chromosomal DNA showed that all contained considerable chromosomal amplification of the integration plasmid. One transformant of each transformation, designated MG124, MG125 and MG142, was taken for further analysis. The number of copies of the integrated plasmids in these transformants was approximately 20 (Table 2). The growth rates of the selected integrants MG124, MG125 and MG142 were determined by growth in microtitre plates with SM17 as the growth substrate. Strain SH4352, a wild-type lactococcal strain able to grow on media containing sucrose as the sole carbon source, was used as a reference. From the results summarized in Table 3 it can be concluded that the growth rates of the recombinant strains are significantly lower ($\mu_{max} \approx 0.4\ h^{-1}$) than that of the wild-type strain SH4352 grown in SM17 ($\mu_{max} \approx 0.6\ h^{-1}$). The growth rates of all strains grown in GM17 are comparable to that of the parental strain MG1363 ($\mu_{max} \approx 0.7\ h^{-1}$), except for strain MG142, which shows a significantly lower value ($\mu_{max} < 0.6\ h^{-1}$).

Stability of the multi-copy integrations

The Suc^+ phenotypes and the integrated plasmid copy number of the integrants MG124, MG125 and MG142 were very stable in SM17. No Suc^- colonies were detected and no significant differences in the number of integrated plasmid copies were observed in Southern hybridizations after growth for over 100 generations in SM17 (less than 0.1% Suc^-; data not shown).

When MG124, MG125 and MG142 were subjected to non-selective growth in GM17 for 120 generations, a minor fraction of the colonies (0–2%) initially appeared to have acquired the Suc^- phenotype. However, upon prolonged incubation for 48 h, Suc^+ sectors developed in these colonies, suggesting that the sucrose genes were still present in the initially Suc^- colonies. This was supported by the following experiment: Suc^+ cells from the Suc^+ sectors were grown to colonies and subsequently transferred to SM17 for growth and isolation of chromosomal DNA, which was subjected to Southern blot hybridization. This showed that amplification of the integration plasmid was present to a similar extent to that in the parental transformants (data not shown). Although the number of integrated plasmid copies in the initially Suc^- colonies was not investigated, it is reasonable to assume that the number was insufficient to produce a Suc^+ phenotype and that amplification of the integrated plasmids gave rise to the sectorial Suc^+ phenotype in the initially Suc^- colonies.

At $t = 0$, $t = 60$ and $t = 120$ chromosomal DNA was isolated from the MG124, MG125 and MG142 cultures, as well as from randomly picked colonies from dilutions of these cultures plated onto GM17 plates. An example of a Southern blot analysis of the MG125 cultures and colonies is shown in Fig. 2. The 3.6-kb *Pvu*II fragment indicated by the double arrow represents amplified vector DNA carrying the sucrose genes. The single arrow indicates the border fragments, which both migrate as 2.8-kb fragments. The hybridizing fragments of higher molecular mass are, most likely, the result of incomplete digestion of the chromosomal DNA.

The average numbers of integrated plasmid copies was determined at each time interval for the three cultures and are given in Table 2. MG124 retained the highest number of copies with an average loss of 7.5×10^{-2} copies/generation, resulting in approximately 11 copies after 120 generations of non-selective growth in GM17 medium. In MG125 and MG142 the rate of loss was approximately twice as high. It was concluded that the Campbell-type integrations in the three strains investigated were relatively stable.

Table 2 Number of integrated plasmid copies in *Lactococcus lactis strains MG124, MG125 and MG142 during non-selective growth*

Strain	Number of generations of non-selective growth			Average loss of copies/ generation
	0	60	120	
MG124	20	16	11	0.075
MG125	21	8	3	0.15
MG142	20	5	3	0.14

Table 3 Growth rate (μ_{max}) of strains grown in SM17 and GM17. *NT* not tested. Results are averages of eight measurements

Strain	$\mu_{max} \pm$ SD (h^{-1})	
	SM17	GM17
SH4352	0.64 ± 0.01	0.73 ± 0.02
MG124	0.37 ± 0.02	0.73 ± 0.04
MG125	0.44 ± 0.01	0.67 ± 0.04
MG142	0.34 ± 0.02	0.56 ± 0.02
MG1363	NT	0.75 ± 0.02

Discussion

Stable food-grade integration in the chromosome of multiple copies of a gene of interest can be achieved by consecutive gene replacements at different chromosomal

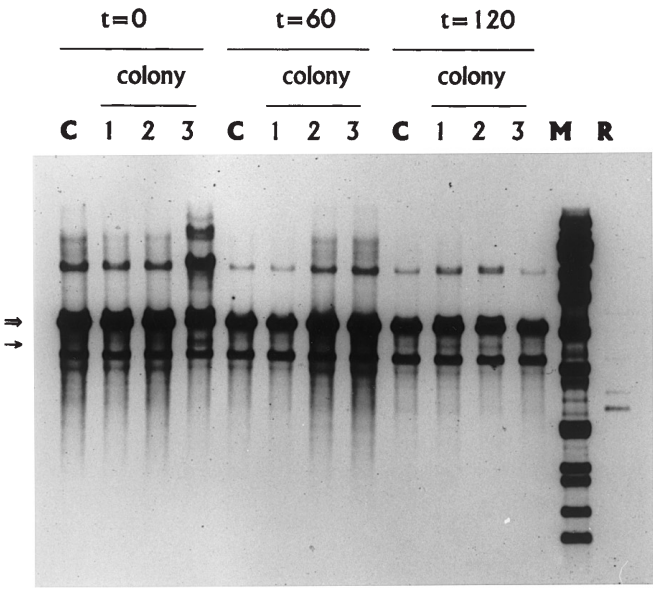

Fig. 2 Southern hybridization analysis of *Pvu*II-digested chromosomal DNA of strain MG125 after growth in GM17 medium. The chromosomal DNA fragment present in pINT125 (see Fig. 1) was used as a probe. The hybridization solution included labelled SPP1 marker DNA digested with *Eco*RI, to visualize the marker (*M*). The sizes (kb) of the marker from bottom to top: 1.0, 1.1, 1.4, 1.5, 1.9, 2.0, 2.7, 3.6, 4.9, 6.0, 7.4, 8.0. **C** DNA from the original culture grown for the indicated number of generations (*t*); *1*, *2*, *3* DNA from cultures of randomly picked colonies at the indicated assay times; *R* chromosomal DNA of strain MG1363

loci, but this is a rather laborious procedure. Nevertheless, the technology to follow this procedure for *L. lactis* is available (Leenhouts et al. 1991b, 1996; Maguin et al. 1992). The present study describes a lactococcal food-grade vector system that can be used in a simple procedure to stably integrate multiple copies of a gene of interest in the chromosome of *L. lactis*. The vector consists entirely of DNA of lactic acid bacteria and integrations occur in the well-characterized *pepX* chromosomal region. This region was chosen for reasons of convenience to demonstrate the feasibility of the approach. In applications it may be desirable to select other chromosomal regions. The two *L. lactis* helper strains LL108 and LL302, which produce the pWV01 RepA protein essential for replication of the Ori^+ vectors, eliminate the need to pass the plasmids through a non-lactococcal bacterial species, a step that may not be acceptable for food-grade vectors. The latter step could also be avoided by using thermosensitive lactococcal Campbell-type integration vectors (Polzin and McKay 1992; Biswas et al. 1993). However, stable maintenance of the integrated plasmids usually requires continuous growth at the non-permissive temperature, which may not be desirable for certain (food) applications or may prevent optimal growth, thus impairing the production of the desired gene product.

A single chromosomal copy of the *P. pentosaceus* sucrose genes was not sufficient to obtain Suc^+ *L. lactis*, probably as because of low expression of these genes in this host. Other work indeed showed an extremely low level of expression of the sucrose genes in *L. lactis* (to be published elsewhere). Extensive chromosomal amplifications have been observed before in *L. lactis* when a poorly expressed antibiotic resistance marker was used (Leenhouts et al. 1989; Simons et al. 1993).

The amplified integrated plasmid copies in the strains obtained in this study were stable during growth in medium containing sucrose as the sole carbon source. However, a significant reduction in the growth rate was observed under these growth conditions, which could be undesirable for some industrial applications. Growth in glucose- or lactose-containing media may be preferable. Although growth with, for instance, glucose as the only carbon source resulted in a gradual loss of integrated plasmid copies, strain MG124 had retained 11 copies after 120 generation of non-selective growth.

Strain MG124 displayed a higher degree of stability during non-selective growth than strains MG125 and MG142. Although pINT124 contains a smaller chromosomal DNA fragment (0.8 kb) than do pINT125 and pINT142 (1.5 kb), it is unlikely that the size difference causes the difference in stability. The excision of plasmid copies probably occurs by recombination with the entire plasmid (approximately 6 kb in each case) as a substrate. Since in *L. lactis* the recombination frequency between homologous fragments larger than 2.5 kb does not increase with increasing size (Biswas et al. 1993), other factors must cause the observed differences. The presence of actively transcribed promoters is a known cause for elevated levels of recombination (Biswas et al. 1993). The integration fragment used to make MG124 is devoid of promoter activity, while the fragment used to construct MG142 does contain active promoters (Mayo et al. 1991). Whether the chromosomal fragment used in pINT125 contains an active promoter is currently unknown.

A possible drawback of the sucrose integration system may be its limited applicability in traditional dairy applications. Although not tested in the present study, dairy strains are likely to contain pWV01-related plasmids, which may interfere with transformability, integration and stability. Curing of plasmids before the integration event is a feasible strategy, but could lead to the loss of desired properties. However, the present system may meet the requirements for applications such as the production of enzymes or peptides applied in food-related processes or in vaccine development.

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