

# Efficient System for Directed Integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* Chromosomes via Homologous Recombination

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**An efficient method is described for the generation of site-specific chromosomal integrations in *Lactobacillus acidophilus* and *Lactobacillus gasseri*. The strategy is an adaptation of the lactococcal pORI system (K. Leenhouts, G. Venema, and J. Kok, *Methods Cell Sci.* 20:35–50, 1998) and relies on the simultaneous use of two plasmids. The functionality of the integration strategy was demonstrated by the insertional inactivation of the *Lactobacillus acidophilus* NCFM *lacL* gene encoding  $\beta$ -galactosidase and of the *Lactobacillus gasseri* ADH *gusA* gene encoding  $\beta$ -glucuronidase.**

*Lactobacillus acidophilus* and related lactobacilli represent an important group of lactic acid bacteria which are commonly found in association with the human gastrointestinal tract and are delivered as health-promoting bacteria in foods and probiotics. Recent research efforts have focused on expanding the use of probiotic lactobacilli to include biotechnological applications such as enzyme and vaccine delivery systems (reviewed in reference 12). The results to date are promising, but successful implementation of this technology depends on the ability to identify and genetically engineer proper candidate strains. Unfortunately, efficient integration systems are not available for many important lactobacilli. In order to study gene function and chromosomally stabilize expression cassettes, an integration strategy is needed for thermophilic lactobacilli that is independent of transformation frequency, based on a broad-host-range replicon, and stably maintains integrants at the desired temperature (37°C).

Conditionally replicating plasmids based on the broad-host-range lactococcal pWV01 replicon have been used to make a variety of integrations into lactococcal chromosomes. Law et al. (4) described an integration strategy for *Lactococcus* that utilizes pWV01-derived vectors from which the *repA* gene has been removed. These “Ori<sup>+</sup>” integration vectors replicate only in strains providing *repA* in *trans*. One method to establish these vectors in a desired host strain is to supply *repA* from a second temperature-sensitive helper plasmid. When a DNA fragment with homology to the host chromosome has been cloned on the Ori<sup>+</sup> vector, a subsequent temperature shift selects for loss of the helper plasmid and integration of Ori<sup>+</sup>-plasmid DNA. Despite the potential usefulness of this system, its application to thermophilic lactobacilli was still limited by

the low functional temperature range of the available helper plasmid, pVE6007.

**Creation and temperature sensitivity of plasmid pTRK669.** In order to increase the host range of this system to include thermophilic lactobacilli, we sought to combine the useful features of the Ori<sup>+</sup> vectors with a more suitable pWV01-based helper plasmid. Previous experiments in our laboratory have indicated that the wild-type pWV01 replicon, while not completely temperature sensitive, is moderately unstable at temperatures of >42°C in thermophilic lactobacilli, similar to what has been reported for the related plasmid, pE194, in *Bacillus subtilis* (14). While not suitable for performing traditional temperature-sensitive integration experiments, this feature made it possible to adapt the two-plasmid lactococcal integration strategy for use in *Lactobacillus*. A helper plasmid, based on pGK12, was created to provide *repA* in *trans* for the replication of pORI28-based plasmids (7). This helper plasmid, pTRK669, retains the pWV01 replicon including the origin of replication and *repAC* genes as well as the *cat* gene (Table 1). The erythromycin resistance (Em<sup>r</sup>) marker was removed in order to make the new plasmid compatible with pORI28, which harbors its own Em<sup>r</sup> gene. Plasmids pGK12 and pTRK669 were transformed into *L. acidophilus*, where their stability was monitored during growth at 37 and 43°C in the absence of antibiotic selection (Fig. 1). The results indicate that both plasmids are stable at 37°C but not at 43°C. An almost 4-log reduction of plasmid-containing cells was observed at 43°C over the course of 30 generations. Similar results were obtained using *L. gasseri* cells (data not shown). The ability of pTRK669 to supply RepA for the replication of pORI28 was tested by transferring pORI28 into *L. acidophilus* and *L. gasseri* cells with or without pTRK669. Em<sup>r</sup> clones could only be recovered from cells which also carried pTRK669, indicating that replication of pORI28 was supported by pTRK669.

**Inactivation of the *L. acidophilus lacL* gene.** In order to verify the functionality of the integration system, plasmid pTRK670 was designed to disrupt the *L. acidophilus lacL* gene, encoding  $\beta$ -galactosidase. The primers *bgalF* (5'-GACTGGATCCTGC CGAACGAGCCATGTATG-3') and *bgalR* (5'-GACTGAAT

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Origin or relevant characteristics	Source or reference
<b>Strains</b>		
<i>L. acidophilus</i>		
NCFM	Human intestinal isolate	1
NCK1398	NCFM <i>lacL</i> ::pTRK670	This study
<i>L. gasseri</i>		
ADH	Human intestinal isolate (called strain MS02)	2
NCK1423	ADH <i>gusA</i> ::pTRK685	This study
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>endA1 recA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>deoR</i>	Gibco-BRL <sup>a</sup>
EC1000	RepA <sup>+</sup> MC1000, Km <sup>r</sup> , carrying a single copy of the pWV01 <i>repA</i> gene in the <i>glgB</i> gene; host for pOR128-based plasmids	4
<b>Plasmids</b>		
pGK12	ori (pWV01), Em <sup>r</sup> Cm <sup>r</sup> , RepA <sup>+</sup> , gram-positive shuttle vector	3
pTRK669	ori (pWV01), Cm <sup>r</sup> , RepA <sup>+</sup>	This study
pORI28	Em <sup>r</sup> , ori (pWV01), replicates only with <i>repA</i> provided <i>in trans</i>	4
pTRK670	2.6 kb; pORI28 with 945-bp internal <i>L. acidophilus</i> NCFM <i>lacL</i> fragment	This study
pTRK685	2.4 kb; pORI28 with 777-bp internal <i>L. gasseri</i> ADH <i>gusA</i> fragment	This study

<sup>a</sup> Gibco-BRL, Grand Island, N.Y.

TCCCGGCATAAGATTCGTTTCC-3'), based on the previously reported *lacL* gene of *L. acidophilus* JCM 1229 (Gen Bank no. AB004867), were used to amplify a 945-bp internal region of *lacL* from *L. acidophilus* NCFM. This fragment was then cloned via the *Bam*HI/*Eco*RI sites (underlined) into pORI28, an Ori<sup>+</sup> RepA<sup>-</sup> integration plasmid (4). DNA sequencing confirmed that the sequence of the NCFM fragment was 100% identical to the JCM1229 *lacL* sequence. Plasmid pTRK670 was then introduced by electroporation into *L. acidophilus*(pTRK669). One Em<sup>r</sup>, chloramphenicol-resistant (Cm<sup>r</sup>) transformant, carrying both plasmids, was propagated overnight once at 37°C in MRS broth plus 5  $\mu$ g of erythromycin and 5  $\mu$ g of chloramphenicol per ml. The culture was then transferred three times at 43°C (1% inoculum) in MRS broth plus 5  $\mu$ g of erythromycin/ml (ca. 30 generations). After this enrichment, the culture was plated with a Whitley Automatic

Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England) on MRS-X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside)-galactose plates with either erythromycin, chloramphenicol, or no antibiotic, and incubated for 48 h at 37°C. The results of the plating indicated that the final culture contained  $\sim 10^7$  Em<sup>r</sup> cells per ml and  $< 10^{-7}$  Cm<sup>r</sup> cells per ml. All of the recovered colonies were white, indicating the loss of  $\beta$ -galactosidase activity. Conversely, a control culture, which had been enriched at 37°C instead of at 43°C, still contained a large percentage of LacL<sup>+</sup>, Cm<sup>r</sup> cells. One Em<sup>r</sup> Cm<sup>s</sup> clone, designated NCK1398 was chosen for further confirmation. To confirm the loss of  $\beta$ -galactosidase activity, ONPG (*o*-nitrophenyl- $\beta$ -D-galactopyranoside) assays (11) were performed on log-phase cultures of *L. acidophilus* NCFM and NCK1398. In order to induce  $\beta$ -galactosidase production, cultures were grown with galactose as the sole carbon source. Upon analysis, no measurable  $\beta$ -galactosidase activity could be detected from NCK1398 compared to  $2,652 \pm 167$  U from NCFM. In order to demonstrate that the disruption of  $\beta$ -galactosidase was the result of integration of pTRK670 in the *lacL* gene, Southern hybridizations were performed using the 945-bp *lacL* fragment as a probe (Fig. 2). The *lacL* probe hybridized to an *Eco*RI fragment of ca. 11.5 kb in *L. acidophilus* NCFM. In NCK1398, this band disappeared and was replaced by junction fragments of ca. 5.3 and 8.8 kb due to the presence of a single *Eco*RI site present in the pTRK670 plasmid sequence. In addition to the two junction fragments, another band was observed that corresponded to the amplified copies of the 2.6-kb pTRK670 sequence. Further confirmation was obtained by hybridization to *Hind*III digestions because no *Hind*III sites were present within the pTRK670 sequence. The *lacL* probe hybridized to an ca. 3.2-kb fragment in *L. acidophilus* NCFM. In NCK1398, this band disappeared and was replaced with bands of ca. 5.8, 8.4, and 11.0 kb, corresponding to insertions of one, two, and three plasmid copies, respectively. These results were identical in each of the clones tested, indicating that each individual clone gave rise to a mixed population of cells harboring different numbers of plasmid copies. This variation in plasmid copy number has been observed in other lactic acid bacteria (9,

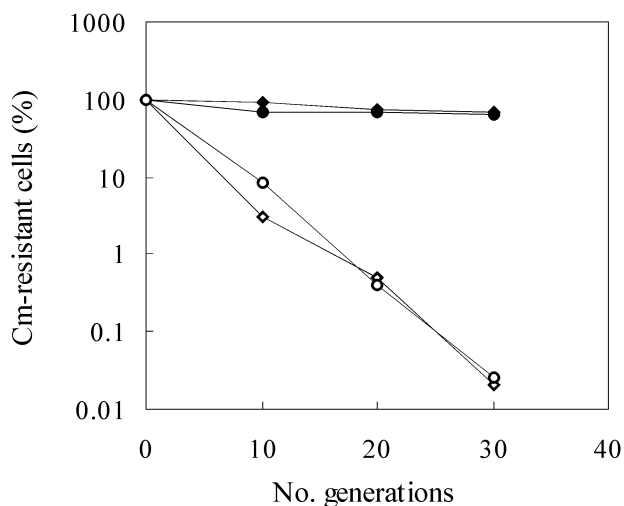


FIG. 1. Stability of pGK12 (◆) and pTRK669 (●) in *L. acidophilus* NCFM at 37°C (closed symbols) and 43°C (open symbols). The percentage of Cm<sup>r</sup> cells in each culture was determined by plating on MRS versus MRS plus chloramphenicol.

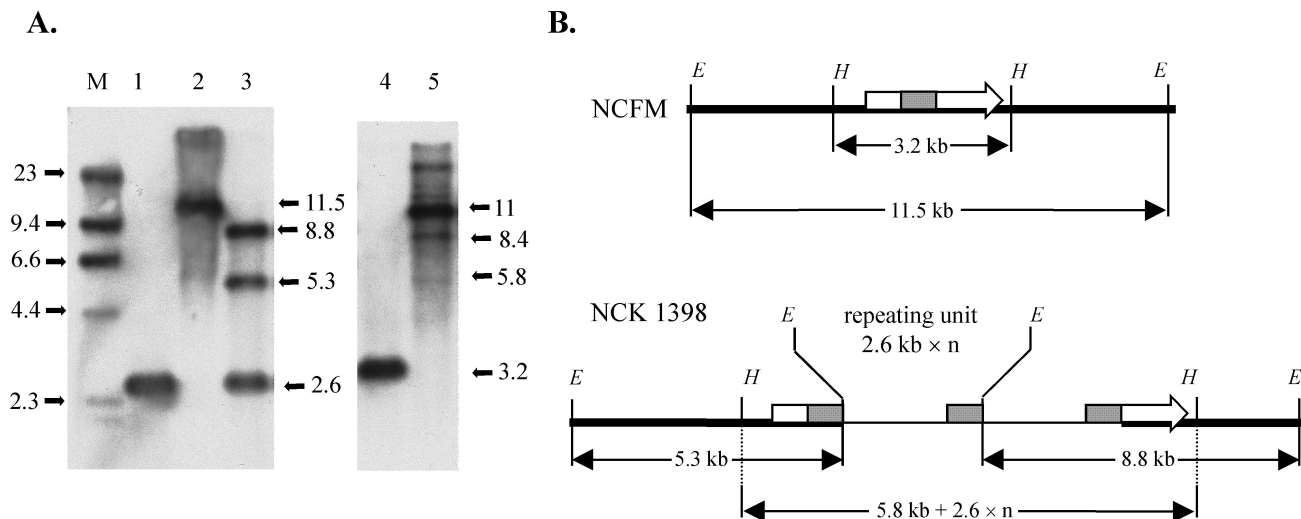


FIG. 2. (A) Southern hybridization analysis of *L. acidophilus* NCFM and NCK1398. Chromosomal DNA was digested with *EcoRI* (lanes 1, 2, and 3) or *HindIII* (lanes 4 and 5) and hybridized with the 945-bp internal *lacL* fragment as a probe. Lane 1, pTRK670; lanes 2 and 4, NCFM; lanes 3 and 5, NCK1398; M, digoxigenin-labeled  $\lambda$ -*HindIII* marker. DNA sizes are indicated in kilobases. (B) Schematic representation of the relevant regions of the NCFM and NCK1398 chromosomes. Chromosomal DNA is represented by a heavy line, plasmid DNA is represented by a thin line, the *lacL* gene is represented by an arrow, and the internal *lacL* fragment is represented by a solid box. The *EcoRI* (*E*) and *HindIII* (*H*) sites are indicated. The repeating unit represents the plasmid DNA present in various copies ("n"). The diagram is not drawn to scale.

10). Leenhouts et al. (8) reported *L. lactis* clones that had generated one, two, or three chromosomal plasmid copies after the insertion of a pORI-type plasmid. This amplification of plasmid sequences has generally been attributed to recombinatory activity between the flanking DNA regions of homology that result from campbell-like integration and is influenced by a number of factors, including the nature and location of the insertion event.

**Inactivation of the *L. gasseri gusA* gene.** The complete sequence of the *L. gasseri* ADH *gusA* gene, encoding  $\beta$ -glucuronidase, has been reported recently (13). A 777-bp internal region of the *gusA* gene was amplified by PCR using the primers GUS1F (5'-GACTTCTAGAACAGTTGACGAATACACAGAT-3') and GUS1R (5'-GTGAATTCAGGCGATGAGAAGAAGATAATG-3'). This fragment was then cloned into pORI28 as an *XbaI/EcoRI* fragment using the sites added to the 5' ends of the primers (underlined). The resulting plasmid, pTRK685, was then transferred by electroporation into *L. gasseri* ADH(pTRK669), and the integration strategy was carried out as described above. The results were similar to those obtained for *lacL*; only  $Em^r$   $Cm^s$  colonies were obtained, and disruption of the *gusA* gene was evidenced by the formation of only white colonies. One clone, designated NCK1423, was subjected to further testing. Confirmation of the  $GusA^-$  phenotype was obtained by performing  $\beta$ -glucuronidase assays (13) on stationary-phase *L. gasseri* ADH and NCK1423 cells. No measurable  $\beta$ -glucuronidase activity could be detected with NCK1423 compared to  $97.2 \pm 11$  U in ADH. Integration of plasmid DNA into the *gusA* gene was confirmed by Southern hybridization (Fig. 3). The results of the Southern hybridization revealed that integration had occurred within the *gusA* gene. However, unlike the previous integration into *L. acidophilus lacL*, the number of plasmid DNA sequences integrated into the *gusA* gene appeared to be fixed at two copies.

**Stability of integrations.** To determine the stability of the integrated plasmids, NCK1398 and NCK1423 were propagated in MRS broth in the absence of antibiotics for ca. 50 generations and plated on media with the appropriate chromogenic substrate, i.e., either X-Gal or X-Gluc (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid). Loss of the integrated plasmid was assessed by restoration of the  $LacL^+$  or  $GusA^+$  phenotypes, as indicated by the formation of blue colonies. After 50 generations, the percentages of revertants were 5.36% for *lacL* and 0.72% for *gusA*, corresponding to frequencies of 0.11% and 0.01% per generation, respectively. These results indicated that, while plasmid DNA is stably integrated in both strains, the integrated DNA is ca. 10-fold more stable in the *gusA* gene of *L. gasseri* NCK1423.

**Advantages of the system.** The advantages of the method reported here over other integration strategies for lactobacilli are that (i) it is not dependent on transformation efficiency, (ii) it allows growth of mutant strains at preferred growth temperatures, (iii) it allows efficient recovery of stable integrants, and (iv) it is likely to function across a broad range of species. While this study has focused on the use of a single integration event to insertionally inactivate functional genes, a number of other applications are envisioned. For example, this strategy can be easily modified to construct and integrate expression cassettes into preferred sites in the chromosome. However, the primary disadvantage of the current design is that plasmid DNA and antibiotic markers remain integrated in the chromosome, leading to potential complications in food-grade applications. Recently, the pORI system has been used to create unlabeled gene replacements in *L. lactis* and *B. subtilis* (7) and to create a food-grade multiple-copy integration system (6) that has been used to overexpress proteins in *Lactococcus* (5). It is expected that the protocol described here could be easily modified to facilitate similar strategies in lactobacilli.

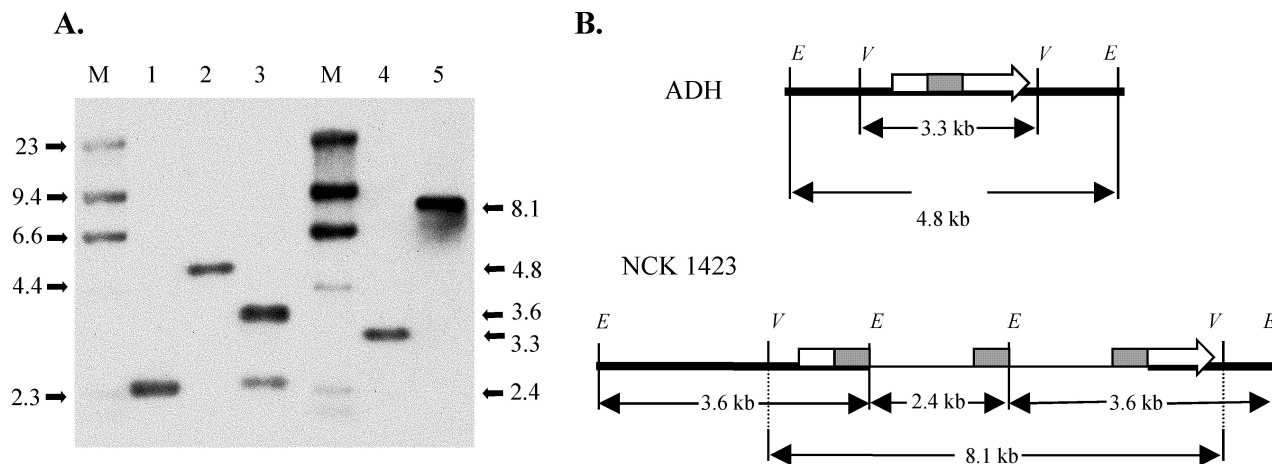


FIG. 3. (A) Southern hybridization analysis of *L. gasseri* ADH and NCK1423. Chromosomal DNA was digested with *Eco*RI (lanes 1, 2, and 3) or *Eco*RV (lanes 4 and 5) and hybridized with the 777-bp internal *gusA* fragment as a probe. Lane 1, pTRK685; lanes 2 and 4, ADH; lanes 3 and 5, NCK1423; M, digoxigenin-labeled  $\lambda$ -*Hind*III marker. DNA sizes are indicated in kilobases. (B) Schematic representation of the relevant regions of the ADH and NCK1423 chromosomes. Chromosomal DNA is represented by a heavy line, plasmid DNA is represented by a thin line, the *gusA* gene is represented by an arrow, and the internal *gusA* fragment is represented by a solid box. The *Eco*RI (E) and *Eco*RV (V) sites are indicated. The diagram is not drawn to scale.

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